Chapter 34
Drosophila: An Invertebrate Model of NF1

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34.1 Introduction

As discussed in preceding chapters, NF1 is a multisystem chronic genetic disease associated with a variety of symptoms. Another oft-cited hallmark of NF1 is its variability and unpredictability. Thus, patients typically only develop a subset of symptoms, and the severity of individual symptoms can vary dramatically between patients. Variable expressivity of a single-gene genetic disorder, like NF1, can be due to several different factors, including incomplete genetic penetrance, the nature of the culprit gene defect, whether or not a patient is a somatic mosaic, and whether or not a given patient carries genetic modifiers or has been exposed to different environments. In the case of NF1, all these factors are believed to play a role. Thus, although individuals carrying *NF1* mutations develop at least some symptoms with complete penetrance, stochastic genetic or epigenetic events may result in partial penetrance of individual symptoms. An example is provided by plexiform neurofibromas, most of which are believed to develop congenitally. The fact that no more than about a third of patients develop these tumors may reflect the limited probability of loss of the wild-type *NF1* allele in tumor progenitors during a restricted developmental period. Secondly, while genotype–phenotype correlations are uncommon in NF1, some notable exceptions exist. First, 5–10 % of patients harbor recurring 1.2–1.4 Mb microdeletions that include the *NF1* gene and several flanking protein-coding and microRNA genes. Microdeletion patients often develop particularly severe symptoms, including mental retardation, dysmorphism, childhood overgrowth, and high numbers of unusually early onset neurofibromas (Kayes et al. 1994; Pasmant et al. 2010). In support of the view that the loss of contiguous genes may cause or modify these defects, patients carrying deletions

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that remove just the *NF1* gene do not exhibit this more severe phenotype. Another example is provided by the finding that 21 unrelated NF1 patients carrying the same single amino acid deletion lacked obvious neurofibromas (Upadhyaya et al. 2007). Beyond effects attributable to the *NF1* gene itself, variable expressivity can also reflect somatic mosaicism in sporadic patients who acquired their *NF1* mutation post-zygotically (Bernards and Gusella 1994). The hypothesis that symptom-specific genetic modifiers play important roles in determining disease outcome received early support from a landmark study, which analyzed the presence or absence of five binary symptoms, as well as the severity of three quantitative symptoms amongst 175 patients in 48 NF1 families, including six monozygotic (MZ) twin pairs. This study found high correlation coefficients for four binary and two quantitative traits (neurofibroma and café-au-lait spot burden) for the MZ twins, lower scores for first-degree relatives, and lower values still for more distant family members (Easton et al. 1993). The hypothesis that unlinked modifier genes control specific NF1 symptoms has received additional support from subsequent work (Rieley et al. 2011; Szudek et al. 2002). Environmental factors are another potential cause for variable expressivity. In the case of NF1, the clearest example of a nongenetic factor affecting disease progression is the observation that neurofibromas increase in size and/or number during pregnancy (Roth et al. 2008).

The identity of modifier genes may provide clues to mechanisms responsible for disease symptoms, and since modifier genes perform rate-limiting functions, human modifiers represent prevalidated therapeutic targets. However, when the *NF1* gene was identified in 1990, the human genome sequence and other tools to efficiently survey cohorts of differentially affected patients for potential modifiers were not available. Thus, our strategy to identify NF1 modifiers involved the identification of a highly conserved *Drosophila NF1* (*dNF1*) ortholog, the generation of mutants, the analysis of phenotypes, structure–function studies, and genetic screens. The remainder of this chapter summarizes what has been learned so far and how this knowledge may be relevant to those interested in human NF1.

34.2 Identification and Structure of *dNF1*

The human *NF1* gene sequence immediately suggested a function for the encoded protein as a GTPase-Activating Protein (GAP) for Ras (Xu et al. 1990). It also became apparent that, whereas neurofibromin shares a functional ~360 amino acid catalytic domain with RasGAPs from several species, it exhibited more extensive similarity over almost half of its length with the budding yeast *Inhibitor of Ras Activity-1* and *-2* (IRA1/2) proteins (Ballester et al. 1990). These findings prompted us to test whether *NF1* orthologs existed in invertebrate species amenable to genetic analysis. No *NF1* ortholog exists in the nematode, *Caenorhabditis elegans*. However, the *dNF1* gene of the fruit fly, *Drosophila melanogaster,* was found to predict a protein 55 % identical and 69 % similar to human neurofibromin over its entire 2,802 amino acid length (The et al. 1997). As shown in Fig. 34.1, the IRA-related central segment of *Drosophila* neurofibromin is most similar to the human protein, but conserved regions also exist both up- and downstream. Reflecting the smaller

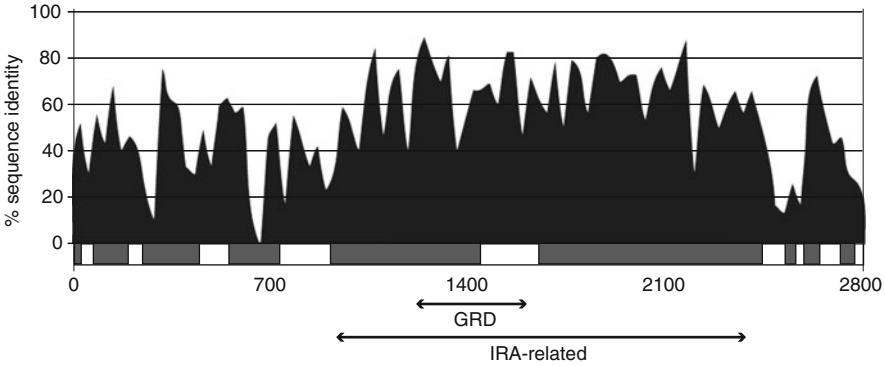

Fig. 34.1 Diagram showing the degree of amino acid sequence identity between *Drosophila* and human neurofibromin. The alternate *gray/white boxes* below the graph indicate the size and location of 18 *dNF1* exons. GRD: GAP-related domain. The extent of the protein segment related to *S. cerevisiae* IRA1 and IRA2 proteins is also indicated

size of the *Drosophila* genome, the *dNF1* gene is more compact than its human counterpart. Thus, while human *NF1* consists of 60 exons distributed over ~283 kb of DNA and harbors three other genes in its introns, *dNF1* comprises 18 exons (alternate white/gray boxes in Fig. 34.1) and spans only 13 kb. At two locations, *dNF1* mRNA is alternatively spliced. The position of alternatively spliced *dNF1* exon 14 corresponds closely to where exon 43 is alternatively spliced in man (Thomson and Wallace 2002). However, while human exon 43 has been suggested to provide a nuclear localization signal (Vandenbroucke et al. 2004), whether alternative splicing of the (not obviously related) 30 amino acids predicted by *dNF1* exon 14 has functional consequences, remains to be determined. At the exact location of alternatively spliced human exon 48a, the penultimate *dNF1* coding exon 17 splices to three different final exons, yielding mRNAs predicting proteins with three distinct C-termini. Again, whether these C-terminal isoforms are functionally different remains unknown. In human NF1, inclusion of alternatively spliced exon 23a in the GAP-related domain (GRD) reduces the catalytic activity of the encoded protein (Andersen et al. 1993). However, the location of exon 23a does not correspond to a splice site in *dNF1*. Finally, we note that disease-associated missense mutations disproportionally affect residues conserved in *Drosophila* NF1. For example, the approximately 1,200 amino acid segment upstream of the GRD shares 46 % overall sequence identity between man and fly, but 35 of 47 missense mutations in this region (74 %) affect residues conserved in *Drosophila*.

34.3 *dNF1* Mutants and Phenotypes

No classical *dNF1* mutations existed. In their absence, we mobilized a flanking *P* transposon to generate two *dNF1* disrupting de novo integrations. Both $dNF1^{P1}$ and $dNF1^{P2}$ alleles behave as molecular and genetic nulls. Given that expression of

oncogenic Ras in fly eyes causes severe defects (Bishop and Corces 1988), it seemed puzzling at first that homozygous loss of *dNF1* did not cause any abnormal patterning. However, both mutants exhibited a 15–20 % reduction in linear dimensions during all phases of postembryonic development, and adult flies had a reduced tendency to fly away when released (The et al. 1997). Since these and other *dNF1* phenotypes may be affected by the genetic background, and because the $dNF1^{P1}$ allele also deleted adjacent *E*(*spl*) complex genes, we made additional alleles by chemical mutagenesis of isogenized flies. Among three new alleles, $dNF1^{E4}$ is a C1045Y missense mutant, and $dNF1^{E1}$ and $dNF1^{E2}$ are Q370* and Q1062* nonsense mutations (Walker et al. 2006).

Beyond invoking Ras-independent functions, at least two other factors may explain why loss of *dNF1* and expression of constitutively active Ras do not cause similar defects. First, the *Drosophila* genome contains five RasGAP genes (*Gap1*, *Vap*, *dNF1*, *CG1657*, *CG42684*) and two plexin-related potential RasGAPs. The relatively subtle *dNF1* phenotypes may also reflect the apparent restriction of *dNF1* expression to the nervous system (Walker et al. 2006). While loss of *dNF1* does not obviously affect viability, fertility, or patterning, several macroscopic, behavioral, and biochemical phenotypes have been identified (Fig. 34.2). Interestingly, the initially identified *dNF1* defects were not sensitive to genetic manipulations that affect Ras signaling strength. Rather, they were restored by increasing, and enhanced or mimicked by reducing, signaling through the cAMP-dependent Protein Kinase A (PKA) pathway (Guo et al. 1997; The et al. 1997). The suggested link between *dNF1* and adenylyl cyclase (AC)/PKA signaling has motivated much subsequent research, and while there is no doubt that loss of *dNF1* somehow affects AC/PKA signaling, we and others have reached conflicting conclusions as to whether *dNF1* affects this pathway directly or indirectly. Before discussing what might explain this discrepancy, the following sections first briefly describe the *dNF1* phenotypes illustrated in Fig. 34.2.

34.3.1 Postembryonic Growth Deficiency

dNF1 embryos are of normal size, but mutants are 15–20 % smaller than controls during all subsequent larval, pupal, and adult stages. By measuring adult wing cell densities, this defect was shown to reflect a reduction in cell size rather than in cell number. However, smaller mutant eyes consist of fewer normal-sized ommatidia, indicating that different tissues respond differently to loss of *dNF1*. Mosaic analysis provided the first indication that reduced wing growth involves a non-cell-autonomous mechanism (The et al. 1997). This conclusion is further supported by evidence that *dNF1* expression is largely restricted to the nervous system (Walker et al. 2006) and by findings that neuronal re-expression of either fly or human NF1 sufficed to restore *dNF1* growth (Tong et al. 2002; Walker et al. 2006). The *dNF1* growth defect was not suppressed by heterozygous loss of *Ras1* or the Ras exchange factor *Son-of-sevenless* and not enhanced in mutants carrying a gain-of-function *Raf*

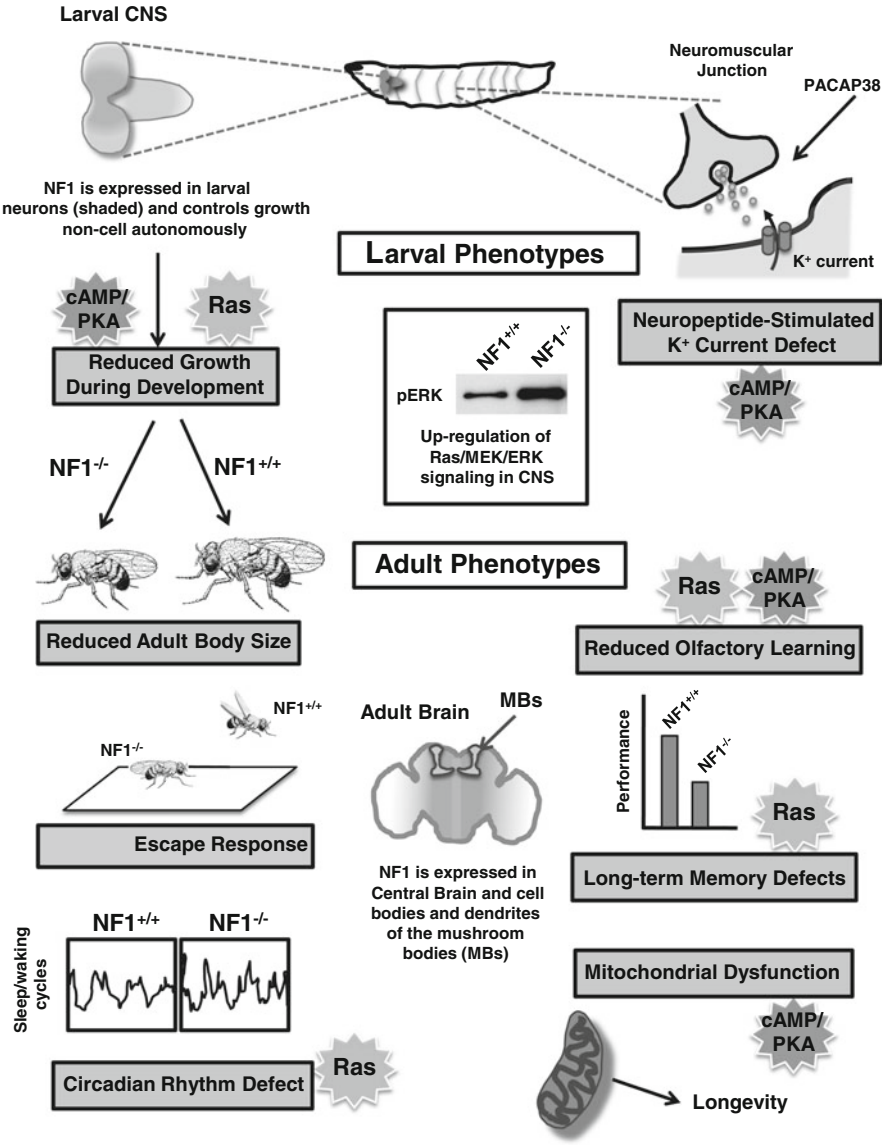

Fig. 34.2 Larval and adult homozygous null *dNF1* phenotypes. *dNF1* larvae lack a neuropeptide-induced rectifying K^+-current at the body wall neuromuscular junction. Larvae, pupae, and adults are 15–20 % smaller than isogenic wild-type controls, and larval and adult *dNF1* brain phospho-ERK levels are approximately threefold higher than in controls. Adult dNF1 flies lack normal day/night rhythmic locomotor behavior and exhibit a reduced olfactory associative learning/short-term memory performance, as well as deficits in middle-term and long-term memory. The diagram depicting the adult brain shows the location of the mushroom bodies (MBs), considered the insect equivalent of the vertebrate hippocampus. MBs play essential roles in olfactory learning and preferentially express several AC/PKA pathway proteins. Although *dNF1* is expressed in MBs, whether its learning/memory-related functions involve cells within or outside of MBs remains controversial (Buchanan and Davis 2010; Gouzi et al. 2011)

allele. Rather, hypomorphic PKA catalytic subunit mutations phenocopied the *dNF1* size defect and expression of a constitutively active PKA transgene throughout development partially restored *dNF1* growth (The et al. 1997).

Organism size is a function of growth rate and duration. In insects, whose growth occurs mostly during larval development, the former is controlled by insulin-like peptides and the latter by a hormonal cascade that culminates in the timed release of the molting hormone, ecdysone. No conclusive evidence implicating *dNF1* in either pathway has yet been found (Walker et al. 2006). Among the various *dNF1* phenotypes, the growth defect is the only one readily amenable to genetic analysis. Another reason for focusing on this defect is that reduced growth is a common symptom of human NF1 and related RASopathies (Szudek et al. 2000).

34.3.2 Behavioral Deficits

dNF1 mutants exhibit subtle behavioral defects. When flies are released, ~15 % do not fly away, even after repeated prodding (The et al. 1997). When adult flies are tapped down in their vials, mutants take longer to climb back up (Tong et al. 2007). No anatomical basis for these defects has been identified; mutants responded normally to visual or olfactory cues (The et al. 1997) and display normal locomotor activity (Williams et al. 2001). Like the reduced growth phenotype, the abnormal climbing response has been attributed to an AC/PKA signaling defect (Tong et al. 2007).

34.3.3 Neuropeptide-Stimulated K^+-Current Defect

Loss of *dNF1* does not affect *sevenless*-mediated Ras signals controlling photoreceptor cell development or torso/Ras signaling in the embryo (The et al. 1997). Hence, to analyze whether *dNF1* affects other Ras pathways, we collaborated with Dr. Yi Zhong, who had reported that a rectifying K^+ current elicited in *Drosophila* larval body wall neuromuscular junction preparations by the mammalian neuropeptide PACAP38 required intact Ras and *rutabaga* AC pathways (Zhong 1995). Electrophysiological analysis showed that *dNF1* mutants lack the PACAP38-elicited current, which was restored by manipulating AC rather than Ras signaling (Guo et al. 1997). An unanswered question remains as to how mammalian PACAP38 stimulates a response in the absence of an obvious *Drosophila* PACAP receptor ortholog.

34.3.4 Circadian Rhythm Defect

The circadian clock controls various rhythmic behaviors, including daily changes in locomotor activity. When analyzed in constant darkness, *dNF1* mutants showed normal cycling of the *period* and *timeless* clock genes but were either completely

($NF1^{P1}$) or nearly completely ($NF1^{P2}$) arrhythmic. Normal rhythmic behavior was restored upon *elav*-GAL4-driven pan-neuronal expression of UAS-*dNF1*, but not by restricted expression in central brain lateral neurons, the site of the circadian clock. Antibody staining revealed a circadian oscillation in phospho-ERK near nerve terminals containing Pigment Dispersing Factor, a secreted output of clock cells, and *dNF1* arhythmicity was restored by mutations that attenuate Ras/ERK signaling. Thus, excess Ras/ERK signaling in *dNF1*-deficient neurons affects a circadian clock output pathway and circadian locomotor activity (Williams et al. 2001).

34.3.5 Associative Olfactory Learning and Short/Middle-Term Memory Defects

Drosophila is widely used to study molecular and cellular circuits responsible for learning and memory (Davis 2005). Because analysis of the neuromuscular signaling defect implicated dNF1 in a pathway involving the *rutabaga* AC, a well-known learning mutant, and because NF1 is associated with learning deficits in both man and mouse (Hyman et al. 2005; Silva et al. 1997), the Zhong group analyzed *dNF1* mutants in a Pavlovian associative learning assay that uses electrical shocks to teach adult flies to choose between two equally aversive odors. In this test, $NF1^{P1}$ and $NF1^{P2}$ mutants both exhibited a significantly reduced learning/short-term memory performance. Arguing that developmental defects do not explain this deficiency, adult expression of a hsp70-*dNF1* transgene suppressed the learning deficit. Further work showed that beyond short-term (3 min) memory, 3- and 8-h middle-term memory retention was also affected in flies lacking *dNF1*. Again implicating abnormal AC/PKA signaling, both learning and memory defects were suppressed by increased PKA expression (Guo et al. 2000).

34.3.6 Long-Term Memory Defect

Excess Ras/ERK signaling underlies the reduced performance of $Nf1^{+/-}$ mice in the Morris water maze, which assesses spatial learning ability (Costa et al. 2002; Cui et al. 2008). By contrast, defective AC signaling appears responsible for the *dNF1* olfactory learning deficit (Guo et al. 2000). Unlike the short-term olfactory learning assay, performance in the water maze requires protein synthesis-dependent long-term memory (LTM). Arguing that this difference may explain the different conclusions reached, *dNF1* null mutants were found to have an LTM defect that, unlike the short-term learning and memory deficits, appeared Ras dependent (Ho et al. 2007). More recent work by others further supports roles for dNF1 in short-term (3 min), middle-term (3 h), and 24-h LTM, and specifically in memory acquisition but not decay (Buchanan and Davis 2010).

34.3.7 Mitochondrial Dysfunction

dNF1 mutants have reduced life spans and increased vulnerability to heat or oxidative stress, in association with reduced mitochondrial respiration and elevated reactive oxygen species (ROS) production. By contrast, *dNF1* overexpression increased life span and improved reproductive fitness, increased resistance to heat and oxidative stress, accompanied by increased mitochondrial respiration and a 60 % reduction in ROS production. Like other *dNF1* defects, these phenotypes were restored by pharmacological or genetic manipulations that increase cAMP/PKA signaling. Counteracting ROS with catalytic antioxidants also restored normal life span to homozygous *dNF1* null mutants (Tong et al. 2007).

34.4 Does *dNF1* Affect AC/PKA Signaling Directly or Indirectly?

As summarized above, genetic evidence strongly supports a link between dNF1 and AC/PKA signaling. A functional link is further supported by biochemical evidence, including the observation that GTPγS-stimulated but not basal AC activity is reduced in $dNF1^{P1}$ and $dNF1^{P2}$ brain membrane preparations (Guo et al. 2000). Moreover, GTPγS-stimulated AC activity and cAMP levels were also found to be reduced in day E12.5 $Nf1^{-/-}$ murine brain extracts (Tong et al. 2002). Thus, while defective AC signaling appears to be an evolutionarily conserved, albeit recessive, *NF1* phenotype, these results do not reveal how neurofibromin affects AC activity. Consistent with the view that non-cell-autonomous mechanisms may be involved, GTPγS-stimulated AC activity was not only reduced in *Drosophila* brain but also in abdominal tissue (Guo et al. 2000), where *dNF1* may not be expressed (Walker et al. 2006). Also compatible with a non-cell-autonomous mechanism, the dNF1/PKA-mediated effect on mitochondrial respiration was detected in experiments with mitochondria isolated from whole *dNF1* mutant flies (Tong et al. 2007; Walker and Bernards 2007).

In experiments aimed at identifying how dNF1 affects AC/PKA signaling, we and others have reached conflicting conclusions. Thus, work by the Zhong group led them to postulate the existence of two dNF1-dependent AC pathways, beyond the canonical Gαs-dependent pathway. The first NF1-requiring pathway, stimulated by serotonin and histamine, depended upon Gαs, whereas a novel AC pathway involved the EGF receptor, NF1, and Ras, but not Gαs. Using transgenic flies expressing human *NF1* deletion and point mutants, the authors reported that NF1-mediated Ras regulation was essential for the novel EGF receptor-stimulated AC pathway, but not for NF1/Gαs-dependent neurotransmitter-stimulated AC activity. Moreover, providing the clearest evidence that NF1 may have Ras-independent functions, a C-terminal segment of human neurofibromin that did not include the GRD was capable of rescuing the AC-dependent organismal growth (Hannan et al. 2006) and learning defects (Ho et al. 2007).

Our conflicting conclusion that excess neuronal Ras/ERK activity is the proximal cause of *dNF1* growth and learning phenotypes is based on the results of two studies. The first focused on the *dNF1* growth defect. In structure/function studies similar to those performed with human *NF1* transgenes (Hannan et al. 2006), we found that large *dNF1* segments other than the GRD were dispensable for growth regulation, that several GAP deficient *dNF1* point mutants did not restore growth, and that expression of a truncated protein representing just the dNF1 GRD was sufficient to suppress the growth defect. Moreover, the growth detect was also suppressed by neuronal expression of a *Drosophila* p120RasGAP ortholog. To investigate why in earlier studies, loss of *Ras1* or other canonical Ras pathway components did not modify the *dNF1* growth defect, we tested a comprehensive set of Ras pathway single and double mutants for their ability to restore *dNF1* growth. As a molecular correlate, we also analyzed the ability of these mutants to restore the elevated larval and adult brain phospho-ERK level. None of the single mutants modified either phenotype, arguing that the tested Ras pathway components are either not involved, or not rate limiting in the pathway leading to ERK activation in *dNF1* brain. Supporting the latter conclusion, some double mutants that did restore normal ERK activity also rescued the growth deficiency at least partially (Walker et al. 2006). Thus, the proximal cause of the non-cell-autonomous *dNF1* organismal growth defect is excessive neuronal Ras/ERK signaling (Walker et al. 2006).

34.4.1 Drosophila *Alk/Ras Signaling May Be Responsible for* dNF1 *Growth and Learning Defects*

Further evidence implicating abnormal Ras/ERK signaling as the primary cause of *dNF1* defects comes from work that began with the observation that overexpression of either the *Drosophila Alk* (*dAlk*) receptor tyrosine kinase or of its activating ligand *jelly belly* (*jeb*) phenocopied both *dNF1* growth and olfactory learning phenotypes. This result was not unexpected, since previous work had identified *dAlk* as an activator of Ras/ERK signaling in vivo (Loren et al. 2001). However, attenuating dAlk expression or activity, either through the use of *dAlk* mutant alleles, *dAlk* shRNA constructs, expression of a dominant-negative *dAlk* transgene, or through pharmacological inhibition, rescued *dNF1* growth, olfactory learning, and brain ERK over-activation phenotypes. In support of the hypothesis that *dAlk* is a rate-limiting activator of *dNF1*-regulated Ras/ERK signals that promote organismal growth and that limit olfactory learning, *dNF1* and *dAlk* expression overlaps extensively in both larval and adult brain. Moreover, *dAlk*-GAL4 driven neuronal UAS-*dNF1* expression sufficed to restore all tested defects (Gouzi et al. 2011). Finally, although a role for dAlk as a negative regulator of learning appears unusual, recent work indicates that murine Alk may have a similar role (Weiss et al. 2012).

Whether excess Alk/NF1/Ras signaling contributes to human NF1 defects is an obvious next question. Intriguingly, loss of *NF1* expression is common and

associated with a worse prognosis in neuroblastoma (Holzel et al. 2010), a significant proportion of which shows *ALK* amplification or gain-of-function *ALK* mutations [reviewed by Azarova et al. (2011)]. ALK overexpression and *NF1* loss have similarly been implicated in glioblastoma (Powers et al. 2002; Verhaak et al. 2010). Finally, mammalian ALK is activated, either directly (Stoica et al. 2001, 2002) or indirectly (Perez-Pinera et al. 2007) by two related secreted ligands, pleiotrophin and midkine. Suggesting the intriguing possibility that inappropriate ALK signaling may contribute to NF1 tumorigenesis, midkine is overexpressed in human NF1 and has been shown to act as a mitogen for NF1 tumor cells (Mashour et al. 2001, 2004).

34.5 Conclusions

Our results have led us to conclude that excess neuronal Ras/ERK signaling is the root cause of most, if not all, *dNF1* defects. However, several important questions remain. Perhaps the most vexing unresolved issue is how *dNF1* affects AC/PKA signaling. One possible mechanism is that loss of *dNF1* precipitates one or more neuroendocrine or neurotransmitter signaling defects. Since hormones and neurotransmitters often signal through AC-coupled receptors, such defects might be restored by increased AC/PKA signaling. We note that in this model, defective AC/PKA signaling does not necessarily involve *dNF1*-requiring neurons. However, although no results to date formally exclude the possibility that *dNF1* affects AC/PKA signaling cell autonomously, our working hypothesis remains that cross talk between distinct neuronal populations may be involved in generating the various Ras/ERK- and AC/PKA-dependent *dNF1* phenotypes. Ongoing genetic screens for dominant genetic modifiers of *dNF1* phenotypes, beyond *dAlk* and *jeb*, experiments to identify the site of action of modifiers, and studies to pinpoint the cells in which increased AC/PKA signaling restores *dNF1* defects, may eventually reveal the neuronal circuits via which *dNF1* exerts its various functions, and provide further clues to molecular and cellular pathways responsible for human NF1 symptoms.

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