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14. ABSTRACT: This proposal aims to improve the understanding of NF1 associated cognitive deficits and the domains of the protein through genetic studies in the two main NF1 model systems, Drosophila and mice. We plan to enhance our understanding of Nf1 functions other than regulation of Ras/MAPK signaling as also a means of rationalizing the variability of phenotypic manifestations of the disease. Our main objective towards that is to establish the role(s) of the N-IRA and the LBR Nf1 domains in learning and memory and identify neuronal circuits and molecular mechanisms requiring functionality of these domains. We aim to test the following hypotheses: First, Nf1 mutations outside the GAP domain affect learning and memory with distinct spatiotemporal requirements from loss of function mutations. Second, these mutations outside the GAP domain do not affect growth as null mutations do. Finally, do these point mutations affect the same or distinct biochemical pathways as null mutations?					
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1. Introduction:

Neurofibromatosis 1 (NF1) is a multisymptomatic disorder with highly variable deficits, especially as it pertains to cognitive defects, characterizing most children with the disease and susceptibility to tumor formation. Evidence from patients and models including *Drosophila* suggest that distinct point mutations or small deletions often present distinct pathologies if the mutations are outside the prominent GAP domain which has dominated the functional assignment of the protein thus far. In fact, particular point mutations in patients have been linked to solely or predominantly cognitive deficits without tumor formation and others primarily with tumor formation, with little or no cognitive deficits.

Loss of the highly conserved *Drosophila* *dNf1* ortholog results in recessive organismal size reduction and deficits in associative learning and memory among other deficits that broadly resemble human NF1 symptoms. We have shown that null *dNf1* mutations, as well as adult specific abrogation of *dNf1* in the CNS results in learning and memory deficits, indicating that the protein is acutely required for learning and memory. We also revealed functional interaction between *dNf1* and the Receptor Tyrosine Kinase Alk, a negative regulator of olfactory associative learning and memory in the fly. In fact, genetic or pharmacological abrogation of its activity restored the reduced size of null mutants and ameliorated their learning deficits. Recent findings indicate that *Nf1* is required within particular neurons to negatively regulate GABA synthesis in a PKA independent, but Ras1-dependent manner.

Recent characterization of a novel point mutation (*dNf1*^{E4}) lying outside the GAP domain revealed that it presents dominant learning and memory deficits, but has minor effects on the size of mutant homozygotes and the activated MAPK in their CNS. Importantly, it also does not respond to Alk-inhibition based phenotypic amelioration. Moreover, ongoing analyses indicate that unlike for the null mutants, the effects of this mutation are developmental in origin and proteomic analyses of head lysates revealed unique proteomic profiles of *dNf1*^{E4} animals, distinct from both controls and the null mutations. This likely underlies the distinct presentation of the point mutation from the nulls. Interestingly the *dNf1*^{E4} mutation lies in the little characterized amino-terminal IRA domain, where some human point mutations preferentially associated with cognitive deficits, Leopard and MPNST syndromes also lie.

Collectively, this evidence led us to hypothesize that *the Nf1 protein has tissue or neuronal circuit-specific functions engaging distinct molecular interactors and signalling pathways than suggested by its prominent GAP domain. These distinct functions are conferred by protein domains such as the N-IRA and may underlie the phenotypic variability common in this disease.*

To address this hypothesis with the aim of understanding the basic mechanisms of phenotypic variability and cognitive deficits towards development of targeted/personalized treatments we aim to:

The first aim is to investigate whether mutations in the N-IRA and LBR domains of *Nf1* affect learning and memory in flies and mice alike, a first step towards establishing the functionality of these domains in the processes. Are the mutations dominant (as the C1045Y is in flies), or recessive as the null mutants in flies, but dominant in mice? This aim is supported by our results from the C1045Y mutation in flies and the existence of human mutations with, but also without overt cognitive defects in humans.

Our second aim is to determine the neuronal circuits implicated in these putative learning deficits. This is motivated by our results, indicating that the *Nf1* GAP domain-mediated Ras activity modulation is required outside the MBs, the neurons typically implicated in associative learning in flies, although *dNf1* is highly enriched within these neurons (Gouzi, Moressis et al. 2011).

The third aim addresses the molecular pathways affected by these point mutations relative to the Alk-dependent Ras signaling hyperactivation, apparently leading to excess GABA release in the null mutants. This is supported by the data on the learning deficits of the C1045Y mutants that appear Alk activity independent.

The fourth aim is to proteomically profile the newly generated mutant flies and mice relative to controls and null mutations to obtain a global initial understanding of similarities and/or differences of their effects as a first approximation of broad molecular mechanisms affected differentially or in common and towards devising rational ameliorative strategies for patients.

2. Keywords:

Neurofibromatosis 1,

Learning disability

Phenotypic variability

Mutations and functionalization of human mutations

Anaplastic Lymphoma Kinase (ALK)

MAPK signaling

GABAergic signaling

Drosophila melanogaster

mouse

3. Accomplishments:

Time line: Although the project was approved to start in April 2020, the actual start date was September 1, when contracts of the people employed were signed. Therefore, actually we are in the 9th month since the project started. There were two reasons for this delay:

First, due to the COVID pandemic in Greece the Research Centre was underworking until late June.

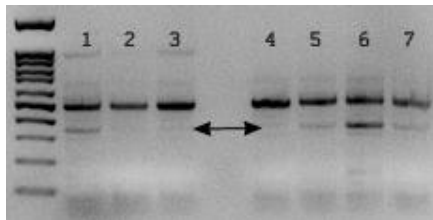
Second, Greek law mandates that Research Institutions cannot start charging salaries or consumables in a program “on credit” offered by a contract, but only after actual funds are in the Institutes account and after central approval by the Relevant Ministry. In addition, since Fleming never had a contract with DoD before, it took a while to establish the methodology of requesting funds via the voucher system, which added to the time delay before start. In addition, COVID measures since before December 2020 that have been relaxed since late April/early May attenuated the projected progress of the project. This included not being able to hire a graduate student until April 1 2021, as the Institution did not allow the presence of new people in the labs. Nevertheless, significant milestones were covered as detailed below:

The first aim is to investigate whether mutations in the N-IRA and LBR domains of Nf1 affect learning and memory in flies and mice alike, a first step towards establishing the functionality of these domains in the processes. Are the mutations dominant (as the C1045Y is in flies), or recessive as the null mutants in flies, but dominant in mice? This aim is supported by our results from the C1045Y mutation in flies and the existence of human mutations with, but also without overt cognitive defects in humans.

Per SOW

- Subtask 1: Isolation of the fly **M1035R** mutation and start generation of **R1809C** fly mutant.

We started with the generation of the R1809C fly mutant because we reasoned that it would provide pioneer data for the analysis of the R1809C mouse. We have generated the R1809C fly mutant in the “lipid binding” region of the protein CrispR technology to generate point mutations by injecting the gRNA plasmid and relevant oligos into a CAS9 expressing line. Genomic DNA was amplified from the particular region of the *dNf1* gene of potential CrispR-generated heterozygous mutants. In the gel,



genomic DNA from potential heterozygous mutants was PCR-amplified and cut with a restriction enzyme that cuts within the fragment, if it contains the mutation leading to R1809C. Samples 1, 5, 6 and 7 present the mutation-specific band (indicated by the double headed arrow), while samples 2, 3, 4 are from non-mutated flies. This conclusion was verified by sequencing of the PCR

amplified fragment and shown to contain only the sought mutation for samples 1, 5, 7.

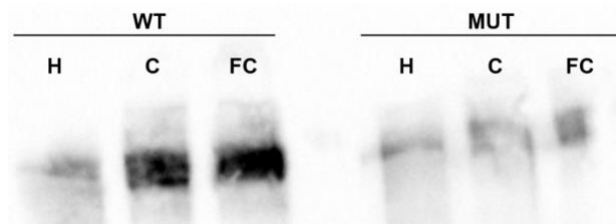
Mutant flies are being backcrossed to normalize the genetic background as necessary for behavioral assessment. We did already observe that unlike the null mutants we have characterized before (Gouzi, Moressis et al. 2011), these R1809C mutant homozygotes do not present significant size reduction, appear normal macroscopically and are fully homozygous viable and fertile.

We have generated the gRNA plasmid and relevant oligos to produce the fly M1035R mutation and these have already been injected in the CAS9 expressing line and we are waiting for potential mutants to screen as above and establish as strains for future behavioral analyses.

- Subtask 2: Breeding and characterization of the **R1809C** mouse.

The R1809C mutant mouse was planned to be generated via CrispR-mediated mutagenesis under an EU grant. The process yielded two founder animals and sequencing indicated one, a female to carry the mutation without any other changes in within a few Kb of the R1809C mutation. The second animal, a male bore the mutation, but it also harbored a 4 base deletion immediately adjacent to it.

Unfortunately, the female mouse bearing only the R1809C point mutation expired for unknown reasons at 35 days of age. The male founder as expected from the sequencing results bore a null mutation in the gene and this was verified by western blots. As demonstrated in the blot the new mutation (MUT), is a null as we do not observe a novel band expected at about 198-200 kD, which is significantly different than the expected >300 kD of the full-length protein (WT). The new mutants do not yield a new band in the tissues sampled (hippocampus-H, cortex-C and frontal cortex-FC) with an antibody against an epitope in the middle portion of the protein. Consistent with the notion that the



new mutant is a null allele, it is homozygous lethal as other established null mouse mutants have been reported to be (Silva, Frankland et al. 1997). The abundance of the protein, which is about half of that in controls, is also consistent with this interpretation.

Ameliorative measures: Since it was determined that the R1809C mouse mutant was not generated, the following measures have been taken to overcome this:

1. a new round of mutagenesis was initiated at no cost to the grant and currently we have around 40 animals under screening for the mutation. Additional potentially mutant animals are generated biweekly and will continue until we have secured the mutant.
2. A publication using a mouse bearing this mutation, but not focusing on its behavioral effects was recently published from the Gutmann lab (Anastasaki, Wegscheid et al. 2020) and Dr Gutmann agreed to transfer the mutant to us when the finish its molecular characterization midsummer 2021. This will serve as a back-up in case our own efforts to generate the mutant will fail. The advantage of making our own mutant is that it will be in a genetic background typically used for the types of behavioral analyses we are planning to perform. In contract, the Gutmann mutant is in a different genetic background and it will be necessary to backcross it, which requires a lot of time.

- Subtask 3: Characterization of Nf1 null mouse cognitive behaviors.

Although we did not obtain the sought after *NF1* mutant, we are breeding the new apparent null heterozygote, to obtain significant numbers to run such mice through our behavioral analysis battery. In the meantime, we have established the benchmark performance of the genetic background strain which we use to generate the R1809C mutant. The benchmarked assays are:

Elevated plus maze, open field and fear conditioning. We are in the process of completing benchmarking for passive avoidance. These assays will be employed to in the first instance to assess the new null mutants when enough animals for a complete experimental set is secured.

- Subtask 4: Generation of M1035R and R1809C transgenes and generation of transgenic lines.

With the partial relaxation of COVID measures in Greece, we managed to recruit a very junior graduate student who has initiated generation of these transgenes. We plan to insert the transgenes in the same

genomic location (attp40), such that all constructs will yield the same expression levels permitting comparison of their potential effects without concern about differential expression levels. This requires subcloning of the *dNf1* cDNA into the proper vector and followed by mutagenesis to introduce the point mutation as we have done before (Papanikolopoulou and Skoulakis 2015). These experiments are ongoing.

- Subtask 5: Isolation of fly M1035 mutant

Nothing to report yet.

Our second aim is to determine the neuronal circuits implicated in these putative learning deficits. This is motivated by our results, indicating that the Nf1 GAP domain-mediated Ras activity modulation is required outside the MBs, the neurons typically implicated in associative learning in flies, although dNf1 is highly enriched within these neurons (Gouzi, Moressis et al. 2011).

- Subtask 1: Learning experiments with M1035R and R1809C fly mutants
- Subtask 2: Learning experiments with M1035R and R1809C transgenics
- Subtask 3: Fear conditioning and passive avoidance experiments with mouse mutant and nulls
- Subtask 5: Brain lysates for MAPK blots and initial proteomic samples

Nothing to report yet, since these depend on completion of tasks from aim 1.

However, we completed the description of the neuronal circuits and defined that the Ras/MAPK pathway, rather than cAMP signaling, which has been proposed for over a decade as essential for dNf1 mediated learning and memory. These results were broadly disseminated by a major publication in the Journal of Neuroscience (preprint of this publication is attached) and the recent (early May) biannual Neurofly-European Drosophila Neurobiology meeting (attached poster presentation).

Opportunities for training and professional development.

The project allowed recruitment of a new graduate student that will work of Drosophila related aims of the project. This will impact significantly her training and professional development towards her PhD.

The technician Maro Loizou has acquired new professionally valuable skills in mouse handling and genotyping including initial screening for mutations

The postdoctoral researcher Dr Georganta just published a major paper as a first author, presented her results in a Major Neurobiology meeting and is preparing a second publication on the C1045Y mutation as prelude to future papers on the fly M1035R and R1809C mutants.

In addition, side-projects anchored on this main project have provided training for one undergraduate summer student.

Planned experiments until the next reporting period.

We plan to stay the course outlined in the SOW.

- Initially we will finish the manuscript detailing the effects of the C1045Y mutation, which provided the major support for the basic premise of this grant. Briefly, the results indicate that C1045Y is a novel developmental dominant mutation that acts as a “gain of function” possibly by negatively affecting

dNf1 interactions with other cellular proteins. This mutation does not affect GABAergic neurotransmission like the loss of function does and we are in the process of examining the effects of cAMP and Insulin-dependent signaling in the phenotype, but also in mitochondrial function as we recently discovered.

Otherwise we will follow the SOW as detailed in the grant and focus on:

Aim 1:

- Subtask 2: Breeding and characterization of the **R1809C** mouse.
- Subtask 3: Characterization of Nf1 null mouse cognitive behaviors
- Subtask 4: Generation of M1035R and R1809C transgenes and generation of transgenic lines.

Aim 2:

- Subtask 1: Learning experiments with M1035R and R1809C fly mutants
- Subtask 2: Learning experiments with M1035R and R1809C transgenics
- Subtask 3: Fear conditioning and passive avoidance experiments with mouse mutant and nulls
- Subtask 5: Brain lysates for MAPK blots and initial proteomic samples

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4. Impact

The results reported in the first publication of this work have a **major impact** on the NF1 research area as indicated below:

Two decades after initially reporting the learning deficit upon Neurofibromin loss we reveal that it is consequent of excess GABAergic signaling to MB neurons. Significantly, dNf1 activity within these non-MB neurons is necessary and sufficient for normal associative learning. We demonstrate that unlike prior suggestions, GABAergic modulation in these neurons does not require cAMP/PKA, but rather regulated Ras signaling. However, a novel Ras1-dependent mechanism in the MBs may affect resident cAMP levels in response to elevated GABAergic stimulation providing a plausible explanation of the unsettled link between cAMP levels and Nf1 function.

Impact on other disciplines:

We believe that our finding will impact other areas of Cognitive Neurobiology, especially in the areas of molecular mechanisms of learning and memory.

Impact on Technology Transfer

Nothing to report yet

Impact on society beyond science and technology

Our findings impact potential ameliorative approaches for the cognitive deficits of NF1 patients by identifying that GABAergic neurotransmission is regulated by the Alk/Ras pathway and provides a potential explanation on the use of Alk inhibitors as drugs to combat disease symptoms.

5. Changes/Problems

We anticipate staying the course outlined in the grant and the SOW. The strategies to ascertain obtaining the mouse R1809C mutant have been outlined above.

We have experienced significant delays due to COVID measures which limited the lab time spend on the project and did not permit until recently the recruitment of a graduate student for the project.

We do not anticipate significant changes on expenditure, but we do foresee requesting a no-cost extension at the end of the grant period to permit accomplishing the goals detailed.

No significant changes are anticipated in use of vertebrate animals, biohazards or other agents.

6. Products

Nothing to report except the establishment of a web site we use to upload publications and data:

https://www.synapse.org/#!Profile:3425179/projects_syn25720502

7. Participants & Other Collaborating Organizations

People that have worked on the project

Name:	<i>Efthimios Skoulakis, PhD</i>
Project Role:	<i>PI</i>
Researcher Identifier (e.g. ORCID ID):	<i>000-0001-5113-6192</i>
Nearest person month worked:	<i>8</i>
Contribution to Project:	<i>Oversees work, organizes experiments and analyzes data. Prepares publications and reports.</i>

Name:	<i>Maria-Eirini Georganta, PhD</i>
Project Role:	<i>postdoc</i>
Researcher Identifier (e.g. ORCID ID):	<i>000-0001-5610-6144</i>
Nearest person month worked:	<i>8</i>
Contribution to Project:	<i>Generation of mouse and fly CrispeR mutants, behavioral and molecular experiments in both species, data evaluation and reporting. Graduate student oversight</i>

Name:	<i>Maro Loizou</i>
Project Role:	<i>Animal Technician</i>
Researcher Identifier (e.g. ORCID ID):	<i>-</i>
Nearest person month worked:	<i>8</i>
Contribution to Project:	<i>Mouse and fly husbandry, genotypic and molecular screening for mutants</i>

Name:	<i>Kalliopi Atsoniou</i>
Project Role:	<i>Graduate student</i>
Researcher Identifier (e.g. ORCID ID):	<i>-</i>
Nearest person month worked:	<i>1</i>
Contribution to Project:	<i>Generation of wild type and mutant transgenes for fly transformation. Behavioral analysis upon Ras overactivation in flies.</i>

Nothing to report regarding changes in other **support of the PI or key personnel**.

There is no **involvement of other organizations in the project**.

8. Special Reporting requirements

NONE

9. APPENDICES

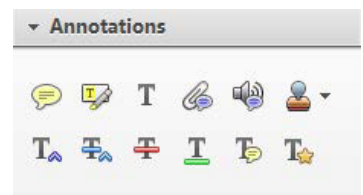
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Behavioral/Cognitive

Associative Learning Requires Neurofibromin to Modulate GABAergic Inputs to Drosophila Mushroom Bodies

AQ:au **Eirini-Maria Georganta, Anastasios Moressis, and ^{ID}Efthimios M.C. Skoulakis***

AQ:or Biomedical Sciences Research Centre "Alexander Fleming," Institute for Fundamental Biomedical Research, Vari, 16672, Greece

AQ:A Cognitive dysfunction is among the hallmark symptoms of Neurofibromatosis 1, and accordingly, loss of the *Drosophila melanogaster* ortholog of Neurofibromin 1 (dNf1) precipitates associative learning deficits. However, the affected circuitry in the adult CNS remained unclear and the compromised mechanisms debatable. Although the main evolutionarily conserved function attributed to Nf1 is to inactivate Ras, decreased cAMP signaling on its loss has been thought to underlie impaired learning. Using mixed sex populations, we determine that dNf1 loss results in excess GABAergic signaling to the central for associative learning mushroom body (MB) neurons, apparently suppressing learning. dNf1 is necessary and sufficient for learning within these non-MB neurons, as a dAlk and Ras1-dependent, but PKA-independent modulator of GABAergic neurotransmission. Surprisingly, we also uncovered and discuss a postsynaptic Ras1-dependent, but dNf1-independent signaling within the MBs that apparently responds to presynaptic GABA levels and contributes to the learning deficit on the mutants.

Key words: cAMP; *Drosophila*; GABA; learning; neurofibromatosis 1; Ras1

Significance Statement

Two decades after initially reporting the learning deficit on Neurofibromin loss, we reveal that it is consequent of excess GABAergic signaling to mushroom body (MB) neurons. Significantly, dNf1 activity within these non-MB neurons is necessary and sufficient for normal associative learning. We demonstrate that, unlike prior suggestions, GABAergic modulation in these neurons does not require cAMP/PKA, but rather regulated Ras signaling. However, a novel Ras1-dependent mechanism in the MBs may affect resident cAMP levels in response to elevated GABAergic stimulation providing a plausible explanation of the unsettled link between cAMP levels and Nf1 function.

Introduction

Neurofibromatosis Type 1 (NF1) is an autosomal dominant, multisymptomatic, and characteristically clinically variable disorder and the most prevalent of the neurocutaneous syndromes (Yap et al., 2014; Nix et al., 2020). NF1 is the result of mutations in Neurofibromin, a regulatory protein of multiple cellular processes, including Ras inactivation via its GTPase-activating domain and apparently activation of adenylyl cyclase by a yet unknown mechanism (Diggs-Andrews and Gutmann, 2013). Neurofibromin is a large ubiquitous cytoplasmic protein, but

most abundant in the brain, the spinal cord, and the peripheral nervous system; 50%–80% of children with NF1 manifest impairments in executive functions, attention, language, visual perception, and learning (Shilyansky et al., 2010a; Diggs-Andrews and Gutmann, 2013). However, the molecular and cellular circuitries whose perturbation underlies these cognitive dysfunctions remain poorly understood, but results from animal models have yielded valuable mechanistic insights (Diggs-Andrews and Gutmann, 2013).

Loss of the conserved *Drosophila* dNf1 ortholog results in organismal size reduction, circadian and associative learning, and memory deficits (Walker et al., 2012) and behavioral inflexibility (King et al., 2016), in accord to human pathology. Intrinsic mushroom body (MB) neurons are central to olfactory learning and memory in *Drosophila* (Davis, 2005; Hige, 2018). The MBs are bilateral clusters of ~2500 neurons in the dorsal posterior brain, with their dendrites situated ventrally to their somata and their fasciculated axons projecting anteriorly and bifurcating to form medial (β , β' and γ) and dorsal (α and α') lobes (Tanaka et al., 2008). Unexpectedly, transgenic restoration of dNf1 in the adult *Drosophila* CNS, with explicit exclusion of the MBs, appears sufficient to reverse the associative learning deficits of dNf1 null mutants (Gouzi et al., 2011). Given the cardinal role of MBs for associative learning (Hige, 2018) and prior reports

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Author contributions: xxx.

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The authors declare no competing financial interests.

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implicating these neurons in the deficient learning of the mutants (Buchanan and Davis, 2010), it was rather surprising that dNf1 appeared required outside the MBs for the process. In accord with this observation, rescue of the learning deficit and reduced size of the mutants depended on attenuation of the receptor tyrosine kinase, anaplastic lymphoma kinase (dAlk) outside the MBs (Gouzi et al., 2011). However, the neurons housing this dAlk-dNf1 functional interaction remained elusive. Therefore, as neurons projecting to the MBs with roles in learning and memory have been described (Tanaka et al., 2008; Chen et al., 2012), we aimed to identify the neuronal circuit where dNf1 is necessary and sufficient for negatively reinforced associative learning in *Drosophila*.

The growth deficiency of *dNf1* homozygotes depends on cAMP/PKA signaling within the neuroendocrine cells of the *Drosophila* larval ring gland (Walker et al., 2013). However, the implication of this signaling pathway in cognitive deficits in flies and mice (The et al., 1997; Guo et al., 2000; Tong et al., 2002; Brown et al., 2010; Diggs-Andrews and Gutmann, 2013), remained controversial as the learning deficits of *Nf1*^{+/-} mice appear entirely attributable to excessive Ras activity (Costa and Silva, 2002; Li et al., 2005). We address this discordance by investigating whether cAMP signaling cascade is implicated autonomously or nonautonomously in neurons where we define that dNf1 is required for associative learning.

Materials and Methods

Drosophila culture and strains. *Drosophila* crosses were set up in standard wheat-flour-sugar food supplemented with soy flour and CaCl₂ and cultured at 25°C and 50%-70% humidity with a 12 h light/dark cycle. The *dNf1*^{E2} mutant has been described previously (Walker et al., 2006; Gouzi et al., 2011). Transgenic fly strains used in this work were as follows: UAS-*dNf1* (Walker et al., 2006), UAS-*Nf1*^{RNAi} (Bloomington Drosophila Stock Center #25845), UAS-mCD8::GFP (Lee and Luo, 1999), UAS-*Gad*^{RNAi} (Vienna Drosophila RNAi Center #32344), trans-Tango (UAS-myrGFP.QUAS-mtdTomato-3xHA; Bloomington Drosophila Stock Center #77124) (Talay et al., 2017), UAS-*PKA*^{CI} (Bloomington Drosophila Stock Center #35554), murine UAS-*PKA*^{*} (Walker et al., 2013), UAS-*Alk*^{RNAiKK} (Vienna Drosophila RNAi Center #KK107083), UAS-*Alk*^{DN} (Bazigou et al., 2007), UAS-*Ras1*^{RNAi} (Vienna Drosophila RNAi Center #28129), UAS-*Ras2*^{RNAi} (Vienna Drosophila RNAi Center #6225), UAS-*r^{erm}* (Martin-Blanco, 1998), UAS-*r^{RNAi}* (Bloomington Drosophila Stock Center #43121), UAS-*Dsor*^{RNAi} (Vienna Drosophila RNAi Center #40026), UAS-*Ras1*^{V12} (Karim and Rubin, 1998), TubGal80^{ts} (McGuire et al., 2004), and the MB-specific Gal80 (MB-Gal80), which suppresses expression in the MBs (Krashes et al., 2007). The Gal4-driver lines used in this study were as follows: Leo-Gal4 (Messarito et al., 2009), OK72-Gal4 (Bloomington Drosophila Stock Center #6486) (Acebes and Ferrús, 2001), *c739*-Gal4 (Bloomington Drosophila Stock Center #7362), *Gad*-Gal4 (Bloomington Drosophila Stock Center #51630), *Alk(38)*-Gal4 (Gouzi et al., 2011), *Cha*-Gal4 (Bloomington Drosophila Stock Center #6798), NP5288-Gal4 (Kyoto Drosophila Genetic Resource Center #104937), *c316*-Gal4 (Bloomington Drosophila Stock Center #30830), G0431-Gal4 (Chen et al., 2012), *Or83b*-Gal4 (Bloomington Drosophila Stock Center #23909), *Ras1*-Gal4 (Gouzi et al., 2011), *Elav[C155]*-Gal4, and *c772*-Gal4 (Bloomington Drosophila Stock Center #6494). All strains were backcrossed into the Cantonised-*w¹¹¹⁸* isogenic background for six generations.

Behavioral analyses and conditioning. Conditioning and testing were performed under dim red light at 24°C–25°C and 73%–80% humidity. All animals were 2–6 d old, collected under light CO₂ anesthesia 1 d before testing, and kept in food vials in groups of 50–70 at 25°C or 18°C as appropriate for strains with Gal80^{ts}-dependent temporal restriction of transgene expression (TARGET). For experiments using the TARGET system, all animals were raised at 18°C and transgene expression was induced maximally by placing 3- to 5-d-old adults at 30°C for 48–72 h.

All flies were transferred to fresh vials 1 h before testing and acclimated to the training temperature (25°C) and darkness for 30–60 min before training.

Olfactory learning in the negatively reinforced paradigm coupling aversive odors as conditioned stimuli (CS⁺ and CS⁻) with the electric shock unconditioned stimulus (US) was used to assess learning as described previously (Gouzi et al., 2011). For training, 50–70 flies of both sexes were loaded into a training tube, lined with an electrifiable grid, where they received the following sequence of stimuli: 30 s of an odor paired with 6 pulses of 90 V electric shock (CS⁺), 30 s of air, 30 s of a second odor with no electric shock pulses (CS⁻), and finally 30 s of air. We used benzaldehyde (Sigma Millipore) and 3-octanol (OCT) (ACROS Organics), as standard odorants. Two groups of animals of the same genotype were trained simultaneously, one to avoid benzaldehyde, the other 3-octanol, while the complementary odorant was used as the respective control. The animals were transferred to a T-maze apparatus immediately and allowed to choose between the two odors converging in the middle for 90 s. Because the time between testing and US/CS coupling is 3 min, the initial performance assessment is that of 3 min memory, which we refer to as learning. Performance was measured by calculating an index (PI), as the fraction of flies that avoided the shock-associated odor minus the fraction that avoided the control odor reflected learning because of one of the conditioning stimuli and represented half of the performance index. One performance index was calculated as the average of the half-learning indexes for each of the two groups of animals trained to complementary conditioning stimuli and ranges from 100 to 0, for perfect to no learning, respectively. All behavioral experiments were conducted in a balanced design, where all genotypes involved in an experiment were tested per day and the experimental set was replicated at least once with flies from different crosses and a different time period (biological replicates).

Drug administration. The phosphodiesterase-4 inhibitor rolipram (4-[3-(cyclopentylloxy)-4-methoxyphenyl]-2-pyrrolidinone) was from Cayman Chemicals and was dissolved in DMSO as per the manufacturer's instructions and mixed into brewer's yeast paste to a final concentration of 1 mM; ~60 flies of both sexes were placed in plastic vials containing 0.5 ml of this paste and allowed to feed *ad libitum* for 5 h before training (Kanellopoulos et al., 2012). Food dye was added to monitor homogeneity and to ensure that flies were actually ingesting the food; ~1 h before conditioning, flies were transferred into normal fly-food vials. The no drug groups were fed with the same yeast paste, but containing DMSO instead of drug. No differences in learning performance have been observed in flies fed with DMSO, relative to no DMSO controls (Gouzi et al., 2011).

Immunohistochemical analysis and data processing. Whole-mount adult (2- to 6-d-old) brains were dissected in cold PBS, fixed in 4% paraformaldehyde for 20 min at room temperature and permeabilized using 0.3% Triton X-100 in PBS (PBST). Samples were blocked in 10% normal goat serum (diluted in 0.3% PBST) for 90 min at room temperature, and incubated with primary antibodies for one or two overnights at 4°C. Samples were washed (PBST) 3 times for 15 min each at room temperature and incubated with secondary antibodies for 2 h at room temperature or overnight at 4°C. The samples were then washed again at least 3 times for 15 min at room temperature and mounted on a slide using Dako fluorescent mounting medium.

The primary and secondary antibodies were diluted in blocking solution. The primary antibodies were used as follows: mouse anti-GFP (3E6, Invitrogen, 1:400), rabbit anti-GABA (A2052, Sigma Millipore, 1:50), rabbit anti-GFP (A-6455, Invitrogen, 1:400), mouse anti-HA (sc-7392, Santa Cruz Biotechnology, 1:400), rabbit anti-Leo (1:1000) (Skoulakis and Davis, 1996), and mouse anti-FasII (1:5) (Developmental Studies Hybridoma Bank). The secondary antibodies used were as follows: goat anti-mouse and anti-rabbit conjugated with Alexa-Fluor secondary antibodies (1:400, all from Invitrogen). Confocal laser microscopy was performed using the Leica Microsystems TCS SP8 Confocal Microscope. Serial optic sections of 0.75 μm thickness were obtained from the fixed whole-mount adult brain samples. For the representative images shown, each experiment has been successfully reproduced at least 3 times and was performed on multiple days. Images were formatted using Fiji software and Adobe Photoshop CS2.

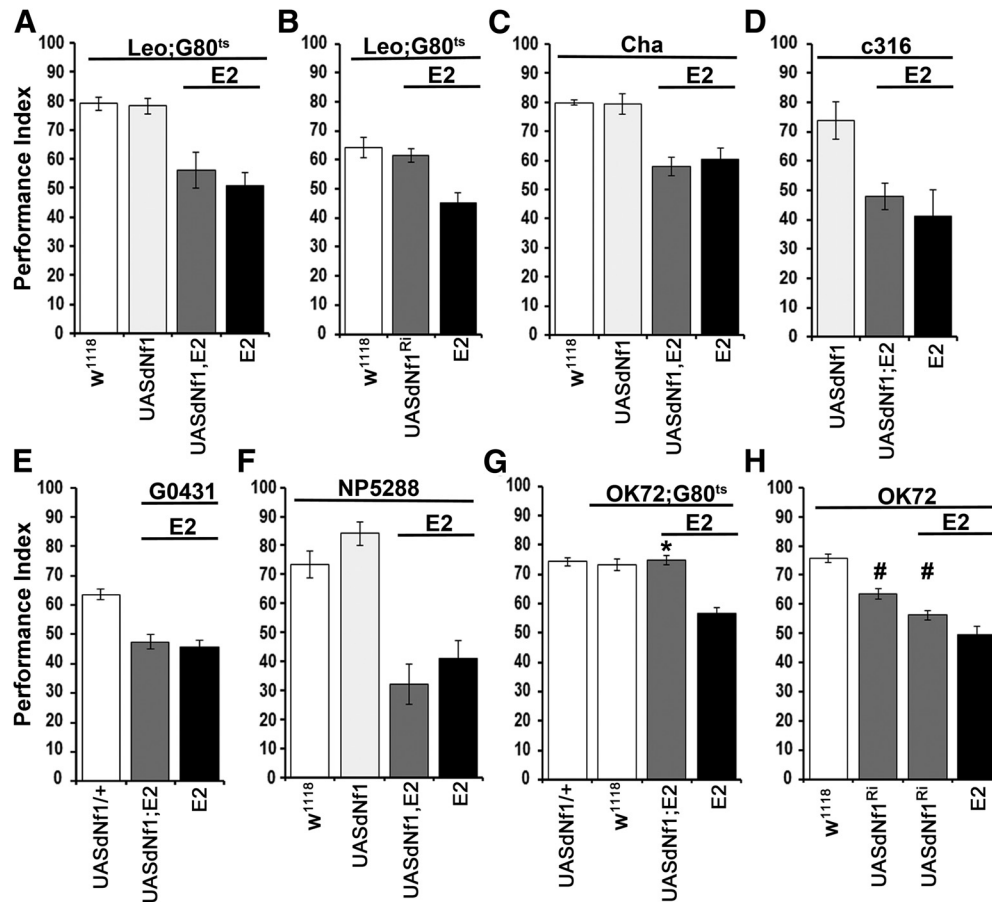


Figure 1. Adult-specific requirement of dNf1 in OK72 neurons for olfactory associative learning. Performance assessment 3 min after conditioning in control (white bars), mutant (black bars), experimental (dark gray bars), and transgene (UAS-dNf1)-expressing (light gray bars) animals. The genotypes are indicated below each bar. Data are mean $\pm$ SEM. *Statistically significant difference of the experimental from the mutant flies. #Statistically significant difference of the experimental from the control flies. G80^{ts} indicates the ubiquitously expressed temperature-sensitive Gal4 repressor Tub-Gal80^{ts}. **A**, Adult-specific expression of an Nf1 transgene throughout the MBs under Leo-Gal4 does not restore learning in *dNf1*^{E2} homozygotes. **B**, RNAi-mediated (UASdNf1^{Ri}) dNf1 attenuation in MBs (gray bar) does not affect learning. **C**, Restoration of dNf1 expression within cholinergic neurons under Cha-Gal4 (gray bar) does not reverse the learning deficit of *dNf1*^{E2} homozygotes (black bar), which remained significantly different from that of controls (white bar). The UAS-Nf1 transgene-expressing flies (light gray bar) presented the same learning performance with control flies. **D**, UAS-Nf1 expression in dorsal paired medial neurons under c316-Gal4 does not restore learning to *dNf1*^{E2} homozygotes. **E**, UAS-Nf1 expression in dorsal anterior lateral neurons under the G0431-Gal4 driver does not restore associative learning to mutant homozygotes. **F**, UAS-Nf1 expression in antennal lobe projection neurons, including the APL, marked with the NP5288-Gal4 driver does not restore associative learning to mutant homozygotes. **G**, Adult-specific UAS-Nf1 expression in neurons marked with the OK72-Gal4 driver (gray bar) reverses the associative learning deficit of *dNf1*^{E2} nulls. **H**, RNAi-mediated dNf1 abrogation (UASdNf1^{Ri}) in OK72 neurons in control flies and *dNf1*^{E2} heterozygotes (gray bars) induces significant learning deficits.

Western blot analyses. Single female fly heads at 2–5 d after eclosion were homogenized in 1 $\times$ Laemmli buffer (50 mM Tris, pH 6.8, 100 mM DTT, 5% 2-mercaptoethanol, 2% SDS, 10% glycerol, and 0.01% bromophenol blue) and loaded per lane. Proteins were transferred to PVDF membrane and probed with the following antibodies: mouse anti- β -tubulin E7 (Developmental Studies Hybridoma Bank) at 1:500, mouse anti-pERK (Sigma Millipore) at 1:2000, and rabbit anti-ERK (Cell Signaling Technology) at 1:2000. Appropriate HRP-conjugated secondary antibodies were applied at 1:5000. Proteins were visualized with chemiluminescence (ECL Plus, GE Healthcare).

Statistical analysis of behavioral data. Untransformed (raw) behavioral data were analyzed parametrically with the JMP 7.1 statistical software package (SAS Institute) as described previously (Moressis et al., 2009). Following initial ANOVA, least square means (LSM) contrast analyses were performed between the experimental group (in black throughout all figures) and its genetic or treatment controls, using $\alpha = 0.01$ unless mentioned otherwise (§). The level of significance was adjusted for the experiment-wise error rate. Detailed results of all planned comparisons (LSM contrast analyses) are reported in the text.

Results

dNf1 is required in OK72 neurons for normal olfactory associative learning

Initially, the finding that dNf1 function is required for negatively reinforced associative learning outside the MBs (Gouzi et al., 2011) was independently confirmed. Adult-specific expression of a UAS-dNf1 transgene (Walker et al., 2006) under the strong pan-MB driver Leo-Gal4 (Messaritou et al., 2010) failed to restore learning in *dNf1*^{E2} null mutants (Fig. 1A; ANOVA: $F_{(3,33)} = 9.7133$, $p < 0.0001$; subsequent LSM: LeoGal4/+;E2,Gal80^{ts}/UASdNf1,E2 vs LeoGal4/+;E2,Gal80^{ts}/E2, $p = 0.4050$). Conversely, adult-specific RNAi-mediated dNf1 attenuation in the MBs did not impair associative learning (Fig. 1B; ANOVA: $F_{(2,30)} = 10.177$, $p = 0.0005$; subsequent LSM: LeoGal4/+;UASdNf1^{Ri}/E2,Gal80^{ts} vs LeoGal4/+;Gal80^{ts}/+, $p = 0.5653$). Therefore, although present in their dendrites (Gouzi et al., 2011), clearly dNf1 presence in the MBs is not necessary for normal olfactory associative learning.

To search for neurons within the adult CNS where dNf1 is necessary and sufficient for normal associative learning, we

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introduced Gal4 drivers marking neurons implicated in the process (Güven-Ozkan and Davis, 2014) to *dNf1^{E2}* null mutants and asked for learning deficit reversal on UASdNf1 expression therein. Initially, dNf1 was restored within cholinergic neurons, but this did not reverse the learning deficit of null homozygotes (Fig. 1C; ANOVA: $F_{(3,29)} = 15.7783$, $p < 0.0001$; subsequent LSM: ChaGal4;UASdNf1;E2/E2 vs ChaGal4;E2/E2, $p = 0.5369$). Furthermore, dNf1 restoration within the dorsal paired medial MB intrinsic neurons (Waddell et al., 2000) under c316-Gal4 (Fig. 1D), or the dorsal-anterior lateral MB extrinsic neurons (Fig. 1E) under G0431-Gal4 (Chen et al., 2012) did not rescue the learning deficit of *dNf1* nulls (ANOVA: $F_{(2,17)} = 6.2708$, $p = 0.0105$; subsequent LSM: c316Gal4/+;UASdNf1;E2/E2 vs c316Gal4/+;E2/E2, $p = 0.5079$ and ANOVA: $F_{(2,57)} = 20.2124$, $p < 0.0001$; subsequent LSM: G0431Gal4/UASdNf1;E2/E2 vs G0431Gal4/+;E2/E2, $p = 0.6117$, respectively). Finally, we assayed neurons marked by NP5288-Gal4, which include antennal lobe projection neurons, presynaptic to the MBs and lateral horn but also the GABAergic anterior-paired-lateral (APL) neurons (Tanaka et al., 2008), known to be involved in associative learning (Liu and Davis, 2009). However, UASdNf1 expression therein failed again (ANOVA: $F_{(3,22)} = 17.0161$, $p < 0.0001$; subsequent LSM: NP5288Gal4/+;UASdNf1;E2/E2 vs NP5288Gal4/+;E2/E2, $p = 0.2886$) to restore learning in *dNf1^{E2}* homozygotes (Fig. 1F).

Having excluded the MBs and main MB-associated neurons involved in dNf1-mediated learning, additional available Gal4 drivers were screened, including OK72-Gal4, where surprisingly, adult-specific dNf1 reinstatement fully restored associative learning to the mutants (Fig. 1G; ANOVA: $F_{(3,46)} = 18.9052$, $p < 0.0001$; subsequent LSM: OK72Gal4/UASdNf1;E2, Gal80^{ts}/E2 vs OK72Gal4/+;E2, Gal80^{ts}/E2, $p < 0.0001$). This outcome demonstrates that dNf1 loss in the mutants does not result in developmental defects underlying their learning deficit. Corroborating this interpretation, RNAi-mediated dNf1 attenuation in OK72-marked neurons precipitated a strong learning deficit (Fig. 1H; ANOVA: $F_{(3,65)} = 37.0918$, $p < 0.0001$; subsequent LSM: OK72Gal4/+ vs OK72Gal4/+; UASdNf1^{RNAi}/+, $p < 0.0001$; OK72Gal4/+ vs OK72Gal4/+; UASdNf1^{RNAi}/E2, $p < 0.0001$). The RNAi-mediating transgene appears to be inefficient, as on its own yielded a partial deficit (LSM following ANOVA above: OK72Gal4/+;UASdNf1^{RNAi}/+ vs OK72Gal4/+;UASdNf1^{RNAi}/E2 $p = 0.0071$ and vs OK72Gal4/+; E2/E2 $p < 0.0001$), while in combination with one *dNf1^{E2}* mutant allele phenocopied the learning deficit of the null homozygotes (LSM: OK72Gal4/+;UASdNf1^{RNAi}/E2 vs OK72Gal4/+;E2/E2 $p = 0.0187$). Consequently, the RNAi-mediating transgene is used in null heterozygous background henceforth. Significantly, the collective results demonstrate that dNf1 is acutely necessary and sufficient within adult OK72-marked neurons to support normal olfactory learning.

dNf1 is acutely required in adult non-MB OK72-marked neurons to support learning

OK72-Gal4 is expressed, although in a restricted pattern, in embryonic, larval, and adult MBs (de Haro et al., 2010), and we observed significant levels of GFP immunoreactivity primarily in α/β MB neurons (Fig. 2A1,A2), in the fan-shaped body (Fig. 2A3) and two large clusters of lateral neurons with axons appearing to project to MB dendrites (Fig. 2A3,A4). Other prominent GFP-positive structures included the MB dendrites (Fig. 2A4) and a constellation of neurons in the superior medial protocerebrum (SMP; Fig. 2A3,A4).

The OK72 driver is also expressed in a group of 50–70 cells in the third antennal segment and antennal lobe glomeruli VM1, VM4, and DL1 (Devaud et al., 2003), as indicated in Figure 2A1, A2. However, UASdNf1 expression under Or83b-GAL4, which marks ~80% of olfactory neurons and the antennal glomeruli (Wang et al., 2003), did not rescue (ANOVA: $F_{(2,35)} = 15.5027$, $p < 0.0001$ subsequent LSM: Or83bGal4/UASdNf1;E2/E2 vs UASdNf1/+;E2/E2 $p = 0.5235$) the *dNf1^{E2}* learning deficit (Fig. 2B). Hence, rescue under OK72-Gal4 was not consequent of protein restoration in olfactory neurons and glomeruli, in accord with the lack of olfactory, or footshock sensory deficits in the mutants (Gouzi et al., 2011).

Because OK72 marks α/β MB neurons, dNf1 may still be necessary therein, although not sufficient for learning. To address this possibility, the UASdNf1 transgene was expressed under OK72Gal4 but coexpressing the constitutive Gal4 suppressor MB-Gal80 (Krashes et al., 2007), which eliminated expression in MBs (Fig. 2C). Significantly, dNf1 restoration in adult non-MB OK72-marked neurons, fully rescued the learning deficit (ANOVA: $F_{(4,49)} = 17.6434$, $p < 0.0001$ subsequent LSM: OK72Gal4/MBG80;UASdNf1;E2/E2, Gal80^{ts} vs MBG80/+;UASdNf1;E2/E2, $p < 0.0001$), just as it did when expressed in all neurons marked with this driver (LSM: OK72Gal4/UASdNf1;E2, Gal80^{ts}/E2 vs OK72Gal4/+;E2, Gal80^{ts}/E2, $p < 0.0001$, but note that $p = 0.3148$ compared with OK72Gal4/MBG80;UASdNf1;E2/E2, Gal80^{ts}), as shown in Figure 2D. Therefore, dNf1 expression within the MBs is neither necessary nor sufficient for olfactory associative learning, but rather it is required within other types of OK72 marked neurons for the process.

This conclusion was independently verified with c739-GAL4, which is preferentially expressed in the α/β MB neurons, but also in a number of unidentified neurons distributed throughout but especially in the posterior and dorsomedial CNS (Fig. 2E1, E2). Use of this driver was reported to improve learning in *dNf1* mutants (Buchanan and Davis, 2010) and consistently, it partially rescued the learning deficit of *dNf1^{E2}* homozygotes (Fig. 2F; ANOVA: $F_{(3,46)} = 21.5259$, $p < 0.0001$ subsequent LSM: c739Gal4/UASdNf1;E2/E2 vs c739Gal4/+;E2/E2, $p = 0.0006$). However, *dNf1^{E2}* homozygotes expressing UASdNf1 under c739, but not in the MBs (Fig. 2E3,E4), presented the same level of rescue (Fig. 2F; LSM after ANOVA above: c739Gal4/MBG80; UASdNf1;E2/E2 vs c739Gal4/+;E2/E2, $p = 0.0004$), as mutant flies expressing the transgene under the full c739 pattern. Notably, c739 expression outside the MBs includes cells in the vicinity of OK72-marked neurons, including the SMP cluster (Fig. 2E2,E4), which likely account for the learning improvement, rather than expression in the MBs. Collectively, then, dNf1 expression in a subset of OK72-Gal4 marked neurons outside the MBs is necessary and sufficient to support olfactory learning.

GABAergic dysregulation within OK72 neurons underlies the learning deficits on dNf1 loss

Given the complex OK72 expression pattern, lack of a functional requirement for dNf1 in cholinergic neurons for learning (Fig. 1C) and reports from *Nf1^{+/-}* mice and humans (Cui et al., 2008; Shilyansky et al., 2010b), we asked whether any of the OK72-marked neurons are inhibitory, expressing glutamic acid decarboxylase (GAD), the enzyme requisite for GABA production (Kulkarni et al., 1994). Importantly, restoring dNf1 expression in GABAergic neurons of null homozygotes fully reversed their deficient learning (Fig. 3A; ANOVA: $F_{(2,39)} = 42.4689$, $p < 0.0001$ subsequent LSM: GadGal4/UASdNf1;E2/E2 vs GadGal4/+;

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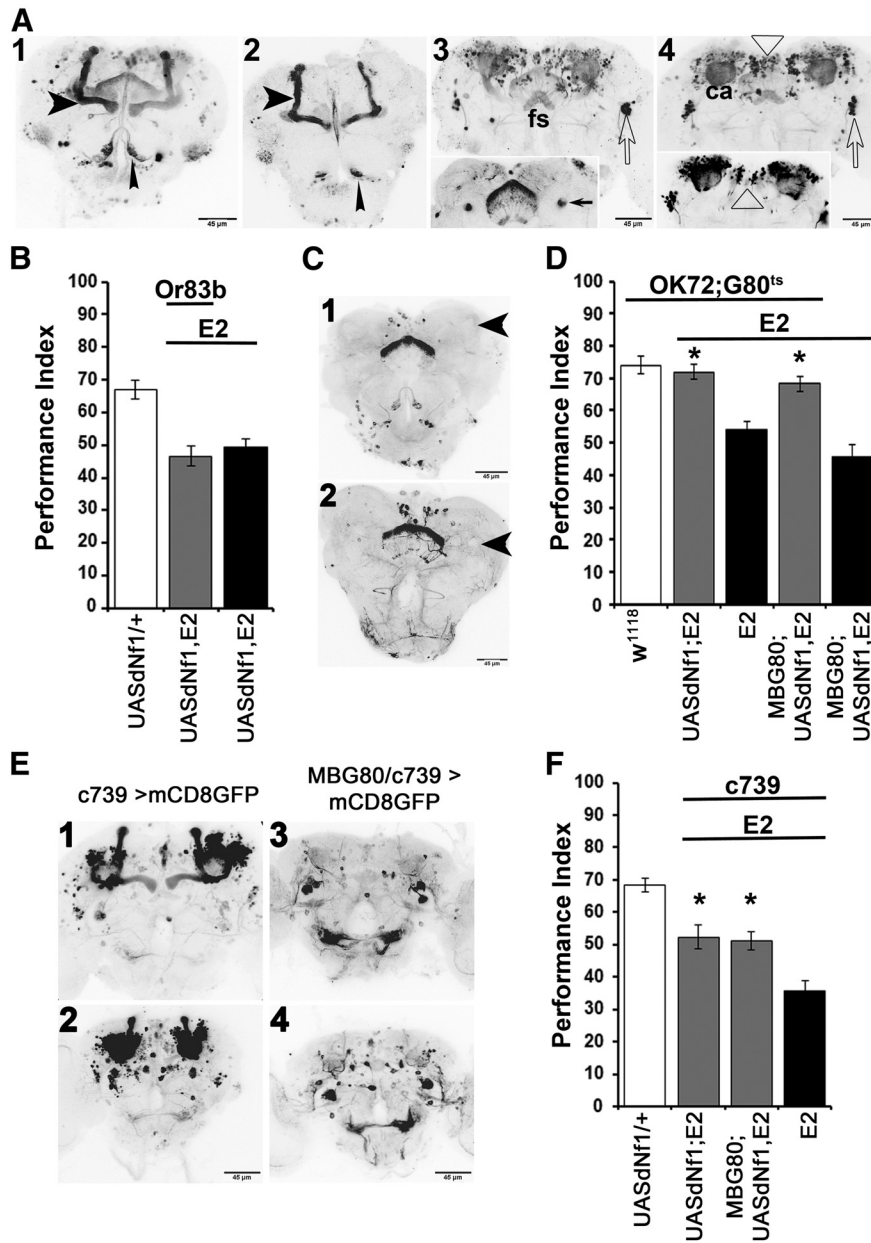


Figure 2. MB-independent requirement of dNf1 in OK72 neurons for olfactory associative learning. **A**, Grayscale inverted confocal images of adult brains stained with anti-GFP driven by the OK72-Gal4 driver, arranged from anterior to posterior (**A1–A4**): **A1**, AL glomeruli (narrow arrowhead) and MB lobes (wide arrowhead). Note the constellation of positive neurons between the vertical MB lobes in the center of the brain. **A2**, A single confocal section at the anterior of the brain clearly indicating expression in the $\alpha\beta$ MB lobes (wide arrowhead) and AL glomeruli (narrow arrowhead). **A3**, A mid-frontal view of a brain revealing the constellation of positive neurons at the SMP, as well as symmetrical clusters of lateral neurons (open arrow pointing to the right cluster). The fan-shaped body (fs) is indicated with the staining pattern flanked by the MB pedunculi, better revealed in the single confocal section in the insert (arrows). **A4**, A posterior view of the brain reveals the MB dendrites/calyses (ca), the posterior SMP neurons (open triangle), and the lateral clusters (open arrow). The single confocal plain insert focuses on the posterior SMP neurons (open triangle). **B**, UAS-Nf1 expression in olfactory receptor neurons and antennal lobe glomeruli marked with the Or83b-Gal4 driver do not restore associative learning to *dNf1*^{E2} homozygotes. **C**, Anti-GFP stained grayscale inverted confocal images of adult brains of OK72-Gal4/MBG80 driving UAS-mcd8GFP in an anterior (**C1**) and mid-posterior (**C2**) view. Although staining remains in the fs, antennal lobe, and SMP neurons, the area occupied by the MBs does not express GFP (large arrowheads). **D**, Adult-specific dNf1 expression within the OK72 marked neurons, excluding the MBs, restores associative learning in *dNf1*^{E2} homozygotes. MBG80 encodes a Gal80 repressor expressed constitutively within MB neurons. The performance of OK72-Gal4/MBG80; UAS-Nf1,dNf1^{E2}/TubG80^{ts},dNf1^{E2} (second gray bar) was significantly different from that of animals not expressing the transgene (black bars), but not from controls (white bar). *Statistically significant difference of the experimental from the mutant flies. **E**, Confocal images of adult brains stained with anti-GFP driven by the c739-Gal4 driver in anterior (**E1**) and posterior (**E2**) views, illustrating strong MB expression as well as a constellation of non-MB GFP-positive neurons, especially in the posterior of the brain (**E2**). These non-MB neurons appear prominently in the anterior (**E3**) and mostly in the posterior (**E4**) of c739-Gal4/MBG80 brains where GFP expression in the

E2/E2, $p < 0.0001$). This strongly suggests that dNf1 loss from OK72-marked GABAergic neurons underlies the learning deficit of the null animals. To validate this notion, GAD was abrogated in OK72 neurons of adult *dNf1*^{E2} homozygotes, which in accord with the hypothesis fully restored their deficient learning (Fig. 3B; ANOVA: $F_{(2,32)} = 15.6612$, $p < 0.0001$ subsequent LSM: OK72GAL4/+;UASGad^{RNAi}/Gal80^{ts} vs UASGad^{RNAi},E2/E2, $p < 0.0001$). Therefore, at least a subset of the OK72-marked neurons must be GABAergic.

Although somewhat depressed, learning was not significantly different in adult WT flies expressing the UAS-Gad^{RNAi} transgene under OK72-Gal4;Gal80^{ts} compared with nonexpressing siblings (Fig. 3C; ANOVA: $F_{(5,69)} = 6.5681$, $p < 0.0001$ subsequent LSM: OK72GAL4/+;UASGad^{RNAi}/Gal80^{ts} Uninduced vs Induced, $p = 0.7104$). Hence, reduction or loss of GABA from GABAergic OK72-marked neurons does not underlie the learning deficit of the null *Nf1* mutant homozygotes. Collectively, the results indicate that GABA production or release is elevated in OK72 neurons on dNf1 loss. However, GABAergic activity from OK72-marked neurons does not limit learning in control animals, as activity of the also GABAergic APL neurons does (Liu et al., 2009). It is unlikely that dNf1 regulates APL-mediated GABAergic activity since its expression therein in the mutants did not restore learning (Fig. 1F). Therefore, it is likely that the OK72-marked GABAergic neurons define a novel circuit, which in addition to the APL are involved in *Drosophila* associative learning.

Immunohistochemical experiments revealed GABA and OK72-Gal4-driven GFP colocalization in a small subset of cells within the lateral horn and a distinct group in the SMP (Fig. 3D). This is not consequent of reduced anti-GABA penetration as demonstrated in the respective panel inserts (Fig. 3D; anti-GABA). Therefore, these specific GABAergic OK72 neurons represent the minimal number of cells where dNf1 is required for normal olfactory associative learning. Unfortunately, we have been unable thus far to identify a

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MBs is eliminated. **F**, c739-Gal4;UAS-Nf1,dNf1^{E2}/dNf1^{E2} animals (first gray bar) express transgenic dNf1 in c739 neurons, and this partially restores learning performance in *dNf1*^{E2} nulls equally well as in animals expressing the transgene under c739 at the exclusion of the MBs (second gray bar). *Statistically significant difference of the experimental from the mutant flies.

Gal4 driver restricted to these SMP neurons to formally test this assertion. However, we traced the synaptic connections of OK72-marked neurons, taking advantage of the trans-TANGO system of trans-synaptic circuit analysis (Talay et al., 2017). Presynaptic neurons are marked by mcd8GFP expression and express a trans-synaptic ligand that activates mtdTomato expression in post-synaptic neurons.

The distributed expression of OK72-Gal4 under TANGO resulted in a complex expression pattern (Fig. 3E1,E3). It is interesting that neurons of the MB core appeared to be presynaptic (Fig. 3E1, green), most likely to other MB neurons via lateral connections. Since dNf1 expression or abrogation within MB neurons did not rescue the learning deficit of null mutants or precipitate any learning deficiency, respectively, such potential synaptic connections do not contribute to the phenotype. It should be noted that expression levels were not as high as reported (Talay et al., 2017), with some neurons marked with OK72Gal4 directly driving mcd8GFP expression (Fig. 2A), but not visible under TANGO. This was most notable with the somata of lateral neurons projecting to the MBs (Fig. 2A3,A4). This may result from weak expression of the driver within these neurons, or a resolution limitation of the TANGO system, but some of the driver-marked neurons could be presynaptic to (Fig. 3E2a) and in contact with MB dendrites (Fig. 3E2b). This agrees with the maximum projection image in the posterior of the brain, where presynaptic OK72-marked neurons appear to synapse with the postsynaptic MB dendrites (calyces) (Fig. 3E2c). Therefore, at least one set of OK72-marked neurons appear to impinge on the MBs, and we posit that these are likely GABAergic. This is strongly suggested by the importance of the MBs for associative learning, the role of GABAergic neurons in the process (Liu et al., 2007; Liu and Davis, 2009), and the apparent functional upregulation of GABA in a subset of OK72 neurons, on *dNf1* loss (Fig. 3A,B).

To address this possibility, brains expressing TANGO under OK72-Gal4 were stained with anti-GABA. Although the antibody does not stain GABAergic projections, but rather mostly cell bodies (Fig. 3D, anti-GABA stained inserts), limited colocalization was observed in the SMP area (Fig. 3E4a; and magnified in Fig. 3E4b), consistent with the notion that some OK72-expressing presynaptic neurons in that area are GABAergic.

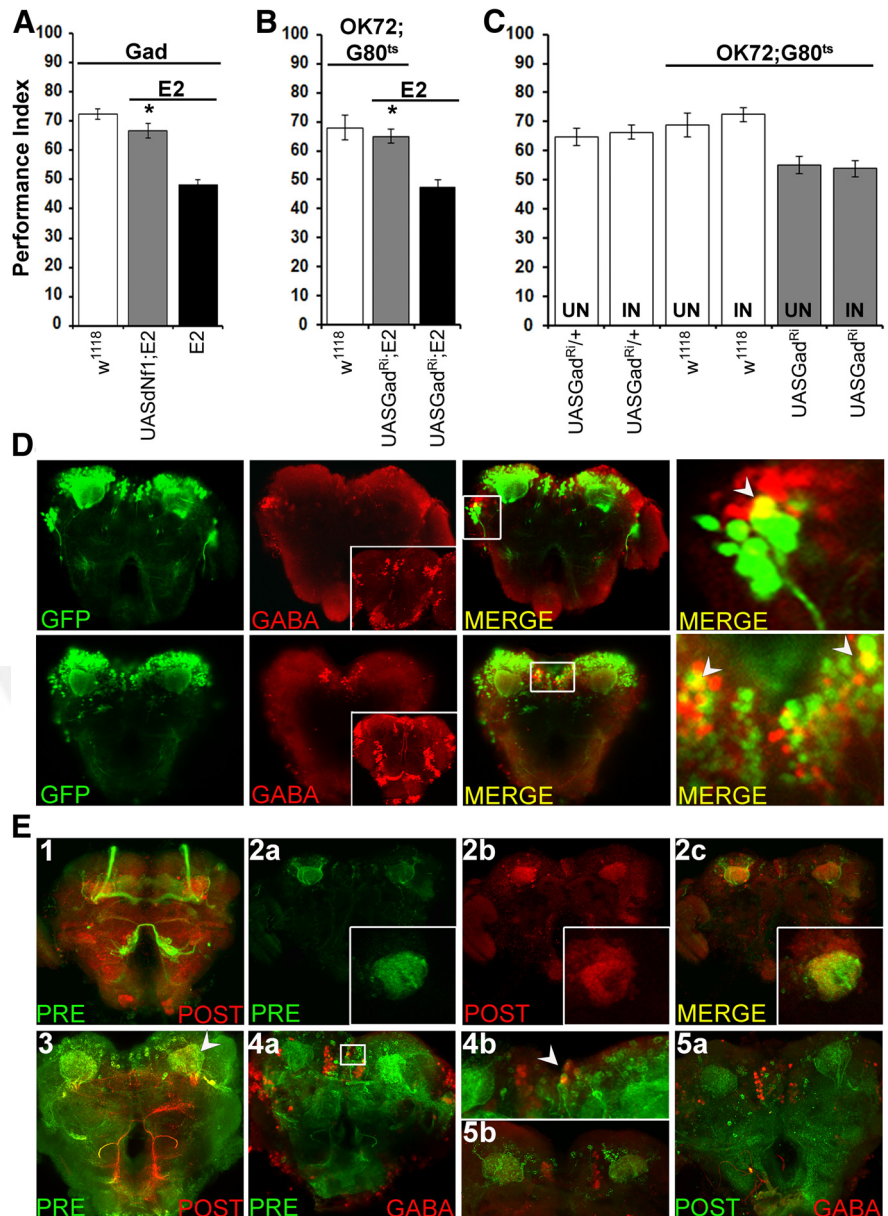


Figure 3. Olfactory associative learning requires dNf1 in GABAergic OK72-marked neurons. **A–C**, Performance was assessed within 3 min after conditioning in control (white bars), mutant (black bars), and experimental (gray bars) animals. The genotypes are indicated below each bar, representing the mean $\pm$ SEM. *Statistically significant difference of the experimental from mutant flies. *G80^{ts}* indicates the ubiquitously expressed temperature-sensitive Gal4 repressor Tub-Gal80^{ts}. **A**, Expression of the UAS-Nf1 transgene within GABAergic neurons restores normal learning to *dNf1^{E2}* nulls. **B**, Adult-specific *Gad* abrogation (*UASGad^{Ri}*) within OK72 neurons reverses the learning deficit of *dNf1^{E2}* homozygotes to levels not significantly different from that of controls. **C**, RNAi-mediated abrogation of *Gad* (*UASGad^{Ri}*) in OK72 neurons of adult WT flies does not alter learning at the restrictive (18°C, UN) or the permissive temperature (30°C, IN), respectively (gray bars). White bars represent the control genotypes, raised at both temperatures. **D**, All images are at 40 $\times$ magnification. Size bar indicates relative size. Anti-GFP staining (green), anti-GABA staining (red), and merging of the two (yellow) in representative optical sections from fly brains where GFP expression is driven by OK72-Gal4. The insets in the GABA panels are maximum projections to demonstrate ample penetration of the anti-GABA antibody not obvious in the optical sections used for colocalization. Colocalization of GFP and GABA immunofluorescence is detected laterally (top) and the SMP (bottom). Right panels (both top and bottom), Magnifications of the respective marked boxes, showing colocalization in single cells. **E**, In flies bearing the trans-Tango components, driving ligand and myrGFP expression under OK72-Gal4 (green) results in HA-mtdTomato expression in postsynaptic MB dendrites (red). Anterior (**E1**) and posterior (**E3**) view of maximum projections of presynaptic and postsynaptic neurons under OK72Gal4-driven TANGO. **E2a–E2c**, Representative optical sections of presynaptic (OK72; green) and postsynaptic (red) neurons at the dendritic level, where yellow represents their interaction. Insets, Higher magnification of confocal images from different brains at the level of MB dendrites. **E4a**, Posterior view maximum projection of OK72 presynaptic neurons (green) and GABA-positive neurons (red). White box represents the area magnified (80 $\times$) in **E4b** with the white arrow indicating colocalization. **E5a**, Posterior view maximum projection of postsynaptic to OK72-marked neurons (green) and GABAergic ones (red), clearly showing no colocalization. **E5b**, Area in the posterior of a different brain than in **E5a** verifying independently the lack of GABA (red) colocalization with postsynaptic to OK72-marked neurons (green).

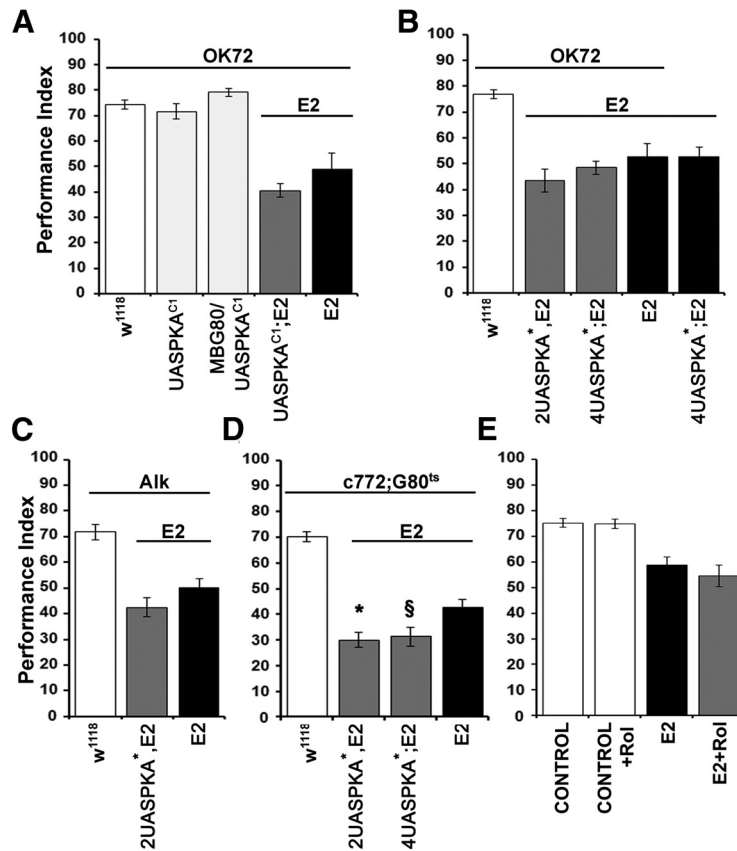


Figure 4. The cAMP/PKA pathway is not implicated in Nf1-related learning deficit within OK72 neurons. Performance assessment after conditioning of control (white bars), mutant (black bars), experimental (dark gray bars), and transgene-overexpressing (light gray bars) animals. The genotypes of all animals are indicated below each bar. Data are mean $\pm$ SEM. G80^{ts} indicates the ubiquitously expressed temperature-sensitive Gal4 repressor Tub-Gal80^{ts}. **A**, Expression of a constitutively active *Drosophila* PKA transgene in OK72 neurons (dark gray bar) does not rescue the *dNf1*^{E2} learning deficit (black bar) relative to controls (white bar). The second and third bars (light gray) represent learning of WT animals expressing the UAS-PKA^{C1} transgene in OK72 neurons, including or excluding the MBs, respectively, and are indistinguishable from the control. **B**, Expression of a constitutively active murine PKA* transgene with 2 (first gray bar) or 4 (second gray bar) UAS elements in OK72 neurons does not reverse the learning deficit of *dNf1*^{E2} mutant homozygotes (black bars). The performance of both null mutants (black bars) and PKA* transgene-expressing mutant animals (gray bars) is significantly different from that of control flies (white bar). **C**, Pan-neuronal expression, under the Alk-Gal4 driver, of a constitutively active murine PKA* transgene with 2 UAS elements (gray bar) fails to reverse the learning deficiency of *dNf1*^{E2} homozygotes (black bar). **D**, Expression of a constitutively active murine PKA* transgene with 2 (first gray bar) or 4 (second gray bar) UAS elements in adult MBs does not rescue the learning deficit of *dNf1*^{E2} homozygotes. *Statistically significant difference of the experimental from mutant flies. \$Statistical difference of the respective experimental group from the mutant flies ($p = 0.0277$). **E**, Rolipram administration (1 mM) to *dNf1*^{E2} null homozygotes (gray bar) does not restore learning, which remains indistinguishable from that of untreated mutant flies (black bar). Treated and untreated control flies (white bars) perform significantly different from the mutant groups. Control flies are OK72-Gal4 homozygotes, whereas the genotype of E2 mutants is OK72-Gal4;E2 homozygotes.

This was confirmed with staining for the TANGO postsynaptic component of the driver with anti-HA (Fig. 3E5a,5b). The punctate postsynaptic pattern (Talay et al., 2017) of neurons postsynaptic to OK72 is evident, and GABAergic neurons clearly do not colocalize with them. Collectively, the results suggest that GABAergic OK72-marked neurons are likely presynaptic to the MBs.

cAMP signaling is not required in OK72 neurons for Nf1-mediated learning

To explore potential mechanisms used within OK72-marked neurons for Nf1-mediated learning, we manipulated signaling pathways therein in the context of Nf1 null mutants. Prior studies have suggested that Nf1 regulates cAMP levels both in

Drosophila and mouse (Guo et al., 1997; The et al., 1997; Tong et al., 2002; Hannan et al., 2006). Indeed, the learning deficits of *Drosophila* Nf1 null mutants have been attributed to disrupted adenylyl cyclase function and compromised cAMP-mediated signaling (Guo et al., 2000). Disrupted cAMP signaling was also reported causative of the reduced size of *dNf1* null homozygotes (Walker et al., 2013).

To address whether compromised cAMP signaling within OK72 neurons underlies the learning deficits of *dNf1* nulls, we expressed therein constitutively active *Drosophila* (Fig. 4A; PKA^{C1}) and murine (Fig. 4B; PKA*) PKA catalytic subunit transgenes, reported to rescue the small size of mutant homozygotes (Walker et al., 2013). However, neither fly (Fig. 4A; ANOVA: $F_{(4,58)} = 34.7329$, $p < 0.0001$ subsequent LSM: OK72GAL4/UASPKA^{C1};E2/E2 vs OK72GAL4/+;E2/E2, $p = 0.073$), nor murine transgenes reversed the *dNf1*^{E2} deficient learning, even when expressed at variable levels (2UAS vs 4UAS) for the murine transgenes (Fig. 4B; ANOVA: $F_{(4,45)} = 28.6341$, $p < 0.0001$ subsequent LSM: OK72GAL4/4UASPKA*;E2/E2 vs 4UASPKA*+/+;E2/E2, $p = 0.3272$ and OK72GAL4/+;2UASPKA*;E2/E2 vs 4UASPKA*+/+;E2/E2, $p = 0.0845$). Failure of these constitutively active PKA catalytic subunit transgenes to rescue the learning deficit of *dNf1* nulls strongly suggests that signaling via PKA is not required within OK72-marked neurons for dNf1-mediated learning.

Alternatively, PKA activity may be compromised in *dNf1*^{E2} homozygotes in neurons not marked by OK72, such as the MBs, where it is known to be required for normal learning (Skoulakis et al., 1993). Such MB-specific kinase activity could in principle explain the reported reversal of the *dNf1* learning deficit with acute global PKA* expression (Guo et al., 2000). We addressed this possibility using the strong Alk-GAL4 driver, which highly overlaps the dNf1 endogenous pattern in the adult CNS (Gouzi et al., 2011), to drive the constitutively active murine 2UASPKA*

transgene, but again failed to rescue the *dNf1*^{E2} learning deficit (Fig. 4C; ANOVA: $F_{(2,29)} = 21.2205$, $p < 0.0001$ subsequent LSM: AlkGAL4/+;2UASPKA*;E2/E2 vs AlkGAL4/+;E2/E2, $p = 0.1286$). Moreover, acute constitutive murine PKA activity within adult MBs under c772Gal4; Gal80^{ts} also failed to rescue the *dNf1*^{E2} learning deficit and in fact depressed learning further (Fig. 4D; ANOVA: $F_{(3,31)} = 23.2169$, $p < 0.0001$ subsequent LSM: c772GAL4/+;2UASPKA*;E2/E2, Gal80^{ts} vs c772GAL4/+;E2, Gal80^{ts}/E2, $p = 0.005$ and c772GAL4/4UASPKA*;E2, Gal80^{ts}/E2 vs c772GAL4/+;E2, Gal80^{ts}/E2, $p = 0.0277$). This strongly argues against a model of PKA activation within the MBs in response to dNf1-mediated signals from OK72 neurons.

Finally, we aimed to investigate the possibility that learning in *dNf1*^{E2} flies may require cAMP elevation independent of PKA

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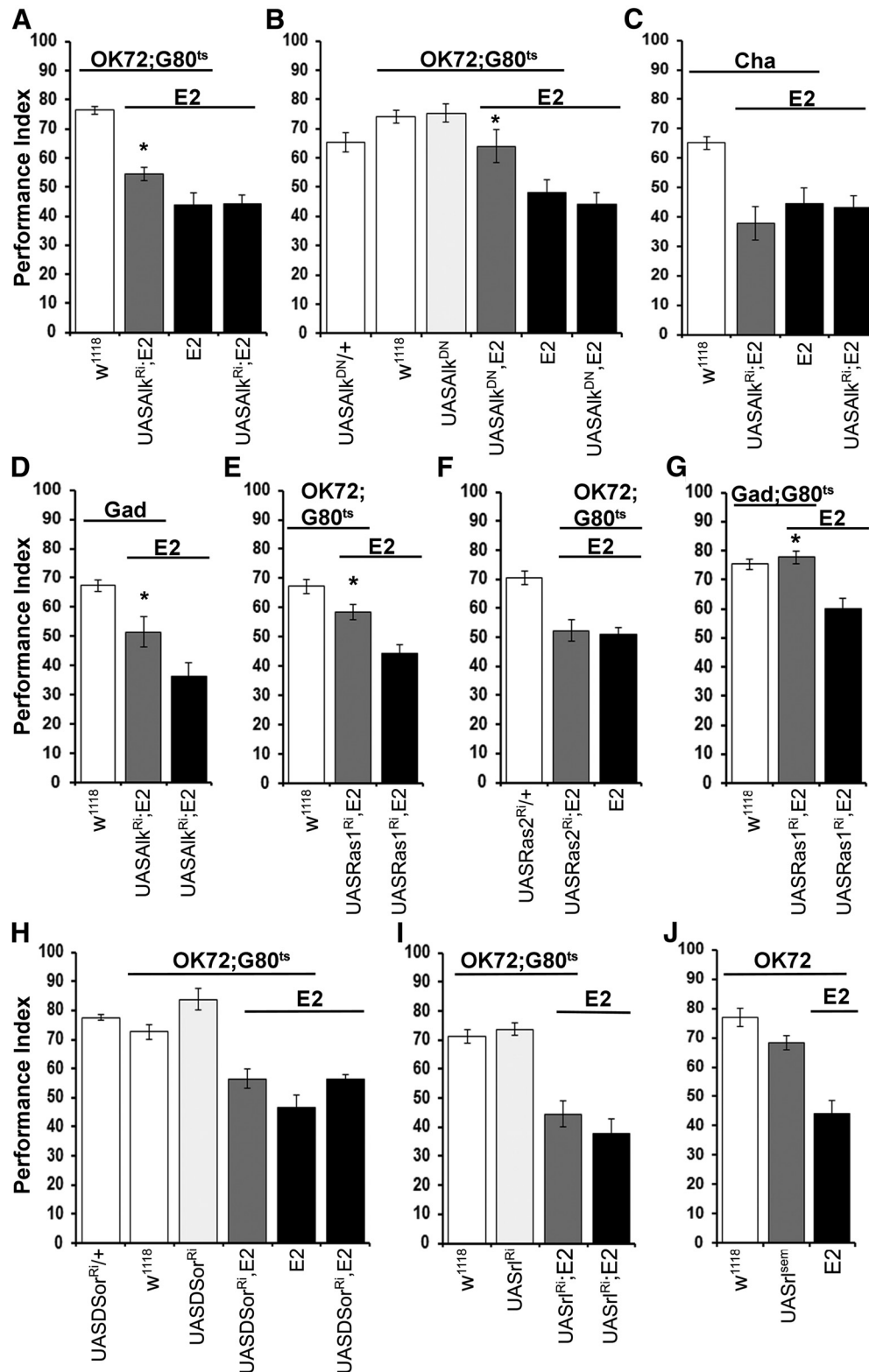


Figure 5. dNf1 engages Alk and Ras1 in OK72-GABAergic neurons to mediate learning. Performance assessment after conditioning of control (white bars), mutant (black bars), experimental (dark gray bars), and transgene-overexpressing (light gray bars) animals. The genotypes of all animals are indicated below each bar. Data are mean ± SEM. *Statistically significant difference in the performance of the experimental from mutant flies. *G80^{ts}* indicates the ubiquitously expressed temperature-sensitive Gal4 repressor Tub-Gal80^{ts}. **A**, Adult-induced dAlk abrogation in OK72 neurons ameliorates the *Nf1^{E2}* learning deficit. The performance of UASAlk^{Ri}-expressing mutants (gray bar) is significantly different from that of the nulls (black bars). **B**, Adult-induced dAlk inhibition in OK72 neurons rescues *Nf1^{E2}* learning deficits. ANOVA indicated significant effects on UASAlk^{DN} expression (dark gray bar), compared with *Nf1^{E2}* homozygotes (black bars), but not compared with controls (white bars). UASAlk^{DN} expressing WT flies (light gray bar) perform equally to control animals. **C**, dAlk suppression specifically in cholinergic neurons (gray bar) does not alter the learning deficit of *dNf1^{E2}* homozygotes (black bars). The performance of UASAlk^{Ri}-expressing mutant flies (gray bar) is significantly different from that of control animals (white bar). **D**, RNAi-mediated dAlk abrogation in GABAergic neurons (gray bar) improves the learning deficiency of *Nf1^{E2}* null flies. **E**, Adult-specific dRas1 abrogation in OK72 neurons of null mutants ameliorates their learning deficiency. **F**, Adult-specific abrogation of dRas2 within OK72 neurons does not reverse the learning deficit of *dNf1^{E2}* homozygotes. **G**, RNAi-mediated adult-specific

activation, or require regulated activation of the endogenous PKA. To that end, we raised global cAMP levels using the phosphodiesterase inhibitor rolipram, shown to be effective in elevating cAMP levels and restoring learning in *dFmr1* deficient flies (Kanellouopoulos et al., 2012). However, systemic cAMP elevation did not alter the learning deficit of the null mutants (Fig. 4E; ANOVA: $F_{(3,46)} = 15.0271$, $p < 0.0001$ subsequent LSM: E2+rolipram vs E2, $p = 0.2819$). Collectively, these results do not support the notion that compromised cAMP levels, or PKA signaling consequent to *dNf1* loss, are causal of the associated learning deficits.

dAlk-mediated signaling via Nf1/Ras1 within OK72-GABAergic neurons is essential for associative learning

Previous work has shown that abrogation of the receptor tyrosine kinase dAlk in the adult CNS at the exclusion of the MBs proved sufficient to reverse the learning deficit of *dNf1* null mutants (Gouzi et al., 2011). Given the role of GABAergic OK72-marked neurons in the *dNf1*^{E2} learning deficit, we investigated whether these are the neurons where dAlk abrogation reverses the deficient learning of the nulls. We used two different transgenes to attenuate dAlk activity within adult OK72 neurons:

F5 a UASAlk^{RNAi} (Fig. 5A) and the dominant negative form UASAlk^{DN} (Fig. 5B) (Bazigou et al., 2007). Indeed, reduction of dAlk expression in these neurons largely (Fig. 5A; ANOVA: $F_{(3,50)} = 45.2064$, $p < 0.0001$ subsequent LSM: OK72GAL4/UASAlk^{RNAi};E2,Gal80^{ts}/E2 vs UASAlk^{RNAi}/+;E2/E2, $p = 0.0031$), albeit not completely, restored olfactory learning in *dNf1* nulls. However, complete rescue of the learning deficit was achieved by adult-specific expression of the much stronger (Gouzi et al., 2011) dominant negative transgenic dAlk^{DN} protein in OK72 neurons (Fig. 5B; ANOVA: $F_{(5,58)} = 10.1953$, $p < 0.0001$ subsequent LSM: OK72GAL4/+; UASAlk^{DN},E2/E2,Gal80^{ts} vs UASAlk^{DN},E2/E2, $p = 0.0005$). Moreover, suppressing dAlk levels specifically within cholinergic (Fig. 5C) neurons did not rescue the learning deficit (ANOVA: $F_{(3,28)} = 12.6667$, $p < 0.0001$ subsequent LSM: ChaGAL4/UASAlk^{RNAi};E2/E2 vs UASAlk^{RNAi}/+;E2/E2, $p = 0.3847$). In contrast, dAlk attenuation in GABAergic neurons improved learning in *dNf1*^{E2} flies (Fig. 5D; ANOVA: $F_{(2,46)} = 16.7035$, $p < 0.0001$ subsequent LSM: GadGAL4/UASAlk^{RNAi};E2/E2 vs UASAlk^{RNAi}/+;E2/E2, $p = 0.0104$). These results support the notion that dAlk-dependent compensation of *dNf1* loss is specific to OK72-marked GABAergic neurons.

dAlk is a typical receptor tyrosine kinase, transducing signals through the Ras/ERK pathway (Gouzi et al., 2005; Palmer et al., 2009). The main function ascribed to Neurofibromin is negative regulation of Ras signaling (Xu et al., 1990; Costa et al., 2002), potentially intercepting activated dAlk signals (Gouzi et al., 2011). Therefore, we investigated the possibility that hyperactivation of one of the *Drosophila* Ras proteins within OK72 GABAergic neurons underlies the deficient learning of *dNf1* nulls. Indeed, RNAi-mediated adult-specific attenuation of Ras1 within OK72 neurons partially rescued the learning deficit of null homozygotes (Fig. 5E; ANOVA: $F_{(2,49)} = 19.2928$,

$p < 0.0001$ subsequent LSM: OK72GAL4/+;UASRas1^{RNAi}, E2/E2,Gal80^{ts} vs UASRas1^{RNAi},E2/E2, $p = 0.0007$, but vs OK72GAL4/+;Gal80^{ts}/+, $p = 0.0257$). In contrast, attenuation of Ras2 did not alter the *dNf1*^{E2} learning deficit (Fig. 5F; ANOVA: $F_{(2,31)} = 14.9055$, $p < 0.0001$ subsequent LSM: OK72GAL4/UASRas2^{RNAi};E2,Gal80^{ts}/E2 vs OK72GAL4/+;E2,Gal80^{ts}/E2, $p = 0.7144$). Furthermore, complete rescue of the *dNf1*^{E2} learning deficit was attained on adult-specific Ras1 attenuation in GABAergic neurons (Fig. 5G; ANOVA: $F_{(2,59)} = 14.5783$, $p < 0.0001$ subsequent LSM: GadGAL4/+; UASRas1^{RNAi},E2/E2,Gal80^{ts} vs UASRas1^{RNAi},E2/E2, $p < 0.0001$). Therefore, Ras1 overactivation on *dNf1* loss within OK72-marked GABAergic neurons underlies the resultant associative learning deficit, most likely by increased GABA production and/or release to MB dendrites.

Loss of *dNf1* results in neuronal ERK overactivation underlying the reduced body size of mutant homozygotes (Walker et al., 2006), while pan-neuronal expression of constitutively active ERK (UAS-*r^{sem}*) (Martin-Blanco, 1998) yielded smaller animals (Gouzi et al., 2011). Conversely, pan-neuronal dAlk overexpression in adult *Drosophila* elevated phospho-ERK and RNAi-mediated dAlk abrogation reduced the activated kinase in head lysates (Gouzi et al., 2011). Therefore, it is possible that *dNf1* loss leads to hyperactivation of the Ras/ERK cascade within OK72 neurons precipitating GABA elevation and deficient learning. However, RNAi-mediated attenuation of the fly MEK ortholog Dsor (Fig. 5H), or of the MAPK Rolled (rl) (Fig. 5I), did not reverse the learning deficit of null homozygotes (Fig. 5H; ANOVA: $F_{(5,41)} = 21.7710$, $p < 0.0001$ subsequent LSM: OK72Gal4/+;UASDsor^{RNAi},E2/E2,Gal80^{ts} vs UASDsor^{RNAi},E2/E2, $p = 0.9482$ and Fig. 5I; ANOVA: $F_{(3,45)} = 30.2013$, $p < 0.0001$ subsequent LSM: OK72GAL4/UASrl^{RNAi},E2,Gal80^{ts}/E2 vs UAS-rl^{RNAi}/+;E2/E2, $p = 0.1971$, respectively). In agreement with these results, expression of the constitutively active ERK rl^{sem} within OK72 neurons did not yield deficient learning in otherwise WT animals (Fig. 5J; ANOVA: $F_{(2,40)} = 26.2560$, $p < 0.0001$ subsequent LSM: OK72GAL4/UASrl^{sem} vs OK72GAL4/+;E2/E2, $p < 0.0001$). Therefore, unless the RNAis have very little efficacy, hyperactivation of MEK and ERK within OK72 neurons does not appear to account for the learning deficits of *dNf1* nulls.

Collectively, the results suggest that signals, originated by dAlk and transduced to Ras1 to trigger downstream effectors, are intercepted and regulated by *dNf1* within OK72 neurons. Moreover, Ras1 effectors other than the canonical MEK and ERK are implicated either in regulation of GAD activity or GABA release within these neurons. Experiments to define these effectors and their mode of action on GABA production or release are currently ongoing.

Ras1 hyperactivation within the MBs of *dNf1* nulls attenuates learning

Because Ras1 abrogation under OK72 improved learning of *dNf1* homozygotes (Fig. 5E), we investigated whether its effects were solely because of its activity in GABAergic neurons (Fig. 5G), or the MBs where Ras1 appears preferentially expressed (Fig. 6A). Importantly, Ras1 attenuation in OK72-marked neurons with exclusion of the MBs resulted in significant rescue of the *dNf1*^{E2} learning deficit (Fig. 6B; ANOVA: $F_{(3,37)} = 18.7081$, $p < 0.0001$, subsequent LSM: OK72GAL4/MBG80; UASRas1^{RNAi},E2/E2,Gal80^{ts} vs OK72GAL4/+;E2,Gal80^{ts}/E2, $p = 0.0007$), similar to that when Ras1 was also abrogated in the MBs (Fig. 6B; LSM following initial ANOVA

dRas1 attenuation in GABAergic neurons fully rescues the *dNf1*^{E2} learning deficiency. **H**, Adult-specific RNAi-mediated abrogation of MEK (UASDsorRi) in OK72 neurons fails to rescue the learning deficit of *dNf1*^{E2} homozygotes (dark gray bar). **I**, Adult specific RNAi-mediated abrogation of MAPK (UASrlRi) in OK72 neurons does not restore the learning deficits of *dNf1*^{E2} homozygotes. **J**, Overexpression of a constitutively active MAPK transgene (UASrlsem) in OK72 neurons does not precipitate learning deficits in WT flies.

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above: OK72GAL4/+; UASRas1^{RNAi},E2/E2, Gal80^{ts} vs OK72GAL4/+;E2, Gal80^{ts}/E2, $p < 0.0001$). However, reversal of the learning deficit appeared more efficient when the OK72 pattern included the MBs (Fig. 6B; LSM after ANOVA above: OK72GAL4/MBG80; UASRas1^{RNAi},E2/E2, Gal80^{ts} vs OK72GAL4/+; Gal80^{ts}/+, $p = 0.0005$ and OK72GAL4/+; UASRas1^{RNAi},E2/E2, Gal80^{ts} vs OK72GAL4/+; Gal80^{ts}/+, $p = 0.0542$). Therefore, we investigated whether Ras1 activity is required within the MBs in addition to GABAergic neurons. Remarkably, acute abrogation of Ras1 in adult MBs under the broad MB driver LeoGAL4 ameliorated the deficient learning of *dNf1*^{E2} nulls (Fig. 6C; ANOVA: $F_{(2,34)} = 16.9274$, $p < 0.0001$, LSM: LeoG4/+;UASRas1^{RNAi},E2/E2, Gal80^{ts} vs UASRas1^{RNAi},E2/E2, $p = 0.0003$), which was recapitulated under an independent more restricted MB driver (Fig. 6D; ANOVA: $F_{(2,33)} = 6.6027$, $p < 0.0041$, LSM: c772G4/+;UASRas1^{RNAi},E2/E2, Gal80^{ts} vs c772GAL4/+;E2, Gal80^{ts}/E2, $p = 0.0312$). However, Ras1 attenuation in the MBs of control animals did not affect learning (Fig. 6E; ANOVA $F_{(2,42)} = 0.0883$, $p = 0.9156$), even after reduced training conditions (Fig. 6F; ANOVA $F_{(2,47)} = 0.1663$, $p = 0.8473$). This indicates that Ras1 is hyperactivated specifically within the MBs of the *dNf1* nulls and this attenuates learning even when excessive GABA from OK72 neurons is ameliorated. However, Ras1 in the MBs does not appear to play a role in learning of control animals. In agreement with this notion, adult-specific expression of an ERK hyperactivating (Fig. 6H), constitutively active Ras^{V12} protein in the MBs precipitated a significant learning deficit in otherwise WT flies (Fig. 6G; ANOVA: $F_{(2,22)} = 21.7134$, $p < 0.0001$, LSM: c772GAL4/+;UASRas1^{V12}/Gal80^{ts} vs UASRas1^{V12}/+, $p < 0.0001$ and vs c772GAL4/+;Gal80^{ts}/+, $p = 0.0002$).

Together, the data suggest Ras1 hyperactivation in the MBs of *dNf1* nulls, possibly in response to GABA signals from presynaptic OK72 neurons, which ostensibly inhibits learning. Interestingly, an atypical Ras-mediated pathway regulating cAMP levels by suppressing $G\alpha_s$ activity has been reported in vertebrate neurons (Anastasakis and Gutmann, 2014) and a similar activity in *Drosophila* head lysates (Hannan et al., 2006). Therefore, hyperactivated Ras1 may lower cAMP levels and its attenuation in neurons using this pathway, as MBs appear to be, would elevate intracellular cAMP. Collectively then, regulated Ras1 activity is required within OK72-marked GABAergic neurons to regulate synthesis or release of GABA. In

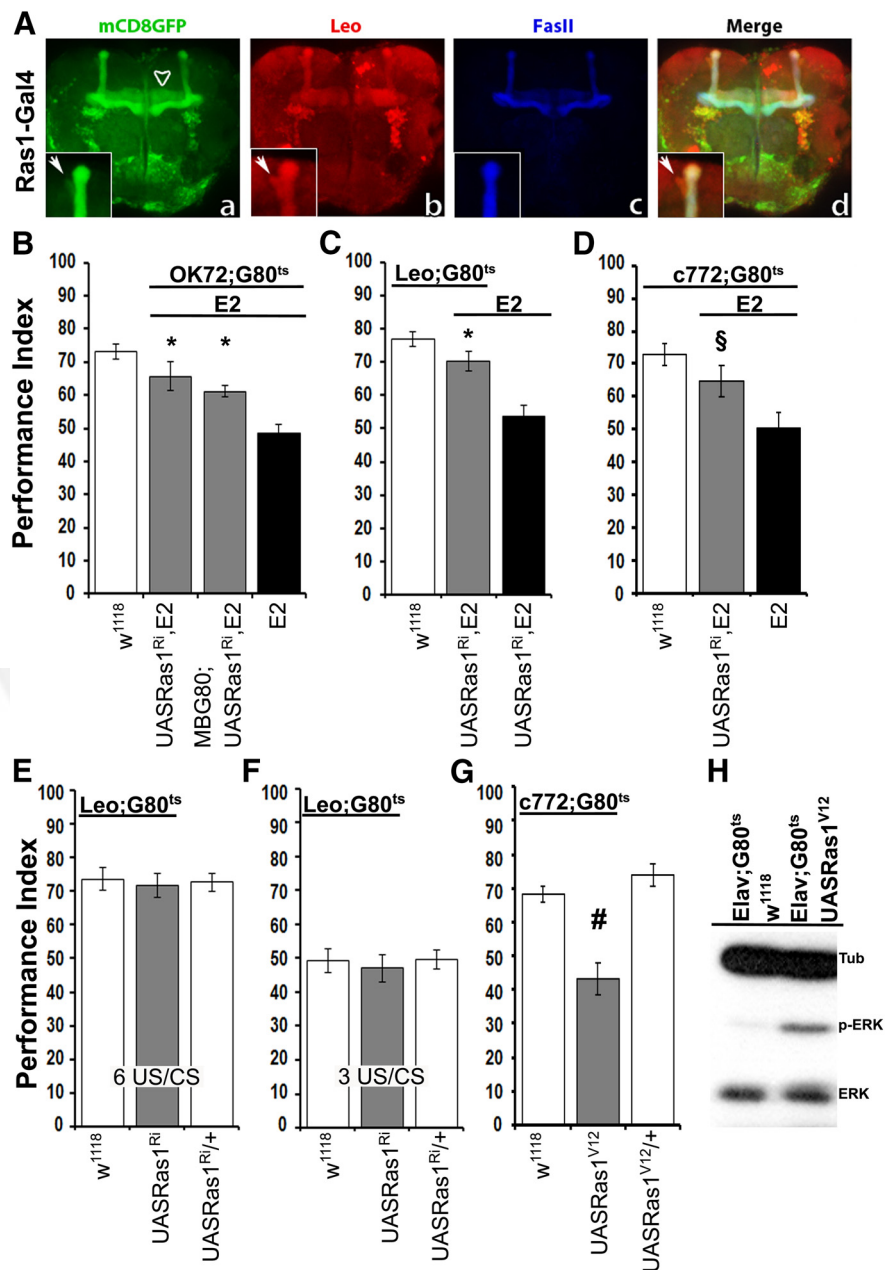


Figure 6. Ras1 hyperactivation in the MBs contributes to the learning deficiency of *dNf1* nulls. **A**, Confocal images illustrate the distribution of Ras1 in the MBs of adult flies. Ras1-Gal4 drives the expression of UAS-mCD8GFP, especially in the α/β MB lobes (**Aa–Ad**), the γ lobe (**Aa**, arrowhead), and at lower levels in the α'/β' lobes, where it colocalizes with Leo that marks all MB lobes (**Aa,Ab,Ad**, arrow in the marked boxes). Marked boxes represent higher magnification of the α/α' lobes. **B–G**, Performance assessment after conditioning in control (white bars), mutant (black bars), and experimental (gray bars) animals. The genotypes of all animals are indicated below each bar. Data are mean $\pm$ SEM. G80^{ts} indicates the ubiquitously expressed temperature-sensitive Gal4 repressor Tub-Gal80^{ts}. **B**, Adult-specific dRas1 abrogation within the OK72 marked neurons at the exclusion of MB expression (second gray bar) partially restores learning in *dNf1*^{E2} homozygotes. *Statistically significant difference of the experimental from mutant flies. **C**, dRas1 abrogation in MB neurons (Leo-Gal4 driver) during adulthood rescues the *dNf1*^{E2} learning deficits. *Statistically significant difference of the experimental from mutant flies. **D**, dRas1 abrogation in MB neurons (c772-Gal4 driver) during adulthood improves the *dNf1*^{E2} learning deficit. \$Statistical difference of the experimental from the mutant flies ($p = 0.0312$). **E, F**, Adult-specific abrogation of Ras1 (UASRas1^{RNAi}) in MB neurons does not yield learning deficits in control flies. The performance of animals with reduced Ras1 within MBs was not significantly different from that of controls both after 6 (**E**) and 3 (**F**) CS-US pairings. **G**, Overexpression of the constitutively active Ras1 (UASRas1^{V12}) in the MBs of WT adult flies yields learning deficits. #Statistically significant difference of the experimental from the control groups. **H**, Representative Western blot of head lysates from adult flies expressing pan-neuronally a constitutively active form of Ras1 (UASRas1^{V12}) under Elav-Gal4;Gal80^{ts} using an antibody against phosphorylated ERK (p-ERK), revealing elevated active kinase compared with control animals. The amount of total-ERK protein is not affected. The genotypes of animals used are indicated above the immunoblot. Tubulin levels serve as loading control.

addition, GABA levels cell autonomously within MB neurons may be linked to, or acting in parallel to reported (Liu and Davis, 2009), or novel signaling pathways suppressing olfactory learning.

Discussion

We reveal GABAergic neurons apparently presynaptic to the MBs (Fig. 3D,E), where dNf1 is acutely required to support normal associative learning (Figs. 1G,H, 2D). Collectively, our results indicate that excess GABA, either because of elevated GAD activity (Fig. 3A,B) or GABA release on dNf1 loss, inhibits MB-mediated associative learning. This broadly agrees with results from *Nf1*^{+/-} mice that also present increased GABAergic neurotransmission to the hippocampus, PFC, striatum, and amygdala and apparently underlies the deficits in visual-spatial, working memory, social learning, and attention they present (Costa and Silva, 2002; Cui et al., 2008; Shilyansky et al., 2010b; Diggs-Andrews and Gutmann, 2013; Molosh et al., 2014; Omrani et al., 2015).

The increase in GABAergic neurotransmission in *dNf1* nulls depends on dAlk activation (Fig. 5A,B,D) and Ras1 engagement (Fig. 5E,G) within these OK72-marked neurons, but it is MEK- and ERK-independent (Fig. 5H–J). This suggests that a variant to the canonical Ras/ERK cascade is operant within these neurons modulating GABA levels. Similarly, an Alk (Weiss et al., 2017a, 2017b) and Ras-dependent cascade results in ERK-mediated increase in GABAergic neurotransmission to the hippocampus in *Nf1*^{+/-} mice (Costa et al., 2002; Cui et al., 2008). The molecular mechanism(s) underlying the elevated GABAergic neurotransmission to *Drosophila* MBs is under investigation, but identification of the OK72-marked inhibitory neurons housing them is a significant step toward their elucidation. These neurons are distinct from the also GABAergic APL neuron (Fig. 1F), whose activity normally suppresses the CS⁺ and is inhibited by learning (Liu et al., 2009; Liu and Davis, 2009).

Although as shown herein, these GABAergic OK72-marked neurons are cardinal to the dNf1 loss-mediated learning deficit, their normal function is less clear. GABAergic neurotransmission from the APL is thought to contribute to the fidelity and effectiveness of the CS⁺ (Liu and Davis, 2009). Consistent with this limiting inhibitory role, GAD reduction in APL neurons enhanced olfactory associative learning (Liu and Davis, 2009). However, GAD abrogation within GABAergic OK72-marked neurons did not affect learning in control flies (Fig. 3C), suggesting that normally they do not limit the process. Rather, activation of dAlk, a negative regulator of learning and memory (Gouzi et al., 2011, 2018) and Ras1 within these OK72-marked neurons may regulate GABA release modulating, but not suppressing, MB activation. We propose that hyperactivation of dAlk/Ras1-mediated signaling on dNf1 loss results in dysregulated, excessive, or persistent GABA levels, altering the excitatory to inhibitory balance of inputs to the MBs, thus impairing learning. Ongoing experiments address this hypothesis.

Significantly, our results demonstrate that constitutive activity of murine, or *Drosophila* PKA does not reverse the GABAergic excess of OK72 neurons (Fig. 4A,B) to rescue leaning in *dNf1* null homozygotes. This strongly suggests that dNf1 loss does not result in decreased cAMP levels and consequently of endogenous PKA activity, at least in OK72 neurons. This conclusion is further supported by elevation of cAMP levels via rolipram-dependent Dnc phosphodiesterase inhibition (Kanellopoulos et al., 2012), which also did not ameliorate the deficient learning of *dNf1*^{E2} homozygotes (Fig. 3E). In genetic corroboration of the pharmacological results, broad expression in the fly CNS of

constitutively active PKA also did not suppress the learning deficit of *dNf1* nulls (Fig. 4C). Furthermore, acute activated PKA expression within adult MBs did not improve the deficient learning of the null mutants (Fig. 4D). These results argue against the notion that dNf1 loss precipitates a cAMP/PKA deficit within the fly CNS underlying the compromised associative leaning, in discord with suggestions from prior reports (Guo et al., 1997, 2000).

The collective evidence strongly supports the model whereby dNf1 is required within GABAergic OK72-marked neurons to directly or indirectly regulate the levels of inhibitory neurotransmission to the MBs. Loss of dNf1 therein presents excess GABA to the MBs, resulting in suppression of olfactory associative learning. However, Ras1 appears not to be hyperactivated only within GABAergic OK72-marked neurons of *dNf1*^{E2} nulls (Figs. 5E,G, 6B), but also in their MBs (Fig. 6C,D) and contributes to their deficient learning. This notion is supported by the constitutively active Ras^{V12} transgene, which suppressed learning when acutely expressed in the MBs of control animals (Fig. 6G). Ras1 hyperactivation has been shown to result via PKC ζ activation in suppression of G α_s and therefore reduced cAMP in vertebrate cultured neurons (Anastasaki and Gutmann, 2014), suggesting that this may also occur in the MBs of *dNf1*^{E2} homozygotes. Interestingly, an inhibitory role for Ras/Raf signaling for protein synthesis-dependent memory formation in MBs has been described recently (Noyes et al., 2020).

Importantly, however, Ras1 activity in the MBs does not appear regulated by dNf1 because its abrogation therein did not phenocopy the learning deficits (Fig. 1B). In accord, dNf1 restoration within the MBs does not rescue the learning deficit of *dNf1* nulls (Fig. 1A). It is then possible that dNf1-independent Ras1 hyperactivation within the MBs may result from excessive GABAergic activity, or the resultant imbalance of inhibitory to excitatory inputs into these neurons. Together, dNf1 loss appears to result in Ras1 hyperactivation within the MBs, which is not regulated by dNf1 within these neurons and could result in reduced cAMP levels. However, constitutively active PKA expression in the MBs (Fig. 4D), or rolipram treatment (Fig. 4E), did not reverse the deficient learning of the mutants. Indeed, strong activated catalytic PKA expression in *dNf1*^{E2} MBs appeared to reduce learning below that of null homozygotes (Fig. 4D). Collectively, these results indicate that GABAergic inhibition to the MBs on dNf1 loss must be relieved preceding, or concurrently with this putative MB-specific PKA elevation. Predictions and mechanistic aspects of the proposed model are currently under investigation.

Importantly, the learning defects of homozygotes for another null allele, *dNf1*^{P2}, were reported reversed by acute expression of a constitutively active murine PKA subunit (hs-PKA*) expressed throughout the fly (Guo et al., 2000) and suggested to overcome the low cAMP levels in this mutant (Guo et al., 2000; Tong et al., 2002). The model proposed above offers reconciliation of our data and these reports noting the following. Reversal of the learning deficits of null *dNf1* mutants with the constitutively active PKA has been obtained only under the heat shock promoter, which results in massive transgene expression throughout the fly, even without induction (Guo et al., 2000), probably because of position effects. The massive expression of the hs-PKA* transgene likely suffices to bypass the increased GABAergic inhibitory effects and elevate neuronal PKA activity within the MBs above a threshold that yields significant learning. Such levels of transgene induction are not likely in a tissue-limited manner and under Gal80^{ts}, which appears to decrease UAS-driven transgene

expression by $\geq 50\%$ (Papanikolopoulou et al., 2018), under the conditions used herein. Although additional experiments are required to explore this hypothesis, it provides a putative resolution of a long-standing controversy, probably pertinent to vertebrate models where dopaminergic elevation of cAMP appears altered on Nf1 loss (Diggs-Andrews et al., 2013).

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1

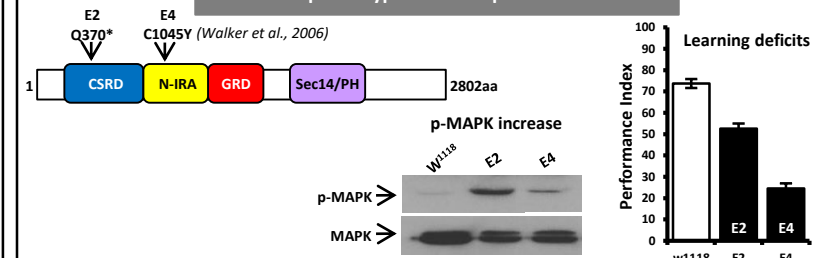
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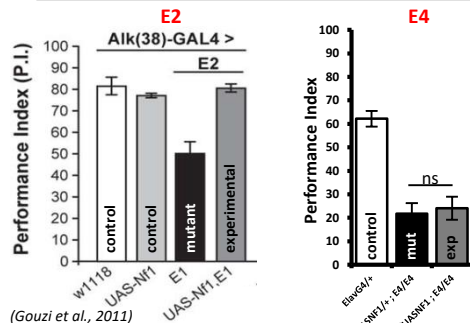
Abstract:

Neurofibromatosis type 1 (NF1) is an autosomal dominantly inherited multisymptomatic disorder that affects 1:3000 individuals and results from mutations in the neurofibromin (*Nf1*) gene. Although the disease is fully penetrant, NF1-associated deficits are highly variable and significantly what underlies NF1-associated cognitive problems remains poorly understood. These include executive and higher-order functions, motor coordination and mainly learning and attention impairments. Neurofibromin is expressed in almost all tissues but most highly in the brain, spinal cord, and the peripheral nervous system. Despite its large size, the only well understood evolutionary conserved function of Nf1 is its role as a GTPase-activating protein (GAP) for Ras, through its GAP-related domain (GRD). Previous and current work from our and other labs in the *Drosophila* NF1 model have shown that loss of neurofibromin results in organismal size reduction and deficits in associative learning and memory, thus resembling human NF1 symptoms. We reveal a novel GABAergic neuronal subset, outside the Mushroom Bodies, where dNf1 is acutely required to support normal associative learning. The deficient learning of *dNf1* null flies depends on dAlk activation and Ras1 engagement within these neurons, strongly suggesting its assignment to loss of GRD function. On the other hand, point mutations outside the GRD domain show distinct spatiotemporal effects and implicate impairment of different molecular mechanisms than those upon total dNf1 loss. These results posit that distinct *Nf1* mutations affect differentially known and novel functions of the protein, possibly in a cell-type-specific manner, thus contributing to the variability of NF1 pathologies.

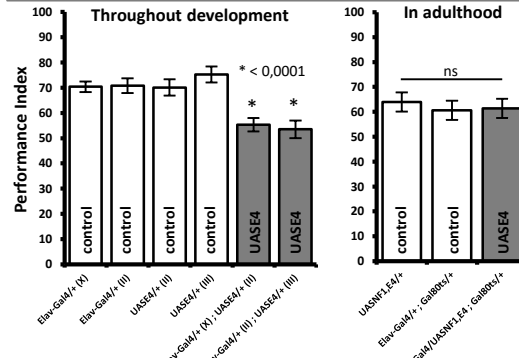
1. Distinct phenotypes of *Drosophila* Nf1 mutants



2. Does pan-neuronal dNf1 expression restore the learning deficits of *dNf1* mutants?

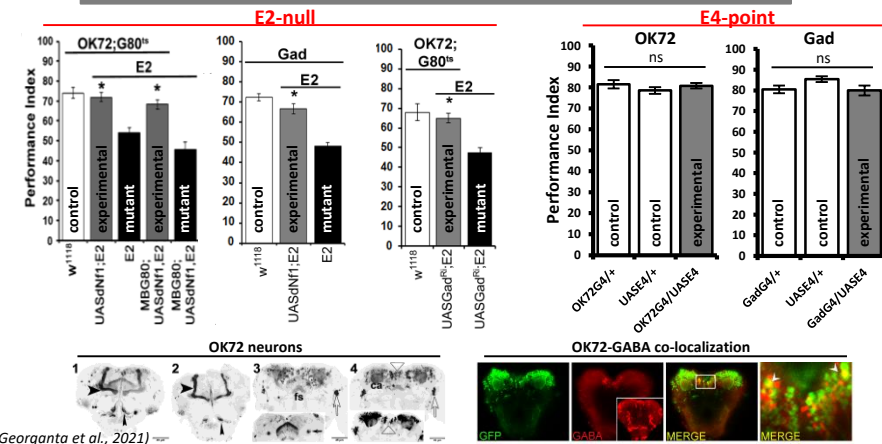


3. Pan-neuronal expression of a transgene bearing the C1045Y mutation (UASE4) in control flies ...

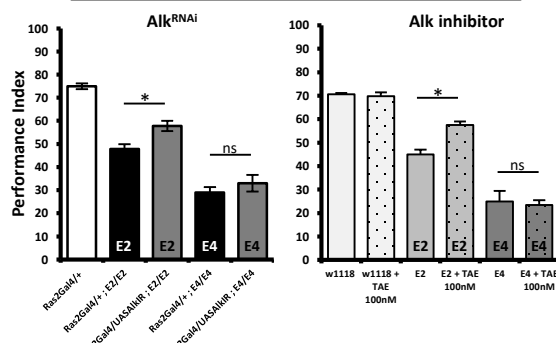


... precipitates learning deficits if expressed throughout development but not if expression is acute and limited to adult animals.

4. E2-null and E4-point *dNf1* mutations do not affect the same neurons

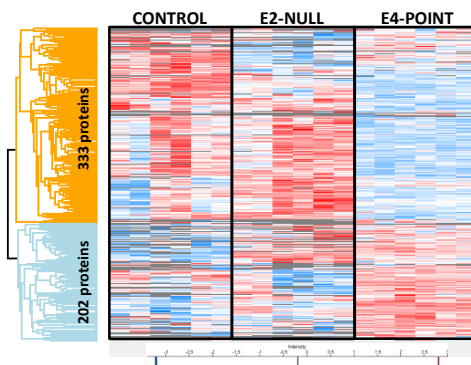


5. E2-null and E4-point *dNf1* mutations do not affect the same molecular mechanisms



Genetic (*Alk^{RNAi}*) and pharmacological (TAE/Crizotinib) inhibition of Alk rescue the *dNf1^{E2}* but NOT the *dNf1^{E4}* learning deficits.

6. Whole brain proteome analysis in *Nf1^{E2}* null and *Nf1^{E4}* point mutants



The E4 point mutant has distinct effects on the brain proteome than total loss of dNf1.

Conclusions and perspectives

- Our results, concerning the differential effects of *dNf1^{E2}* null and *dNf1^{E4}* point mutants, resemble the NF1 disease heterogeneity.
- dNf1* is required in a subset of adult GABAergic OK72-marked neurons, outside the MBs, for normal olfactory associative learning.
- C1045Y (E4) is a novel developmental dominant mutation with distinct functional and spatiotemporal effects than total loss of dNf1.
- Our data argue that mutations in more understudied domains of Nf1 (outside the GRD) could affect Nf1-regulated neuronal function, manifesting as cognitive deficits.
- We hypothesize that point mutations in the *Nf1* gene contribute significantly to the variability of NF1-associated phenotypes.

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