National and Kapodistrian
UNIVERSITY OF ATHENS

"ALEXANDER FLEMING"
Biomedical Sciences Research Center

Neuron specific functions of neurofibromin in learning & memory

Master Thesis

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M. Sc. Program: Molecular Biomedicine

ATHENS, Sept 2018-2019

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Acknowledgements

The experiments for the present dissertation thesis were conducted at the Skoulakis Lab, of the Neuroscience division of B.S.R.C “Alexander Fleming”. During this endeavor, I received valuable help and support from many people whom I would like to pay tribute to.

First of all, I wish to sincerely thank my supervisor, Research Professor Efthimios Skoulakis, thanks to whose guidance and support I managed to approach the required research subjects and successfully complete my experiments.

I would also like to express my gratitude to the Associate Professors Antonis Stamatakis and Christos Consoulas, for their eager participation in the Evaluation Committee of this thesis.

Special acknowledgments to the Postdoctoral Scientist Eirini Georganta, who has supported and guided me throughout my journey, for all the help and the implicit trust she put on me, as we collaborated on the project.

I could not omit to especially thank the Phd Candidate Ergina Vourkou as well, for all her valuable advice, support and patience.

Last but not least, I would like to warmly thank all the members of the Lab, for the perfect collaboration and the beautiful and intellectual atmosphere that was cultivated among us and made the every-day life in the lab very pleasant.

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1. Introduction

1.1. Neurofibromatosis Type 1 (NF1)

Neurofibromatosis Type 1 (NF1) is a common genetic disorder, affecting multiple organs, including the nervous system. It is an autosomal dominantly inherited syndrome that affects 1 out of 3000 individuals worldwide. It results from loss-of-function mutations in the *Nf1* gene that resides on human chromosome 17 (17q11.2). *Nf1* consists of over 60 exons and produces a differentially spliced mRNA that is expressed in almost every human tissue, though it appears highly abundant in the brain, the spinal cord and the peripheral nervous system (PNS).

Nf1 encodes a large cytoplasmic protein, Neurofibromin (Nf1), consisting of 2818 amino acids. Nf1 is widely expressed during embryonic development, while its adult expression pattern in neurons, Schwann cells and oligodendrocytes is established at least one week after birth (Wallace *et al.*, 1990; Yap *et al.*, 2014). Due to its large size, the *Nf1* gene exhibits high rates of mutagenesis, thus causing varying clinical symptoms in NF1 patients. Among several domains, it contains a cysteine-rich domain (CSRD), a leucine repeat domain (LRD) and a RAS-GAP domain (GRD) (Fig. 1).

Figure 1: Neurofibromin structure and function (Diggs-Andrews and Gutmann, 2013)

Nf1 is a ubiquitously expressed protein, which functions both as a RAS-GTPase Activating Protein [GAP-related domain (GRD)], a critical negative regulator of RAS activity (Xu *et al.*, 1990) and controversially as an activator of adenylyl cyclase (AC) (H. F. Guo *et al.*, 2000)(Fig. 2). In mammals, its role as a RAS- GAP has been most clearly implicated in the regulation of neuronal function. Loss of Neurofibromin in neurons results in constitutive increases in Ras intracellular signaling (Li, Cui, Steven A. Kushner, *et al.*, 2005) and has been shown to lead to decreased levels of cyclic adenosine monophosphate (cAMP) as well, both in glia and in neurons of animals but also patients (Guo *et al.*, 1997; The *et al.*, 1997; H.-F. Guo *et al.*, 2000). It should be noted, however, that although these studies support a functional relationship between Nf1 and cAMP levels, the exact mechanisms by which Nf1 regulates cAMP

levels and through them Protein Kinase A (PKA) activity and whether these are direct or indirect remains unclear.

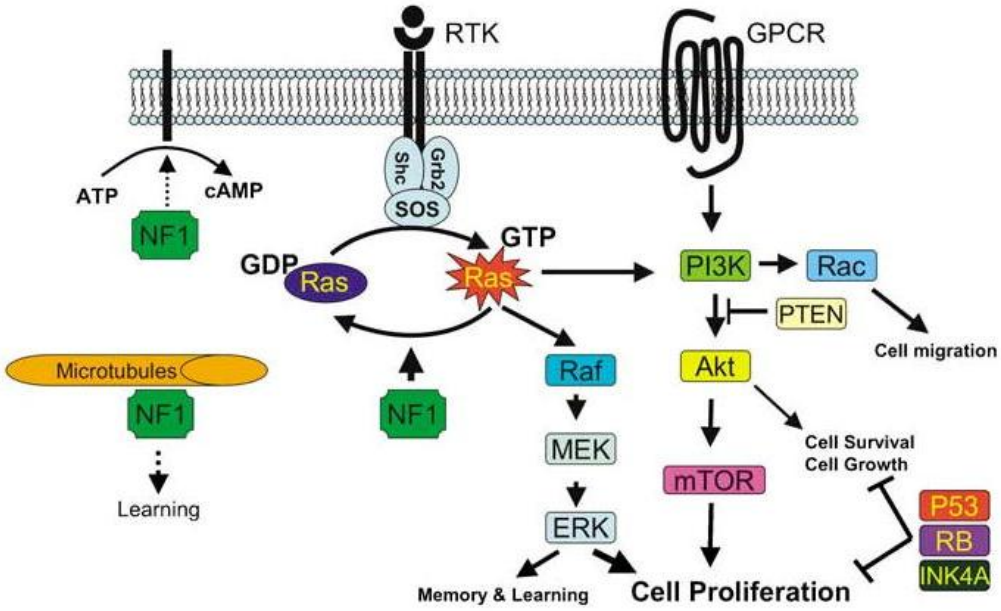


Figure 2: Representation of NF1 interactions with the Ras and PI3K pathways. NF1 constrains Ras activity in the normal cell by accelerating the hydrolysis of the GTP-bound active Ras, producing inactive GDP-bound Ras. Upon Receptor Tyrosine Kinase (RTK) activation, Ras guanosine exchange (GDP to GTP) promotes Ras activity. Activated Ras, in turn, stimulates its downstream effectors, including MEK/MAPK and PI3K/Akt/mTOR. These kinases then induce a number of downstream processes, including transcription of immediate early genes, which lead to short- and long-term changes in neuronal function. Loss of NF1 expression leads to elevated Ras activity, dysregulated cell growth and tumorigenesis. NF1 may also associate with microtubules and modulate the cAMP-PKA signaling pathway (Le & Parada, 2007).

NF1 is a multisystem chronic genetic disease associated with a variety of symptoms. A hallmark of NF1 is its variability and unpredictability. Thus, although there are some established criteria for NF1 diagnosis, patients typically only develop a subset of symptoms, and the severity of individual symptoms can vary dramatically between patients. Variable expressivity of a single-gene genetic disorder, like NF1, can be due to several different factors, including the nature of the culprit genetic defect, whether or not a patient is a somatic mosaic, and whether or not a given patient carries genetic modifiers or has been exposed to different environments. In the case of NF1, all these factors are believed to play a role. Thus, although

individuals carrying *Nf1* mutations develop at least some symptoms with complete penetrance, stochastic genetic or epigenetic events may result in partial penetrance of individual symptoms (Upadhyaya and Cooper, 2013) . Individuals affected by NF1 are heterozygous for the *Nf1* gene mutation, as homozygous mutations appear to be lethal (Friedman, 1999).

Patients exhibit a wide clinical spectrum, but the main phenotype of the disease is the development of benign (neurofibromas) and malignant tumors involving the peripheral and central (to a lesser extent) nervous systems. The latter constitute the main cause of morbidity and mortality. NF1 patients also present skin lesions with café au lait spots, skeletal and cardiovascular dysplasias and shorter stature (Lee and Stephenson, 2007; Monroe, Dahiya and Gutmann, 2017). Furthermore, 50-70% of children with NF1 manifest specific cognitive impairments, including deficits in executive functions, attention, language, visual perception, and learning as well as autism spectrum disorder-like traits (Shilyansky, Lee and Silva, 2010; King *et al.*, 2016). Thus, NF1 is considered the most common monogenic disorder affecting cognitive function. Although important progress has been noted to date in understanding the mechanisms implicated in the development of tumors involving Nf1 loss in the central and peripheral NS, whether the different cognitive deficits that NF1 patients exhibit, result from deregulation of one or more molecular pathways and if such a deregulation occurs in the same or different neuronal circuits remains obscure, resulting in lack of pharmaceutical treatment. Therefore, the potential contribution of the implicated molecular pathways to the cognitive deficits of Nf1-mutant individuals has to be studied in a more targeted fashion within specific neuronal circuits and requires use of animal models for their elucidation.

Animal models recapitulate some behavioral features of the NF1 phenotypic spectrum since they exhibit reduced performance in learning and memory assays (H.-F. Guo *et al.*, 2000; Costa *et al.*, 2001; Ho *et al.*, 2007; M. E. Buchanan and Davis, 2010; Gouzi *et al.*, 2011; Georganta *et al.,* in preparation). For the study of learning and memory impairments the animal models that are mainly used are heterozygous $Nf1^{+/-}$ mice (Costa *et al.*, 2002a; Cui *et al.*, 2008a) and homozygous $dNf1^{-/-}$ *Drosophila melanogaster* flies (H. F. Guo *et al.*, 2000). There is also a contribution of a single paper that recapitulates the learning and memory deficient phenotype in zebra-fish

mutants (Wolman *et al.*, 2014). In *Nf1*-mutant mice, learning disruptions have been associated with increased Ras activity, since it has been shown that they can be reversed upon its genetic or pharmacological inhibition (Costa *et al.*, 2002a; Li, Cui, Steven A Kushner, *et al.*, 2005). Although heterozygous $Nf1^{+/-}$ mice might not perfectly recapitulate human pathologies, studies have indicated that learning disabilities are possibly related to impairments in GABA-ergic neurotransmission in the hippocampus (critical center for many forms of learning and memory). However, the identity and the interconnections of the affected neurons remain to a great extent unclear, since progress is limited by the complexity not only of the cognitive disorders and the molecular mechanisms underlying them, but also of the mouse animal model itself.

1.2. Drosophila melanogaster as a model organism

Life Cycle and general characteristics

Drosophila melanogaster is a species of fly in the family Drosophilidae. The species is known generally as the common fruit fly or vinegar fly. Under optimal growth conditions at 25 °C, the *D. melanogaster* lifespan is about 50 days from egg to death. The developmental period for *Drosophila* varies with temperature. It increases at higher temperatures due to heat stress. Under ideal conditions at 25 °C is 10 days whereas at 18 °C it takes 20 days. The *Drosophila* life cycle is comprised of the following developmental stages (Fig. 3):

1. Embryogenesis: It is a fast process completed 24h after fertilization of the oocyte by the male sperm. From a one cell embryo, a syncytial embryo is rapidly developed.
2. Larval stage: There are three larval stages (3 instars) which take altogether about 4 days. During larval growth, most cell types are already differentiated and functional. Therefore, many biological questions can be addressed already at the larval stage.
3. Pupal stage: After encapsulation of the 3d instar larva, pupal stage starts and lasts around 4 days. Many larval structures are lysed and new structures are formed.
4. Adult life: Adult fly emerges upon eclosion of the pupal case. Lifespan is around 30 days, although this is variable according to temperature.

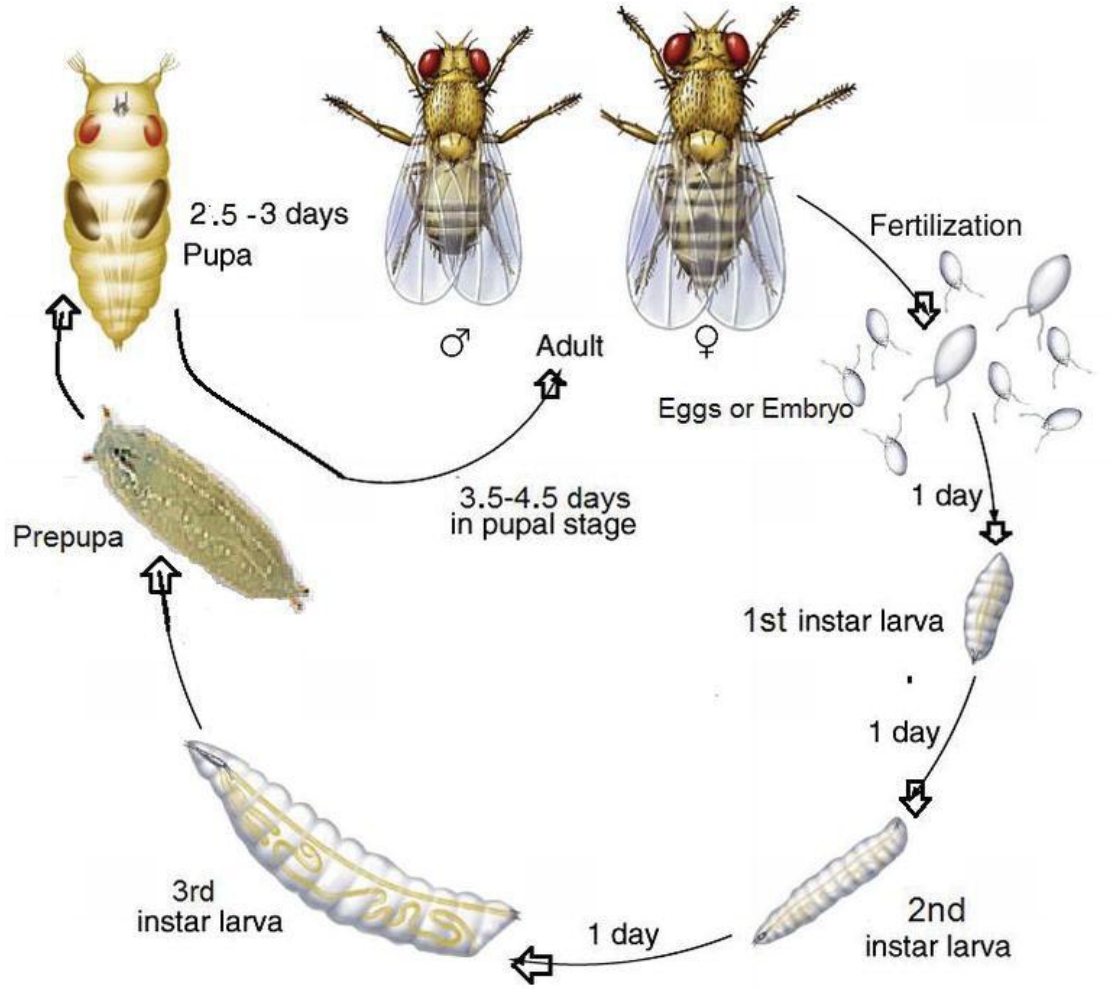


Figure 3: Drosophila life cycle (Perveen, 2018)

The well-established animal model of *Drosophila melanogaster*, offers many advantages for experimental study and has been used in research for more than 100 years. With a DNA content 50 times greater than that of Escherichia coli bacteria, 30 times smaller than that of mammals and a cellular structure that is by far simpler than the respective of vertebrates, it offers a balance of complexity and simplicity. Functional and comparative genomic studies have also revealed a high degree of gene conservation between flies and other species. Sequencing of the fly genome, largely completed in 2000, suggests that *Drosophila* has approximately 13600 genes. Most of these have homologs in other eukaryotes. Nearly 75% of human disease related genes have been estimated to have functional orthologues in Drosophila (Pandey and Nichols, 2011; Yamamoto *et al.*, 2014). Overall identity at the nucleotide or amino acid sequence between *Drosophila* and mammals is approximately 40% between homologues. Regarding the conserved functional

domains of proteins, identity may be more than 80%. The availability of its genome sequence greatly facilitates comparisons between the Drosophila and other model organisms. In contrast to mammalian models, the generation of fly mutants is easy, cheap and fast. Moreover, the pool of publically available stocks that can be readily utilized to induce gain- and loss-of-function is enormous. *Drosophila* has only four pairs of chromosomes, three autosomes and one pair of sex chromosomes, breeds quickly, and provides a large number of offspring. The above, combined with the ability to perform highly detailed molecular and genetic analysis and genome manipulations by creating transgenic animals, qualifies *Drosophila* as an ideal system for the study of genes and signaling cascades. It is worth mentioning that the last two decades *Drosophila* research has contributed to our understanding of nervous system development (Doe, 2008; Hartenstein *et al.*, 2008), growth cone guidance and target recognition (Dickson, 2002), exocytosis and endocytosis at synapses (Bellen, Tong and Tsuda, 2010), synapse remodeling (Collins and DiAntonio, 2007) and the neural circuitry underlying conserved behaviors such as courtship (Villella and Hall, 2008), diurnal rhythms and sleep (Crocker and Sehgal, 2010), aggression (Kravitz and Huber, 2003; Dierick and Greenspan, 2006), addiction (Devineni and Heberlein, 2010; Kaun, Devineni and Heberlein, 2012)and learning and memory *in vivo* (McGuire, Deshazer and Davis, 2005; Skoulakis and Grammenoudi, 2006).

The UAS-GAL4 and TARGET expression systems as genetic tools

In *Drosophila*, a wide range of genetic tools is available. There are two main strategies to study human diseases using the Drosophila model: forward and reverse genetics. In forward genetics, mutations are induced at random, and flies are screened for a phenotype of interest. In a reverse genetic approach, mutations are generated in Drosophila homologues of human genes to characterize their phenotypes. There are several approaches to knockdown or knockout genes in Drosophila. One of the most important genetic systems used in reverse genetic approaches is the GAL4-UAS-system. This system has two parts: the Gal4 gene, encoding the yeast transcription activator protein GAL4, and the UAS (Upstream Activator Sequence), an enhancer to which GAL4 specifically binds to activate gene

transcription. As shown in Figure 4, if one of two transgenic lines of the P generation bears the GAL4 driver in a known spatial or temporal pattern, and the other, the responder, contains a UAS dependent transgene, a part of the progeny of the F1 generation will bear the GAL4-UAS line, which will allow for the transgene to be expressed. It should be mentioned at this point that, in the absence of GAL4, the UAS- target gene is silent. In order to achieve temporal control of the respective transgene expression by using the GAL4 system, the TARGET (Temporal And Regional Gene Expression Targeting) technique is being utilized. This technique relies on the ability of a temperature-sensitive version of the GAL80 yeast protein ($GAL80^{TS}$), to bind at 18°C to the GAL4 protein, thus preventing it from activating transcription during development. Adult flies bearing the $GAL80^{TS}$ are subsequently transferred to 30°C, thus allowed to express the target gene (Fig. 5).

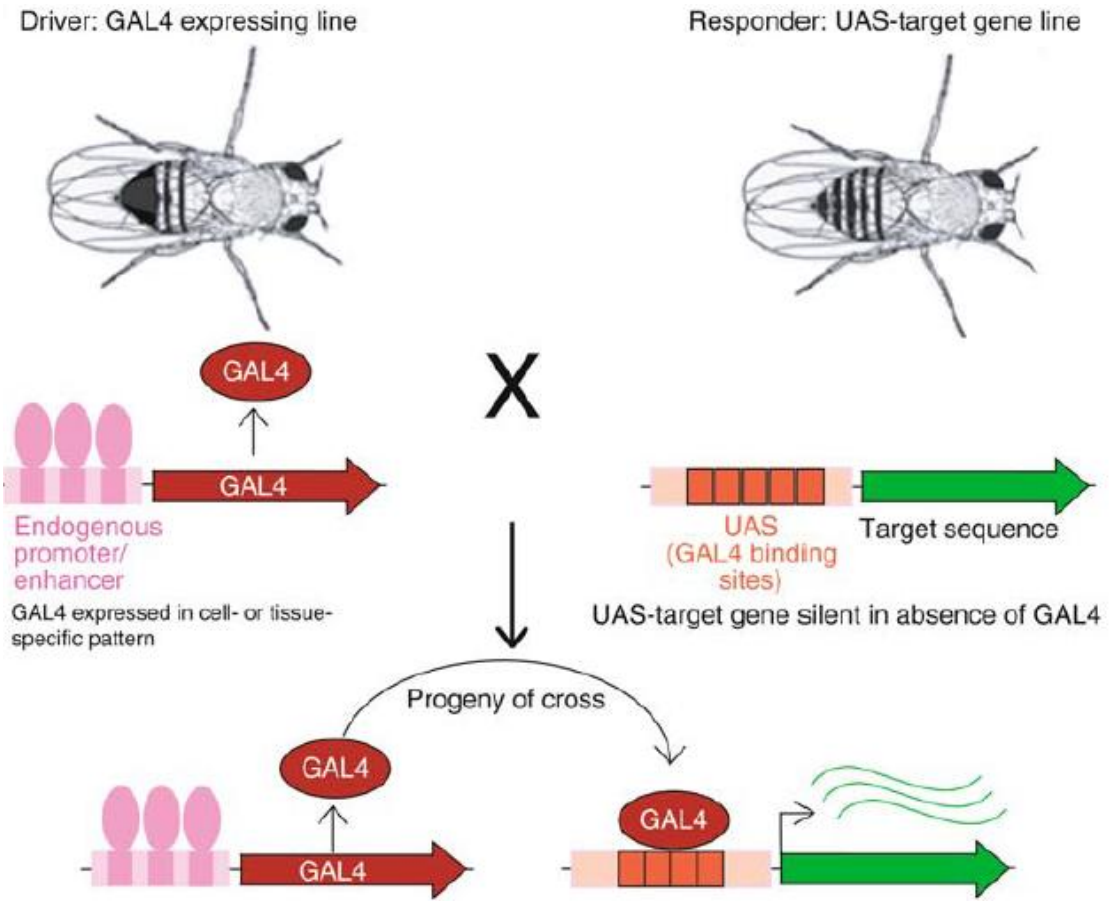


Figure 4 : GAL4 drives expression of UAS-target gene in cell- or tissue- specific pattern (Elliott and Brand, 2008).

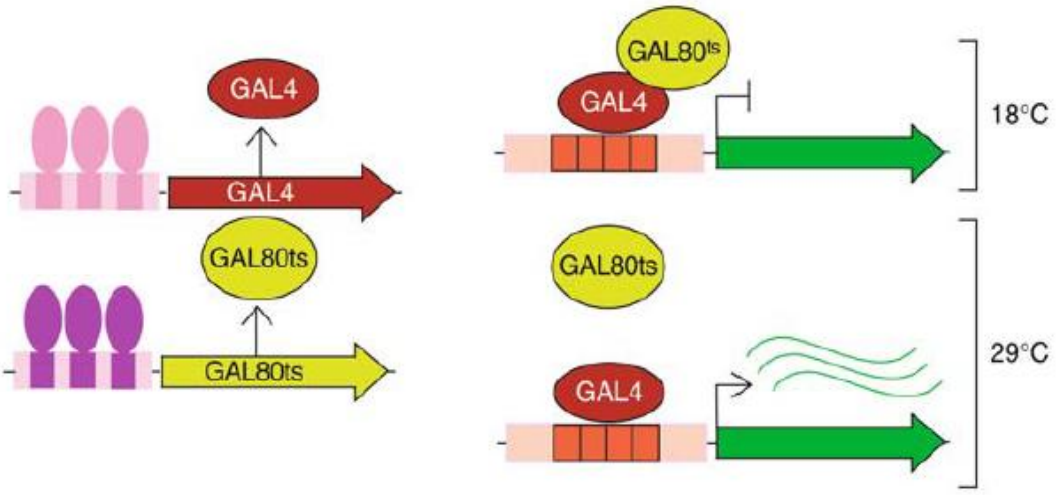


Figure 5: Temporal Control of the GAL4 system (Elliott and Brand, 2008).

Gene silencing using RNA interference (RNAi)

RNA interference (RNAi) provides a powerful reverse genetics approach to analyze gene functions both in tissue culture and *in vivo*. During the biological process, RNA molecules inhibit gene expression or translation, by neutralizing targeted mRNA molecules. It is the simplest way to affect gene function quantitatively. Transgenic RNAi has been widely used to study gene function in somatic tissues. Importantly, in *Drosophila* RNAi is cell-autonomous, and because of this, targeted expression of RNAi constructs using the GAL4-UAS system (Brand and Perrimon, 1993) can be used for cell- or tissue-specific interrogation of gene function. Indeed, this approach has been used extensively. To date, transgenic RNAi lines have been shown to be potent in all somatic tissues, including neurons and muscles.

An important issue with regard to *Drosophila* RNAi screens in tissue culture concerns false positives that occur from sequence specific off-target effects (OTEs) (Kulkarni *et al.*, 2006; Perrimon and Mathey-Prevot, 2007). OTEs can be avoided by selecting sequences that do not contain 19 nucleotides or longer cross-hybridizing stretches to other genes or tri-nucleotide CAN (CA[AGCT]) repeats. In this regard, a number of software tools are available for identifying the most common off-target sequences so that they can be excluded from RNAi constructs. In vivo, although it is

difficult to fully evaluate the rates of false positives and negatives in general, as it depends on the reagents used, the specific Gal4 driver used, and the temperature at which the flies are screened, the consensus is that OTEs appear negligible if sequences that avoid potentially problematic sequences are used (Dietzl *et al.*, 2007; Ni *et al.*, 2008, 2009).

Drosophila melanogaster as a model organism for brain disorders

The fly brain is estimated to contain 90,000 neurons, a million-fold less than the typical human brain (Meinertzhagen, 2010), but with a similar complexity of different neural cell types. Flies and mammals use the same neurotransmitters (GABA, Glutamate, Acetylcholine), share biogenic amines like dopamine and serotonin, and have numerous neuromodulatory peptides. Like mammals, flies have sodium channels that propagate action potentials, and the same families of potassium and calcium channels regulate membrane potential. In both systems, information passes between neurons at specialized contact points called synapses, and these synapses have common protein architecture. Thus, insights about the nervous system obtained in *Drosophila* are often relevant for other species (Meinertzhagen, 2010).

In combination with genome-wide genetic screening, genome-wide analyses with deep sequencers, such as RNA-seq and ChIP-seq, and metabolomics analyses, *Drosophila* is now commonly used as a model to study several human diseases including brain diseases, with the aim of identifying novel biomarkers or therapeutic targets for human diseases together with the screening of candidate substances for their treatment (Pandey and Nichols, 2011). In most of these invertebrate transgenic models, some aspects of human disease are reproduced. Anatomic divergence between the fruit fly and humans is apparent, which may be not sufficient to recapitulate some morphological features of neurological disease but fundamental molecular pathways are highly conserved (Rubin and Lewis, 2000). Functional analysis of human disease genes including high-throughput pharmacological screens, as well as behavioral assays, has become available in *Drosophila.*

The *Drosophila* central nervous system (CNS), is composed of a bilaterally symmetrical brain with two cell types, neurons and glia, both originating from neural progenitors named neuroglioblasts. Although the CNS of the fly is considerably simpler than that of vertebrates, it operates on the same fundamental principles as the mammalian equivalent. The neurodevelopmental pattern is conserved among the organisms thus allowing for a better understanding of the function of a gene involved in a disease. Drosophila is now used in the study of various human diseases related to the central and peripheral nervous systems such as neurodegeneration, Alzheimer's disease, Parkinson's disease, tauopathies, Prion diseases, Huntington's disease, X-linked spinobulbar muscular atrophy, Amyotrophic lateral sclerosis (ALS), triplet repeat expansion disease, sleep disorders, seizure disorders, neurodevelopmental (NF1, FXS) cognitive and psychosis disorders (Pandey and Nichols, 2011) and Charcot-Marie-Tooth disease, as well as epilepsy, tumors (NF1, NF2, Tuberous Sclerosis), trauma and others (Jeibmann and Paulus, 2009).

1.3. Learning and Memory in *Drosophila melanogaster*

Although the *Drosophila* brain has only 100 000 cells, it produces complex behaviors and sustains various forms of learning and memory, which are highly amenable to analysis using current genetic methods. Moreover, *Drosophila* studies allow us to investigate different concepts of memory that may eventually be generalized to other species.

The identification of genes, molecules and neuronal circuits implicated in behavioral phenomena such as learning, memory formation and retrieval, as well as forgetting, is of extreme importance in order to understand how the brain processes external information and how that differs between healthy and diseased individuals. Learning refers to the way by which the acquisition of new information is achieved, while memory is defined as the procedure by which this new knowledge is encoded, stored and later on recalled (Kandel, Schwartz and Jessell, 2000). Learning and memory constitute fundamental skills for the survival of a living organism. It has been shown that Drosophila exhibits numerous types of learning, but the most

robust and useful one –in terms of analysis- seems to be that of olfactory learning. In a Pavlovian olfactory learning and memory paradigm, flies learn to associate an odor (conditioned stimulus: CS+) with the negative reinforcement of mild, electric footshock (unconditioned stimulus: US). Memory of this learned association is tested in a T-maze, in which successfully trained flies tend to avoid the punished odor.

Early phases of memory, short-term memory (STM) and middle-term memory (MTM), are consolidated into 2 types of longer lasting forms of memory in *Drosophila*: long-term memory (LTM) and anesthesia-resistant memory (ARM). ARM is a consolidated form of memory that has been named after its characteristic resistance to cold-shock anesthesia (Tully and Harbor, 1994). The dissection of memory phases, as has been described, is shown in Figure 6. Long-term memory has been dissected into two distinct forms: protein synthesis-dependent LTM (PSD-LTM) and protein synthesis independent LTM (PSI-LTM). Both of them can be generated after aversive conditioning involving multiple conditioning trials, but while PSD-LTM requires intervening rest intervals (spaced conditioning), PSI-LTM is generated without intervening rest periods (massed conditioning). This PSI form of LTM has also been referred to as ARM. ARM is gradually produced shortly after training and reaches asymptotic levels between 1 and 2 h after training. It can be detected until 24 hrs after training. LTM on the contrary, can last up to 7 days in *Drosophila* (Tully and Harbor, 1994). It should be mentioned that ARM has been described only in *Drosophila* and snails so far (Yamada *et al.*, 1992).

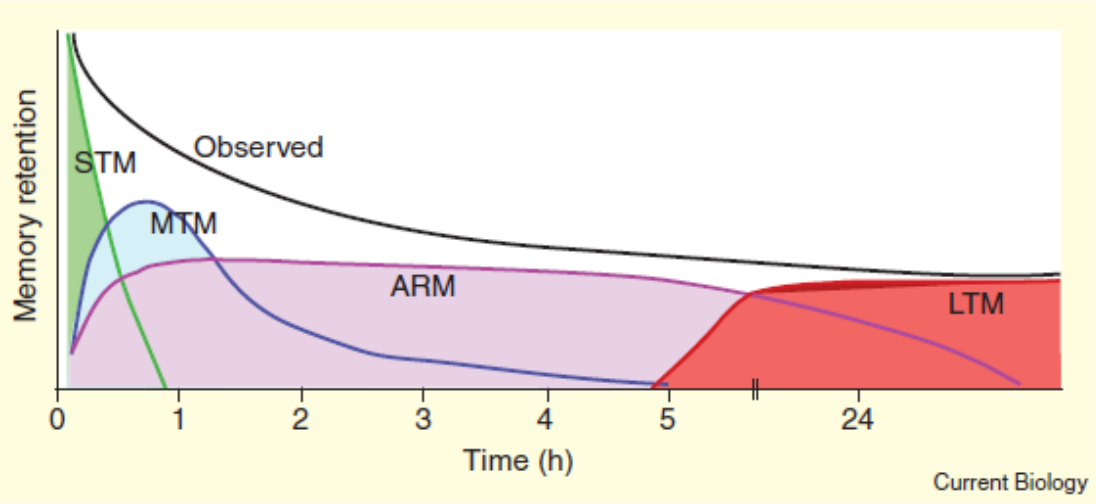


Figure 6: Dissection of memory phases. At the behavioral level, the observed decay of memory appears relatively seamless (black). Experimental disruptions in numerous animal species including humans, however, reveal temporally, mechanistically and anatomically distinct phases underlying memory retention. In *Drosophila*, at least four mechanistically distinct phases have been described. These are short-term memory (STM; green), middle-term memory (MTM; blue) anesthesia-resistant memory (ARM; purple) and long-term memory (LTM; red) (Margulies, Tully and Dubnau, 2005).

ARM could also be generated after a single training session, following an anesthesia-sensitive (ASM) phase of memory formation. However, massed repetitive training (10 sessions) can produce even stronger memory retention that could last even up to three days, still retaining its PSI identity, thus differentiating it from the conventional PSD-LTM (Tully and Harbor, 1994)

Long-lasting memory can be genetically dissected into functionally and mechanistically distinct ARM and LTM phases, which co-exist in normal flies. This genetic dissection of memory suggests not only sequential steps in the processing of olfactory memory but also parallel steps, because LTM and ARM appear to form independently of each other, thus clearly establishing that they are distinct. However, an alternative hypothesis has been proposed (Tully and Harbor, 1994) in which ARM and LTM are not processed in parallel, but instead are 'mutually exclusive'. In this alternative model, ARM forms after massed training but not after spaced training and LTM forms only after spaced training. The authors of this study suggest that ARM actually prevents LTM after massed training, and likewise LTM induction inhibits formation of ARM after spaced training.

Olfactory memory formation is mediated principally by the olfactory nervous system (Davis, 2004, 2005). *Drosophila* receives olfactory input through olfactory receptor neurons located in the antennae and maxillary palps and transmit this information to the antennal lobe (AL). Odor information is further processed by local interneurons in the AL and projection neurons (Pn) then convey the information to the mushroom body neuron (MBn) dendrites and to an area of the brain known as the lateral horn (Fig. 7). Numerous lines of evidence have pointed to MBn as critical centers for olfactory memory formation (Heisenberg *et al.*, 1985; Davis, 1993; de Belle and Heisenberg, 1994).

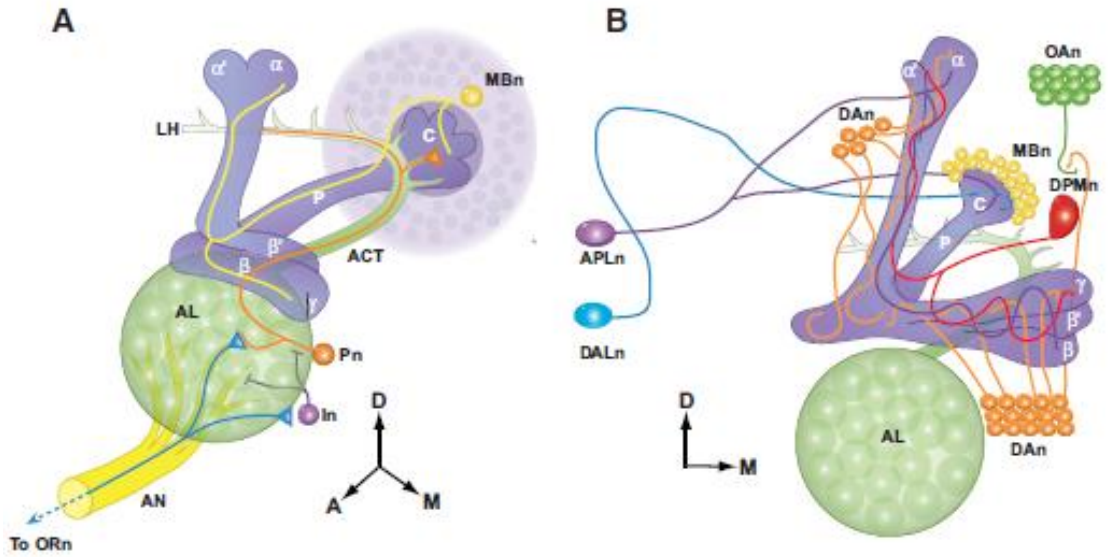


Figure 7: Olfactory nervous system of Drosophila. A schematic diagram of the olfactory nervous system components within the fly's right brain hemisphere viewed from an anterior and slightly dorsal perspective. (D) Dorsal, (A) anterior, (M) medial. *(A)* Olfactory information is conveyed from olfactory receptors neurons (ORn) to the antennal lobe (AL) via the antennal nerve (AN). The ORn axons synapse with projection neurons (Pn) in discrete glomeruli as well as interneurons (In). Pn carry this information to the mushroom body neurons (MBn), forming synapses with the MBn in the calyx (C) at the posterior edge of the brain, as well as the lateral horn (LH). Each MBn sends a single axon anterior through the peduncle (P). Near the anterior face of the brain, MBn neurites turns to form one or more lobes (vertical: α or α'; horizontal: β, β', or γ) according to the MBn cell type (α/β, α'/β', or γ). *(B)* MB extrinsic neurons that are involved in learning and memory. (APLn) Anterior paired lateral neuron, (DPMn) dorsal paired medial neuron, (DAn) dopaminergic neurons, (DALn) dorsal anterior lateral neurons, (OAn) octopaminergic neurons. (Davis, 2011).

Odors used as the conditioned stimulus (CS+) for olfactory associative conditioning in flies are conveyed and processed through the circuitry described above and depicted in Figure 7. As far as the US is concerned, the pathway for aversive conditioning is mediated by G-protein coupled DA receptors expressed by the MBn (Connolly *et al.*, 1996; Schwaerzel *et al.*, 2003; Kim, Lee and Han, 2007). The CS and US coincidence integration in the MBn occurs, at least in part, through the activity of an adenylyl cyclase encoded by the rutabaga (rut) gene (Tomchik and Davis, 2009), a gene that has been implicated in a number of functions, including learning and memory, behavior, and cell communication. Rutabaga mutants display defects in olfactory memory. Newer studies have shown that there are multiple types of MBn, and that information presented to the MBn is further modified by anterior paired lateral neurons (APLn), dorsal paired medial neurons (DPMn), and subsets of Dopamine neurons (DAn).

Mushroom Bodies as a center for olfactory learning and memory

The mushroom body (MB) consists of thousands of intrinsic and extrinsic neurons. Kenyon cells, the second-order olfactory interneurons in Drosophila, constitute the majority of the intrinsic neurons (2500 Kenyon cells per hemisphere). Their cell bodies form a pair of quadruple clusters at the dorsal posterior cortex. Their extensive dendritic arborizations contribute to the globular structure beneath the cell bodies, called the calyx, in which the collaterals of the olfactory projection neurons terminate (Stocker *et al.*, 1990). The axon bundle of the Kenyon cells further project anteriorly through the pedunculus to the lobes. The lobes of the MB are considered as the main output site of Kenyon cells, but also receive many inputs from extrinsic neurons (Ito *et al.*, 1998; Johard *et al.*, 2008; Tanaka, Tanimoto and Ito, 2008). Various behavioral and physiological functions that the MB is known to support range from olfactory learning to decision making under uncertain conditions. Its structural heterogeneity may anatomically reflect the organization of circuits that are required to achieve an array of distinct behavioral functions in one brain structure. *Drosophila* Kenyon cells are roughly classified into three subtypes by their projections in the lobes: the γ, α'/ β', and α/β lobe neurons in order of birth (Crittenden *et al.*, 1998; Jefferis *et al.*, 2002). The γ-neurons project only to the medial lobe, while the α'/ β', and α/β neurons bifurcate at the anterior end of the pedunculus to project to the medial and vertical lobes (Crittenden *et al.*, 1998; Ito *et al.*, 1998). In addition to their morphological distinction, these subtypes are differentiated with respect to their gene expression, neurotransmitter systems, connectivity to extrinsic neurons, and behavioral functions (Strausfeld, Sinakevitch and Vilinsky, 2003; Keene and Waddell, 2007; Tanaka, Tanimoto and Ito, 2008).

MBs integrate olfactory input with punishment or reward and are thought to be part of the driving force for the behavioral response. The activity of these neurons contributes to different temporal phases of memory. Blocking synaptic transmission from the MBn impairs the expression of olfactory memory, consistent with the model that many of the plastic events underlying the representation of olfactory memories occur within the MBs themselves or at prior nodes of information flow within the olfactory nervous system (Dubnau *et al.*, 2001; McGuire, Le and Davis,

2001). Moreover, flies completely lacking MBs, turned out to show no olfactory memory (de Belle and Heisenberg, 1994), although abilities to mediate olfactory memory (shock reactivity, olfaction, and locomotor behavior) were intact. Together, these data strongly implicate the MBs in associative olfactory memory.

1.4. Nf1 and *Drosophila melanogaster*

The *dNf1* gene of the fruit fly, residing on chromosome 3, was found to predict a protein 55 % identical and 69 % similar to human neurofibromin over its entire 2,802 amino acid length. The IRA-related central segment of Drosophila neurofibromin is most similar to the human protein, but conserved regions also exist both up- and downstream. While loss of this highly conserved *Drosophila dNf1* ortholog, does not obviously affect viability, fertility, or patterning, several macroscopic, behavioral, and biochemical phenotypes have been identified. In particular, *dNf1*-null mutants present 15%-20% organismal size reduction in linear dimensions during all developmental stages (Fig. 8), deficits in associative learning and memory (Georganta *et al.,* in preparation; Guo *et al.*, 2000), as well as defects in circadian rhythms, escape response, and activity across the day/night cycle (Williams *et al.*, 2001; King *et al.*, 2016), thus resembling human NF1 symptoms. Figure 9 summarizes and describes phenotypes of *Nf1*-null mutant flies that have been reported, along with the indicative molecular pathways possibly implicated in each one.

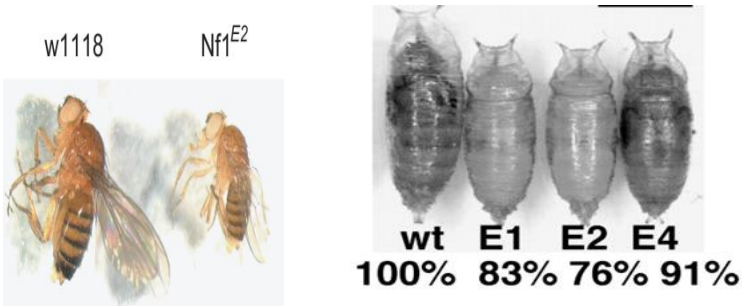


Figure 8: Size Reduction in *Nf1* mutant flies. (Walker *et al.*, 2006)

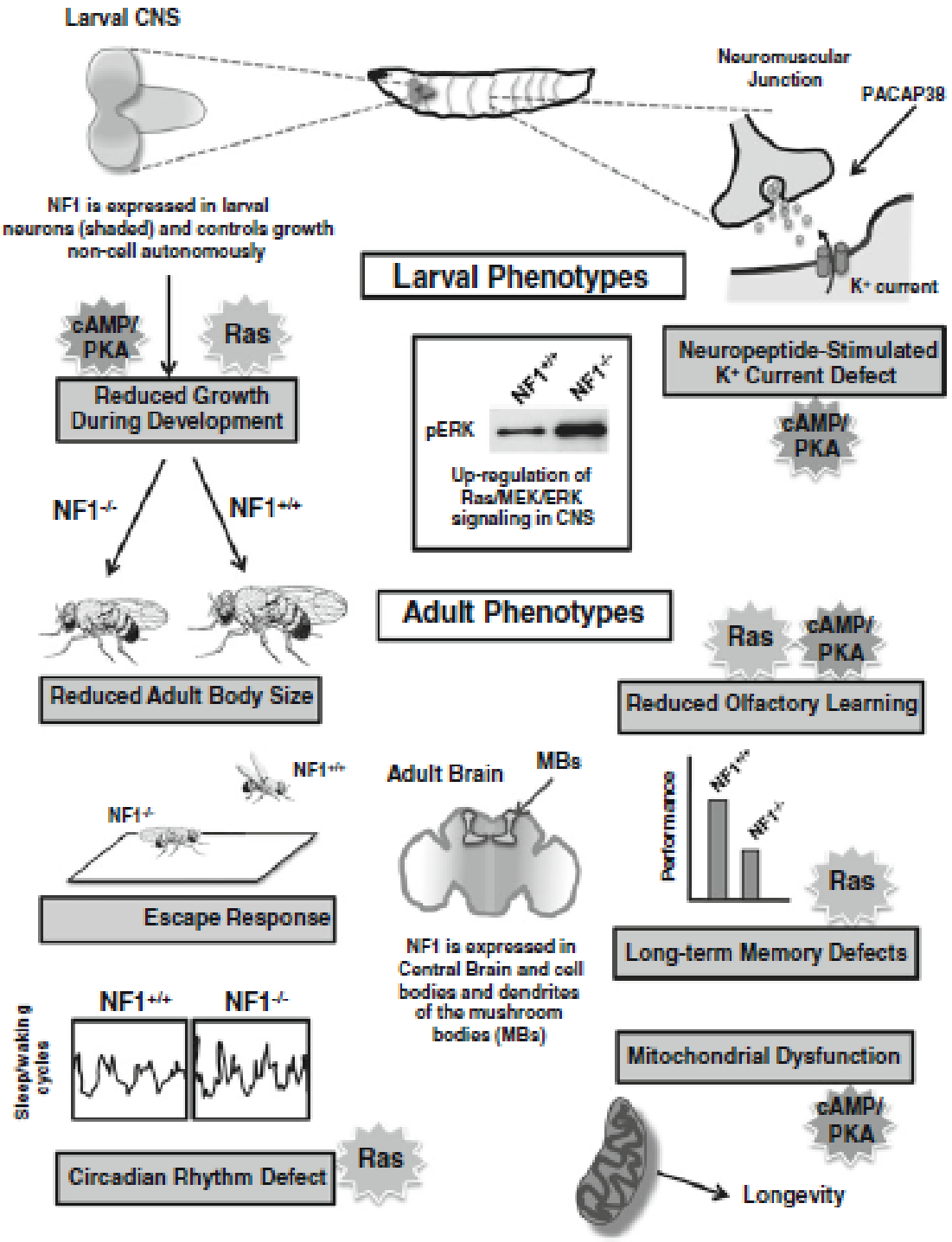


Figure 9: Larval and adult homozygous null dNF1 phenotypes. dNf1 larvae lack a neuropeptide induced rectifying K^+ -current at the body wall neuromuscular junction. Larvae, pupae, and adults are 15–20 % smaller than wild-type controls, and larval and adult dNF1 brain phospho- ERK levels are approximately threefold higher than in controls. Adult dNf1 flies lack normal day/ night rhythmic locomotor behavior and exhibit a reduced olfactory associative learning/short-term memory performance, as well as deficits in middle-term and long-term memory. The diagram depicting the adult brain shows the location of the mushroom bodies (MBs), considered the insect centre of learning and memory. MBs play essential roles in olfactory learning and preferentially express several AC/PKA pathway proteins (M. E. Buchanan and Davis, 2010; Gouzi *et al.*, 2011).

dNf1 is widely expressed in the brain and appears to be very abundant in the dendrites and cell bodies of the Mushroom Bodies (MBs). Previous work from the Skoulakis lab has unexpectedly shown that pan-neuronal expression of a *dNf1* transgene, **with explicit exclusion of the MBs**, is sufficient to fully reverse the associative learning deficit of *dNf1* null mutants (Gouzi *et al.,* 2011). A consequential study has recently revealed the neuronal circuit, outside the MBs, where *dNf1* is essential and sufficient for normal associative learning and the implicated signaling pathway therein (Georganta *et al.,* in preparation). Evidence suggests that this circuit is GABAergic and *dNf1* loss results in excess GABA production (Georganta *et al.,* in preparation). This GABAergic neuronal circuit is marked by the OK72 driver and is located in the lateral horn (LH) and a group of cells in the superior medial protocerebrum (SMP). In accordance to this finding, studies in Nf1 deficient mice, suggest a role for GABAergic hippocampal neurotransmission in learning impairments (Costa *et al*., 2002; Cui *et al.,* 2008)

Moreover, findings indicate that learning deficits of *Nf1*-deficient flies can be fully reversed upon inhibition of the Alk receptor tyrosine kinase in the same GABAergic neurons, thus suggesting a functional interaction of dAlk and dNf1 therein. It should be mentioned that Receptor Tyrosine Kinases (RTKs) are common upstream activators of Ras signaling and that Alk has been shown to act as a negative regulator of olfactory learning and memory in neurons outside and inside MBs, respectively (Gouzi *et al.,* 2011; Gouzi *et al.*, 2018).

Experiments performed later in mice, recapitulated the above evidence, supporting the functional interaction of Alk with Nf1 for normal learning (Weiss, S. J. Weber, *et al*., 2017a; 2017b). In particular, they demonstrated that the cognitive disorders observed in Nf1 mutant mice, are improved after both genetic and pharmacological inhibition of Alk (Weiss, S. J. Weber, *et al*., 2017; Weiss, S. Weber, *et al*., 2017). These findings suggest that Alk inhibitors could be applied to NF1 patients as well, for their pharmacological improvement.

Alk kinase, as a typical RTK, is responsible for its signal transduction via the Ras/MAPK pathway, both *in vitro* and *in vivo* (Gouzi *et al.*, 2005). Previous studies have shown that loss of dNf1 resulting in neuronal ERK overactivation underlies the reduced body size of mutant homozygotes (Walker *et al*., 2006). On the other hand,

pan-neuronal increase of dAlk levels or activity in adult Drosophila yielded significantly elevated phospho-ERK levels in head lysates (Gouzi *et al*., 2011). Furthermore, phospho-ERK levels were reduced in flies with RNAi-mediated dAlk abrogation, or expressing the dominant negative dAlk transgene (Gouzi *et al*., 2011). Thus, dAlk modulates ERK activation in the adult Drosophila CNS, probably by engaging the Ras/ERK cascade.

As expected, further experiments from our lab have proved the specific implication of Ras1 in Nf1-associated learning in OK72 GABAergic neurons, since dRas1 abrogation therein could rescue the learning deficiency of *Nf1* null mutants (Georganta *et al*., in preparation). On the other hand, the same study has shown that the cAMP signaling is not required in OK72 neurons for Nf1-mediated learning. Remarkably, the afore mentioned learning impairments can be fully reversed either genetically or pharmacologically in adult flies, indicating that loss of Nf1 from the OK72-GABAergic neuronal circuit is not responsible for important developmental disorders therein The *dNf1* mutant alleles on which both previous studies of the lab and the current one have been focused are the $Nf1^{E2}$ and $Nf1^{E4}$. $Nf1^{E2}$ displays a nonsense mutation upstream of the catalytic GRD domain (Fig. 10), truncating the protein after 369 amino acids, thus generating a null mutant. $Nf1^{E4}$ is a C1045Y missense mutation, in a conserved part of neurofibromin, outside the GRD domain, that also harbors two disease associated missense mutations (Wu *et al.*, 1996; Kluwe, 2003). Interestingly, the effects of the d$Nf1^{E4}$ point mutant appear much more severe on associative learning, than those of the null mutant (Georganta *et al*., in preparation), although milder on the body size (Fig. 8).

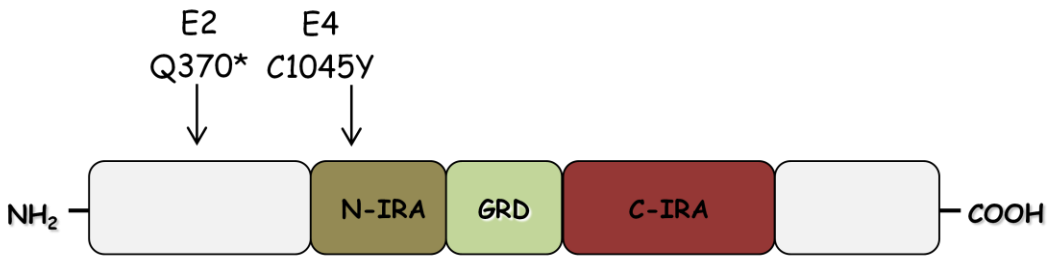


Figure 10: Location of *dNf1*^{E2}, and *dNf1*^{E4} mutations.

2. Aim of master thesis

Significant progress has been noted up to date regarding the understanding of the oncogenic mechanisms that involve Nf1 in the CNS and PNS. On the contrary, the neuronal circuits and mechanisms that malfunction upon Nf1 loss and are responsible for the phenotype of cognitive impairments remain relatively unknown resulting to lack of appropriate drugs. Therefore, the study of these neuronal circuits and mechanisms underlying this pathology is of great importance and requires the usage of model organisms for the delineation of their nature.

The complexity of the signaling cascades implicated in NF1 pathophysiology, combined with the lack of drugs to target Nf1 directly, highlight the pressing need to distinguish NF1 cognitive phenotypes and attribute them to specific types of neurons. We hypothesize that the complexity and variety of NF1 cognitive symptoms, reflect a functional disruption of Nf1 that affects distinct biochemical pathways and molecular mechanisms in different neurons of the CNS that are related to the processes of learning and memory.

dNf1 expression is enriched in MBs, however, whether its learning/memory-related functions involve cells within or outside of MBs remains controversial. As far as learning is concerned, previous work of the lab has shed light into which neurons require the expression of Nf1 for normal learning performance. However, the identity and connections of the neurons, where Nf1 is required for normal memory, remain to a great extent unknown. Therefore this dissertation thesis, aims to the delineation of the neuronal circuits where the presence of Nf1 is essential for normal associative long term memory (LTM) in *Drosophila*. In particular, this dissertation thesis aims:

1. To characterize the memory phenotype of *dNf1* mutants and
2. To identify the respective neuronal circuits to which the expression of Nf1 is essential for normal olfactory memory.

Given the highly conserved function of Nf1, this new knowledge is expected to contribute to better comprehension of the complexity that characterizes cognitive deficits in NF1 patients and most importantly, to the development of novel and targeted treatment approaches.

3. Materials and methods

3.1. *Drosophila* strains and culture

Drosophila raising and crossing was set up in standard wheat-flour- sugar food supplemented with soy flour and $CaCl_2$ and cultured at 25°C and 50% humidity with a 12h light/dark cycle. For experiments using the TARGET system (flies bearing $Gal80^{ts}$), all animals were raised at 18°C until adulthood and transgenes were induced maximally by placing 3–5-day old flies at 30°C for 72 h.

The $dNf1^{E2}$ and $dNf1^{E4}$ mutants have been described previously ((Walker *et al.*, 2006; Gouzi *et al.*, 2011)

Transgenic fly strains used in this work were:

- UAS-dNf1 (Walker *et al.*, 2006)
- UAS-Nf1RNAi (Vienna Drosophila RNAi Center #109637),
- Gal80ts (McGuire, Mao and Davis, 2004)
- UAS-mCD8::GFP (Lee and Luo, 1999)
- The MB-specific Gal80 (MB-Gal80), which suppresses expression in the MBs (Krashes *et al.*, 2007)

Gal4-driver lines used in this work were:

- Leo-Gal4 /NP0863 (Kyoto Drosophila Genetic Resource Center #112369) (Messaritou *et al.*, 2009)
- MB247-Gal4 (Bloomington Drosophila Stock Center #50742)
- Elav[C155]-Gal4 (Bloomington Drosophila Stock Center #458)
- Elav-Gal4 (chr 2) (Bloomington Drosophila Stock Center #8765)

3.2. Behavioral experiments and conditioning

Behavioral tests were performed under dim red light at 23–25 °C and 70%–78% humidity. All animals were 2–6 days old if raised at 25°C and 2-10 days if raised at 18°C. They were subsequently collected under light CO_2 anesthesia, one day prior to training and kept in food vials in groups of 50–70 at 23 –25 °C or 18°C as appropriate for strains with $Gal80^{ts}$ temporal restriction of transgene expression. They were transferred to fresh vials 1–1.5 hours before training. Olfactory learning and memory in the negatively reinforced paradigm coupling equally aversive odors as conditioned stimuli (CS+ and CS-) with the electric shock unconditioned stimulus (US) was used to assess learning and memory. The aversive odors used were benzaldehyde (BNZ) and 3-octanol (OCT). Two groups of animals of the same genotype were trained simultaneously, one to avoid BNZ, the other OCT, while the complementary odorant was used as the respective control. 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 due to one of the conditioning stimuli and represented half of the performance index. One performance index was calculated as the average of the half-learning indeces 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. The animals were kept at the training temperature (25 °C) for 30 min before training.

Odorant preparation

Benzaldehyde (x2):

1. In a 1.5 tube are added:

- 950 µl Isopropyl myristrate (Isopropyl myristrate 98%, Cat. No. 17,247-2, SIGMA-ALDRICH)
- 50 µl Benzaldehyde

2. Quick Stirring by light vortexing
3. Solution is transferred into the appropriate experimental glass vial

Octanol (x2):

1. In a 1.5 tube are added:

- 500 µl Isopropyl myristate
- 500 µl Octanol

2. Stirring by light vortexing
3. Solution is transferred into the appropriate glass vial

- *Concentration of Odors may require adjustment according to bias*

Each odor is placed in a specific place in the behavior room and connected to specific tubing that allows the respective odor to be delivered to the chamber with the flies. The air flow should be set to 500 ml/min. Odors are connected to each maze approximately ½ hour before the experiment (for priming).

LTM Protocol (Spaced training)

Training:

In this paradigm, the same principle as in learning applies. However the number of CS/US pairings is fixed at 12 and the number of rounds for each training cycle is 5. Moreover the setup is different since it involves 4 tables instead of mazes. Each table has four clips where the tubes containing the flies are placed. The vacuum should be connected with a two way silicone tube (splitter) that can be attached on the fly tubes.

Depending on the number of ns used, the number of cycles is set. For one training cycle of one table 4 vials of flies (50-70 flies each) -2 ns- are required and correspond to 4 training tubes:

1. Flies are transferred from each vial to one of each training tube on the table.
2. Splitter is connected with the four tubes and the vacuum
3. An "Octopus" of crocodile clips is used to connect the training tube grids with alligator clips to the square pulse stimulator (Grass Instruments)
4. Odors are connected to the training tubes in a way that the 1st and the 3rd tube -as you go from left to right- would receive Benzaldehyde whereas the 2nd and the 4th would receive Octanol

5. Electric shock is turned on (90 V, 1 pulse, delay 4)
6. 12 CS/US pairings are delivered (~1 min)
7. Shock is turned off and odors disconnected from tubes. Flies are allowed to rest for 30 sec
8. Odors are connected to the fly tubes in a way that this time that the 1st and the 3rd tube -as you go from left to right- would receive Octanol whereas the 2nd and the 4th would receive Benzaldehyde.
9. The flies are allowed to receive the odors in that setup without getting electric shock for 1 min
10. Flies are allowed to rest for 30 sec
11. Steps from 4 to 10 are repeated 4 more times for each table with an interval of 15 minutes in-between.

Testing:

1. Flies are brought to the central chamber of the maze and connected with vacuum
2. Benzaldehyde is connected to the right and Octanol to the left arm of each maze
3. Flies are brought to center of the maze at the choice point and are allowed to choose between the two odors for 1.5 min
4. Flies are transferred from each arm to a different falcon tube
5. Flies are sacrificed by quick freezing at -30°C and subsequently counted

3.3. Statistical analysis

Untransformed (raw) data were analyzed parametrically with the JMP 7.1 statistical software package (SAS Institute Inc., Cary, NC) as described before (Moressis *et al.*, 2009). Following initial ANOVA, planned multiple comparisons were performed, using a=0.01. The level of significance was adjusted for the experimentwise error rate. Data are shown as mean ± S.E.M.

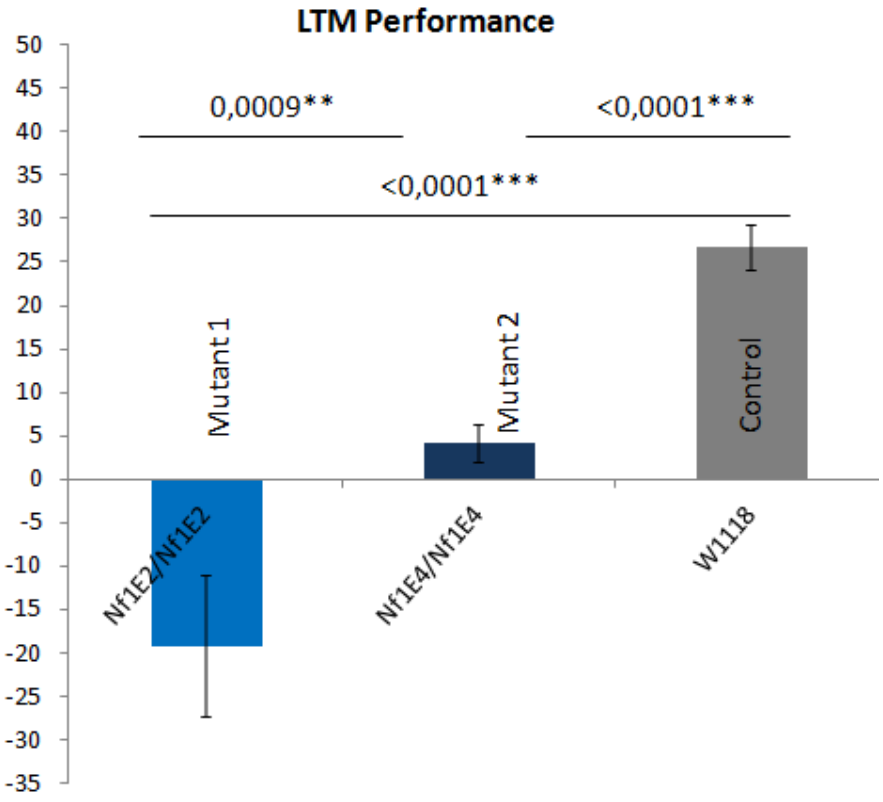
3.4. Immunohistochemical analysis and data processing

Whole-mount adult (2-6 days old) brains were dissected in cold PBS, fixed in 4% paraformaldehyde for 20 min at room temperature (RT) 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 RT, and incubated with primary antibodies for overnight at 4^0C. Samples were washed (PBST) three times for 15 min at RT and incubated with secondary antibodies for two hours at RT or for overnight at 4^0C. The samples were then washed again three times for 15 min at RT 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, Molecular Probes, 1:250). The secondary antibodies used were: Goat anti-mouse, conjugated with Alexa-Fluor secondary antibodies (1:400, all from Molecular Probes). Confocal laser microscopy was performed using the Leica 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 three times and was performed on multiple days. Images were formatted using Fiji software or Adobe Photoshop CS2.

4. Results

It is known from previous results of the Lab that both $Nf1^{E2}$ and (especially) $Nf1^{E4}$ mutant flies present learning impairments. While the expected Performance Index (PI) for the Control flies lies between 70-80 for learning, the *Nf1* mutant flies exhibit a PI of 30-50. The experimental hypothesis was that *Nf1* mutant flies would possibly demonstrate impairments in memory as well, which could be potentially reversed after either pan-neuronal or mushroom body-specific expression of the *Nf1* transgene. In order to validate this hypothesis, we tried to assess the LTM performance of these flies. Based on the data regarding the learning performance, we would expect from the *Nf1* mutants to display impairments -compared to the control groups- in memory as well, like in learning. Unfortunately, the LTM performance of the homozygous $Nf1^{E2}$/ $Nf1^{E2}$ mutant flies could not be assessed, since possibly for some metabolic or other reason homozygous flies for the mutant allele suddenly stopped being produced. However the hypothesis seems to be recapitulated even with a small number of flies (Histogram 1), as preliminary data.



Histogram 1: Long Term Memory (24h) performance of *Nf1*^{E2} and *Nf1*^{E4} mutant flies. The Performance index (PI) of the LTM performance. Values represent the mean ± standard error for each genotype PI = (# CS^- flies - # CS^+ flies) / (# total flies) x 100. PI = 100 => all flies correctly chose the correct odor (CS-). PI = 0 => random 50:50 distribution.

If this result is indicative, it is intriguing that the memory deficient phenotype of the null mutants seems to be more severe than that of the point mutants, which is the opposite of what occurs for learning and would be important to be elucidated in future studies.

To confirm that no anatomical differences are observed between the Nf1 null mutant and control flies, because of the absence of Nf1 during development, either in the MBs or in neurons outside the MBs, we performed confocal microscopy and compared the expression of GFP under the pan-neuronal Elav-Gal4 driver between the two strains (Fig. 11).

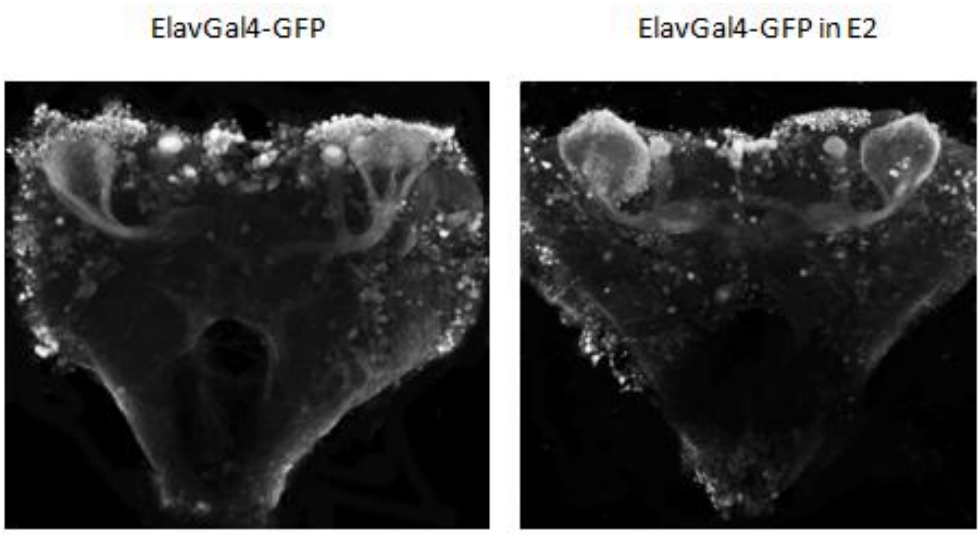


Figure 11: *Nf1*^{E2} mutants do not seem to display anatomical developmental impairments

Published work suggests that Nf1 displays a role inside the MBs for normal LTM, and particularly in specific neuronal subsets known as α/β lobes (Monica E Buchanan and Davis, 2010). Nf1 is actually widely expressed in the dendrites of the MBs (Jean Y. Gouzi *et al.*, 2011), strongly indicating its probable postsynaptic role in memory formation. Taking into consideration the data acquired up to this point regarding the role of Nf1 in learning, in OK72-GABAergic neurons, a major question that arose was whether Nf1 is required in neurons inside or outside the MBs for normal LTM

performance. To that end, we attempted to investigate if the expression of the Nf1 transgene in the MBs, rescues the LTM deficits of the *dNf1* mutant flies. In order to set up the necessary crosses for the generation of the appropriate strains that will bear the *Nf1* transgene and express it in a spatio-temporal and tissue specific manner, we took advantage of the GAL4-UAS and the TARGET system (Elliott and Brand, 2008), as well as of the variety of neuron specific and pan-neuronal drivers. The use of this system, allows for the characterization of a phenotype as either developmental or as adult specific.

Initially, we aimed to study the LTM phenotype only in adult flies, as it's been shown for learning deficits that they can be reversed in adult organisms, thus demonstrating that Nf1 loss does not cause significant developmental disruptions, at least in the neurons where Nf1 is required for normal learning (Jean Y. Gouzi *et al.*, 2011; Georganta *et al.,* in preparation). Therefore, based on bibliography arguing that Nf1 is essential within the MBs for normal associative LTM (M. E. Buchanan and Davis, 2010), as well as on the fact that MBs are considered the center of learning and memory, we aimed to rescue the LTM deficient phenotype of null mutants by expressing the Nf1 transgene specifically in the MBs of adult flies with two different MB- specific drivers, Leo (Messaritou *et al.*, 2009)(Fig. 12) and MB247 (Aso *et al.*, 2009).

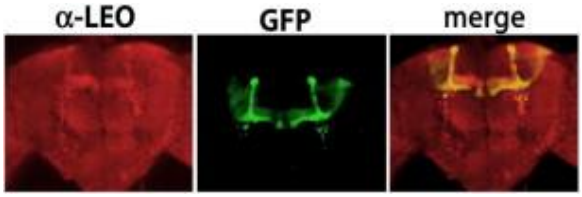
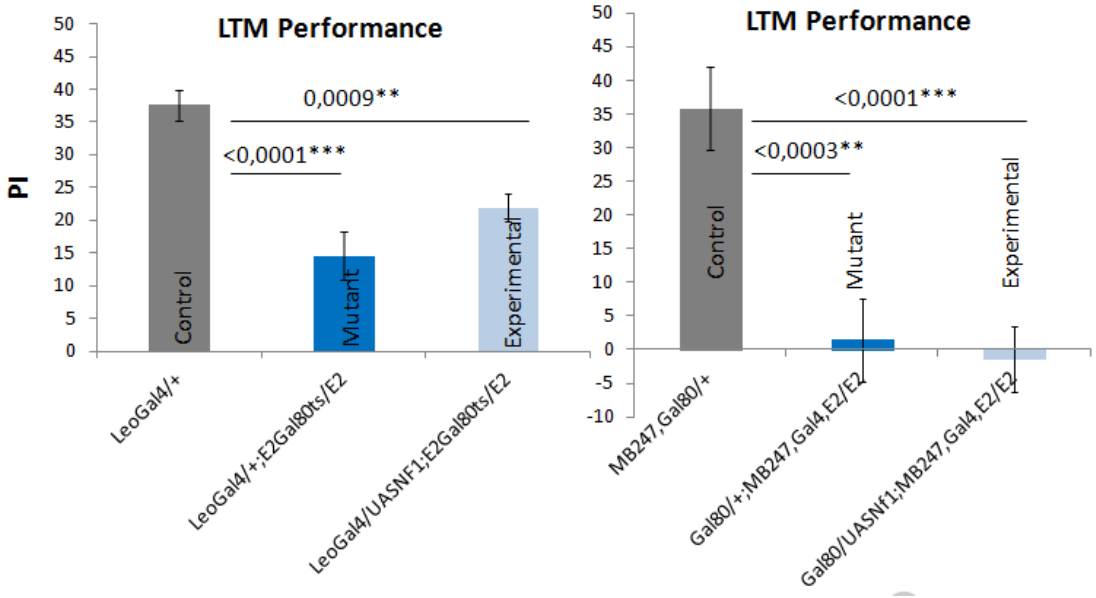


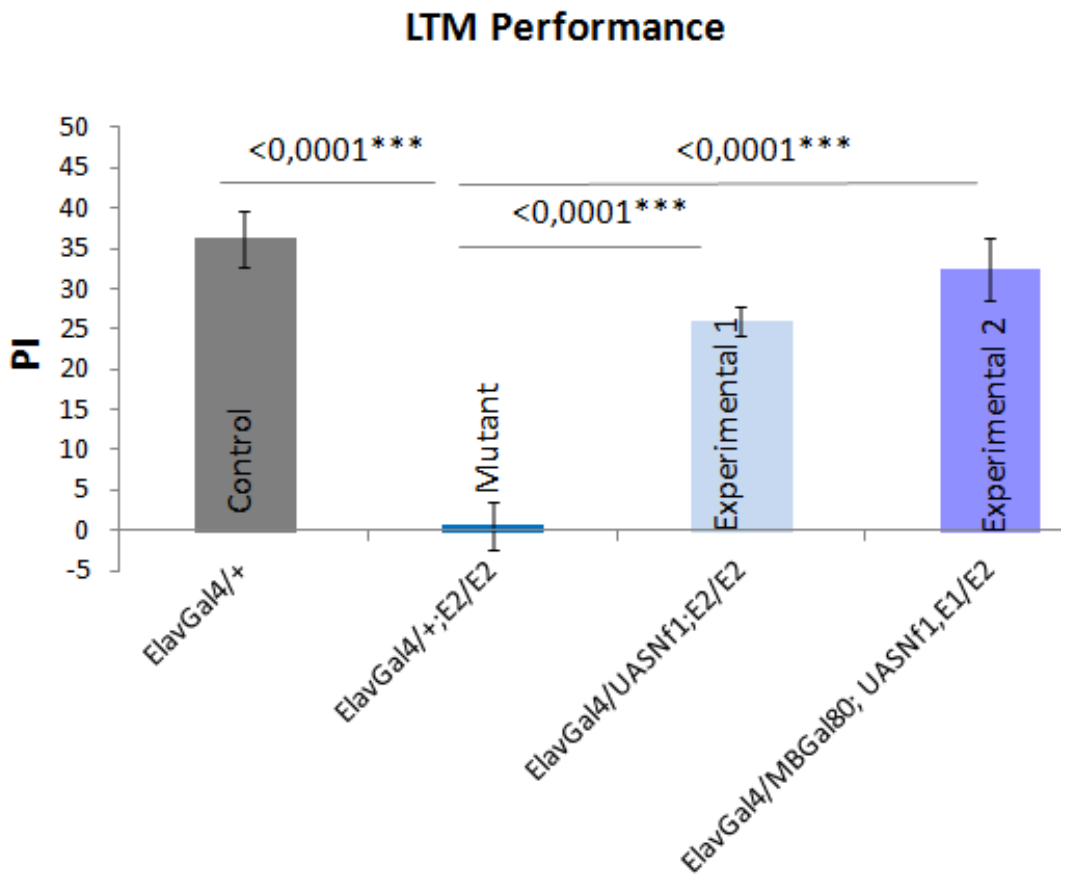
Figure 12: Expression pattern of Leo MB driver (Messaritou *et al.*, 2009)

In contrast to our hypothesis no rescue was achieved (Histogram 2), indicating that either Nf1 is not required within the MBs for normal LTM, but elsewhere as in learning, or that as far as LTM is concerned, Nf1 is required in the MBs developmentally.



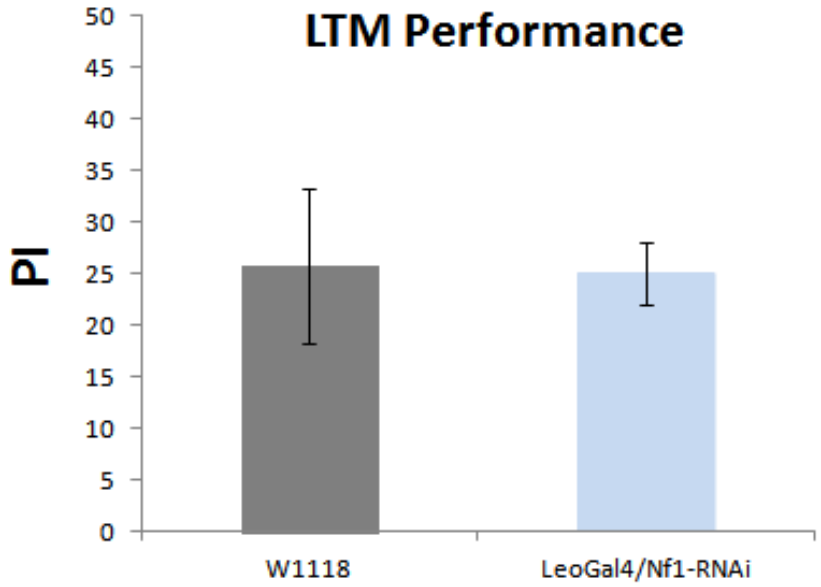
Histogram 2: Expression of the Nf1 transgene in the MBs (Leo driver - left graph and MB247 driver – right graph) of adult *Nf1^{E2}* mutant flies does not rescue the LTM deficit. Performance assessment 24 hours after negatively reinforced olfactory conditioning in control flies (grey bar), null mutants (dark blue bar) and flies expressing the transgene in the MBs (light blue bar), that have been subjected to 48 hours induction in 30°C before training. The Performance index (PI) for LTM is shown. Values represent the mean ± standard error for each genotype. The genotypes of all animals are indicated below each bar. G80^{ts} denotes the ubiquitously expressed temperature sensitive Gal4 repressor Tub-Gal80^{ts}. ***The w^{1118} strain of flies was used as a Wild Type (+/+) control. *The Progeny of choice was selected with the help of chromosomal Balancer markers such as TM3, TM6 & Cyo, that provide the flies with a distinct phenotype.***

In order to elucidate which of the above is the case, we consequently expressed the Nf1 transgene pan-neuronally during development, expecting to achieve full rescue of LTM, for the confirmation that Nf1 is required during CNS development to support normal memory formation. Full rescue of the LTM deficient phenotype of mutant flies was achieved, even when expressing the transgene pan-neuronally and excluding the MBs (Histogram 3). These results provide evidence that the expression of Nf1 inside the MBs during development is dispensable for normal LTM and thus the failure to rescue the phenotype when expressing the transgene in the MBs of adult flies (Histogram 2), seems to support the interpretation that Nf1 is not essential in the MBs for normal LTM.



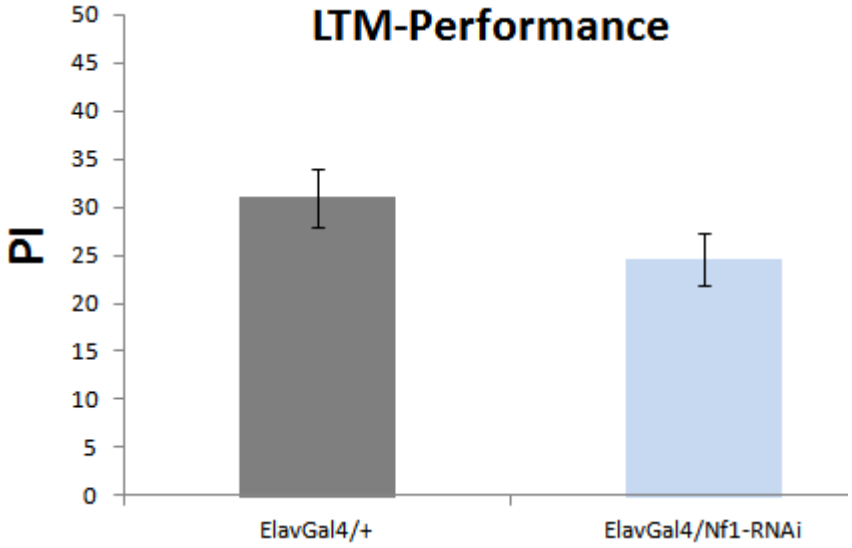
<u>Histogram 3:</u> Pan-neuronal & pan-neuronal excluding the MBs expression of the Nf1 transgene, during development, restores the LTM deficient phenotype of *Nf1^{E2}* mutant flies. Performance assessment 24 hours after negatively reinforced olfactory conditioning in control (grey bar), mutant (dark blue bar), pan-neuronally expressing the transgene –experimental 1- (light blue bar) and pan-neuronally expressing the transgene excluding the MBs –experimental 2- (purple bar) flies, that have been subjected to overnight induction in 30°C before training . The Performance index (PI) for LTM is shown. Values represent the mean ± standard error for each genotype. The genotypes of all animals are indicated below each bar.

Subsequently, we tried to recapitulate these results by deleting Nf1 with Nf1-RNAi in wild type flies during development, at first in the MBs and subsequently pan-neuronally. Deletion of Nf1 in the MBs was not expected to evoke a deficient phenotype based on previous results, and it did not (Histogram 4).



Histogram 4: Deletion of Nf1 in the MBs does not show an LTM deficient phenotype. Performance assessment 24 hours after negatively reinforced olfactory conditioning in control (grey bar) and pan-neuronally expressing the Nf1-RNAi (light blue bar) flies that have been subjected to overnight induction in 30°C before training. Depicted are the Performance indeces (PI) of LTM. Values represent the mean ± standard error for each genotype. The genotypes of all animals are indicated below each bar.

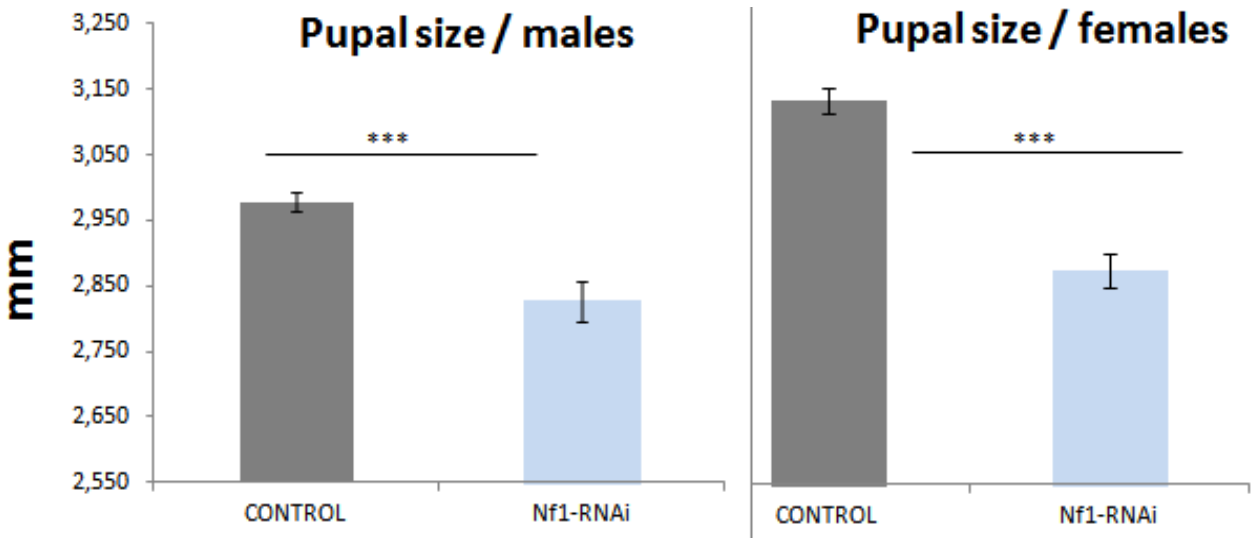
Pan-neuronal deletion of Nf1 however should – as a proof of principle- recapitulate the phenotype of null mutant flies and elicit an LTM-related deficit. Unexpectedly, once more it did not (Histogram 5), prompting us to doubt the efficiency of the particular RNAi.



Histogram 5: Pan-neuronal deletion of Nf1 does not show an LTM deficient phenotype. Performance assessment 24 hours after negatively reinforced olfactory conditioning in control (grey

bar) and pan-neuronally expressing the Nf1-RNAi (light blue bar) flies that have been subjected to overnight induction in 30°C before training. Depicted are the Performance indeces (PI) of LTM. Values represent the mean ± standard error for each genotype. The genotypes of all animals are indicated below each bar.

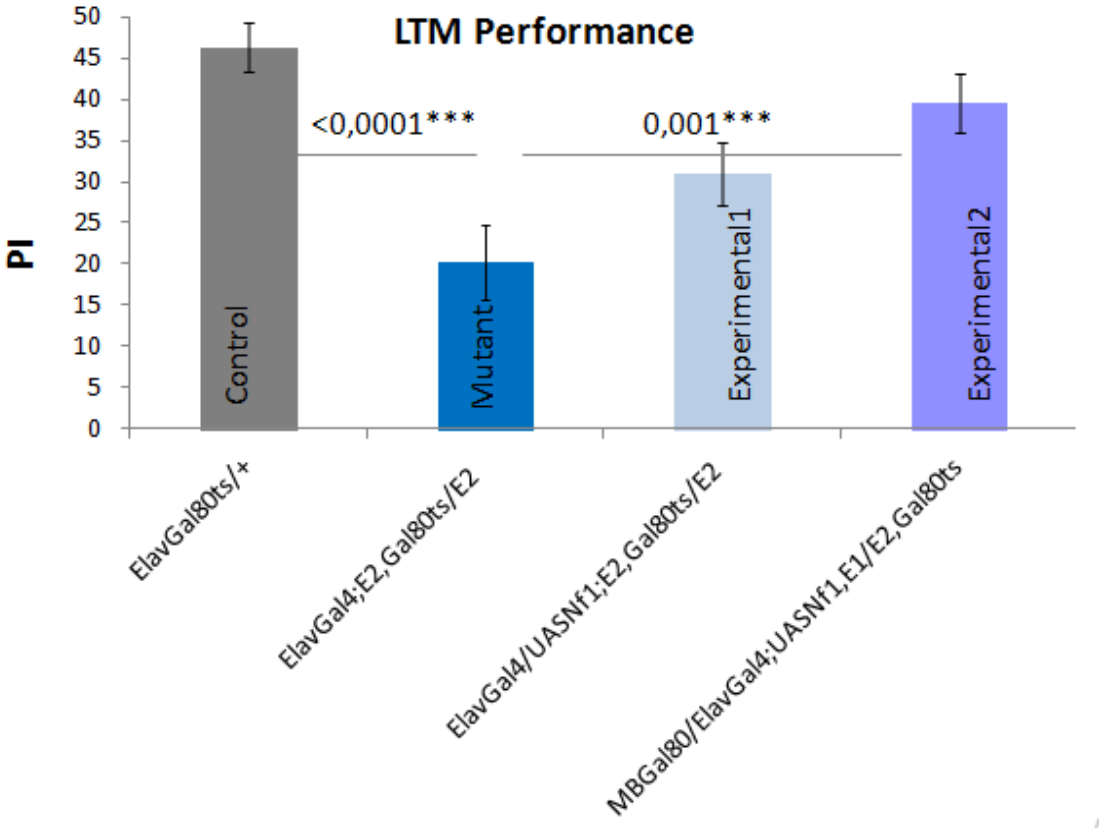
Unfortunately, since there is no commercially available antibody for dNf1, we cannot test this hypothesis with a Western blot analysis of protein expression levels. Thus, we measured the size of the pupae for males and females separately which were expected to be significantly smaller than controls if the RNAi would be effective. In both cases expression of the Nf1-RNAi under a Tubulin driver (expressed in every cell) resulted in pupae of significantly smaller size (Histogram 6), suggesting that the level to which the particular Nf1-RNAi used was expressed, was sufficient to affect molecular pathways implicated in growth but not sufficient to affect pathways associated with LTM formation.



Histogram 6: Tubulin-driven expression of Nf1-RNAi results in pupae size reduction.

To confirm the previous results and hypothesis we also expressed the UAS-*dNf1* transgene both pan-neuronally and pan-neuronally excluding the MBs, this time in adult flies. Unexpectedly, while the expression of the Nf1 transgene pan-neuronally and excluding the MBs, resulted in full rescue of the mutant LTM deficient

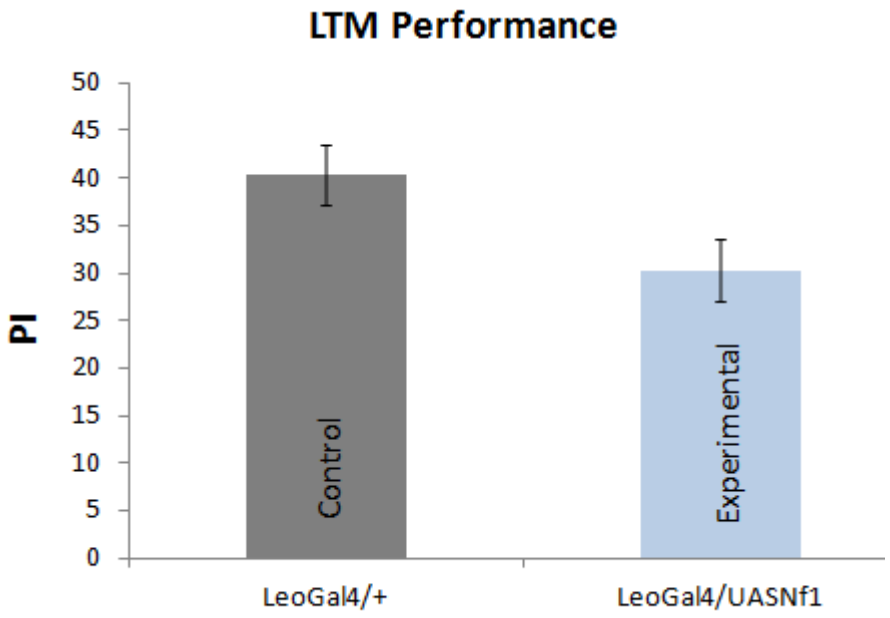
phenotype, its expression pan-neuronally including the MBs exhibited no rescue (Histogram 7).



Histogram 7: Pan-neuronal expression of the *Nf1* transgene excluding the MBs rescues the LTM deficient phenotype of adult *Nf1*^{E2} mutant flies but pan-neuronal expression including the MBs does not. Performance assessment 24 hours after negatively reinforced olfactory conditioning in control (grey bar), mutant (dark blue bar), pan-neuronally expressing the transgene –experimental 1- (light blue bar) and pan-neuronally expressing the transgene excluding the MBs –experimental 2- (purple bar) flies, that have been subjected to 48 hours induction in 30°C before training . Depicted appears the Performance index (PI) of the LTM performance. Values represent the mean ± standard error for each genotype. The genotypes of all animals are indicated below each bar. G80^{ts} denotes the ubiquitously expressed temperature sensitive Gal4 repressor Tub-Gal80^{ts}.

This puzzling result urged us to speculate that perhaps since we are unable to control the amount to which the transgene is expressed, we cause over-expression that is harmful if it occurs inside the MBs. If this hypothesis were valid the no rescue phenotype of the pan-neuronal expression could be explained. Again, since there is no commercially available antibody for dNf1, we cannot test this hypothesis with a Western blot analysis of protein expression levels. However, to validate our

hypothesis, we expressed the Nf1 transgene in the MBs of wild type flies that express endogenous Nf1, during development, to see if over-expression may result in LTM defects. Although the p-value is close to borderline, the final results indicate that there is overall no significant difference in the LTM performance when over-expressing the transgene in the MBs compared to the control group. This result fails to explain the no-rescue phenotype when expressing the transgene pan-neuronally in adult flies (Histogram 8), leading us to the hypothesis that acute over-expression of the transgene in the MBs of adult flies might be the restricting factor for normal LTM, which is not the case when Nf1 is absent during development possibly due to functional compensation that might have occurred by unknown mechanisms. This hypothesis remains to be tested, along with other issues that need to be elucidated in future studies, which are discussed below. An alternative explanation is that the driver may not be optimal for this and another pan-MB driver needs to tested to verify this result independently.



Histogram 8: Over-expression of the Nf1 transgene in the MBs of control flies during development does not cause defects in LTM. Performance assessment 24 hours after negatively reinforced olfactory conditioning in control (grey bar) and over-expressing the transgene in the MBs (light blue bar) flies, that have been subjected to overnight induction in 30°C before training. Depicted are the Performance indeces (PI) of the LTM performance. Values represent the mean ± standard error for each genotype. The genotypes of all animals are indicated below each bar.

5. Discussion

Collectively, these results strongly indicate a role for Nf1 outside the MBs for normal olfactory associative LTM. This is contradictory to previous work stating that Nf1 is required in specific compartments of the MBs (α/β lobes) for normal LTM (Monica E Buchanan and Davis, 2010). The same study supports that the learning impairments of Nf1 mutants can be fully reversed upon expression of the Nf1 transgene therein as well. This inconsistency has been explained by the fact that the c739-Gal4 driver that Buchanan and Davis use in the aforementioned publication, is not only expressed in the α/β lobes of the MBs but also in other extrinsic neurons including GABAergic local interneurons of the Antennal lobe (Wilson, 2005; Chen *et al.*, 2012), the Superior Medial Protocerebrum (SMP) (Kahsai, Martin and Winther, 2010) and the Dorsal Anterior Lateral neurons (DALn) (Chen *et al.*, 2012). As described above, a group of cells in the SMP are part of the OK72-GABAergic circuit shown by our Lab to require the expression of Nf1 within it, for normal associative learning (Fig. 13) (Georganta *et al.*, in preparation). In fact, the attempt of our lab to recapitulate the results of Buchanan and Davis failed, since both the expression of the WT transgene using the c739-Gal4 driver and its expression solely in c739 neurons aside from the MBs, only partially and equally rescued the learning deficient phenotype of null mutants, thus indicating that the learning improvement under c739 is likely a consequence of UAS-*dNf1* expression in these SMP cells and not the MBs.

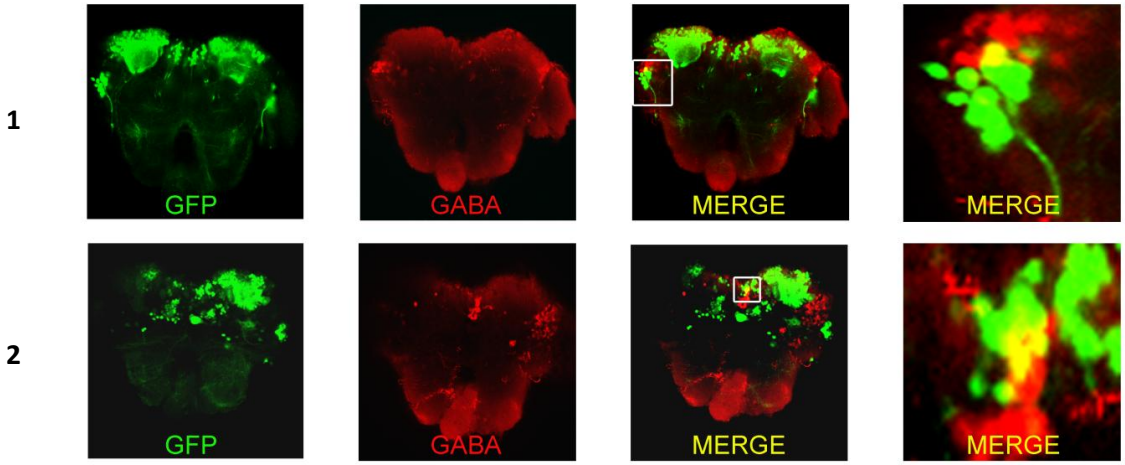


Figure 13: Co-localization of GABAergic and OK72-marked neurons within the lateral horn (line 1) and a group of cells in the SMP (line 2). These specific GABAergic OK72 neurons represent the minimal number of neurons where dNf1 is required for normal olfactory associative learning (Georganta *et al*., in preparation)

Based on the data arguing for a role of Nf1 outside the MBs for normal LTM, we hypothesize that the prerequisite for normal LTM is functional Nf1-mediated learning and therefore, that the circuits where the expression of Nf1 is essential and sufficient for normal LTM might, most possibly and at least partially, coincide with the OK72-GABAergic circuit. Thus, the rescue of the LTM deficient phenotype of mutants upon expression of the transgene in the α/β lobes that Buchanan and Davis observe, might as well be a result of the non MB specific nature of the c739-Gal4 driver. A nice way to validate this hypothesis would be to use this driver in order to express the Nf1 transgene wherever c739 is expressed but excluding the MBs (c739-Gal4 + MBGal80), in mutant flies, aiming to rescue the LTM impairments of the latter, as it has been done for learning.

It is intriguing that although the MBs are considered the main center of learning and memory in drosophila, both Nf1-dependent learning and Nf1-dependent memory, seem to take place in neurons outside the MBs, but probably projecting to them. A study published in 2012 by Chen *et al.,* surprisingly shows that blocking protein synthesis in two DAL neurons but not in the MBs, impairs LTM. In particular, memory retention was impaired by blocking neural output in DAL during retrieval but not during acquisition or consolidation. These findings suggest an extra-MB memory circuit in Drosophila: LTM consolidation (MB to DAL), storage (DAL), and retrieval (DAL to MB) (Chen *et al.*, 2012). The above evidence, combined with the expression pattern of the c739-Gal4 driver that includes DAL neurons, might provide an alternative hypothesis - other than that arguing that Nf1 is required for LTM in neurons coinciding with the OK72-GABAergic circuit - that puts in the forefront DAL neurons as a key player among the neurons outside the MBs, where Nf1 is required for normal associative LTM.

The description of the novel neuronal circuit where dNf1 presence is necessary and sufficient for normal olfactory associative learning marked by the OK72 driver

involves GABAergic neurons and requires normal GABA synthesis therein (Georganta *et al*., in preparation). In accordance to this finding, studies in *Nf1* deficient mice, suggest a role for GABAergic hippocampal neurotransmission in learning impairments (Costa *et al.*, 2002b; Cui *et al.*, 2008a). In particular, they associate the NF1 learning deficits with excessive Ras activity which leads to impairments in long-term potentiation (LTP) caused by increased GABA-mediated inhibition. Recent results of our lab also demonstrated an implication of Ras1 in Nf1-mediated learning in the aforementioned GABAergic neurons. Evidence demonstrated that the over-activation of Ras1 upon Nf1 loss, most likely leads to increased production and release of GABA, since Gad RNAi within this OK72- GABAergic circuit fully reverses the learning deficits of null mutant flies (Georganta *et al*., in preparation). Genetic and pharmacological experiments performed in mice and Drosophila have previously supported a role for the Ras-GTPase activating domain (GRD) of Nf1, which functions to downregulate Ras activity in Protein Synthesis Dependent memory (Silva *et al.*, 1997; Costa *et al.*, 2002c; Li, Cui, Steven A Kushner, *et al.*, 2005; Guilding *et al.*, 2007; Ho *et al.*, 2007; Cui *et al.*, 2008b). These data strongly indicate that impaired GABAergic neurotransmission due to Ras over-activation could underlie the Nf1-dependent LTM deficits as well, in neurons outside the MBs. However, the controversial, as far as learning is concerned, association of Nf1 with the cAMP/PKA pathway should also be investigated as a possible mechanism implicated in Nf1-mediated LTM.

It is crucial that the experiments performed for this thesis arguing that Nf1 is not required inside the MBs for normal LTM should be recapitulated with the use of alternative pan-neuronal drivers as well as of additional functional Nf1-RNAis to phenocopy the LTM deficits resulting from loss of Nf1 in neurons outside the MBs. Moreover the exact neuronal circuits where the expression of Nf1 is necessary and sufficient for normal LTM are expected to be unraveled by future studies, in order for these results to be solidified. In particular and to summarize the above, the attempt to rescue the LTM deficient phenotype in the neurons where c739 is expressed except for the MBs, is crucial in order to overcome the inconsistency with previously published data (M. E. Buchanan and Davis, 2010). Since the c739-marked neurons overlap with (a) OK72-marked neurons in a group of cells within the SMP

[Fig. 14(5)], (b) certain GABAergic neurons including the group of cells in the SMP [Fig.13, 14(5)] and (c) DAL neurons, it is very likely that the rescue in LTM observed by the study of Buchanan and Davis derives from the expression of the UAS-*dNf1* transgene in some or all of these overlapping neurons.

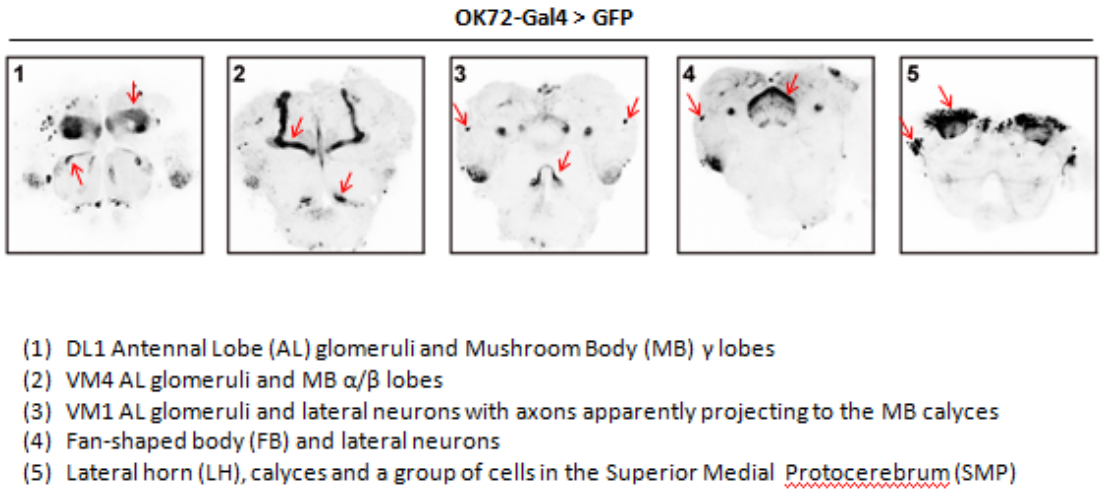


Figure 14. Detailed characterization of the OK72-Gal4 expression pattern (Georganta *et al.*, in preparation)

Thus it is important to investigate whether the expression of the transgene in (1) GABAergic neurons other than those coinciding with the OK72-GABAergic circuit, (2) non GABAergic neurons marked by the OK72 driver, as well as in (3) DAL neurons, rescues the LTM deficits of null mutants and subsequently proceed to the narrowing down, aiming to identify the minimal number and types of neurons required for normal Nf1-mediated LTM and whether they crosstalk or coincide with the OK72-GABAergic circuit.

Last but not least, Alk has been shown to act as a negative regulator of olfactory learning and memory in neurons outside and inside the MBs, respectively. Taking that into consideration, the question that arises is whether Alk is functionally interacting with Nf1 in the neuronal circuits where Nf1 is required for normal memory formation, like it does in the respective circuit responsible for Nf1-mediated learning. Genetic inhibition of Alk in the specific circuits that are expected to unravel (where Nf1 is required for normal LTM) in Nf1 mutant flies, aiming for the rescue of

the LTM deficient phenotype, would shed light to that question. Although Alk acts as a negative regulator of memory only inside the MBs (Gouzi *et al*., 2018), and the results of this thesis propose a role for Nf1 outside the MBs for normal memory, it is possible that Alk and Nf1 functionally interact in a non-cell autonomous manner to regulate LTM. Thus, it would be very interesting and informative to investigate whether Alk inhibition inside the MBs rescues the LTM deficient phenotype of Nf1 mutants.

In conclusion, the results of this thesis strongly support for the first time a role of *dNf1* outside the MBs for normal olfactory memory. Considering the fact that the expression levels of Nf1 are elevated in the dendrites of MB neurons, an important question that rises and needs to be addressed concerns the role of Nf1 therein. MBs might be considered as the center of learning and memory, that being their most prominent function, but display other functions as well where Nf1 might be involved, including habituation. Unpublished data of our lab along with a recent independent study (Fenckova *et al.*, 2019), argue for a role of the MBs both in shock and jump habituation respectively. People suffering from autism spectrum disorder show reduction in habituation to a variety of stimulus and NF1 patients have been reported to exhibit autism spectrum disorder- like traits. Therefore, it would be particularly interesting and significant to investigate whether loss of Nf1 in the MBs affects the different types of habituation.

For cognitive syndroms like Nf1, an essential question is whether all cognitive deficits stem from loss of Nf1 protein within a particular neuronal subset or multiple neuronal circuits. A second question is whether the protein engages a single (and which) signaling cascade, or distinct ones subserving its cognitive functions within one or different neuronal populations. Therefore, the comprehension of the molecular mechanisms and neuronal circuits that are disrupted due to *Nf1* loss, leading to the manifestation of multiple and various cognitive deficits is highly essential for the development of novel and targeted treatment approaches. Further experiments are required to shed light into the mechanisms and the molecules implicated in Nf1-mediated LTM.

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