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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.

15. SUBJECT TERMS

null mutations

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TABLE OF CONTENTS

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

Year three progress is added to that of year 2 and is written in blue colored letters

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.

In addition, the lead postdoctoral scientist was on maternity leave June/22 till Dec 20/22 and although the technician and temporary personnel produced a lot of relevant data as necessary, progress on the mouse part of the project was significantly delayed.

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.

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

As outlined in the previous report we have generated by CrispR methodology the **R1809C** fly mutant and we have progressed in its characterization (see below).

Regarding the **M1035R** (Leopard) we have just verified the mutation (after 2 unsuccessful attempts) and initiated characterization.

Unfortunately, sequencing of the presumed M1035R mutants verified initially be restriction analysis revealed the presence of a small deletion, which renders the strain mutant, but not containing exclusively the point mutation sought.

To expand the analysis of the various functional domains of Nf1 we have initiated generation of additional mutations, namely the **M1149V** and **L1423E**, which are currently in progress of getting generated by injecting the gRNA plasmid and relevant oligos into a CAS9 expressing line. Together, these patient-borne CrispR-generated mutations (presented in the adjacent table), which present selectively, or predominantly behavioral deficits will address distinct functional domains of the protein that mediate these pathologies. All mutations are verified for the presence of novel restriction sites due to the silent changes in the sequence at the level of the gRNA plasmids and by sequencing to verify the mutation and ascertain no additional alterations surrounding the induced mutations.

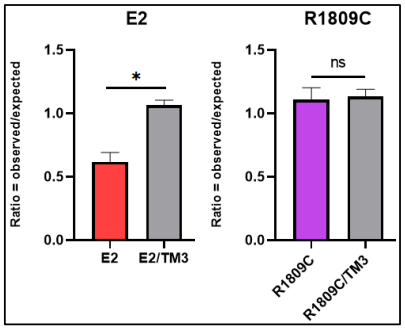
Genotype	Phenotype	Reference
Arg1809Cys (Sec14/PH)	**Learning disabilities**, Noonan like features, CALMs	Pinna et al., 2014; Rojnueangnit et al., 2015
Met1035Arg (N-IRA)	**Learning disabilities**, LEOPARD-like presentation	Wu et al., 1996
Met1149Val(TBD)	Mild Noonan-like phenotype (pigmentary manifestations)	Koczkowska et al., 2020
Lys1423Glu(GRD)	Mild form of NF1 (decreased incidence of neurofibromas)	Koczkowska et al., 2020

The relevant gRNA plasmids along with OSSN were injected and the following are the yields to date from 3 independent runs per construct. As can be seen from the table here, we have screened by PCR followed by restriction digest 681 independent lines without obtaining a single *bona fide* mutant line. Recognizing the importance of these and additional such single point mutations for this and future "structure/function" studies reflecting the human mutations, we have reconsidered the strategy to obtain these mutations. The fact that CrispR strategies are not standardized and seem to present different efficiencies per gene and sought mutation does not help assure success by general admission. However, in sequencing the presumed mutants, which turned out to harbor additional mutations, it became evident that polymorphisms in the *Nf1* sequence of the recipient strain where the novel mutations were to be induced may have interfered with the CrispR mutagenesis process. Given the size of the *Nf1* gene and its known mutability, the frequency of the polymorphisms uncovered is not unreasonable and could in fact account for failing to obtain 3 /4 CrispR mutations we set out to generate.

CONSTRUCT	K1423	M1149	M1149V
injected larvae	551	553	623
Adults	335	364	386
survived/Fertile	187	198	296
positive	0	1	2
confirmed positive		0	0

Nevertheless, before proceeding with another round of mutageneses we are sequencing the genomic areas at least 500bp on both sides of the targeted sequence to ascertain that the sequences of the gRNAs and ssODN are inclusive of potential such polymorphisms. Sequencing has been initiated and is expected to be completed within the month.

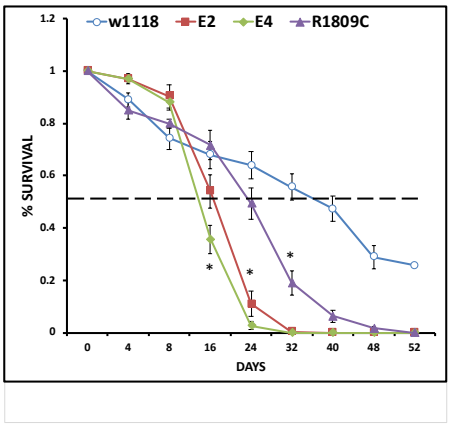
Characterization of **R1809C**



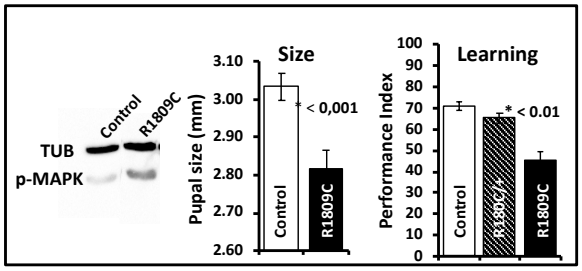
A. Vital functions/ strength of the mutation.

Genetic characterization of the novel R1809C mutant demonstrated that unlike the null mutant E2 used by most labs, the point mutant does not present lethality, hence it represents a milder mutation as shown in the adjacent figure. We calculate the average (±SEM) viability of mutant homozygotes versus that of their heterozygous siblings (gray bars). Significant differences are indicated by the asterisks.

Clearly all R1809C homozygotes are viable in accord of the patient data reported in the relevant references on the table, positing that this mutation is characterized ostensibly largely by cognitive symptoms, not the more common stronger pathologies associated with the disease. This is independently supported by estimating longevity of the mutant under standard laboratory conditions. Clearly the R1809R mutant (purple line) survives longer than the rest of the mutants tested alongside, but not as long as the control (w1118 strain). Significant differences are indicated by the asterisks and the first significant difference relative to controls arises for day 32 in the R1809 animals, but significantly earlier for the other two mutants. The dashed line gives the day of 50% population attrition due to lethality. It is evident that the 50% attrition date for the R1809C mutant flies is day 26, while it is day 18 for the null mutant E2 and day 12 for the point mutant E4 (C1045Y)! Therefore, the R1809C mutation is indeed mild in two different measures of potentially compromised vital processes.



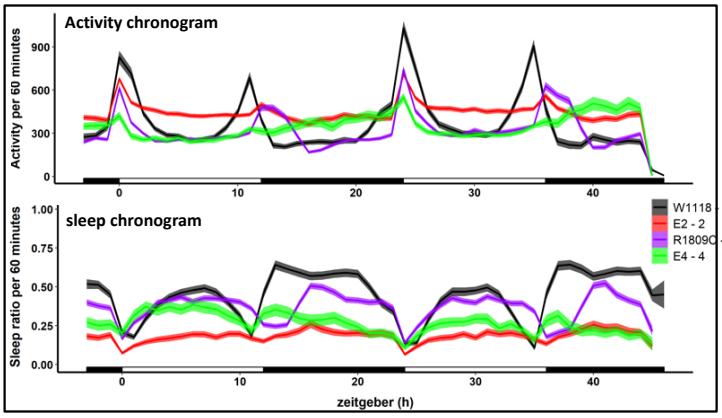
B. Biochemical, Cognitive and other behavioral phenotypes.



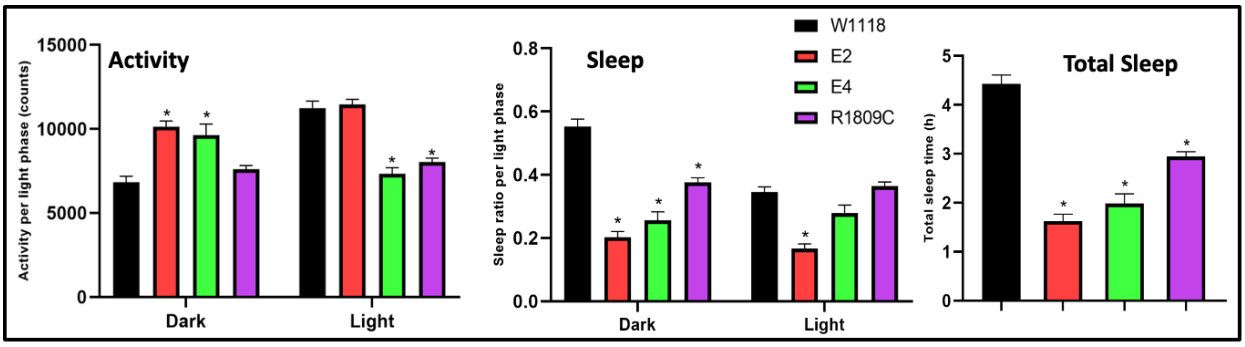
Initial characterization of the R1809 fly mutant demonstrate mildly elevated Ras signaling (pMAPK), significantly decreased size of the animals and significantly deficient learning as indicated in the adjacent figure. We are in progress building up strains to attempt rescue of the phenotype and to try pharmacological rescue as we have done in the past for the null mutant.

Novel behavioral assessments of old and new mutants.

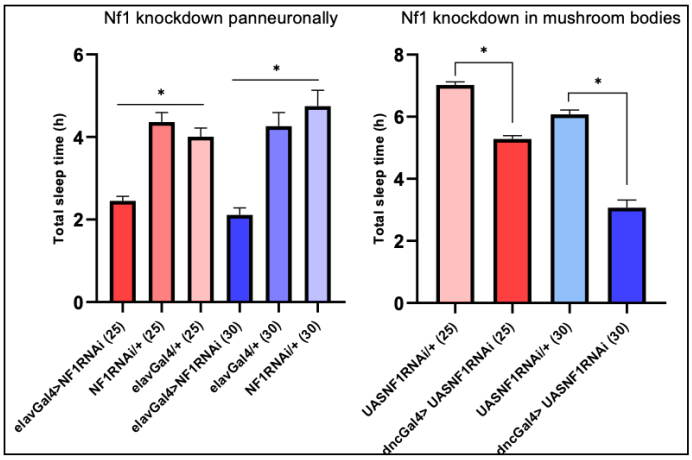
Reduced **sleep and hyperactivity** are typical of Neurofibromatosis 1 patients and to that end we monitored these two complementary in part behavioral outputs in Drosophila *Nf1* mutants. Activity and sleep were assessed using Trikinetics DAM 5M multibeam monitors, which allow much more accurate assessment of activity and sleep times. Each chronogram below*)* is the average activities (or sleep) based on assessment of 32 male flies per genotype and experiment in at least four independent experiments (total n> 128 individuals). The width of the lines indicates the standard error of the mean (SEM). It is rather obvious that compared to the w1118 control animals (black), the overall activity of the nulls (red), lacks periodicity, and remains high even in periods of predominant rest in the middle of day and at night. Similarly, the C1045Y(E4) mutants present not obviously periodic, but overall low



activity, whereas the R1809C animals present good, but not perfect periodicity and hypoactivity compared to controls (upper panel activity chronogram). Both null and C1045Y(E4) mutants present highly reduced sleep without obvious periodicity and is enhanced during the mid-day (siesta) and at nighttime as is the case for control animals. While the R1809C animals sleep less than controls they appear to overall retain sleep rhythmicity (lower panel). Quantification of total activity per light phase clearly indicates that unlike for R1809C, the nulls and C1045Y(E4) are hyperactive during the dark phase in comparison to controls (star: $p<1x10^{-5}$), but

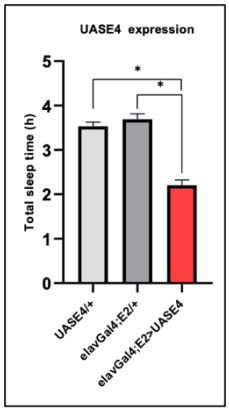


whereas the nulls remain hyperactive during the day C1045Y(E4) and R1809C are hypoactive. This is also reflected in total sleep time during the night which is significantly less than controls for all mutants, but only during the day sleep is significantly reduced only in the nulls. Overall however, sleep is significantly reduced compared to controls.



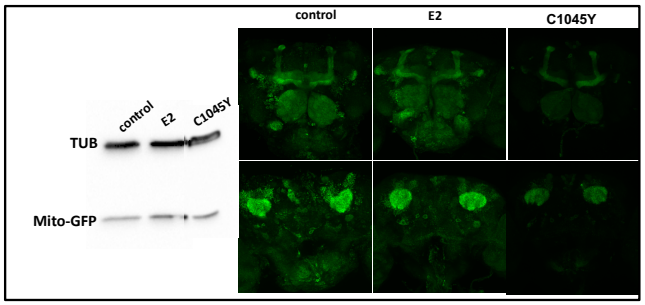
To independently verify the results above, we knocked down Nf1 either pan-neuronally or with mushroom body neurons and to ascertain maximal induction of the RNAi-mediating transgene expression the experiment was conducted either at 30C or at the typical temperature of 25 C. In both cases and at both temperatures total sleep was highly significantly reduced in the mutants and the effect is mostly localized during the nighttime (not shown).

These results offer systematic proof of principle regarding the activity/sleep deficits of the mutants and establish the experimental and analytical methodology and parameters necessary to be assessed to evaluate potential pharmacological rescue of the activity/sleep deficits. Experiments with the R1809C mutation are currently in progress.

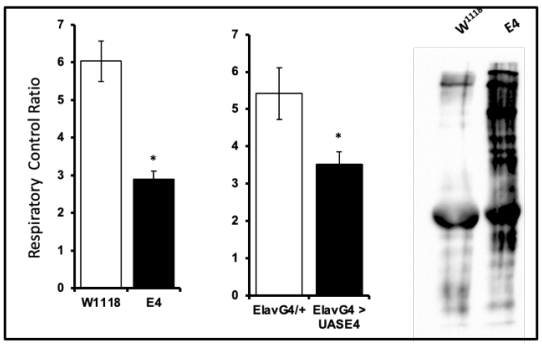


The C1045Y (E4) point mutant however presents highly unusual behavior, being relatively hypo active, but also registering marginal levels of sleep, possibly a reflection patient reports of extreme fatigue and fragmented sleep (Leschziner, Golding et al. 2013, Vassallo, Mughal et al. 2020). This deficient respiration phenotype is also present in the CNS of otherwise wild type animals for Nf1, but expressing throughout their CNS a UAS Nf1 transgene bearing the C1045Y mutation (UASE4). In fact, pan-neuronal expression of this transgene resulted in significantly reduced sleep in animals with 50% reduction in the dosage of the wild type protein, but not in animals retaining two wild type copies (not shown). These results present further evidence of distinct mechanistic effects of these mutations on a number of neuronal circuits.

One potential explanation for these difference and phenotypes might be consequent of the mitochondrial deficit that appears specific to the C1045Y (E4) mutant and has not been reported yet for any Nf1 mutation. A transgenic mitochondrial-targeted GFP (Mito GFP) is significantly reduced in the C1045Y mutant either by western blot (left panel) or by confocal microscopy of the entire brain of E2 nulls versus the C1045Y mutants.

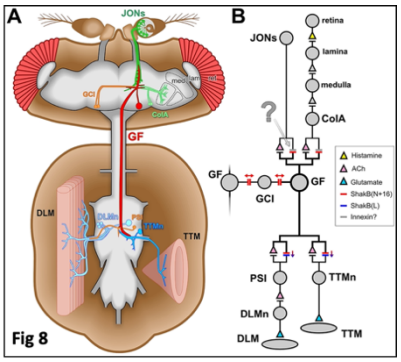


This indicates significantly reduced mitochondria and this could be either due to reduced biogenesis or increased mitophagy. Nevertheless, estimation of mitochondrial functionality/ respiration of brain mitochondria shows significant reduction in the C1045Y mutants (below) and highly elevated oxidized proteins in their brain lysates. We are planning to explore further this novel phenotype and investigate whether it underlies the unique cognitive and behavioral deficits associated with this mutant. For example, we will inhibit mitophagy in the mutant and



initially examine whether the sleep and mobility phenotypes are ameliorated as the easiest and fastest phenotypes to analyze. The effects of the R1809C mutation appear marginal if any at all, but the respiration analysis in these mutants is still ongoing. It should be noted however, that null mutants do not present respiration differences and as seen above the mitochondrial abundance-dependent GFP signal is not affected. Hence we have predicted that R1809C mutant will also not present any differences, but this will have to be verified.

Electrophysiology: The system is based on the visually (predator) evoked response that results in jumping and initiating flight and its well detailed and studied circuitry is detailed in *Fig 8*. Visual input evokes the sequential activation of apparently cholinergic neurons in the fly visual system. A bundle of columnar ColA interneurons, output to the bilaterally symmetrical pair of giant fiber (GF) interneurons. GF follower neurons are the tergotrochanteral motoneurons (TTMns) that innervates the TTM, jump muscle and the peripherally synapsing interneuron (PSI), which outputs to five dorsolongitudinal muscle motoneurons (DLMns). DLMns supply the large indirect dorsal longitudinal muscles (DLMs). Electrical stimulation of the eye, emulating the shadow of a predator/danger, drives activation of the TTM and DLMs where the response is measured. What we measure typically are the following four functional parameters:

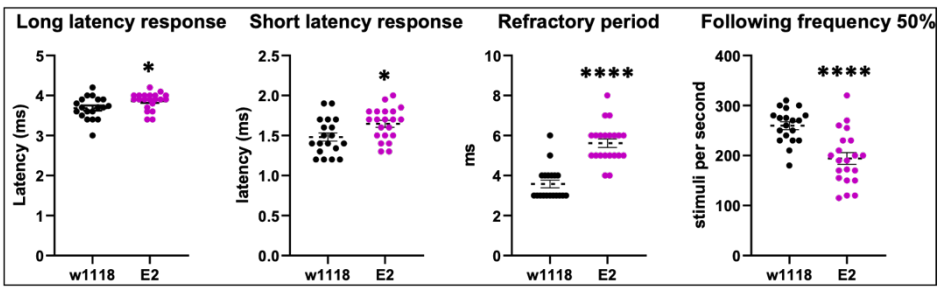


(1) **Short latency response (SLR)**, an estimation of the time required for the signal initiated by electrical stimulation in the GFs to activate the target muscle

(2) **Refractory period (RP)** is a measure of the minimum time interval between a pair of stimuli that can successfully generate corresponding muscle responses.

(3) **Following frequency 50% (FF50)**, a measure of GFS synaptic fidelity, tested by recording the number of evoked muscle action potentials in response to three high-frequency stimulations.

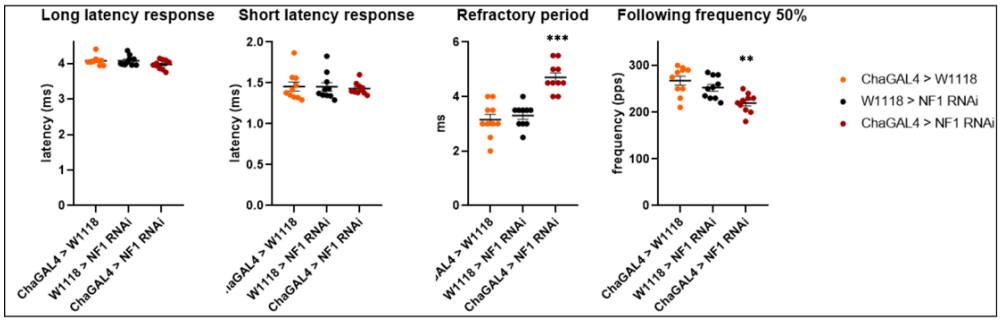
(4) For **long-latency responses (LLRs)**, GF neurons were activated synaptically by indirect GF stimulation. Their activation is achieved by low strength (7–9 V) electrical pulses delivered through electrodes positioned in more external eye layers. The LLR is a plastic response and subject to habituation.

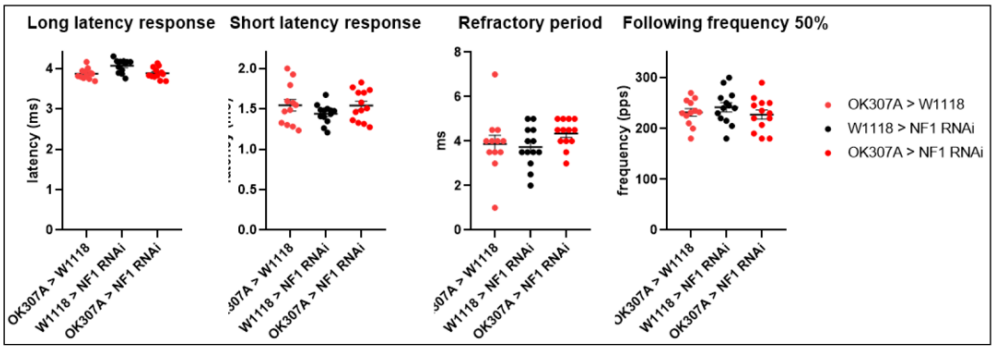


Because electrophysiological characterization of the GF system has not been described before in Nf1 mutants, we initiated this work by investigating the above functional properties initially for the nulls. In fact, the null mutants present a slightly

longer LLR latency than controls (p=0.023) and SLR (p=0.018), but a highly significant increase in refractory period ($p=1.9x10^{-8}$) and a significant decrease in synaptic fidelity ($p= 4.3x10^{-5}$) indicating synaptic dysfunction. The number of animals assessed are greater than 20 in all assays.

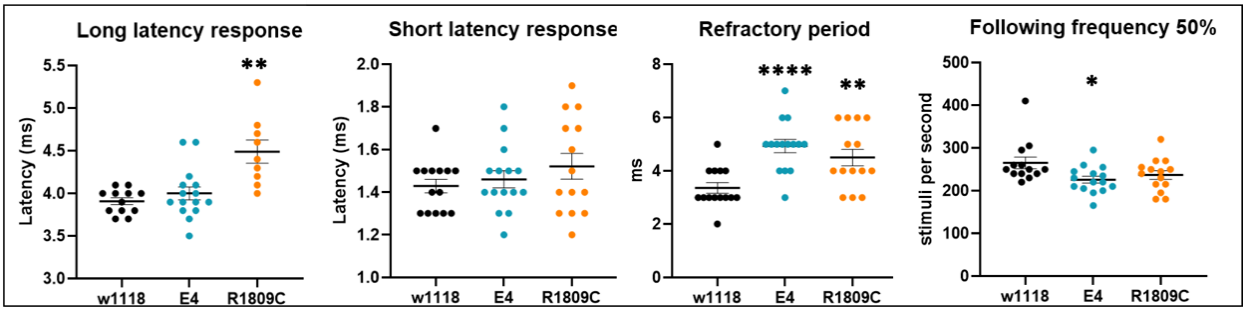
Because the escape response is thought to be mediated by cholinergic neurons, we Nf1 within this neuronal subpopulation using an RNAi encoding transgene. Consistent with the results with the null mutant above, Nf1 attenuation within Cholinergic neurons results in highly significant increase in the Refractory period and significant reduction in the FF50%. In contrast as seen below if Nf1 is abrogated in the Giant Fiber neurons, which are not cholinergic, none of these neurophysiological parameters are affected.





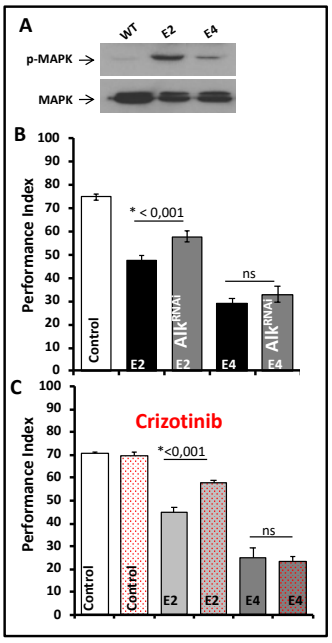
Therefore, Nf1 loss from the cholinergic neurons of the GF escape circuit system at least, results in significant defects in excitatory synaptic transmission. The results are consistent with the interpretation that evoked neuronal activity is affected. This expands significantly our prior findings that Nf1 loss apparently results in enhanced GABAergic activity in particular neurons presynaptic to the Mushroom Bodies, thus affecting associative learning.

Differentiating the other two mutants, LLR was significantly (p=0.0002) increased in the R1809C, but not



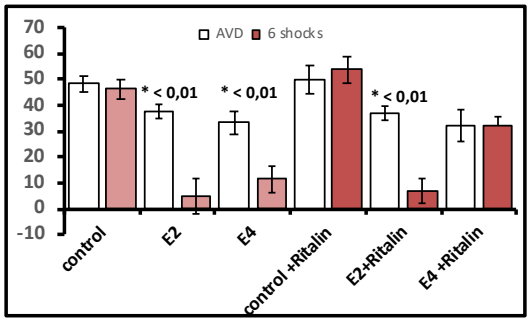
C1045Y(E4) flies and the SLR was unaffected in both. The refractory period was significantly lengthened as for the nulls (p=0.0002 for C1045Y and 0.0065 for R1809C). In contrast to the large decrease in FF50 in the nulls this property was marginally reduced in C1045Y(E4) (p=0.023) but not in R1908C (p=0.1242). These findings once more differentiate the point mutants supporting the notion that they affect different aspects of Nf1 function with respect to the firing properties of Cholinergic neurons. *Significantly, these are the first data on neurophysiology of the adult CNS in Drosophila Nf1 mutants.*

Significant progress on the C1045Y mutant. Briefly as a reminder, C1045Y is a novel developmental dominant mutation that acts as a "gain of function" possibly by negatively affecting dNf1 interactions with other cellular proteins. It is also significant that the E4 mutation is in the amino terminal side of the GAP domain, in a largely uncharacterized, but highly conserved region of the protein in the vicinity of the tubulin-binding domain (TBD), suggested to participate in protein-protein interactions (Anastasaki, 2022).This mutation does not affect GABAergic neurotransmission like the loss of function allele E2 and the learning deficit of C1045Y homozygotes is not rescued by decreasing GABA production in mushroom body efferent neurons. It has a marginal effect on



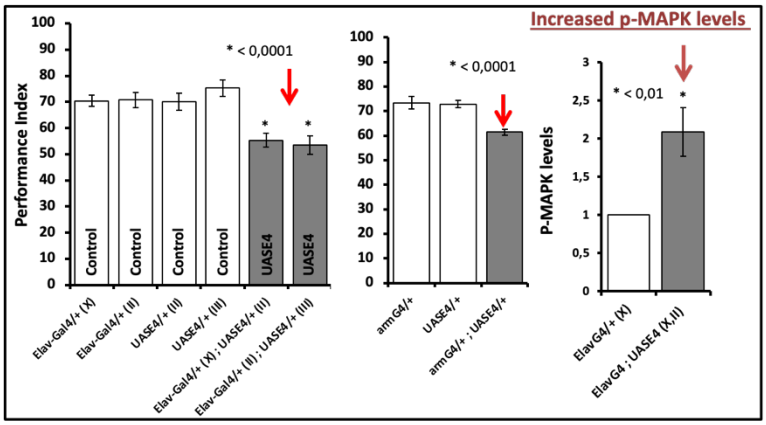
MAPK reregulation compared to the E2 null (panel A in the adjacent figure), and unlike the null it does not respond to genetic (panel B) or pharmacological (panel C) inhibition of the pathway via the Receptor Tyrosine kinase Alk.

To determine potential reasons for the strong learning deficit of the C1045Y mutants, we investigated their habituation properties using the footshock habituation assay we have developed (Acevedo, Froudarakis et al. 2007, Roussou, Papanikolopoulou et al. 2019). Wild type flies typically habituate (reduce their avoidance) after 12-15 45 Volt footshocks (Acevedo, Froudarakis et al. 2007, Roussou, Papanikolopoulou et al. 2019), with avoidance remaining at naïve levels after 1- 10 such stimuli. Premature habituation likely underlies attention deficits by reducing the importance of the recurrent stimulus (Acevedo, Froudarakis et al. 2007, Roussou, Papanikolopoulou et al. 2019), **which is a typical symptom associated with NF1** (Nix, Blakeley et al. 2020).Indeed, as evidenced in the adjacent figure, shock avoidance (open bars) is not altered by prior exposure to 6 shocks in controls (lightly colored bars), but it is in both E2 null and C1045Y mutants, suggesting attention deficits.



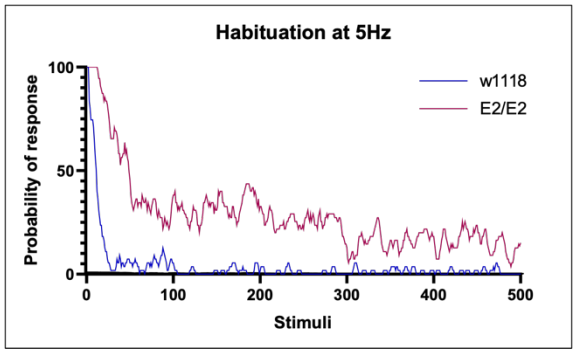
To attempt reversal of this deficit we acutely (4 hrs prior to testing) and C1045Y (E4) mutants, suggesting attention deficits. To attempt reversal of this deficit we acutely (4 hrs prior to testing) fed controls and mutants 2mM Methylphenydate (MPh) typically used to treat individuals with Attention Deficits (ADD and ADHD). The drug did not affect controls, rescued the premature habituation of the C1045Y (E4) mutant, but not of the null E2 (Fig2). As MPh acts mostly as a dopamine and norepinephrine (octopamine in the fly) reuptake inhibitor these results agree with the reduced dopamine hypothesis in NF1 (Nix, Blakeley et al. 2020), but also clearly reflect the symptom variability in patients. However, MPh treatment did not rescue the E4 learning deficits (not shown) and this may be due to the fact that at least in the nulls the deficits underlying learning lies outside the mushroom bodies (Georganta, Moressis et al. 2021), while these neurons are essential to mediate proper habituation (Acevedo, Froudarakis et al. 2007, Roussou, Papanikolopoulou et al. 2019).

To ascertain the dominance of the C1045Y mutation we drove a UASNf1 transgene bearing this mutation (UASE4) throughout the fly, UASE4 expression under two different pan-neuronal drivers resulted in animals harboring significant learning deficits and elevated phosphoMAPK levels, an indication hyperactivation of the Ras/MPK signaling cascade. Because expression of the mutant protein along with the wild type results in a (mild) mutant phenotype, **the results suggest that Nf1 protein likely forms dimers** in accord with published *in vitro* data (Young *et al* 2023). In this scenario the C1045Y mutant protein must dimerize with its wild-type counterpart and render it dysfunctional, a hypothesis we are currently pursuing. However, expression of the transgene specifically in Cholinergic, Dopaminergic, or GABAergic neurons does not precipitate learning deficits further strengthening the notion that this mutation has distinct effects on neuronal function that loss of the Nf1 protein.

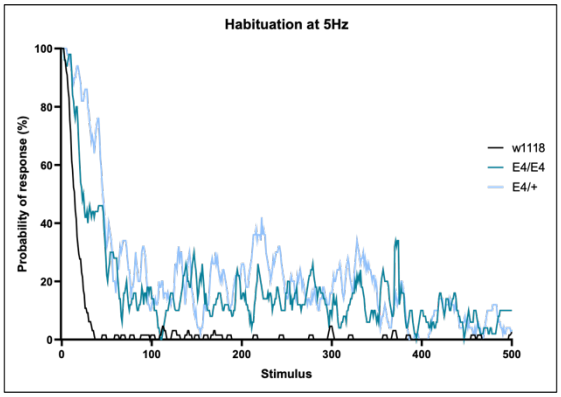


Development and results of habituation in the giant fiber system.

To probe further the habituation deficits of Nf1 mutants we developed electrophysiological protocols of habituation in the giant fiber system. To induce habituation in this circuit, a set of 100-1000 stimuli with an intensity near the top of the LLR range (0.5-1V below the upper threshold) with a frequency of 2Hz, 5Hz or 10Hz is delivered to the fly. The responses to all 100-1000 stimuli/ if the fly responded or not to a specific stimulus are counted and the probability of response per stimulus defined as the number of animals that respond at a certain stimulus. If habituation occurs the probability of response should wane with increasing stimulus number.



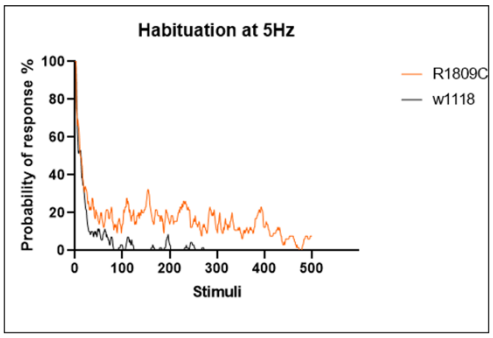
This is demonstrated for control (w1118) and the null animals for stimulation frequency at 5Hz. It is obvious that compared to controls the null animals present highly significantly (Multiple row t-tests Holm-Sidak method, all p's< 0.000001) **delayed habituation** indicating that despite their decreased FF50, they continue synaptic transmission long after control GFs have ceased to activate the muscles, suggesting hypersensitivity.

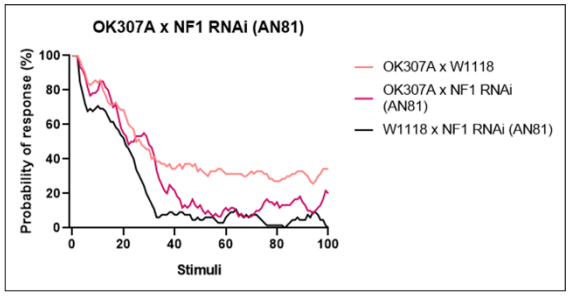


As expected, this is exaggerated at the lower frequency of 2Hz, where a strong deficit also emerged for mutant heterozygotes.

It is also obvious that both C1045Y(E4) homozygotes and heterozygotes present delayed habituation at 5Hz, with the homozygotes presenting the stronger deficit as expected. The phenotype is consistent with the proposed hypersensitivity defect.

However, a much milder phenotype is evident with the R1809C mutants. Collectively, the electrophysiology proof of principle experiments have uncovered a novel phenotype and easily interpretable results based on the extensive knowledge of the circuitry and the neurotransmitters that drive the response. We hypothesize that the delayed habituation phenotype in the Giant Fiber system is driven by the Cholinergic system upstream of the Giant Fiber Neuron itself. In fact, RNAi - mediated attenuation of Nf1 in Cholinergic neurons phenocopied the delayed habituation phenotype of the null mutants. This most likely underlies deficits in evoked neurotransmission or as those seen in the basal characterization of the system (see above), or more likely sustained tonic activity, which attenuates cessation of baseline cholinergic activity necessary for habituation. In contrast, mutants habituate faster (prematurely) in the behavioral footshock assay and this phenotype is consistent with the interpretation of hyperactive (most likely tonicaly) mushroom body neurons, which we have shown drive habituation and are cholinergic (Roussou 2019, Foka 2022). These deficits are amenable to acutely delivered pharmaceutical amelioration because the effect id quantitative and does not require a large number of animals to obtain significant results.

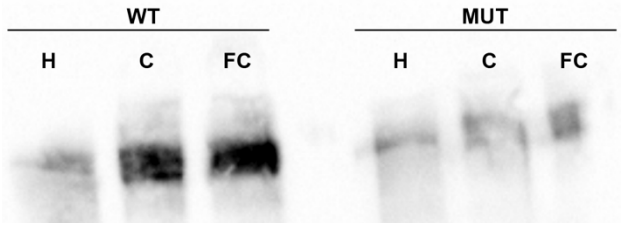




- Subtask 2: Breeding and characterization of the **R1809C** mouse.

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.

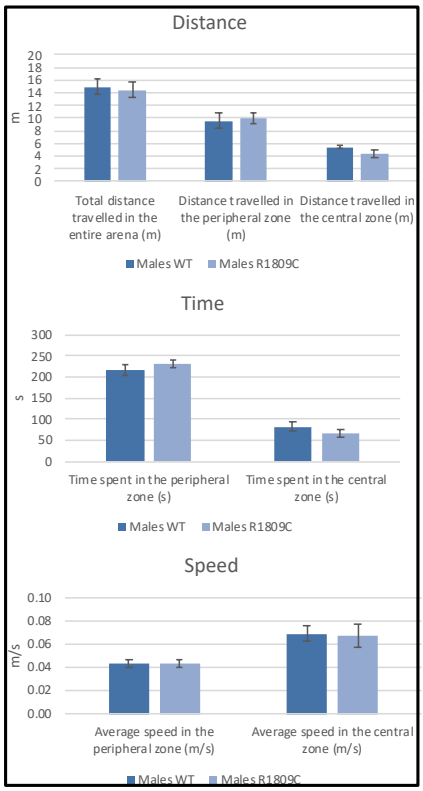


<u>Ameliorative measures:</u>

1. We performed a new round of mutagenesis that yielded two animals harboring the mutation, but now the sequencing results were ambiguous for one and consistent with the sought change in the other, although further characterization is needed by cloning fragments form founders and progeny and sequencing again. *Both new mutant lines are homozygous lethal* as was the original. The present no obvious macrophenotypes (smaller size, aberrant mobility, aggression, fertility) otherwise.

We selected the line with the most consistent with the sought change and after backcrossing for three generations it is expanded to obtain enough animals for behavioral assessment. We have standardized all behavioral assays in the meantime using their genetic control animals (see below).

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. <u>However, due to COVID and other difficulties at the donor lab, processes to ensure the transfer are just under way</u>. This will serve as a back-up of our own mutant mouse 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 contrast, the Gutmann mutant is in a different genetic background, and it will be necessary to go through time consuming backcrosses.



The mouse colony with the Gutmann R1809 line has been established at Fleming and has been expanded to obtain enough animals to set up cohorts for behavioral experiments. This is complicated somewhat and introduces additional delays and expense because the mutant heterozygotes are identified only by sequencing of the genomic fragment encompassing the change. Sequencing of all animals to date indicates that mutant heterozygotes comprise about 50 % of the pups born, which is a deviation from the 66% that is the expected number. This indicates a small degree of lethality associated even with the heterozygotes.

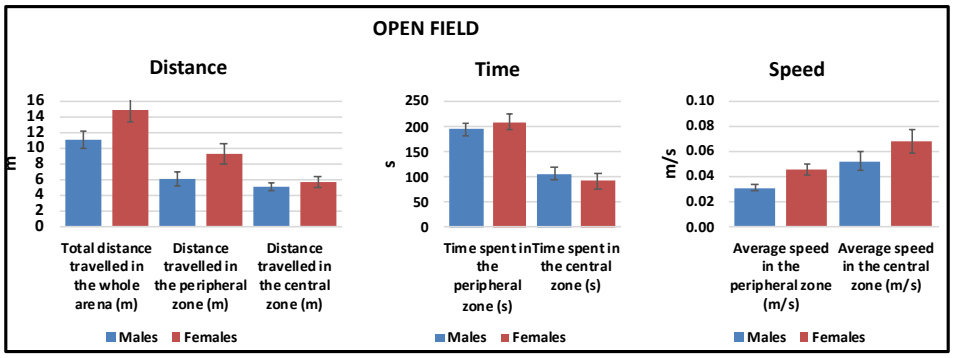
At the moment, the colony is expanded enough to expect an adequate cohort of mutant heterozygotes and their control siblings to be

able to perform the biggest part of the planned behavioral experiments. As seen below (Subtask 3) all behavioral experiments have been satisfactorily benchmarked and therefore the experiments are expected to proceed smoothly.

An initial small cohort of R1809/+ and their controls was interrogated for basal activities required to be within control levels for the cognitive behavior experiments to be meaningful. As seen in the figure all open field activities, thigmotaxis and speed are not different in male mutant heterozygotes and controls. An identical profile was revealed for female animals which is not shown below for economy of space.

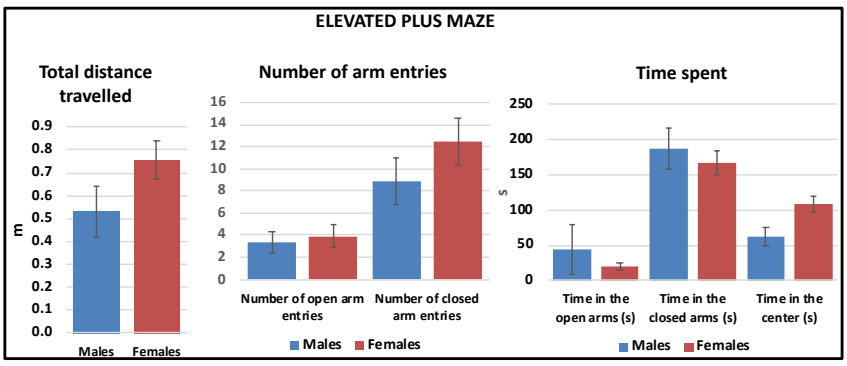
- Subtask 3: Characterization of Nf1 null mouse cognitive behaviors.

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, fear conditioning. and 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. The bechmarking results are presented below for both sexes as indicated.

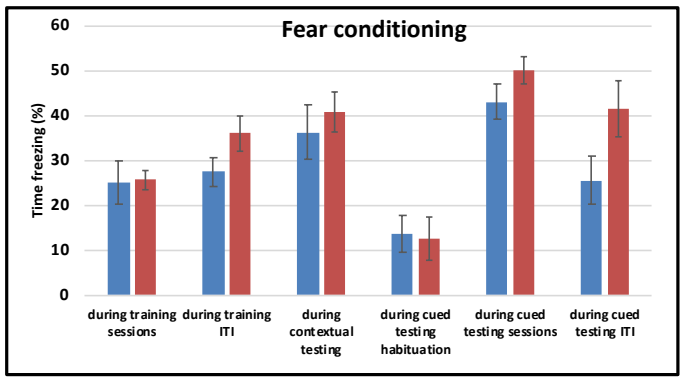


Open field activity measures exploratory activity, and ambulatory ability (Distance travelled and speed in the Different compartments) in addition to fear and anxiety (time spent in the center of the field).

Video recordings and decoding of behavior in novel cages were employed to report the performances in the adjacent figure. We can use this assay to also monitor sleep fragmentation using cages with multiple beams and video recording that we possess.



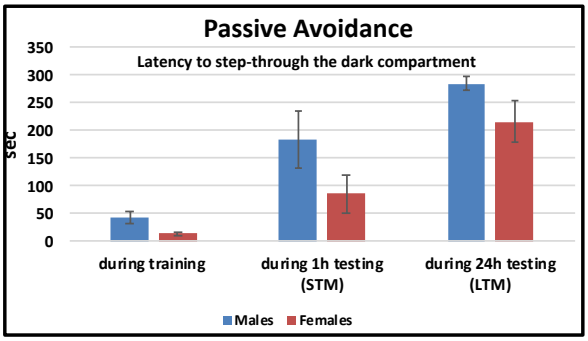
Elevated plus maze. A cross shaped maze with two open arms and two closed arms, which is elevated above the floor. This task exploits the conflict between the innate fear that rodents have of open areas versus their desire to explore novel environments and uses video tracking for recording. The values reported emphasize the fear of the mice for open exposed spaces with marginal sex-specific differences in control animals.



Fear conditioning: is used for measuring aversive learning and memory. A neutral conditioned stimulus (CS) is a steady tone and is paired with mild foot shocks as the aversive unconditioned stimulus (US) After conditioning, the spatial context or the CS (tone) elicits a central state of fear in the absence of

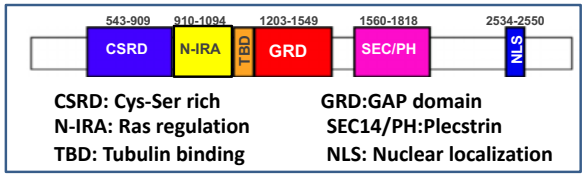
the US (shock) that is expressed as reduced locomotor activity or total lack of movement (freezing) which we use as a measure of hippocampus-dependent learning/memory.

Passive avoidance test: This assay is used to assess learning/memory of the consequences of the subject's actions and assays functionality of the amygdala-hippocampus circuit. The apparatus is composed by a black poorly illuminated compartment and a white illuminated compartment. For conditioning, a mouse is placed in the lit (white) compartment and when it innately crosses to the black compartment it receives a mild foot shock. For testing at various later times the mouse is placed in the lit compartment and its latency to escape to the dark compartment, in other words to passively avoid it, measures memory of the punishment in that compartment.



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

  Although molecular cloning of Nf1 is very challenging due to rearrangements because of its large size and the well-known toxicity of the cDNA, we managed to generate wild type clones in the typical vector used for transgenesis. However, because unlike the C1045Y, the R1809C mutants did not present a dominant phenotype, dominant phenotypes because of transgene expression would not be expected. Therefore, we opted not to spend time and resources on the planned mutagenesis and eliminate this task. Instead to opted to generate additional CrispR-generated mutants as outlined above. Namely the **M1149V** and **M1035R** mutations, which are currently in progress of getting generated. Once generated we will have the null mutation, 2 different mutations in the N-IRA domain (C1045Y and the Leopard syndrome - associated M1035R), M1149V in the tubulin binding domain (TBD), L1423E in the GAP related domain (GRD) and R1089C in the sec14/PH domain <u>all associated with preferential cognitive deficits over tumours in patients</u>.



  This should provide a significant entry point to structure/function analysis of these domains with respect to cognitive and behavioural deficits.

- Subtask 5: Isolation of fly M1035 mutant: **In progress**

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
  See above regarding R1809C results to date.
  See above regarding generation of the M1035R mutant

- Subtask 2: Learning experiments with M1035R and R1809C transgenics
  We have eliminated this subtask as detailed above

- Subtask 3: Fear conditioning and passive avoidance experiments with mouse mutant and nulls

We established the benchmarks for controls as detailed above and we also have benchmarked the active avoidance task in case we need it to monitor amygdala functionality in the mutant.

At the moment the colony is expanded enough to expect an adequate cohort of mutant heterozygotes and their control siblings to be able to perform the biggest part of the planned behavioral experiments. As seen below (Subtask 3) all behavioral experiments have been satisfactorily benchmarked and therefore the experiments are expected to proceed smoothly. We anticipate that there will be enough animals to complete the experiments in early fall 2023.

- Subtask 5: Brain lysates for MAPK blots and initial proteomic samples

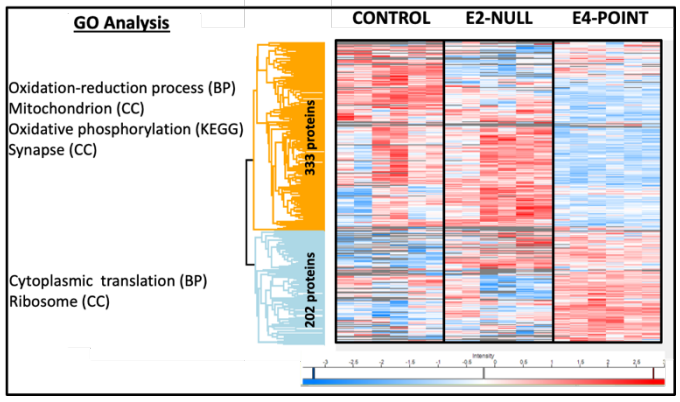
  Nothing to report yet, since these depend on completion of tasks from aim 1.

  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 (*Georganta, E-M, Moressis, A. and Skoulakis, EMC (2021). "Associative learning requires Neurofibromin within a novel neuronal circuit to modulate GABAergic inputs to Drosophila Mushroom Bodies. **J Neurosci.** 41(24) 5274-*5286) the recent biannual Neurofly-European Drosophila Neurobiology meeting (attached poster presentation).

  We do assess MAPK levels in all Drosophila novel mutants we generate (see above) and we have initial assessment of the mouse strains in hand.

***Proteomic analysis and identification of potential interacting or dysregulated proteins relevant to the observed phenotypes*.**

We have presented in the grant proposal an extensive set of proteomic results from brain lysates of the null, C1045Y(E4) homozygotes and control flies. These are presented for reference below:



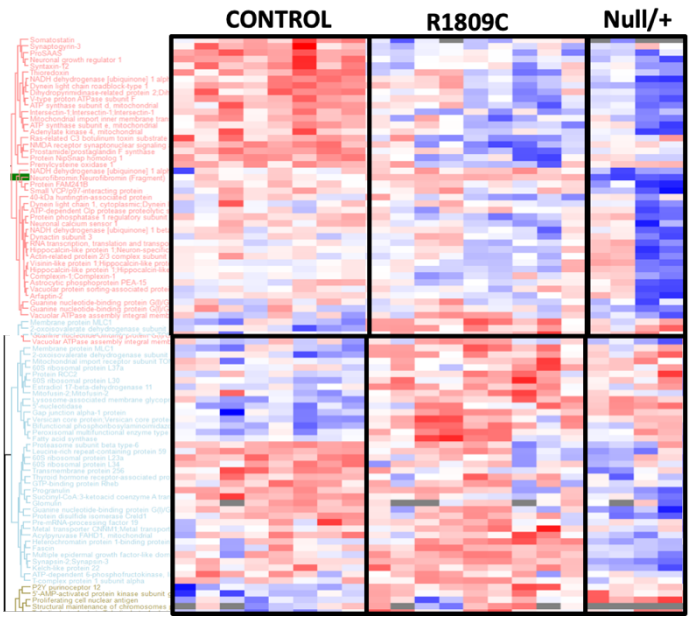
The differences among the mutant genotypes are obvious as are the commonly deregulated proteins compared to controls.

A significant group of proteins, which were in part validated already by the data above, are the mitochondrial proteins, which are specifically downregulated in the C1045Y(E4) homozygotes. Also significantly, and in agreement with the notion that normal synaptic transmission is disrupted in the mutants, proteins that function in regulated neurotransmitter release are dysregulated such as Synaptojanin, Synaptobrevin (nSyb), Syntaxins, Synapsin, Snap25, Snap24 among others. These results make specific predictions regarding genetic interaction and measures to ameliorate the behavioral phenotypes. Proteomic analyses in the R1809C mutant are currently ongoing. In addition, distinct proteins differed both in abundance in C1045Y(E4) homozygotes versus controls and some were different when comparing C1045Y(E4) homozygotes and nulls. In fact, some of these differences involved the synaptic protein mentioned above as well as channels and enzymes required for neurotransmitter synthesis and transport.

One of the likely relevant interesting proteins which is upregulated in the C1045Y mutant and the result has been verified by Western blots is the VAMP-associated protein 33kD (Vap33) which plays a conserved role in synaptic homeostasis, synaptic growth and axonal transport, including neurotransmitter secretion and synaptic vesicle priming (Lloyd *et al* 2000)

To determine whether analogous changes occur in the mouse model, hippocampi from control and mutant heterozygotes were isolated and subjected to proteomic analyses. All samples were taken from the right

hippocampus and the ones shown below are from comparison of hippocampi from male mice, but results were similar in females as well. In this case we also concentrated on changes in the R1809C mouse relative to controls and heterozygous null mutants.



Again the most prominent changes between controls and null heterozygotes involved changes in synaptic proteins (including Synaptogyrin, Syntaxins, Snaps, Synapsins and others. In addition, mitochondrial, but also ribosomal proteins appeared downregulated, suggesting dysregulation at the level of hippocampal energetics and translation, potentially underlying the behavioral phenotypes reported for these mutants.

Significantly, although the hippocampal proteomic profiles of the R1809C heterozygotes appear to fall in line more with the null heterozygotes, there were distinct differences with both controls and null heterozygotes apparent in the accompanying figure, but also in mitochondrial and ribosomal proteins, which appeared downregulated.

We are planning to repeat these experiments with another set of independent samples. Nevertheless, common patterns of dysregulated proteins appear in flies Nf1 null and mice null heterozygotes and initial results suggest similar patterns of change in R1809c heterozygous mice and flies homozygous for the mutation.

In future studies, we are planning to follow up on common proteins and after initial functional characterization in Drosophila, to investigate the mechanistic effects on these proteins of Nf1 loss and the point mutations we are focusing on herein that disrupt learning, sleep and the electrophysiological properties of affected neurons.

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.

Although the graduate student has been supported via another source, all necessary consumables for her project are sourced through this grant.

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

The postdoctoral researcher Dr Georganta just published a major paper as a first author, presented her results in two Major Neurobiology meetings and local meeting of human geneticists where she received a price for her work on Nf1.[Links: https://www.sige.gr/4o-synedrio-sige/ ; https://www.hsfn.gr/meetings/hsn2021/ ; https://neurofly2020.com/].

Dr Georganta was on maternity leave for 6 months during this grant period, but has returned and is leading the effort to publish the findings of this project.

In addition, we hired on a part time basis two early career graduate students for 3 months each and they were exposed to the field, the questions asked and read and presented relevant literature and performed experiments. Unfortunately, due to COVID-related restrictions they left the lab, but one them now has returned and is starting a PhD on the subject funded initially at least by this project.

Moreover, side-projects anchored on this main project have provided training for three undergraduate summer students.

In fact, undergraduate students performed initial pilot experiments on activity and sleep, which we are now capitalizing upon. We have a current Master's student investigating the respiration phenotype for his thesis and another Master's student performing his laboratory rotations on the particular project.

Moreover, we were able to hire a technician that performed the electrophysiology and is training current graduate and Master's students.

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.

  We plan to follow the SOW as detailed in the grant, but we will implement the changes in the SOW presented above

Aim 1:

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

  Colonies of R1809C and null heterozygotes have been established and expanded.

- Subtask 3: Characterization of Nf1 null mouse cognitive behaviors

  Experiments with null and R1809C heterozygotes are initiated and expected to be completed in the next few months

- Subtask 4: **ELIMINATED** .

Aim 2:

- Subtask 1: Learning experiments with M1035R, M1149V and L1423E fly mutants.
  Premature habituation assessment of all mutants
  Sleep fragmentation of all mutants
  These experiments are "on hold" since we did not manage to obtain such mutants. We will repeat the mutagenesis as we posit that these mutants are essential to our understanding of phenotype/genotype effects.

- Subtask 2: **ELIMINATED**

- Subtask 3: Fear conditioning and passive avoidance experiments with mouse mutant and nulls
  Though these experiments have not been initiated yet, as seen above all equipment has been calibrated and procedures benchmarked with the control strain we intent to use for the experiments with the mutants. All values are within accepted ranges
  See above.

- Subtask 5: Brain lysates for MAPK blots and initial proteomic samples
  We only have collected lysates from cortex and hippocampi of mutant and control animals and currently are kept in storage awaiting verification of the mutant status of the animals we have in hand.
  One round of experiments performed and analyzed.
  A second round to confirm the results independently is planned. Confirmation of "hits" is also planned for proteins where antibodies are available (ie synaptic proteins).

References

Acevedo, S. F., E. I. Froudarakis, A. Kanellopoulos and E. M. C. Skoulakis (2007). "Protection from premature habituation requires functional mushroom bodies in Drosophila." Learn Mem **14**(5): 376-384.

Anastasaki, C., M. Wegscheid, K. Hartigan, J. Papke, N. Kopp, J. Chen, O. Cobb, J. Dougherty and D. Gutmann (2020). "Human iPSC-Derived Neurons and Cerebral Organoids Establish Differential Effects of Germline NF1 Gene Mutations." Stem Cell Reports. **14**(4): 541-550.

Foka, K, Georganta EM, Semelidou, O and Skoulakis EMC (2022) Loss of the Schizophrenia-linked Furin protein from Drosophila mushroom body neurons results in antipsychotic-reversible habituation deficits. J Neurosci. **42**(39):7496-511

Georganta, E., A. Moressis and E. Skoulakis (2021). "Associative Learning Requires Neurofibromin to Modulate GABAergic Inputs to Drosophila Mushroom Bodies." J Neurosci. **41**(24): 5274-5286.

Gouzi, J. Y., A. Moressis, J. A. Walker, A. A. Apostolopoulou, R. H. Palmer, A. Bernards and E. M. C. Skoulakis (2011). "Drosophila Alk Tyrosine Kinase Controls Neurofibromin dependent Growth and Learning." PLoS Genet **7**(9): e1002281.

Leschziner, G., J. Golding and R. Ferner (2013). "Sleep disturbance as part of the neurofibromatosis type 1 phenotype in adults." Am J Med Genet A. **161A**(6): 1319-1322.

Lloyd, T.E., Verstreken, P., Ostrin, E.J., Phillippi, A., Lichtarge, O., Bellen, H.J. (2000). A genome-wide search for synaptic vesicle cycle proteins in Drosophila. Neuron **26**(1): 45--50.

Nix, J., J. Blakeley and F. Rodriguez (2020). "An update on the central nervous system manifestations of neurofibromatosis type 1." Acta Neuropathol. **139**(4): 625-641.

Roussou, I., K. Papanikolopoulou, C. Savakis and E. Skoulakis (2019). "Drosophila Bruton's Tyrosine Kinase Regulates Habituation Latency and Facilitation in Distinct Mushroom Body Neurons." J Neurosci. **39**(44): 8730-8743.

Silva, A., P. Frankland, Z. Marowitz, E. Friedman, G. Laszlo, D. Cioffi, T. Jacks and R. Bourtchuladze (1997). "A mouse model for the learning and memory deficits associated with neurofibromatosis type I." Nat Genet. **15**(3): 281-284.

Vassallo, G., Z. Mughal, L. Robinson, D. Weisberg, S. Roberts, E. Hupton, J. Eelloo, E. Burkitt Wright, S. Garg, L. Lewis, D. Evans and S. Stivaros (2020). "Perceived fatigue in children and young adults with neurofibromatosis type 1." J Paediatr Child Health. **56**(6): 878-883.

L. Young, R. Goldstein de Salazar, S.-W. Han, Z. Huang, A. Merk, M. Drew, Darling, J, Wall, V, Grisshammer, R, Cheng, A, Allison, MR, Sale, MJ, Nissley, DV, Esposito, D, Ognjenovic, J, McCormick, F (2023). Destabilizing NF1 variants act in a dominant negative manner through neurofibromin dimerization. Proc Natl Acad Sci U S A 2023 Vol. 120 Issue 5 Pages e2208960120

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.

We have unveiled significant findings regarding neuronal function with the electrophysiology studies in adult flies. We have characterized for the first time to our knowledge deficits consistent with altered synaptic activity in the adult CNS of Drosophila and a significant effect on one novel point mutation on mitochondria number and/or activity. In addition, the behavioral and electrophysiological data on habituation provide a model of attention deficits resented by NF1 patients, which we intent to explore further and screen for pharmaceuticals that may ameliorate the defects. We also anticipate that our sleep analysis emulates another common symptom in patients, that of sleeplessness, especially at night, which we will also explore pharmaceutically for amelioration of symptoms. It should also be noted that although we have not succeeded in generating 3/4 CrispR mutations we aimed to, these mutants are the pioneering ones in a future series of such mutations necessary for a much structure function approach to Nf1. This is expected essential to reveal the hypothesized multiple functions Nf1 appears to be involved in.

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. With the new findings on the effect of at least one Nf1 mutation on mitochondrial function, we anticipate this will impact the Rasopathy field, but also broader, the field of cognitive and behavioral deficits and diseases.
The electrophysiology approach paves the way for similar approaches regarding synaptic activity in the CNS of additional fly models of cognitive deficits, such as Fragile X syndrome, which we have already initiated in the lab.

Impact on Technology Transfer

Nothing to report yet, but we will be exploring task-specific ameliorative drugs in the near future.

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.
The fact that at least one mutation C1045Y is not ameliorated by Alk inhibitors strongly underlies the notion that Nf1 point mutations may in fact affect distinct subsets of the processes the NF1 protein is engaged in, most likely even in a cell type specific manner.

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 **(June 2022**) the recruitment of a graduate student for the project.
We reported delays due to departure of Graduate students and recruitment of new ones. We reported delays due to the 6-month maternity leave of the leading postdoc.

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.
This is based on:
1. The COVID-related delays in the project
2. The difficulty in ascertaining the CrispR-mediated mouse mutation and actually obtaining the alternate mouse from the Guttman Laboratory due to COVID restrictions
3. The leading postdoc Eirini Georganta will be on maternity leave starting June 1 2022. Part of her work will be covered by the newly recruited grad student, but it is unlikely that she will be able to handle the bulk of required work to replace a senior specialized researcher.
We will update this request as time progresses.

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:	*Efthimios Skoulakis, PhD*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*000-0001-5113-6192*
Nearest person month worked:	*12*
Contribution to Project:	*Oversees work, organizes experiments and analyzes data. Prepares publications and reports.*

Name:	*Maria-Eirini Georganta, PhD*
Project Role:	*postdoc*
Researcher Identifier (e.g. ORCID ID):	*000-0001-5610-6144*
Nearest person month worked:	*6*
Contribution to Project:	*Generation of mouse and fly CrispeR mutants, behavioral and molecular experiments in both species, data evaluation and reporting. Graduate student oversight*

Name:	*Maro Loizou*
Project Role:	*Animal Technician*
Researcher Identifier (e.g. ORCID ID):	-
Nearest person month worked:	*12*
Contribution to Project:	*Mouse and fly husbandry, genotypic and molecular screening for mutants*

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

Name:	*Kalliopi Atsoniou*
Project Role:	*Graduate student*
Researcher Identifier (e.g. ORCID ID):	-
Nearest person month worked:	*1*
Contribution to Project:	*Generation of wild type and mutant transgenes for fly transformation. Behavioral analysis of mutant flies for sleep and activity.*

Name:	*Aglaia Pozantzi*
Project Role:	*Graduate student*
Researcher Identifier (e.g. ORCID ID):	-
Nearest person month worked:	*1*
Contribution to Project:	*Behavioral analysis upon Ras overactivation in flies.*

Name:	*Eleni Giannopoulou*
Project Role:	*Graduate student*
Researcher Identifier (e.g. ORCID ID):	-
Nearest person month worked:	*9*
Contribution to Project:	*Electrophysiology.*

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

8. Special Reporting requirements

NONE

9. APPENDICES and PRESENTATIONS

1. Manuscript

At the moment we are completing the last few experiments necessary to submit the following manuscripts:

1. One revealing the behavioral and electrophysiological habituation phenotype of null mutants, genetic and pharmacological reversal and involvement of the Ras cascade.
2. One revealing the behavioral and proteomic effects of the novel mutation C1045Y in comparison to the null, its effects on mitochondrial function.
3. An interspecies study of the effects of R1809C in drosophila and the mouse

2. Poster presentation

   We are planning to present two poster presentations in the upcoming International Hellenic Society of Neuroscience meeting (Nov 23)

3. Talks in English

   The postdoctoral scientist is scheduled to present an oral presentation in the upcoming International Hellenic Society of Neuroscience meeting (Nov 23)