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The Role of NF1 in *Drosophila* Appetitive Long Term Memory

A Dissertation Presented

by

Chunsu Xu

to

The Graduate School

in Partial Fulfillment of the Requirements

for the Degree of

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Abstract of the Dissertation

The Role of NF1 in *Drosophila* Appetitive Long Term Memory

by

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Doctor of Philosophy in **Neuroscience**

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This thesis explores the consequences of mutated neurofibromin gene, NF1, on memory. NF1 disorder is an inheritable genetic disease in humans that is produced by mutation of the gene that encodes the large protein neurofibromin. Mutations in this gene produce fairly non-specific and widespread cognitive defects in human patients, in addition to a variety of other pathologies, such as benign tumors of the peripheral nervous system.

It is unknown how NF1 disorder leads to cognitive deficits. Uncovering the mechanism is hampered by the fact that mutations in the NF1 gene have very unpredictable effects. Even identical (monozygotic) twins who have the same mutation sites can manifest wildly different symptoms. It is unclear why this is the case, but it certainly shows that mutated neurofibromin causes different pathologies in different tissues. Whilst NF1 is widely expressed throughout the body, it is not known if cognitive defects arise from its action in a particular region of the brain or throughout the whole brain. Here I explore this issue using the powerful genetic and behavioral techniques in the model organism, *Drosophila melanogaster*.

I show that normal NF1 protein is required in octopamine neurons. Lacking of correct NF1 gene expression in these neurons leads to impaired long term memory, but not impaired learning. Manipulating the excitability of octopamine neurons after consolidation modulated appetitive LTM. NF1-dependent memory does not require the mushroom body, the insect learning and memory center. This the first time a specific neuron type has been identified as playing a role in the cognitive deficits in *Drosophila* NF1 research.

This thesis is dedicated to my dad, Yi, who guided me to be interested in science and encouraged me to pursue science. He provides me with patience, optimism, positive feedback and unlimited support, and makes me always feel happy with what I am doing.

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Chapter 1

Introduction

There are two broad ways of studying brain function. On the one hand, researchers have explored brain structure and function in normally functioning individuals. On the other hand, researchers have studied individuals with brains perturbed either experimentally, through misadventure, or through disease. Modern techniques available in genetic model organisms, such as flies, worms and mice, are increasingly allowing researchers to blur the distinction between these two approaches.

The modern era of neuroscience was ushered in by Ramón y Cajal, who begun work towards the end of the 19th century. Whilst Cajal was an anatomist, his forceful defense of the neuron doctrine, the idea that neurons are discrete cells, had profound functional implications. Although Cajal worked exclusively on fixed tissue, he had astonishing intuition for the functional nature of his static specimens. Cajal believed neurons were polarised, with information flowing in one direction through them, and he inferred how axonal growth cones developed and grew despite never seeing one move. It wasn't until the mid 20th century that functional analysis of neurons via electrophysiology became practical and widespread. The signature work in the field was conducted by Hubel and Wiesel, who studied the response properties of neurons in the primary visual cortex to visual stimuli.

Working at about the same time as Cajal, researchers such as Ferrier, Schafer, and Munk mapped cortical function through a combination of lesion studies and electrical stimulation. In this manner, areas such as motor cortex, visual cortex, and auditory cortex were identified. Whilst most of this work was performed in dogs, other avenues have produced data from humans such as the case of HM's hippocampal ablations during surgery for epilepsy. Finally, through studying diseases with very specific consequences, such as Parkinson's,

we have been able to learn a huge amount about specific brain circuits.

Our modern understanding of the brain comes from an variety of approaches. The ability to modify the function of specific genes and explore the consequences using behavioral and physiological measures is a technique which has come into its own relatively recently. In this thesis, I explore the consequences of a gene mutation known produce fairly non-specific and widespread cognitive defects in human patients. The gene, NF1, encodes a large protein called neurofibromin. The function of neurofibromin in the brain is unknown and it is also unknown how disease-causing mutations in this gene lead to cognitive deficits. These questions are explored using powerful genetic techniques and behavior in the model organism *Drosophila melanogaster*.

1.1 General introduction

1.1.1 The NF1 disorder, a brief historical review

Neurofibromatosis Type 1 (NF1) is a dominant autosomal genetic disorder affecting approximately 1 in every 3,000 people[1, 2]. The most prominent symptom of the disease are the so-called neurofibromas, which are benign nerve sheath tumors of the peripheral nervous system that can also spread to other tissues. Although named after this type of tumors, NF1 is in essence a highly variable disorder with a range of symptoms which differ between patients. The symptoms of NF1, includes cognitive problems, hypertension, gastrointestinal problems, disfigurement and malignancy including nervous system tumours[3, 4]. To make things worse, the types of symptoms, their severity as well as when they manifest varies even within the same affected family. NF1 is a textbook example of pleiotropy, that when one gene influences multiple, seemingly unrelated phenotypic traits. Thus a better understanding of the NF1 disease, not only offers treatment potentials but also a reward for basic research in understanding the biology of pleiotropic gene.

It has been more than 130 years since the NF1 disorder was discovered. The disease was originally named von Recklinghausen disease by Friedrich von Recklinghausen in 1882[5]. But it was not until 1951 that comprehensive work on understanding the disease commenced. In 1951, Borberg published details of a large Danish patient cohort[6]. In his publication, Dr. Borberg pointed out the existence of tremendous symptom variability within families affected by the NF1 disorder and, in particular, he emphasized the NF1 progression into adulthood and clarified that 'not all of the patients' clinical problems are tumor-related.' Dr. Borberg's work, perhaps providing few therapeutic benefits for the patients themselves, nonetheless laid the groundwork for NF1

disease diagnosis and assessment.

1.1.2 The NF1 gene

Borberg's work was critical in cataloging NF1 symptoms and establishing that these diverse presentations were, in fact, a single underlying disease. Therefore, when molecular genetics and cloning techniques flourished, the molecular basis of NF1 were quickly established. In 1987, a gene underlying the disorder was assigned to the long arm of human chromosome 17 (17q)[7–10]. In 1990 the NF1 gene (the gene was named after the disorder) was cloned and the gene product, neurofibromin, was identified [11, 12]. In 1994, the first NF1 mouse models were published[13, 14].

In the first decade of the 21st century, the NF1 research field has progressed tremendously on two fronts: on the clinical side, pathological assessment and monitoring in the NF1 patients has been established due to advances in MRI techniques. Secondly, on the basic research side, the booming of sequencing and other genomic techniques revealed the complicated molecular biology of the NF1 gene. As we will see, this complicated molecular biology likely underlies symptom variability across patients.

The NF1 gene spans 289,701 base pairs (bp) on the 17th chromosome and encodes 57 exons, making the NF1 gene unusually large. Currently, there is only one known transcription start site and one translational initiation codon for the NF1 gene[15]. The NF1 transcript is a 2,818 amino-acid peptide (mRNA RefSeq accession number NM_001042492.2), which is expressed ubiquitously but with an elevated level in the skin and nervous system [16].

Given the size of the NF1 gene, it perhaps not surprising that about 1,300 different inherited mutations have been reported as a cause of the NF1 disorder[17]. Oddly, even sharing the same mutation in the same family, there are multiple NF1 phenotypes. Investigation into those pathogenic mutations shows poor genotype-phenotype correlation: it is confirmed that only the gross deletions, which constitute 5% of patients, predictably exhibit more severe clinical phenotypes[18]. The remaining 95% of patients, who might have single pair substitutions such as non-sense mutations, or micro-deletions and splicing mutations, have symptoms that can not be easily predicted from the genetics. For instance they may have neurofibromas, optic glioma, or cognitive defects. When in life symptoms manifest can also be variable[19, 20]. One vivid example, shown in Figure 1.1, is the difference between actor Adam Pearson (recently in the Movie 'Under the Skin') with his identical twin brother, Neil Pearson. Adam suffers from facial neurofibroma, whereas Neil, who looks completely normal, suffers from a severe short-term memory deficit. Thus, by and large, NF1 gene mutation screening has not provided a forum for health risk

assessment.

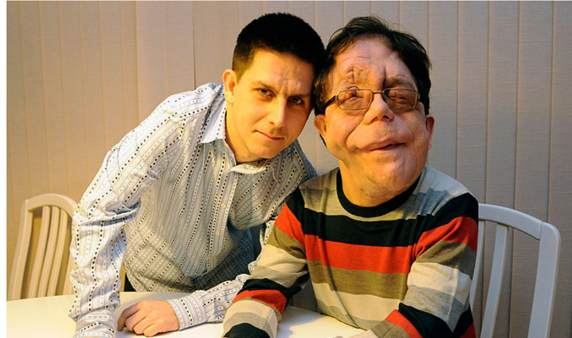


Figure 1.1: Identical (monozygotic) twin brothers, Adam and Neil Pearson, both bear the same NF1 mutation but manifest the complications in different forms. Adam has severe facial tumors and has undergone over thirty medical procedures because of this. Neil is outwardly normal but has impaired short term memory. NF1 disorder affects Neil on the inside and Adam on the outside. Taken from David Lowe, The Sun newspaper, 2011.

1.1.3 Linking NF1 function to phenotype

NF1 has been long hypothesized as tumor suppressor gene since the identification of its Ras-GAP related domain (GRD). Ras, encoded by oncogene RAS, is a small guanine nucleotide binding protein. When binding with GTP, Ras is in active form and transduces downstream signaling involved in cell growth, differentiation and survival; and when binding with GDP, Ras turns into inactive form and terminates downstream signaling[21]. Mutated Ras is often found in malignant tissues, where it demonstrates an inability to be switched to the inactive form[22]. This can cause unintended and overactive signaling inside the cell, even in the absence of incoming signals. Because these signals result in cell growth and division, overactive Ras signaling can ultimately lead to cancer[23].

The function of Ras-GAP, acts as Ras's 'off-switch' by accelerating the hydrolysis of Ras-bound GTP, consequently turning Ras from an active form to inactive form and therefore down-regulates the biological activity of Ras[24]. Thus, the neurofibromas and other tumors common in the NF1 disorder are probably due to the NF1 gene's function as a tumor suppressor.

To describe the full picture of the NF1 disorder, however, the definition of NF1 function as tumor suppressor faces challenges. The first challenge is our understanding of Ras function. To say RAS is an oncogene is more of a statement about the mutation than about the wild-type gene itself. As more studies show that Ras regulates normal cellular growth, differentiation as well

as long term potentiation induction[25], the role of NF1 function needs to be explained under this cellular context as well. The second challenge resides within the large NF1 gene itself: the Ras-GAP coding domain is only one tenth of the total coding sequence of NF1, many domains have been predicted or reported to interact with protein partners other than Ras, and this includes protein kinase A (PKA)[26], protein kinase C (PKC) [27, 28], caveolin-1 [29–31], focal adhesion kinase [32], tubulin [33], amyloid precursor protein[34], syndecan[35], kinesin-1[36], nuclear PML-bodies[37], the UBX-UBD protein ETEA[38] and p97/VCP[39]. A general statement about NF1 gene function seems hard to achieve due to this ever growing list. It is perhaps easy to understand by now that the pleiotropic effect manifested by the NF1 gene is due to its ubiquitous expression and its interaction with many major pathways; and for this reason, it is difficult or even wrong to investigate individual NF1 symptoms without identifying the responsible tissue or cellular types. The challenge faced by NF1 researchers nowadays is how to investigate NF1 gene function within a specific tissue type or cellular type *in vivo*.

1.1.4 NF1 and cognitive defects

Neurofibromin is ubiquitously expressed but with the highest levels being found in the nervous system. Many neuronal cell types, irrespective of neurotransmitter expression, neuronal pathway, or brain region contain neurofibromin[40]. In neurons, this protein appears to be most abundant in dendritic processes. Cells within the central nervous system that express neurofibromin include oligodendrocytes and cortical neurons. In the peripheral nervous system, this protein is expressed in non-myelinating Schwann cells, dorsal root ganglia, and peripheral nerves[16].

Although initially characterized based on the neurofibromas, NF1 disorder has cognitive defects as one of its most common complications. Up to 70% of NF1 children have certain cognitive defects[41], which includes: lower IQ profile, visual-spatial defects, language problems, executive function disorder, learning and memory defects, and attention disorders. The list is getting longer as our diagnosis of NF1 disorder advances and the evaluation of cognitive function in children gets more comprehensive. Moreover, the clinical presentations of cognitive impairment varies from patient to patient, or even within the same family.

The variety of symptoms and their high incidence both suggest the importance of NF1 in the central nervous system. However, what role NF1 plays in the CNS is almost entirely unknown. To understand the role of NF1 in the brain, one question to ask first is whether NF1 gene disorders disturb the brain globally or that regional perturbation leads to different cognitive de-

fects. Since the gene is ubiquitously expressed in the brain, it is conceivable that NF1 gene disorders lead to very broad or global changes in brain function. Alternatively, the pleitropic nature of the NF1 gene may mean that, despite its broad expression, the locations at which it affects brain function are still fairly specifically localized.

Structural imaging studies have provided evidence of abnormal brain development. The most replicated finding in NF1 is increased brain size associated with macrocephaly[42]. However, the relationship between brain volume and cognitive function in NF1 is uncertain[43, 44]. Examination of brain regions such as the corpus callosum and language cortex suggest a link between the size of those regions and level of certain cognitive functions[45, 46]; however, these studies provide little mechanistic insight into the molecular pathology of cognitive defects in NF1.

Contrast to conventional MRI, which focuses on the structure of the brain, functional MRI (fMRI) provides dynamic interrogation of brain activity. Some, but not many, cognitive processes have been investigated under fMRI, for example: phonological processing[47], visual spatial processing[48, 49], working memory and executive functioning[50]. Whilst these results are tantalizing and help localize the effects of the gene mutations, fMRI naturally remains the wrong tool for exploring gene function. In particular, it is very difficult to establish mechanistic relationships between neuro-cognitive phenotypes and genetic mutations in human patients. To investigate the underlying pathology of the disorder, animal models have the great advantage of tissue-specific gene targeting and, coupled with controlled experimental conditions, can help researchers understand the pathogenesis of NF1 from molecular, to cellular, through to whole circuit levels.

1.2 Behavioral genetics

Behavioral genetics is a scientific discipline which studies the role of genetics in the animal and human behavior. The heritage of this discipline, can be summarized by Hermann Ebbinghaus' quote that it has '*a short history, but a long past*'(Ebbinghaus, 1908). The 'long past' for behavior genetics, can traced back as early as 15, 000 years ago, when mankind domesticated wolves into dogs [51]. Domestication, as well as selective breeding for desirable traits, begun in ancient times and continues to this day. Today, behavioral genetics has flourished into a multi-disciplinary field involving psychology, genetics, epigenetics, ethology, molecular biology, and statistics.

1.2.1 Fruit flies as a powerful genetic tool

The fruit fly, *Drosophila melanogaster*, has been a popular animal model to study genetics for more than a century. The *Drosophila* gene mini-white, has the glory of being the first gene associated with inheritance of a specific trait, as its presence was linked to a microscopically observable feature on the fly's polytene chromosome (Fig. 1.2). This discovery by Thomas Morgan strongly supported the chromosome theory, i.e. that chromosomes are the carrier of genetic material, and was made well before the discovery of DNA's double helical structure in the 1953.

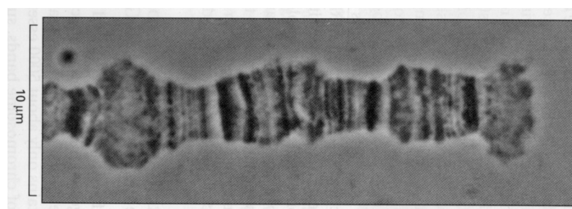


Figure 1.2: A polytene chromosome under the light microscope. Taken from Alberts, *et al.* 1994. Molecular Biology of the Cell. Garland Publishing, New York, NY.

Ever since Thomas Morgan, many genetic discoveries has been made through fruit fly research and in return, an array of genetic and molecular tools have evolved for analysis of gene function.

- *P-element-induced mutant generation.* A P-element is a small non-retroviral transposon identified in flies that has become an unusually useful tool for controlled genetic manipulation[52]. When provided with transposase in the germline, single P-elements can be mobilized therefore causing mutations along the chromosome at high rates. By adding antibiotic resistance genes into the P-element, one can select the progeny of successful jumping events. Moreover, the P-element identifies the location of the induced mutation, allowing rapid mapping, complementation testing, and cloning of the affected gene. And by adding marker gene, such as eye color, one can visually identify the resulting mutant line and facilitate future genetic analysis [53].

- *Spatially controlled gene expression.* The GAL4-UAS system, drives expression of a gene in a tissue-specific pattern[54]. The GAL4 protein is a potent transcriptional activator in yeast; UAS, for Upstream Activation Sequence, is an enhancer to which GAL4 specifically binds to activate gene transcription. By fusing its coding sequence to the P-transposase promoter, different GAL4s depending upon its genomic site of integration, can direct expression in a wide range of patterns in embryos, larvae

and adults. Transgenic flies carrying the target gene are then crossed to a GAL4-expressing line, producing progeny in which the target gene is transcribed in a specific GAL4-dependent pattern. The GAL4 system can be used to express any gene of interest ectopically, including one that might be lethal to the organism.

- *Temporal controlled gene expression.* Gene expression can be triggered via heat-shock promoter. The gene can then be turned on at a specific point by heat-shocking the transgenic animal[55, 56].

- *Spatial and temporal control of gene expression.* The yeast transcription factor GAL4 has been modified into an RU486-inducible form. Upon RU486 feeding, tissue specific gene expression can be induced[57].

- *Double system.* GAL80 is a GAL4 repressor which blocks GAL4 activity by binding to its transcriptional activation domain. By generating different GAL80 lines suppress different GAL4 patterns, spatially refined expression in *Drosophila* can be achieved[58]. Moreover, GAL80 has a temperature sensitive form. At low temperatures (about 18°C) Gal80ts is able to inhibit GAL4 transcriptional activity. At 30°C GAL80ts becomes inactive and cannot inhibit GAL4. This GAL80TS/GAL4 can achieve both temporal and spatial resolution in gene expression[59].

All these points have made *Drosophila* one of the most powerful tools for analysis of gene function. In addition to these tools, the cross-species gene conservation also makes fruit flies a good 'test tube' to study human genes. Now we know many fundamental genes and their products are evolutionarily conserved. It is estimated that about 75% of known human disease genes have a recognizable match in the genome of fruit flies[60] and 50% of fly protein sequences have mammalian homologs[61]. Thus *Drosophila* provide a good start to look into the genetics of human disease.

1.2.2 A fruit fly model to study behavior genetics

Fruit flies exhibit a variety of behaviors: flies can form associative memory to odors, can manifest aggression and mate preference, have circadian rhythm and sleep patterns, food searching behaviors, predator avoidance behaviors, phototactic behaviors, grooming, courtship, etc.

The first demonstration that a mutant can affect behavior was done by Ron Konopka and Seymour Benzer in 1971 using fruit flies. They found mutations on the clock gene, could have either faster or slower rhythms comparing to the wild type which has activity rhythm of 24 hours. Ever since, Benzer and

others have used behavioral screens to isolate genes involved in many aspects of fly behavior, such as vision, olfaction, audition, learning/memory, courtship, and pain[62]. Benzer demonstrated the capability of mutant studies to explore complicated traits like behavior and revolutionized neurobiology by marrying the tradition of behavioral studies with molecular biology. Ever since Benzer, mutants in many animal models have been created, and the neurogenetic field became one of the leading methodologies in modern neuroscience.

With an estimated a hundred thousand neurons in the fly's head, more and more unexpected behaviors and cognitive abilities are published every year: social suppression induced alcohol consumption[63], spatial learning[64], observational learning[65, 66]. A rich behavioral spectrum and unparalleled genetic access has propelled flies as one of the most popular animal models to study behavior genetics.

1.2.3 NF1 in flies

The NF1 gene is very conserved through the animal kingdom. The protein sequence of *Drosophila* NF1 shares 60% sequence identity with that of human NF1[67]. Therefore, *Drosophila* is a good animal model to study the molecular basis of NF1 disease. Like human patients, *Drosophila* mutants manifest many defects: Bernards *et al.* found reduced pupal size and reduced ommatidia number in the NF1 null mutant[67]; Guo and Zhong found NF1 is required for aversive odor associative learning[68]; Williams showed circadian defects in the NF1 null mutant[69]. NF1 work in *Drosophila* has also found evidence of the pleiotropic effects of the NF1 gene, from cellular growth to development to cognitive defects like memory impairment.

At the molecular level, using NF1 mutants in memory research has yielded the following insights:

- NF1 mutants have defects in both learning and long term memory, similar to defects seen in human patients[68, 70].
- NF1 acts acutely in memory formation. The memory impairment is not due to developmental defects[68].
- Signaling partners which were not previously known have been identified via NF1 mutant memory studies: Rutabaga-AC[71] and Anaplastic Lymphoma Kinase (Alk)[72].

To further assist human NF1 studies and to provide insightful guidance for advanced animal models, we need to know which tissues in the NF1 mutants contribute to different defective phenotypes. Functional localization of NF1

mutant effects has been most challenging with human patient studies and other animal models, therefore we are motivated to use *Drosophila* to reveal where in the fly brain NF1 is required for normal memory.

1.3 Fruit flies in memory research

Using fruit flies for memory research can be traced back to Benzer, when in the early 1970s his students Chip Quinn and Bill Harris used an apparatus known as counter current apparatus to shock flies while conditioning them with odor. This assay allowed the isolation of the first learning and memory mutants in the field: dunce, rutabaga and amnesiac [73–75]. Tim Tully, a post-doc in the Quinn lab, modified the original apparatus to make it more closely resemble classical Pavlovian learning. In the process, this also improved learning scores and productivity.

The molecular components for different phases of memory therefore have been dissected with mutants and pharmacological manipulation (see also Chapter 2, p. 13). Evidence from ablation studies through the 60s to early 80s suggested that the mushroom body is required for associative learning in insects[76]; in the 80s, mushroom body mutants in the flies were reported to have learning defects[77]. Combined with the power of the GAL4 system, great leaps have been made in understanding the mushroom body circuitry involved in learning and memory, and revealed where different molecular components for memory are required.And mushroom body has been recognized as olfactory associative learning center in the *drosophila* brain.

1.3.1 Appetitive conditioning

Whilst most fly memory studies to date have used aversive associative conditioning, another olfactory learning paradigm employs sugar reinforcement as a positive unconditioned stimulus[78]. Like aversive conditioning, appetitive memory is also mushroom body dependent. However, appetitive memory memory differs from aversive memory in a variety of interesting ways.

First, appetitive memories are particularly robust in the long term. In the appetitive paradigm, one single session of conditioning produces a long-lasting memory that is equivalent to ten repeated (spaced) trials of aversive conditioning. This indicates that appetitive memories may not be formed in an identical way to aversive memories. This is possibly because forming an association between a food source and odor is more natural for the animals than forming an association between a painful stimulus and odor. In addition, from a practical perspective, the time and labor required to perform appetitive

training is potentially much lower since the conditioning paradigm is shorter (5 minutes compared to 165 minutes).

Second, appetitive memory consolidates within 2 hours after training. Before memory consolidates into a more resistant state, transient cold-shock treatment causes memory impairment.Although the detailed molecular machinery is still no very well known, the effect of of cold shock has been believed to suspend undergoing cellular process, especially like protein synthesis dependent processes. Nontheless, profiling the consolidation time of memory traces by introducing brief cold treatment at various time points after training has been broadly used.In appetitive memory, cold shock treatment performed 2 hours after training has no effect on the performance test 24 hours after tracing. This indicates that appetitive memory rapidly consolidates within the initial 2-hour, whereas in aversive conditioning, the spaced training procedure takes 3 hours to finish. It is known that flies process appetitive LTM using the same parallel and sequential neural circuit mechanism that it uses to process aversive LTM, therefore it is particular intriguing to understand what mechanism such rapid consolidation is.

Third, state dependent retrieval. Formation of the appetitive memory requires flies to be starved before training. The appetitive memory, once formed, requires a starvation state for retrieval. Therefore, the appetitive memory is particular interesting in understanding how memory is regulated by internal state.

1.4 Plan for this thesis

NF1 is an interesting pluripotent gene that is widely expressed and leads to a variety of cognitive defects in people carrying mutations in this gene. Not only is the underlying mechanism of the cognitive defects not known, but it is *even* unknown if the defects originate from brain-wide changes or specific, localized, changes in brain function. Establishing this is a vital first step in understanding how NF1 disorder impairs cognitive function.

Fruit flies are a good choice for studying complex behaviors whilst allowing the effect of precise genetic mutations on those behaviors to be explored. Memory defects are commonly reported in sufferers of human NF1 disorder. Memory is easy to assay in flies and there are robust and widely accepted paradigms for doing so. Thus, the overall goal of this thesis is, firstly, to better characterize the impact of NF1 mutations on memory performance and, secondly, to localize where in the fly brain the deficits originate. Specifically, I focus on appetitive memory as this has not previously been explored in the context of NF1. The thesis is laid out as follows.

Chapter 2: NF1 is required for appetitive long term memory

Does the NF1 mutant have normal appetitive memory? With mutants generated differently as well as their heteroallelic crosses, we found that they all had defective 24 hour memory, yet their learning and 1 hour memory performance normal were normal. Rescue experiments suggest that expressing NF1 selectively in the nervous system is sufficient in normal 24 hour memory. Therefore, we conclude that normally functioning NF1 in the nervous system is required for normal appetitive long term memory.

Chapter 3: Localizing NF1 function

How widely does the NF1 mutation affect neuronal function? We over-expressed NF1 pan-neuronally and found that it enhances both appetitive and aversive long term memory. This enhancement suggests a simpler way to screen for specific neuronal substrate for NF1 function. We therefore selectively over-expressed NF1 in various regions of the brain and found, quite surprisingly, that over-expression in the mushroom body had no effects. Instead, over-expression of NF1 in octopaminergic neurons enhanced memory. We therefore tested if NF1 expression in the octopamine neurons is sufficient to rescue long term memory. Two octopamine GAL4s showed rescue, whereas neither the mushroom body driver, GAL4-OK107, nor the pan-neuronal elav;MB-Gal80 were able to do so. This argues that it is in octopamine neurons that NF1 expression is required for normal appetitive memory. This the first time a specific neuron type has been identified as playing a role in the cognitive deficits in *Drosophila* NF1 research.

Chapter 4: manipulating octopamine neuron activity after consolidation affects long term memory

The identification of octopamine neurons as a substrate for NF1 dependent memory leads us to search for a mechanistic explanation. What does octopamine do during the memory process? To address this we directly manipulated the activity of these cells using Kir to see what happens to appetitive memory. We found a time window, *after* consolidation time, during which octopamine neurons can be inactivated to enhance long term memory. The converse is also true, with activation of octopamine neurons leading to a decrease in LTM. This involvement of octopamine neurons in appetitive memory has not previously been identified.

Chapter 2

NF1 is required for *drosophila* appetitive long term memory

The entry point of the project is to ask if NF1 is necessary for different types of memory. NF1 has been demonstrated to be required for aversive memory, and in this chapter NF1 mutant flies were tested in a different conditioning paradigm–appetitive training. Here I show that NF1 is not required for appetitive learning (immediate memory) but is necessary for normal appetitive long term memory.

2.1 Introduction

2.1.1 Aversive Conditioning

The paradigm

The current aversive paradigm in common use is inspired by Quinn's apparatus but re-designed and improved by Quinn's post-doc, Tim Tully[79]. Quinn's original design leveraged the phototactic response flies possess, to lure them to run sequentially into two tubes. Both tubes were coated with shock-grids and infused with different odors. In only one tube is the shock-grid electrified. The shock is the unconditioned stimulus (US). The odor that is delivered in the presence of the US is known as the conditioned stimulus and denoted as 'CS+', to distinguish it from the odor present in the non-shock tube (the CS-). Flies that experienced the US and CS+ pairing will in future tend to avoid the CS+ odor.

The difference between the fraction of flies avoiding the CS+ minus the

fraction of flies avoiding the CS- is known as the performance index (PI)[80]. In Tully's design, about 100 flies were transfered into a closed chamber and trained by exposing them sequentially to two odors carried by air currents. Shock is delivered only in the presence of the CS+ and not the CS-. To test for conditioned avoidance responses, flies were transported to a T-maze choice point, between converging currents of the two odors. Acquisition of learning was a function of the number of shock pulses received during CS+ presentation. Learning was best when CS+ presentations overlap shock (delay conditioning) and then decreased with increasing CS-US inter-stimulus intervals. Shocking flies immediately before CS+ presentation (backward conditioning) produced no learning. The current protocols which produce saturated learning (95% of trained flies avoided the shock-associated odor CS+) employs 60 volts and 0.2 Hz electrical shock for 1 minute.

Dissecting memory phases

Contrary to many people's intuition, memory formation is actually a very conserved cognitive property across the animal kingdom. Through extensive review of the literature, Tim Tully summarizes the following features shared by memory formation in all kinds of animals[81]:

- Memory immediately after training is sensitive of interruption and its strength is short lived. However, a couple of hours later, the memory will be consolidated into a longer-lasting and more stable form.
- Repeated training sessions with a rest interval between each produces stronger and longer-lasting memory than repeated ones without rest interval. The former is often called as 'spaced training' and the latter is often referred to as 'mass training'.
- Treatments such as electro-convulsive shock or the administration of anesthetics or protein synthesis inhibitors slow or block memory consolidation, thereby producing retrograde amnesia.

Based on the above features, Tully concludes that the basic molecular components underlying such memory formation must be conserved and it is possible to dissect such processes with mutant studies while incorporating manipulations to probe those properties. Typical manipulations include the following:

- To outline the consolidation timing profile, brief cold shock has been adopted as an interruption. Different groups of flies along different time

points after training were exposed to ice for 2 minutes and allowed to recover under room temperature. The 24 hour memories (24 hours after training) of these animals were later tested. The time points after which groups of flies showed normal memory comparing to controls are the time points when memory starts to consolidate[73].

- Spaced and mass protocols have been streamlined for aversive conditioning with automatic training devices, commonly known in the field as 'robots'. In essence, the robots can be programed to perform spaced or massed odor and shock paring. The total training time for the spaced program is 170 minutes and the mass protocol 35 minutes. The only human labor required is loading and unloading the flies. Spaced training produces stronger memory than mass training does.

- Protein synthesis inhibitors cycloheximide (CXM) is used to block protein synthesis dependent memory. The memory performance generated by spaced training is sensitive to CXM treatment whereas the performance due to mass training is not. The difference in performance between two training paradigms is been recognized as long term memory (LTM) whereas the performance due to mass training is defined as amnesia resistant memory (ARM).

Based on the manipulations listed above, Tully *et al.* dissected memory into four phases: learning (also known as immediate memory), short-term memory, middle term memory and consolidated memory. Mutants which disrupt different phases of memory have been identified: such as Dunce, rutabaga (equired for short term memory), amnesiac (for middle term memory) and d-creb for the protein synthesis-dependent component of consolidated memory.

Dissecting the circuits

Mushroom bodies (MB) are bilaterally symmetric multi-lobed brain structures known as the associative memory center of the insect brain. Drosophila mushroom body is required for olfactory associative conditionning. The MB on each side has about 2500 intrinsic neurons and has been categorized into three morphological subsets–$\alpha\beta$, $\alpha'\beta'$, and γ–based on their axonal projections in the region of the MBs called the lobes[82]. Interestingly, the involvement of MB subsets in the memory process is sequential: rutabaga adenylyl cyclase in the γ lobe is required for memory acquisition[83] (of more about adenylyl cyclase see the Discussion); neurotransmission from the $\alpha'\beta'$ subset is required to acquire and stabilize aversive memory but is dispensable during memory

retrieval; and in contrast, the neurotransmission from $\alpha\beta$ neurons is only required for memory retrieval[84].

2.1.2 Appetitive conditioning

The paradigm

The negative reinforcer, electrical shock, can be replaced with a rewarding reinforcer, like sugar. Under these circumstances, flies form a positive association between the CS+ and the US. After the publication of their aversive olfactory training apparatus, Quinn *et al.* replaced the shock grid with a sucrose-coated copper wire, demonstrating the first olfactory reward conditioning paradigm in flies and called the learned preference appetitive memory[78]. Quinn's group found that to acquire the appetitive memory, flies need to be starved, and they also found the appetitive memory decays slower and lasts longer compared to the aversive memory trained side by side. Despite the robust performance and interesting properties produced, appetitive memory in flies was not explored seriously until the beginning of 21st century: Heisenberg's group modified from Tully's apparatus and used a similar Pavlovian reinforcement procedure by replacing the shock grid with sugar-coated paper. They discovered that octopamine is required for appetitive memory[85]. Since then, all sugar reward conditioning has been performed using the same Pavlovian idea with slight variations of reinforcement chamber design[84, 86].

Dissecting molecular components

The preceding bloom in dissecting memory phases with aversive conditioning led by Tully and colleagues in the 1990s offered a lot of mutants as entry points for investigating different memory phases. Reward conditioning studies in the early 2000s, naturally, inherited those insights and also the mutant tools.The Waddell's group[87] revealed several unique properties of appetitive memory:

- The appetitive memory consolidates rapidly within 2 hours after training. Waddell and colleagues used cold shock to interrupt memory consolidation and found the sensitive period is within 2 hour after training.

- The component at 24-hours after training is long term memory (LTM). Waddell and colleagues use both CXM and an inhibitory form of CREB-2b to abolish 24-hour memory. LTM is defined as protein synthesis dependent and CXM and CREB-2b blockade are the hall markers of LTM; meanwhile, aversive LTM mutants *cer* and *teq* also demonstrated 24-hour

defects in reward conditioning. Taken together, Waddell *et al.* conclude that the later memory component is therefore LTM-like in nature.

- The gene *radish* is required for appetitive LTM. The literature for aversive olfactory memory suggests that consolidated memory exists in two forms. One is LTM, introduced by spaced training and is *de novo* protein synthesis dependent, whereas the other form is ARM, which is resulted from mass training, and is protein synthesis independent and requires *radish*[88]. Tully believes the two forms are mutually exclusive[81]. However, the requirement for *radish* in appetitive LTM challenges Tully's exclusive view.

Dissecting circuits

Similar to aversive olfactory memory, subtypes of mushroom body neurons are sequentially involved in the appetitive olfactory memory: neurotransmission from the $\alpha'\beta'$ subset is required to acquire and stabilize appetitive memory but is dispensable during memory retrieval; and in contrast, the neurotransmission from $\alpha\beta$ neurons is only required for memory retrieval[84].

2.1.3 NF1 mutants

Generation of NF1 mutants

The first NF1 mutants were generated by P-element insertion. By mobilizing the nearby P-element, Bernards *et al.* identified two null mutants: NF1^{P1} with a total deletion of the NF1 gene along with adjacent sequences; NF1^{P2} is the same P-element inserted in the first intron of the NF1 gene and causes a frame shift mutation[67]. Later, NF1^{c00617} was identified from the PBac library and studied by Davis' group[89]; Recently, Bernard's group used ethyl methane sulfonate (EMS) to induce point mutations and screen for strains that failed to complement the NF1^{P1} and NF1^{P2} phenotypes. In this process, they identified two non-sense mutations NF1^{E1} and NF1^{E2}. Figure 2.1 is a diagram modified from Bernards' group[67, 90] showing the mutation locations.

NF1 mutants in aversive memory

NF1 is required for aversive olfactory learning and memory in *Drosophila*. Several studies using differently generated null mutants demonstrate consistent learning and memory defects by using the aversive olfactory conditioning paradigm. Our group was the first to show that NF1 mutants have learning defects; and that this defect can be rescued by transient expression of the

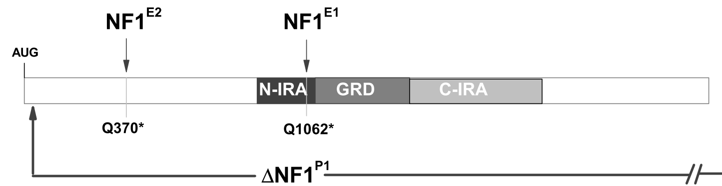


Figure 2.1: Diagram of mutation locations with translational start (AUG) and non-sense mutations (*). The arrow indicates the location of a P-element in NF1^{P2} and point mutations in NF1^{E1} and NF1^{E2}. The extent of the deletion in NF1^{P1} is indicated by the line below the diagram

NF1 construct during the adulthood indicating the defect is not due to developmental effects in the mutant flies[71]. In the following study, our group expands our examination to a different memory phase and identified a long term memory defect in the mutants[70]; meanwhile, Davis' group, using a different mutant, confirmed our findings and brought their focus on the 3 hour memory defects in the NF1 mutant animals[89]. Taken together, NF1 seems to be essential in every stage of aversive memory.

This chapter

NF1 is required for aversive olfactory learning (i.e. immediate memory, tested right after training). And the involvement of NF1 in aversive learning has been thought to be mediated through the AMP/PKA pathway[68, 70]. It is not yet known if NF1 gene is also required in other learning paradigms. Here we use appetitive olfactory learning which uses sugar as unconditioned stimulus, a stimulus more ethologically relevant compared to electrical shock, to test the requirement of NF1 gene. To our surprise, NF1 mutants displayed no significant reduction learning performance but did have impaired LTM.

2.2 Methods

2.2.1 Fly stocks

Flies were raised at 25°C on standard cornmeal agar medium. The NF1 mutants, NF1^{E1} and NF1^{E2}, together with the parental W^{1118} isogenic line were obtained from A. Bernards (Massachusetts General Hospital, Boston, MA). The GAL4 driver line elav-GAL4;NF1^{E1} and Gal80;uas-NF1;NF1^{E2} were obtained from M. Skoulakis (Alexander Fleming Institute, Vari, Greece). The MB-GAL4;NF1^{P1} double lines used for anatomical analysis were generated by performing crosses using double balancers; w/Y;CyO/Sp;TM3Ser/Sb for crossing c739 into NF1^{P2}. The NF1suprE1;OK107-GAL4 line is generated by generations of single pair matings.

2.2.2 Appetitive conditioning paradigm

We followed the protocol published by the Waddell group[91] but with the following specific modifications. The modifications below made it possible to achieve higher PIs, which increases the sensitivity of the assay as it minimizes floor effects in the PI scores.

Preparing the Training Tubes

Cut Whatman 3MM filter paper into two 5.5" by 3.5" rectangles. One rectangle was soaked in water whereas the other one was soaked in saturated sucrose solution. Air dry until none of the filter paper was dripping in liquid, then rolled it lengthwise and slided it into an empty training tube. Put them into 40°C incubator till completely dry. Label the water soaked filter paper 'blank', and this 'blank' tube was used when flies were exposed to a specific odorant without sucrose. Label the sucrose solution soaked filter paper 'sucrose'. This 'sucrose' tube was used when flies were exposed to a specific odorant in the presence of sucrose.

Food Deprivation

Flies need to be starved in order to acquire appetitive memory. The day before conditioning, each genotype is divided into several empty vials with about 80 flies in each. We prefer to starve flies in individual vials rather than big bottles since it yields a more consistent starvation effect across vials. Flies should be starved for about 22 h before training the following day, and a good amount of hydration for the flies is needed. Instead of placing a dampened rectangle of filter paper or a thin layer of 1% agarose, as the Waddell group does, we

add about 1.5ml deionized water into each vial and insert a whole piece of Kimwipe into the bottom of the vial. This provides sufficient water yet not too much vapor being produced to dampen the flies' wings. The Kimwipe is in a cone shape arrangement, providing a greater surface area for the flies yet retains its position and does not fall out of the vial when tapped upside down. Store the flies overnight under the same conditions in which they were raised (typically at 25°C, relative humidity 60%, on a 12-h light/dark cycle).

Appetitive training

The 80 flies in the starvation vial are tapped into the 'blank' training tube and immediately attached to the automatic ('robotic') training apparatus. When attached, constant air current is passing through the training tube. Under computer control, the first odor BA (benzaldehyde), the CS-, will turn on accordingly. The flies in the 'blank' training tube will be exposed to odorant BA for 2 min without sucrose. After 2 min, the air manifold will switch to air for 1 minute, during the last 30 seconds of this period we remove the tube with flies and gently tap the blank tube and transfer flies into the sucrose tube, and immediately connect the sucrose tube into the shock apparatus. This step needs practice with precisely timed movement so that the moment the sucrose tube is connected to the apparatus, the air manifold should just be switching to odorant MCH (methylcyclohexanol), the CS+.[1] After two minutes of CS+ exposure, the air manifold switches to air again. After at least 45 seconds, disconnect the sucrose tube from the apparatus and tap the flies into storage vials.

Prepare the storage vials

Normal food vials are required if testing time points 24 hours after training. Flies must be fed in this normal food vial for 2 hours to ensure survival and fitness and must then again be re-starved in a new starvation tube, prepared as described above. For flies to be tested for 3-hour memory, flies were transfered into new starvation vials without the feeding step. Otherwise, flies were tested immediately after training.

To test for learning, flies were transferred to a choice point where the two odors were presented to them by two converging air currents. Flies were given 120 s to choose between the two arms of the T-maze, from which odors were delivered. At the end of this period flies were trapped inside individual arms,

[1]The requirement for precise timing of manual actions is clearly a bottleneck of the process and a more automated way of doing this is clearly desirable.

anesthetized, and later counted. To eliminate odor bias, the concentrations of the two odors, which are aversive to untrained flies, were calibrated such that untrained flies distributed themselves 50:50 in the T-maze. This procedure reduces the possibility of floor or ceiling effects in the learning scores.

Performance index

Two groups of flies were always trained and tested in one complete experiment; for one group, MCH(methylcyclohexanol) was the CS+ and BA (benzaldehyde) the CS-, whereas for the second group BA was CS+ and MCH was CS-. In this manner, the design is balanced. The 'probability correct' of each reciprocal group was calculated as the number of flies avoiding CS+ minus those avoiding CS- divided by the total number of flies in the T-maze arms. The resulting two probability correct scores are then averaged and normalized to become one performance index (PI), which can range from 0 (a 50:50 distribution reflecting no learning) to 100 (all flies correctly avoid the shock-paired odor). Thus, this final PI constitutes a single independent observation for the purposes of statistical analysis.

Analyses of variance (ANOVA) were followed by planned pairwise comparisons between the relevant groups with a Tukey HSD *post hoc* test. In all cases the symbol '*' indicates $p < 0.05$. The abbreviation 'n.s.' denotes 'not significant' and indicates that $p > 0.05$. The term 'significant' refers to $p < 0.05$.

2.3 Results

We first tested whether NF1 is required for immediate memory (learning) using appetitive conditioning. To our surprise, NF1 mutants displayed no significant reduction in appetitive learning performance (Figure 2.2A–B). In Figure 2.2A, non-sense mutants $NF1^{E1}$ and $NF1^{E2}$ have PIs of 0.6 and showed no difference with their w^{1118} isogenic wild type controls. In Figure 2.2B, the P-element deletion mutant, $NF1^{P1}$, also has a PI of 0.6, and is indistinguishable from the performance of the wild type control 2U (a “Cantonized” white eye isogenic line). It is worth pointing out that the PIs we observe here are exceptionally high compared to almost all published results with appetitive conditioning (which have PIs of around 0.3). The difference is attributable to the precautions we took to keep flies healthy during starvation. These are discussed further on p. 29. In Figure 2.2A, all three mutants scored slightly below wild-type values and so, since the error bars are rather large, it is possible that the difference might reach statistical significance if the sample size were larger. Nonetheless, given the high performance produced in our experiments, it is clear that any potential effect of the $NF1^{E1}$ and $NF1^{E2}$ on learning is small. Combining with the normal learning performance of $NF1^{P1}$, a deficiency line with all coding region of NF1 gene being deleted, we conclude that NF1 is not required for appetitive olfactory learning.

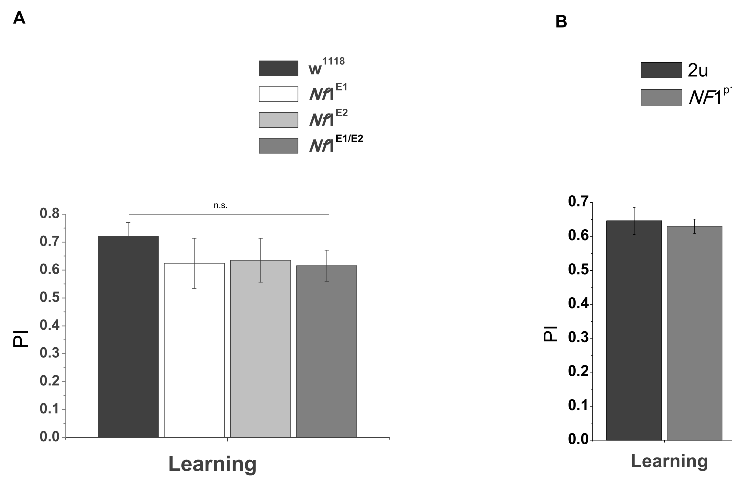


Figure 2.2: **A** NF1 mutants have normal appetitive olfactory learning (immediate memory). In A, there is no significant difference between wild type flies (W^{1118}) and any of the three different NF1 mutants: $NF1^{E1}$, $NF1^{E2}$, and $NF1^{E1/E2}$. In **B**, there is no significant difference between wild type flies, 2u, and the NF1 mutants, $NF1^{P1}$. Bars show mean ± one SEM. In all cases $n = 8$.

The appetitive conditioning offers unique advantage for investigating long term memory (LTM). The initial memory acquired through one session of training, quickly consolidates within 2-hour. Memory at 24-hour after training when tested, its component is LTM[87].

With appetitive memory, we were able to avoid the confound in aversive conditioning that NF1 is involved in several stages of memory formation: learning, 3-hour memory (middle term memory) and long term memory as well. In appetitive conditioning, the NF1 mutant has normal learning; and we tested learning, 1-hour, 3-hour and 24-hour memories for both NF1 mutants and wild type controls. We found no significant difference between the two genotypes in their learning and 1-hour memory performances (Figure 2.3). It is known that appetitive memory is rapidly consolidated within 2-hour after training; therefore, this result strongly suggests that NF1 is required for appetitive LTM only.

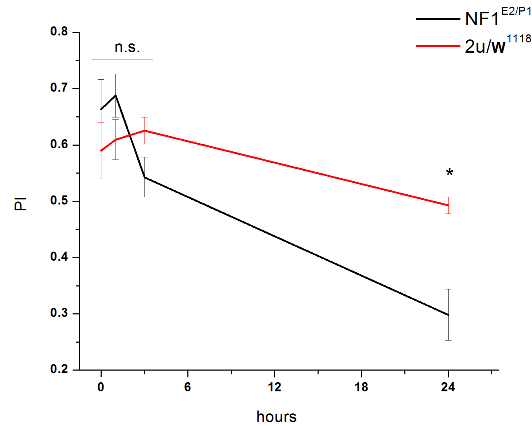


Figure 2.3: Memory curves of Heteroalle mutant NF1$^{E2/P1}$ and its wild type control. NF1$^{E2/P1}$ has normal learning and 3-hour memory compared to the wild type control yet shows LTM defects. Bars show mean ± one SEM. In all cases $n = 8$.

Whilst NF1 does not affect appetitive learning, we found that it has a marked effect on LTM when flies were tested 24 hours after training (Figure 2.4). In every case NF1 mutant animals performed substantially worse than their wild type controls. To exclude the possibility that the defect we observed was due to secondary lesions from the mutagen or P-element, rather than the mutations in NF1 itself, we tested the heteralle mutant form, NF1^{E1} and NF1^{P1}. Our rational is, if the defect was due to a secondary lesion, heterozygotes are not likely to have the same defect. If the defect persists, therefore, it should be purely due to the deficiency of the NF1 gene caused by mutations. Indeed, our heteralle NF1$^{E2/P1}$ animals have significantly lower performance compared to heterozygotes wild type controls. Taken together, the proper functioning of the NF1 gene product is necessary for normal appetitive long term memory.

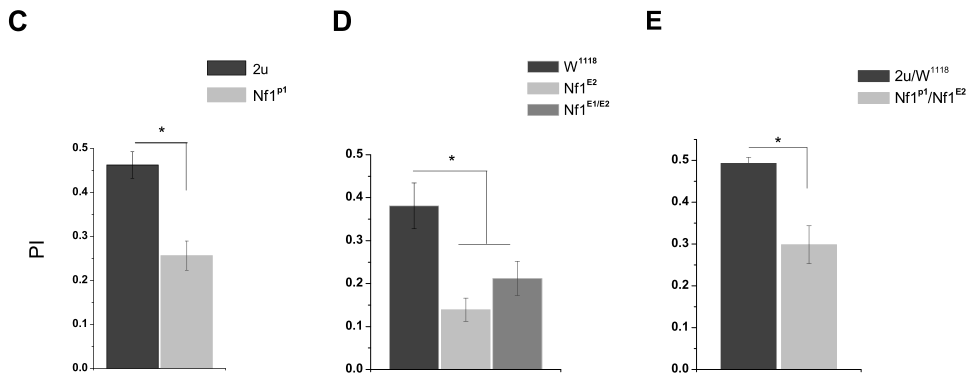


Figure 2.4: C–E. NF1 mutants have defective LTM compared to wild-type controls. In this case flies were tested 24 hours after training. In C, D and E, homozygous mutants as well as heteralle mutants performed significantly worse than to their corresponding controls. Note that in D the ANOVA was performed with 3 groups and the significance asterisk indicates that both mutant groups are significantly different from the wild type. Data from the mutants were not pooled into a single group. Bars show mean ± one SEM. In all cases $n = 8$.

We have shown that the NF1 gene is necessary for normal appetitive LTM. We now want to examine if this requirement is localized within the CNS. We designed experiments to express the NF1 construct in three CNS GAL4 drivers: elav, ras2 and alk(38). Elav is a pan-neuronal driver labeling all neurons, whereas ras2 and alk(38) are not as widespread but are expressed in the majority of CNS neurons[72]. All three drivers were able to restore the LTM to wild type levels compared to the parental controls (Figure 2.5). By using three different, broadly expressing drivers, we demonstrated that not only is NF1 sufficient to rescue the mutant LTM defects, but that the functional localization is within the nervous system and not other tissues. In addition, all parental controls as well as the 'rescue' genotypes have the same learning performance (Figure 2.6). Taken together, the NF1 expression in the CNS is required for appetitive LTM but not learning.

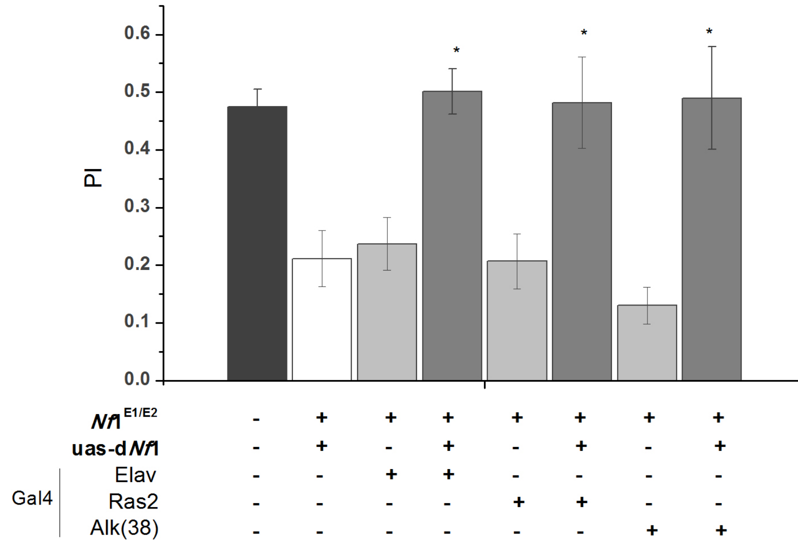


Figure 2.5: LTM defect in NF1 mutants can be rescued by broad expression of NF1 gene in the nervous system. elav, ras2, and Alk(38) are GAL4s labeling extensive neuronal tissues. Restoring the NF1 expression in those GAL4 labeled tissues in the null mutant background rescues otherwise defective LTM performance in the parental controls. Bars show mean ± one SEM. In all cases $n = 8$.

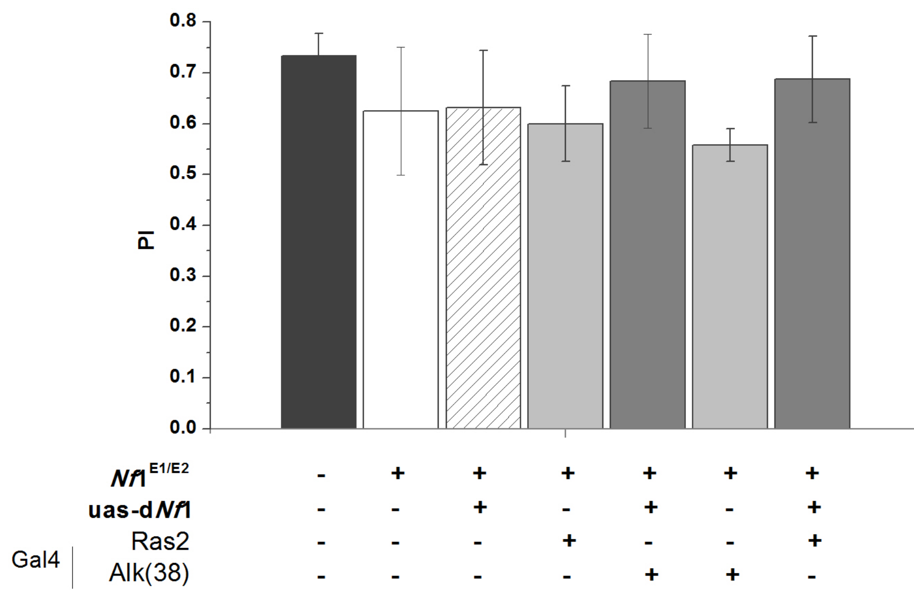


Figure 2.6: All control parental groups as well as LTM rescue groups showed normal learning performance. Bars show mean ± one SEM. In all cases $n = 6$.

The mushroom body (MB) is the associative learning center of the insect brain and is well known for its role in associative olfactory memory. We therefore asked whether the LTM defect can be rescued by restoring NF1 gene expression specifically in the MB. All three GAL4s used previously have MB labeling therefore we have to test more specific drivers to test this question. Here mushroom body GAL4 drivers OK107 and c739 are used to express NF1 in the mutant background. In both cases no rescue effect was observed (Figure 2.7).

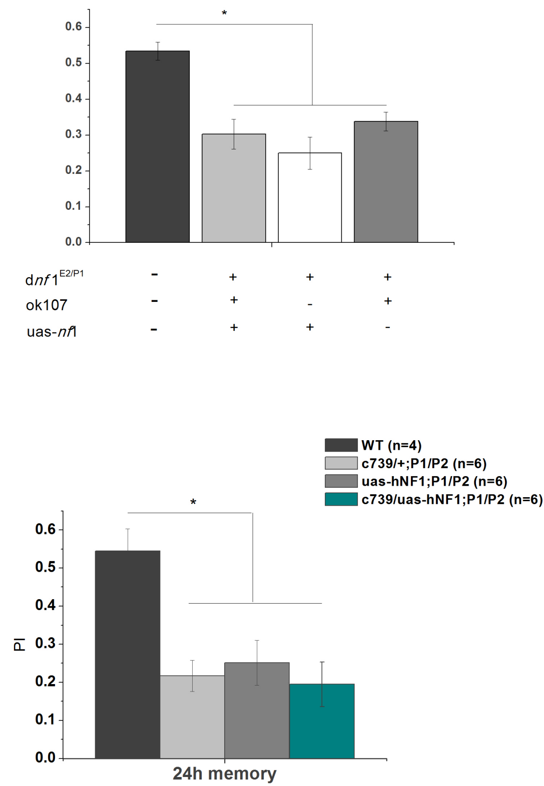


Figure 2.7: Expressing NF1 by mushroom body using GAL4 OK107 (**top**) or GAL4 c739 (**bottom**) does not rescue LTM defects. NF1 driven in the MB by both drivers shows the same LTM defects compared to parental controls. Note that the ANOVA was performed with 4 groups for each panel and the significance asterisks indicate that all three experimental groups are significantly different from the wild type. i.e. the 3 experimental groups were not pooled into a single group for the test. Bars show mean ± one SEM. In all cases $n = 8$.

The above MB experiments do not exclude the possibility that rescue in the MB is necessary but simply not sufficient for NF1-dependent LTM. Therefore we performed 'rescue' experiments with pan-neuronal GAL4 combined with MB GAL80 to *exclude* expression in the MB. We found that LTM was completely restored to wild type levels in these flies (Figure 2.8). This indicates that the NF1-dependent LTM does not require mushroom body; in other words, all the evidence suggests that NF1 is functioning *outside* mushroom body to regulate LTM. Of course this does not exclude the possibility that NF1 is required in MB output or extrinsic neurons not labeled by the drivers OK107 or c739, which label mainly Kenyon cells.

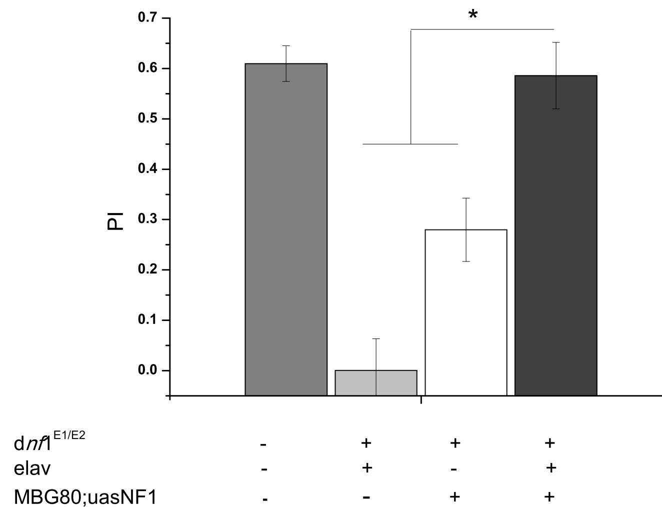


Figure 2.8: Defective LTM is rescued by pan-neuronal drivers which exclude the mushroom body. Mushroom body GAL80 combined with UAS-NF1 construct in the mutant background, crossed with *elav*-GAL4 completely rescues the LTM defects displayed in the parental controls (light grey and white bars). Bars show mean ± one SEM. In all cases $n = 8$.

2.4 Discussion

In this chapter we show that NF1 is required for appetitive LTM, but not learning. Normal NF1 function in the CNS is required for LTM. Normal NF1 function in cells labeled by the OK106 or c739 drivers (which label mainly MB Kenyon cells, and so are commonly referred to as mushroom body lines) is not required for appetitive LTM.

2.4.1 Our starvation details

Almost all the early studies on appetitive memory have PIs in the range of about 0.2 to 0.3[78, 87]. This is very low compared to the learning established in aversive olfactory conditioning, which can be as high as 0.9[83]. It is known that the performance index of appetitive learning depends on the degree of starvation rather than how many sessions of training the animals undergo[78, 92] and therefore the starvation time is critical. However, the required level of starvation depends strongly on genetic background, and hence different labs have established their own starvation regimes. Currently, the starvation time has been decided empirically and this is the biggest hurdle for the appetitive field to bloom despite many interesting features presented by appetitive conditioning for memory study.

In the original protocol by Waddell *at el.*, they described about 5% dead at the end of their starvation session[91]. Although our starvation time is longer than that of the Waddell group (22 to 24 hours comparing to 16 hours), none of our flies would die at the 22 24 hour starvation period. Below are the modifications we made in our starvation procedure and we think those modification are necessary for the high performance we achieved (Figure 2.9).

1. Starve flies in small vials. Small vials instead of big bottle, the number of flies within which is easy to be controlled and therefore the starvation condition is more consistent.

2. Plenty of water should be supplied. Starving flies still have a lot of water intake, and the common protocol with one layer of agar or a couple of filter paper pieces at the bottom of the vials contains about 0.4 ml water at most. We use a whole piece of Kimwipe and it can absorb 1.5 to 2 ml of water.

3. The inner surface of the tube should be dry. Another problem with filter paper laying flat on the bottom is that this generates condensation on the inner surface of container. Starved flies are weaker and can easily

become stuck on the wall due to the condensation. Factors like subtle temperature difference between the water and the incubator (which could fluctuate a lot) make the water contained by a flat surface easily vaporized and subsequently this forms condensation on the vial surface. A whole Kimwipe will absorb more water but with more complex surface form and hence result no condensation.

4. Crowdedness is a big stressor and should be avoided. Another big benefit provided by a tucked-in Kimwipe is that there is a lot of surface area. Flies try to keep a distance from each other, however starvation and dirty vertical (fly excrement) keep flies away from the vertical surface. This results in most flies standing on the bottom towards the end of the starvation session. A Kimwipe provides many more times surface area for flies to stand compared to the bottom of vial/bottle itself. With the same amount of starvation time, our preparation keeps flies alive for much longer comparing to a flat filter paper or agar. This indicates the starvation procedure should be considered to eliminate other stress factor like crowdedness.

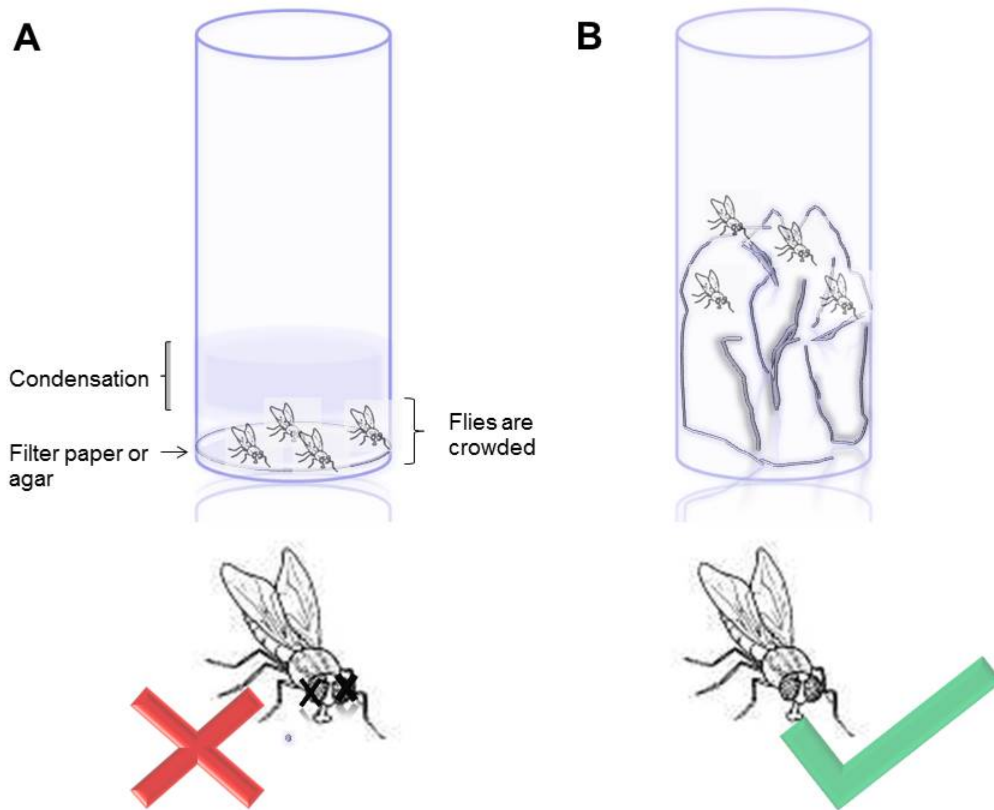


Figure 2.9: Diagrams of starvation factors. **A**. Flat paper/agar surface provides little water but results in condensation and crowdedness. **B**. Cone formed by a piece of Kimwipe provides sufficient water and space and keeps condensation to a minimum. Flies are more active and less stressed under these conditions.

2.4.2 Normal learning in appetitive memory

A previous study in our lab shows that the aversive learning defects in the NF1 mutants were due to impaired PKA signaling. This defect can be rescued by induction of PKA activity. This is the first evidence linking NF1 function in the brain with cAMP signaling where NF1 acts upstream of the pathway[68]. In Guo's study, biochemical assays reveals several interesting observations:

1. NF1 mutant reduces GTPγS-induced AC activity (GTPγS is a G-protein stimulating reagent) indicating that G-protein induced cAMP signaling has a NF1-dependent component.

2. NF1 and rutabaga double mutants have no further reduction on GTPγS induced AC activity: indicating NF1 dependent cAMP level is mediated through rutabaga.

3. NF1 mutant has no impact on calcium level dependent AC activity peak: indicating the calcium activation in Rutabaga is not NF1 dependent.

Taken together, NF1 seems to facilitate the G-protein induced rutabaga activation; without NF1, calcium and G-proteins still synergistically activate rutabaga but with dampened AC activity level. In the appetitive learning, the cAMP levels in NF1 mutants are reduced but still efficient for acquisition of the appetitive memory.

2.4.3 Long term memory defects

The LTM defects in the NF1 mutants are likely due to dis-regulation of Ras. LTM requires *de novo* protein synthesis. A cAMP response element-binding protein (CREB) has been identified as the cellular transcription factor for transcribing the genes required for LTM[93–95]. Many cellular signaling via kinases, like e.g. PKA, CaMKII, CaMKIV, RSK2, MAPK, and PKC, however, can induce CREB. Therefore, the learning-induced LTM requires a specific CREB activation context, i.e. different kinases need to coordinate. LTP studies from brain slice culture suggest the importance of MAPK (Mitogen-Activated Protein Kinase) in synaptic plasticity. MAPK phosphorylates and activates MNK, which, in turn, activates CREB. In addition, MAPK is activated through the Ras-Raf-MEK signaling chain. Human genetic disease studies show that mutations at any level of this signaling cascade cause cognitive problems[96]. NF1 functions as a Ras activity regulator, and depleting NF1 tips the balance towards more active form of Ras and therefore leads to elevated Ras-Raf-MEK-MAPK signaling levels. The high baseline level of

activated MAPK could interfere with learning-induced CREB activation and consequently affect LTM.

Chapter 3

Localizing NF1 function

In the previous chapter we showed that normal NF1 function in the brain is necessary for long term memory, but we were not able to localize this to a sub-region of the brain. In this chapter we identify octopamine neurons as crucial for NF1-dependent long term memory (LTM). Ectopic pan-neuronal expression of NF1 leads to a gain-of-function effect that the memory performance is enhanced. We took advantage of this effect by performing a small GAL4 screen to find where in the brain NF1 is required for normal LTM. Both rescue experiments as well as RNAi knock down confirm the importance of octopamine neurons in NF1-dependent LTM. These data suggest that the engagement of neurofibromin in memory is cell-type specific

3.1 Introduction

The genetic power of *Drosophila* makes it a great model system for a forward genetic screen. The early stages of a forward genetics screen to study a biological process are often referred to as a loss-of-function screen. Briefly, a mutation library is generated by mobilizing P-elements to disrupt gene expression along the chromosome; next, this library is screened against a desired phenotype; then the positive hits are genotyped to discover what gene has been disrupted. The loss-of-function screen, however, has a few caveats: firstly, phenotypes after development might not be revealed due to early lethality. Secondly, the observed phenotype could be due to secondary effects of the insertion but not the inserted gene itself. To overcome those shortcomes, gain-of-function screens have gained popularity. Briefly, the P-element contains a UAS sequence (EP line) at one end so that any gene that it inserts next to can be activated by GAL4. When this EP library is generated, it is possible to simply

cross a desired tissue specific GAL4, and gene which involved in the desired phenotype when forced to be expressed under this GAL4 can be identified. One potential concern with this screen is that the over-expression phenotypes are not always indicative of the normal functions of the gene. Therefore, combining both loss-of-function and gain-of-function approaches will provide for more reliable information about one gene's involvement in the phenotype of interest.

In the previous chapter, we used both mushroom body GAL4 as well as mushroom body GAL80 to rule out the Mushroom body Neuron Keynon cell's involvement in NF1-dependent appetitive LTM. A different report by the Skoulakis group also shows that the intrinsic Keynon cells are not required for NF1-dependent aversive learning[72]. To identify a specific cell types in which NF1 is required for long-term memory out of the whole fly brain is not easy, and the lack of any effective RNAi line to perform specific knock-down narrows down the options for answering the question[1]. Using a mutant study to identify a potential circuit, however, is very time consuming because that every GAL4 needs to be put into the mutant background. Therefore, we start to look into whether NF1 can work as gain-of-function mutant when ectopically driven by tissue-specific GAL4s.

Our previous study revealed two important properties of NF1 function in memory: Firstly, NF1 signaling acts acutely. The attenuated learning performance of the NF1 mutant in aversive conditioning is not due to developmental defects, since inducing NF1 expression post-developmentally can restore the learning performance completely. Secondly, NF1 signaling works in a dosage dependent manner. The degree of learning performance restoration depends on the degree of post-developmental induction[68]. The acute function as well as dosage-dependent effects suggest NF1 could possibly act as a gain-of-function mutant when ectopically expressed in a subset of tissues.

[1]Although effective RNAi lines were not available early on in the project, towards the end we were able to obtain a line and use it experimentally. These experiments are described in the results, below.

3.2 Methods

3.2.1 Aversive olfactory spaced training protocol

Both extended procedures–spaced and mass training–were performed with an automated training system. In brief, about 100 flies were transfered into a closed chamber coated with shock grids; connected to this chamber is a three-channel valve (custom-built by General Valve Corp.) in which fresh air was bubbled at 750 ml/min through. The air flow is switched into one of the three channels, each of which contains air path through distilled water (to add moisture) and the either pure heavy mineral oil (Fisher) alone or with a particular dilution of BA or MCH (Fluka). A circuit board controls the electric shock pulses with odor deliveries. A computer can be programmed to adjust the pairing duration (system custom designed by Island Motion Inc.).

During massed training flies received ten training cycles delivered one right after the other. For spaced training flies received ten training cycles with a 15 min rest interval between each cycle. At the end of training, flies were tapped gently from the training chamber into their usual food vials and stored at 18°C for the duration of 24 hrs. Flies were then transferred to the choice point of the T-maze where the usual 2 min test trial was performed. The test and the performance calculating the same as described in the previous chapter.

Flies were trained and tested with the classical (Pavlovian) conditioning protocol of Tully and Quinn[79]. Briefly, around 100 flies were trapped in a training chamber that is lined with an electrifiable copper grid. Two odors were then delivered to the flies sequentially through air currents, with the first odor (CS+) delivery paired with electric shock (US) but no shock was received with the delivery of the second odor (CS-). Each odor was delivered in an interval of one minute, with a 45s of fresh air after each odor's delivery. This procedure constituted one training cycle.

Extended training procedures were performed with an automated training system in which fresh air was bubbled at 750 ml/min through one of the three channels in a 'bubbler manifold' (custom built by General Valve Corp.). One channel was for 'fresh' air, a second was for benzaldehyde (BA), and the third was for methylcyclohexanol (MCH). Each channel contained two vials, one with 10 ml of distilled water and the other with either pure heavy mineral oil (Fisher) alone or with a particular dilution of BA or MCH (Fluka). Thus all channels contain a water vial in the flow path and the water vial is used to help humidify the air stream.

Switching of bubbler channels and of a relay to deliver electric shock pulses to the flies was computer controlled (system custom designed by Island Motion Inc.). During massed training flies received ten training cycles (as above)

delivered one right after the other. For spaced training flies received ten training cycles with a 15 min rest interval between each cycle. To assay memory retention, flies were tapped gently from the training chamber into their usual food vials and stored at 18°C for 24 hours. Flies were then transferred to the choice point of the T-maze where the usual 2 min test trial was performed.

3.2.2 Behavior analysis

Statistical analyses were performed using KaleidaGraph (Synergy Software). Overall analyses of variance (ANOVA) were followed by planned pairwise comparisons between the relevant groups with a Tukey HSD post-hoc test. Unless stated otherwise, all experiments are n≥8. Unless stated otherwise, the data are shown as means ± one standard error of the mean (SEM) and analyzed by ANOVA with Bonferroni corrected pairwise comparisons in OriginPro 8 (Chicago, IL, USA). In all cases the symbol '*' indicates $p < 0.05$. The abbreviation 'n.s.' denotes 'not significant' and indicates that $p > 0.05$. The term 'significant' refers to $p < 0.05$.

3.3 Results

We first asked what are the effects of pan-neuronal over-expression of NF1 in the fly brain. Figure 3.1 shows enhanced memory performance with *elav*-GAL4 over-expressing NF1 under an *aversive* spaced training protocol. This enhancement is LTM-specific since we did not observe elevated performance with mass training (Fig. 3.2).

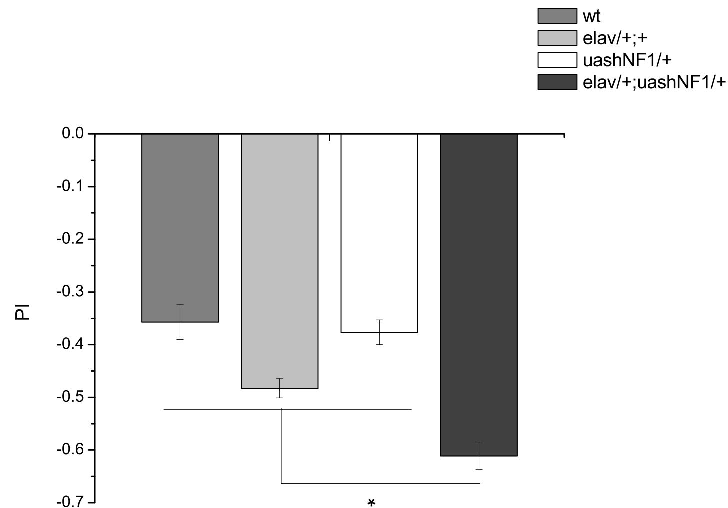


Figure 3.1: Ectopically expressing the NF1 gene enhances 24-hour memory performance after spaced training. To distinguish the performance of olfactory cues that elicit repulsion from those olfactory cues that elicit attraction, the PIs are plotted along the negative Y axis. The human NF1 construct driven by pan-neuronal GAL4 *elav* elevated LTM performance compared to both parental controls as well as wild type control. $n = 8$ for all groups. means $\pm$ SEM.

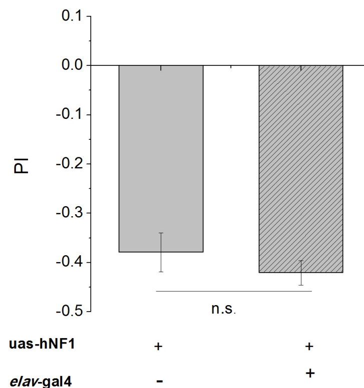


Figure 3.2: Ectopically expressing NF1 gene does not enhance 24-hour memory performance after mass training. Human-NF1 construct driven by pan-neuronal GAL4 *elav* performed the same as the uas parental control. $n = 8$ for both groups. means $\pm$ SEM.

With the same genotype, in Figure 3.3, enhancement has also been observed with appetitive LTM with normal learning performance in Figure 3.4. Thus, we observed a memory enhancement under both aversive and appetitive LTM. However, the aversive LTM training takes almost 3 hours, and the appetitive conditioning takes only several minutes. This is advantageous when performing an LTM screen with many different genotypes.

From here we propose using over-expression to identify potential circuits requiring NF1 function for LTM. We started with some neurotransmitter GAL4s, extrinsic GAL4s, glia GAL4 as well as GAL4s that label the central complex, which was suspected to be involved in LTM. Because each genotype needs to be pre-starved and well timed, we decide to reduce the amount of genotypes but with focus of doing training in a balanced manner, which means training all the genotypes the same day and run them sequential through the same training machine, we reduced all the GAL4 parental controls and only use UAS-humanNF1 heterozygotes as controls. The results of the screen are shown in Figure 3.5. Of the significant positive hits, GAL4 Tdc2 is the most interesting since it enhances LTM robustly and is fairly specific in its expression. The expression of timeless is less clear cut, and so we do not explore it further in this thesis.

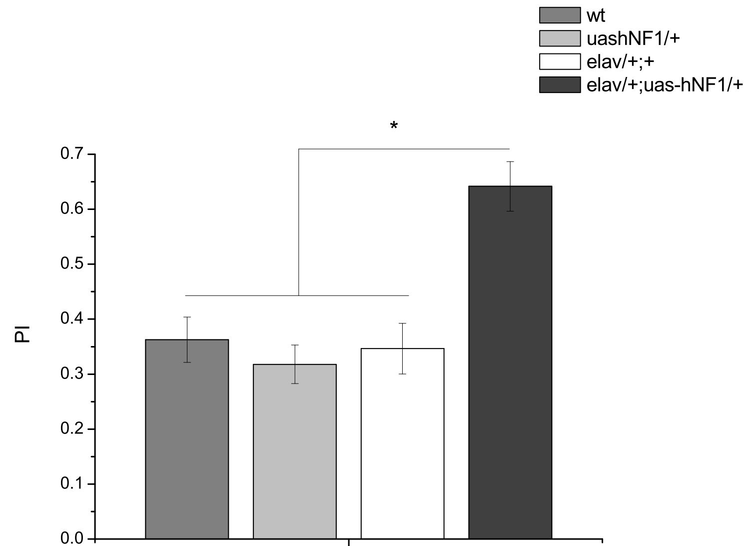


Figure 3.3: Ectopicallly expressing NF1 enhances appetitive LTM. Memory was tested at 24 hours after appetitive training. The human-NF1 construct driven by pan-neuronal GAL4 *elav* has significant elevated LTM performance compared to both parental controls as well as wild type control. The ANOVA was performed on the $n = 8$ for all groups. means $\pm$ SEM.

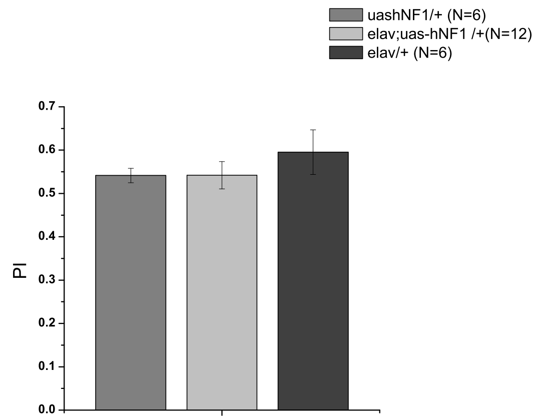


Figure 3.4: Ectopicallly expressing NF1 does not affect appetitive learning performance. Tested right after appetitive training. Human-NF1 construct driven by pan-neuronal GAL4 *elav* has the same performance compared to both parental controls. $n >= 6$ for all groups. means $\pm$ SEM.

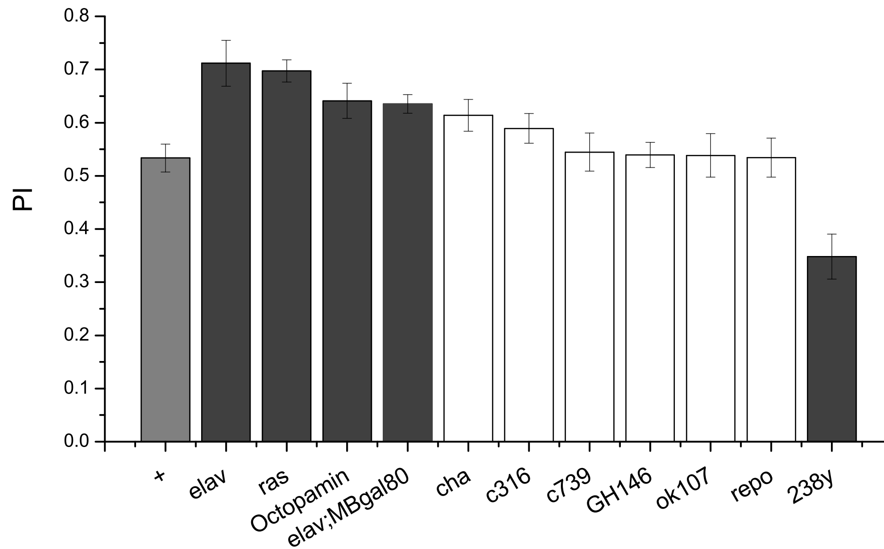


Figure 3.5: Over-expression of NF1 in selected neuronal sub-sets. Grey bars denote are wild type control animals. Black bars denote neuronal populations where over-expression led to a significant ($p < 0.05$) change in LTM compared to controls. White bars denote neuronal populations were over-expression did not lead to a significant improvement in LTM. Statistics were corrected for multiple comparisons. The parental control (+) is UAS-hNf1. elev-GAL4 and ras2-GAL4 are both broadly expressing. Tdc2 octopamine-GAL4 labels octopamine neurons with good specificity. This latter stands out as the most interesting hit due to its specificity. c739 is a mushroom body GAL4, repo is a glial GAL4, and 238Y a central complex GAL4. All three broad neuronal GAL4, elav, ras2 as well as elave;MBgal80 show LTM enhancement, whereas circadian circuit GAL4 timeless as well as octopamine neurons GAL4 Tdc2 when mis-expressing NF1 can also enhance LTM. 238Y is significantly impaired. $n = 8$ for all groups. means ± SEM.

Tdc2-Gal4 is a tyrosine decarboxylase 2 (dTdc2) promoter-directed driver. Tyrosine decarboxylase is an enzyme that catalyzes the chemical reaction to produce tyramine, a first step in octopamine biosynthesis. dTdc2 is expressed in the CNS and innervates the female reproductive tract. Anti-Tdc2 immunostaining has been proved to show remarkably close resemblance to the anti-octopamine staining[97]. The Tdc2-Gal4 targets 8569 neurons in the brain, and only 866 of which are Tdc2-immunonegative[98]. All this indicates Tdc2-gal4 as a fairly specific driver for octopamine neurons. Based on the results of the screen we next put Tdc2-GAL4, as well as another octopamine GAL4, 7088, into the NF1 mutant background and attempted to rescue the phenotype. When we crossed the octopamine GAL4s with uas-NF1, we found it can rescue the LTM defects (Figure 3.6). NP7088 is a gal4 line which has almost completely overlap labeling with Tdc2-gal4 in the central brain[98]. The rescue both specific gal4 lines suggest that the NF1-dependent LTM is functioning in octopaminegic neurons.

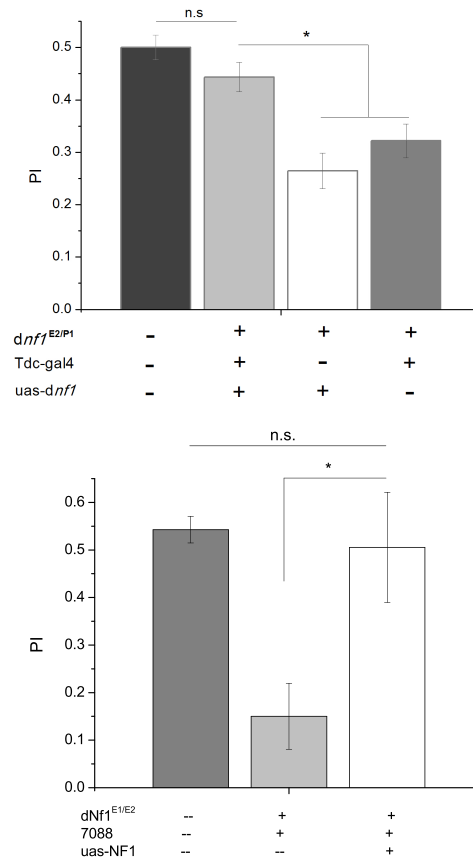


Figure 3.6: Selectively expressing NF1 in octopamine neurons restores LTM defect in the mutant background. **Top** Tdc2, $n = 12$. **Bottom** 7088, $n = 8$. In all cases, memory was tested at 24 hours after appetitive training. In both cases the NF1 construct driven by the octopamine GAL4 has significant higher LTM performance ($p < 0.05$) compared to parental controls but not significantly different from wild type control. means $\pm$ SEM.

Finally, we knocked down NF1 in octopaminegic neurons using RNAi (Figure 3.7), and this resulted in significantly lower performance. This RNAi line was kindly provided by Ni lab from Tsinghua University. Ni lab used VALIUM as an effective and optimized vector for the short hairpin RNA (shRNA) expression[99]. This vector was integrated at genomic attP landing site via phiC31-targeted integration. For best knock-down effect, four target sequences for NF1 gene were selected. Out of four RNAi lines, however, only one lines named 720 gave us a defected memory performance similar to that of NF1 null mutants.

So far we still can not rule out the possibility that the defect we shaw with RNAi knock-down is due to the off-target effect of the RNAi expression. We need another RNAi line targeting different sequence in the NF1 gene to show similar memory defect. And we need to evaluate the knock-down effect by measuring the mRNA level of NF1 by qPCR or the protein level of NF1 with western blot.

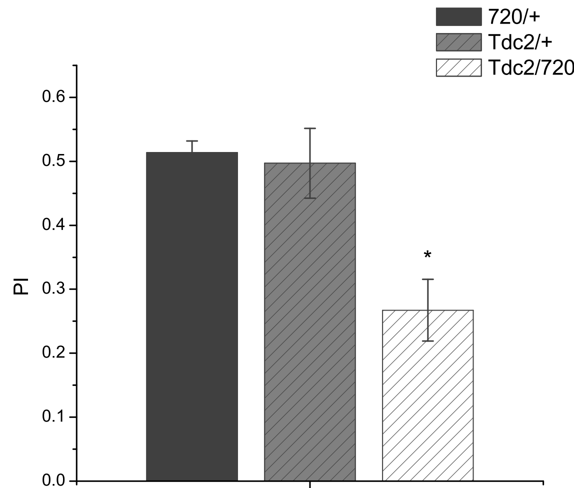


Figure 3.7: Selectively knocking down NF1 in octopamine neurons attenuates appetitive LTM. Memory was tested at 24 hours after appetitive training. The NF1 RNAi line driven by octopamine GAL4 Tdc2 has significant lower LTM performance compared to the parental controls ($p < 0.05$). $n = 8$ for all groups.means $\pm$ SEM.

3.4 Discussion

3.4.1 Controversy

In aversive olfactory conditioning, the involvement of mushroom body in NF1 function is controversial and still not clear. The Davis group found that NF1 is functioning within the mushroom body for both learning and LTM, but they focused on the 3-hour memory defects in the mutants and provided evidence that the $\alpha\beta$ subset of mushroom body is required for NF1-dependent 3-hour memory[89]; whereas the Skoulakis group found that the NF1 aversive learning is not mushroom body dependent[72]. It is worth pointing out that in the Davis group, only one MB GAL4 line, c739, was used to rescue the learning defects. Since c739 labeling is not exclusive to the mushroom body, it is possible that the rescue they observe is due to neurons other than Kenyon cells.

3.4.2 Gain of function screen

Gain-of-function screens in Drosophila are an effective method to identify genes that affect the development of particular structures or cell types[100]. It has been found that about 2–10% of genes result in a discernible phenotype when over-expressed[101]. However, it is not clear to what extent a gain-of-function phenotype generated by over-expression is informative about the normal function of the gene. Thus, very few reports attempt to correlate the loss- and over-expression phenotype for collections of genes identified in gain-of-function screens.

In this chapter, we borrowed the ectopic expression idea from gain-of-function screens but to achieve reverse purpose–to identify specific tissue instead of genes. The reason we can do this is because we found the NF1 gene, when over-expressed, can enhance LTM in either aversive or appetitive conditioning. The molecular basis of this enhancement is still not known. It will be very interesting to use many of the truncated forms of NF1[102] to identify which domain is involved in the LTM enhancement. The enhancement of LTM, also rises a question many people tend to ask: why is there a room for memory enhancement? Likely the improvement in memory comes at a cost, which translates into a decreased fitness for survival of the animal. Potential costs include forming inappropriately strong memories that later deceive the animal or block the formation of new, more relevant, memories. A stronger memory may come at the cost of forming fewer memories.

3.4.3 Octopamine in appetitive memory

Octopamine is a well established neurotransmitter, neuromodulator, and neurohormone in invertebrates. In the fly, octopamine has been implicated in complex behavioral processes and in physiological processes such as ovulation and egg laying. Vertebrates do not have octopamine, but it is believed that norepinephrine is a functional homologue of octopamine. Possibly coincidently, noradrenaline is a key modulator of aggression in mammals and humans whereas Octopamine is also involved in male-male aggression in flies.

Octopamine neurons have also been identified as a channel mediating US information in the appetitive olfactory associative conditioning[85]. So far, studies of octopamine neurons involvement in appetite memory are mainly focused on the acquisition stage: octopamine neurons carry the sweetness information during conditioning [103]; the sweet information is relayed through a specific subset of dopamine neuron to form association[104]. Interestingly, the NP7088 we used in the study, does not relay sweet information as Tdc2-2 GAL4 did[104]. And Tdc2 and NP7088 overlaps mostly in the central brain within the subset of neurons innervating mushroom body.

In our study, we did not observe any learning defects and the requirement of NF1 in the octopamine neurons is for long term memory only. Currently, we do not know how NF1 signaling affects octopamine neuron function, however, studies from octopaminergic type II motor neurons show that the neuronal arbor plasticity of type II neuron requires cAMP and CREB signaling. Giving NF1 interacts with cAMP and CREB both, it is tempting to speculate that NF1 affects the octopamine signaling and consequently affects LTM. It also suggests that octopamine signaling might have another unrevealed role in appetitive long term memory.

Chapter 4

Manipulating octopamine neuron activity

In previous chapter we identified octopamine neurons in which NF1 gene expression is sufficient to rescue mutants' long term memory. However, what does octopamine do in the long term memory process? No implication of its involvement has ever been reported before. Therefore, in this chapter, we focused on octopamine neurons by manipulating their neuronal activity, and in addition, we ectopically express gain-of-function Ras in the octopamine neurons. Our results suggest that octopamine neuron activity during memory retention modulates appetitive long term memory, and that Ras is involved in this process.

4.1 Introduction

4.1.1 What does octopamine do?

Octopamine is an endogenous biogenic amine which acts as a neurotransmitter, neurohormone and neuromodulator in invertebrates[105]. It was first discovered in the octopus salivary glands by Italian scientist Vittorio Erspamer in 1948[106] and hence got its name. Octopamine modulates almost every physiological process in invertebrates studied so far[107]. From peripheral motor organs to sense organs and even processes within the central nervous system. For years octopmaine as neurotransmitter has been widely studied in energy-demanding behaviors in all insects. For example: in the locust, octopamine modulates the jumping activity by making the leg muscles contract more effectively[108]; in the firefly, octopamine release leads to light production

in the lantern[109]; in lobsters, octopamine direct and coordinate with neurohormones to direct postures[110]; in fruit fly, octopamine regulates female sperm storage[111]. The known actions of octopamine (OA) in the central nervous system include desensitization of sensory inputs, influence on learning and memory, or regulation of the 'mood' of the animal[107]. For instance, OA has been studied in regulating aggression in invertebrates, with different effects on different species[112–114]. In honey bee and fruit fly, OA has been implicated a major role in sugar reward learning acquisition. Moreover, studies of alcohol tolerance in fruit flies suggest octopamine regulates alcohol tolerance[115].

4.1.2 OA in olfactory associative learning in *Drosophila*

Appetitive olfactory associative learning requires OA activity. The sweet signal is mediated through OA neurons and the reinforcement signal is relayed via OA to dopamine to deliver the US signal.

Octopamine neurons projects broadly to the brain and the mushroom body is one of their targets. Since MB is required for appetitive memory formation, retention and retrieval, the subset of OA neurons which innervate mushroom body is of particular interest in the appetitive field. There are several subsets of OA neurons that project to the mushroom body, they are: OA-VUMa2, OA-VPM3, OA-VPM4 and OA-VPM5][116]. The driver Tdc-2 labels all of those subset and so does NP7088, with the exception of OA-VPM5. Interestingly, stimulating Tdc2-gal4 labeled neurons with TRPA1 can replace sugar reward yet NP7088 can not[103]. This indicates the innervation of octopamine onto the mushroom body does not serve for US acquisition.

4.2 Methods

4.2.1 memory protocol

Flies in the Kir and TRP experiments (Figure 4.1 and figure 4.2) were raised in 18°C. And when in 18°C, the metabolic rates in flies drop dramatically. To acquire similar level of learning as those raised in 25°C, flies need to be starved at least 2 days. And consequently, the re-starvation has to be extended to a period of 2-day as well. Otherwise specified, the flies were all raised in 18°C, training in 18°C and tested in 18°C. Flies used in Figure 4.3 are raised in 25°C. The starvation and training condition is the same as previous chapter described. For 2-day memory, flies were restarved for 22-hour before testing, that is, flies were kept on food for one day after training, and transfered to starvation tube 22-hour prior to the testing.

4.2.2 fly lines

Kir combined with Gal80^{ts} construct obtained from our colleague Yichun Shuai. Tdc2 gal4, constitutively active Ras V12, and TRPA line have been out-crossed into 2u background.

4.3 Results

Since the involvement of octopamine in memory after acquisition is not known, we thought to test if modulating octopamine activity after learning acquisition affects memory. To manipulate octopamine activity in both directions, TRP channel was used to increase neuronal activity by depolarization; and the inwardly rectifying potassium channels (Kir) was used to shut down neuronal activity. Kir has been reported as the more effective tool to silence neuronal activity compared to Shibire, therefore we combined Kir with temperature sensitive Gal80^{ts} to achieve temporal induction. Our preliminary results shows that, Kir;Gal80 crossed with elav will paralyze flies within 8 hours when the temperature shifts from 18°C to 30°C. Therefore, we chose 8-hour as our heat-shock 'sliding time window' to test *1*, if shutting down octopamine affects memory performance and if so, *2*, can we map out the affecting time period.

Our first result on Tdc2-gal4/Gal80^{ts};Kir/+ flies shows a very interesting and also puzzling discovery. First, blocking octopamine activity can indeed affect memory compared to the no temperature shift group; second, the effect is enhancement and the enhancement only occurs during memory retention (Figure 4.1).

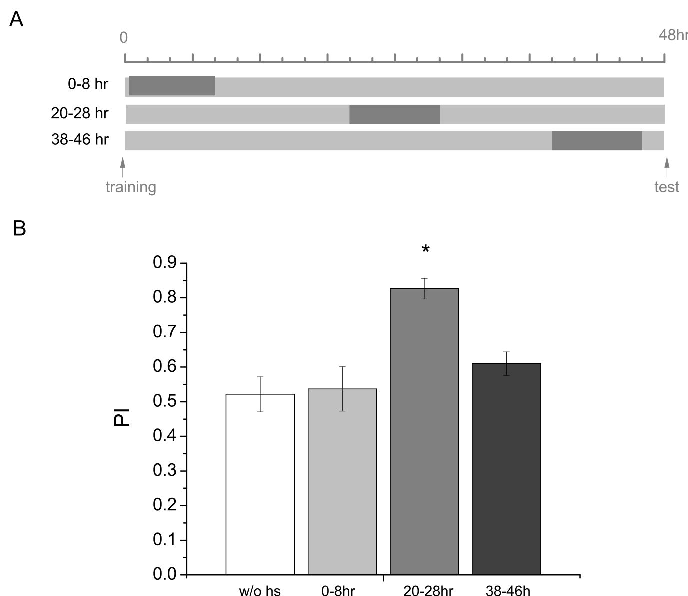


Figure 4.1: A–B: Blocking octopamine neuron activity during memory retention enhances memory. **A** shows three time windows during which Kir is induced in the octopamine neurons. Temperature sensitive gal80^{ts} is combined with Kir to achieve heat shock induction of Kir. Flies were raised, trained and tested all at 18°C; only during the time windows labeled by the darker gray shades were flies were shifted to 30°C. The last time window allows 2 hour recovery before testing in 18°C. **B** Memory was tested 48 hours after training. The results show that only during memory retention, not during consolidation or right before testing, does inhibiting octopamine neuron enhance LTM. $n = 8$ for both groups. means ± SEM.

Because we want to find an efficient time window, when octopamine activity affects memory so we first omitted the corresponding parental controls to reduce the work load. And after we found an effective time window, we fixed on only one heat shock regime and carried out the experiments with parental controls. We found that the enhancement is only occurs in the experimental group not the parental control, indicating the enhancement is not an artifact of temperature shifts. Moreover, we want to know if the opposite effect will happen, when over-activating octopamine neuron with TRP. What we show in Figure 4.2 indicates that the opposite effect does indeed occur: that hyperactivity in octopamine neurons attenuates long term memory. Taken together, we found that octopamine activity levels are inversely correlated with LTM performance.

On another front, the identification of octopamine neuron involvement in NF1-dependent LTM lead to a another question. How does the NF1 signaling change in octopamine neurons affect memory? Our previous result shows

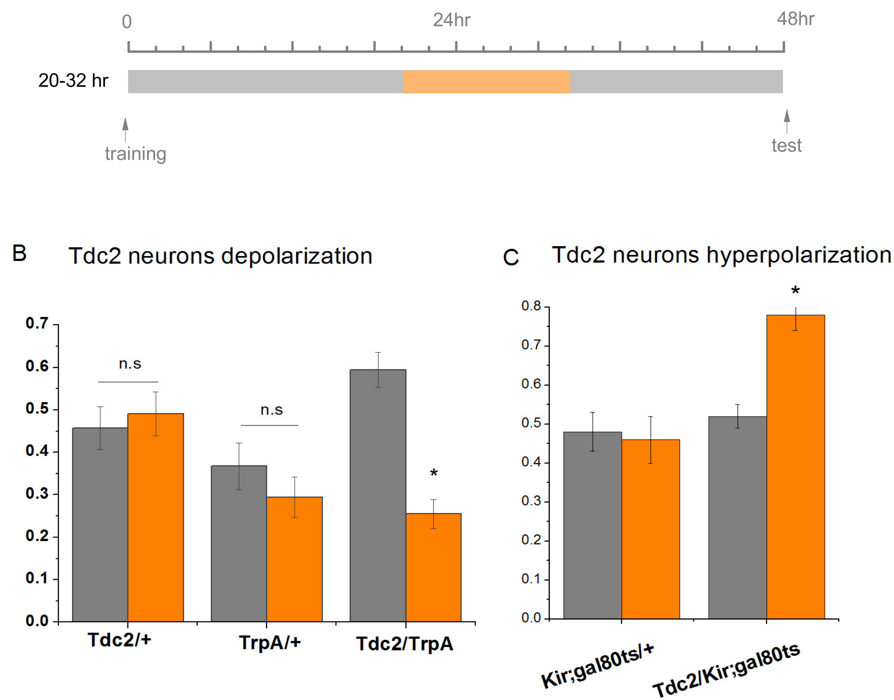


Figure 4.2: A–C: Diagram of heat shock protocol. Flies were raise, trained and tested at 18°C; only during the time window shown in yellow were flies were shifted into 30°C for 12 hours. In **B** and **C**, memory was tested 48 hours after training. **B**. depolarizing octopamine neurons with TRPA channels significantly attenuated LTM performance ($p < 0.05$) whereas heat shock does not introduce any effect on the parental controls. **C**. Hyperpolarizing octopamine neurons with Kir enhanced memory compared to that of parental controls. $n = 8$ for both groups. means ± SEM.

that the Ras-GAP domain of NF1 is required for normal LTM performance in aversive spaced training. Therefore we hypothesize that manipulating Ras signaling in octopamine neurons might affecting appetitive LTM as well. We use Tdc2-GAL4 to drive the constitutive Ras, RasV12. The progeny survived well with no obvious differences observed. We first test learning and 24h memory; what we found is that, although the 24h memory is the same as parental controls, the learning in Tdc2 droved RasV12 flies exhibits higher acquisition to begin with. This enhance learning seems to be due to a stronger starvation effect. Higher learning but resulted in the same strength of memory at 1 day, we reasoned, maybe the memory decayed faster, therefore we keep testing the memory at 2-days after learning. Figure 4.3 shows the performance curve from learning to 1 day and 2 day memory, Tdc2/V12 flies clearly shows a significantly deeper slope compared to that of their parental controls.

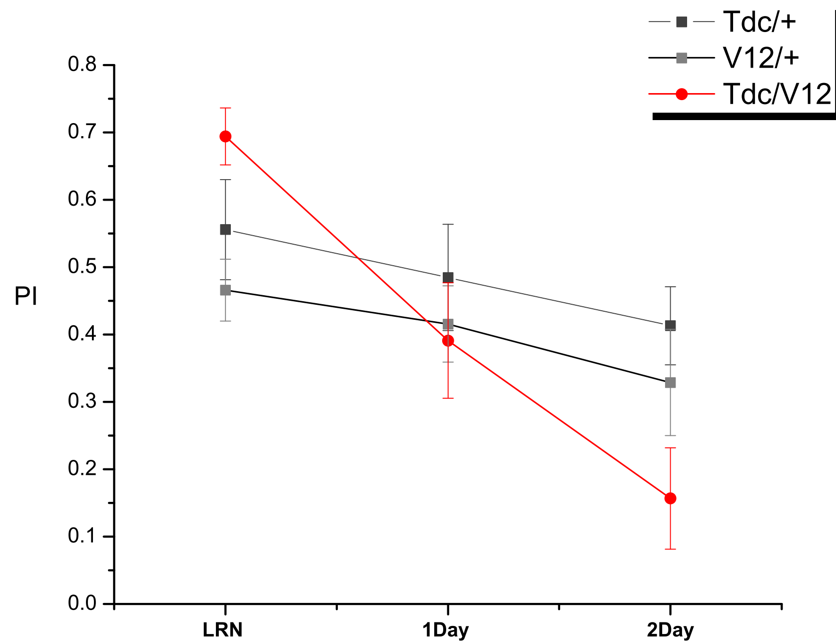


Figure 4.3: Enhanced Ras signaling in octopamine neurons results in more rapidly decaying memory ($p < 0.05$, significant interaction effect of day and genotype in linear regression).

4.4 Discussion

4.4.1 octopamine: acquisition and forgetting

Octopamine mediates the rewarding US. Here we show that it also appears to modulate the decay of LTM. How should we understand the functional heterogeneity within the same neurotransmitter? Perhaps dopamine is be one of the best examples for us looking into this question. Dopamine neurons, which mediate the acquisition of the US in aversive olfactory conditioning, have been identified with another role in labile and consolidated memory[117]. When inhibiting dopamine neurons with shibire blocking after learning, 3h aversive memory is enhanced; and when activating dopamine neurons with TRPA channels, 3h aversive memory reduced. This observation led by the Davis group suggests that dopamine neurons bidirectionally regulate forgetting through activity modulation after learning. By using different dopamine mutants, Berry *et al.* found that dopamine regulated memory decay is mediated through DAMB, a dopamine receptor different from DA which mediates the US signaling in learning acquisition. By narrowing down to a sub-population of the dopamine cluster, DANs, Berry *et al.* did *in vivo* imaging in awake flies and found spontaneous release from the DANs cluster. Based above, Davis group proposes that after this dopamine cluster fulfilled its role in the acquisition of memory by providing a US signal to the MBs predominantly through the dopamine receptor dDA1, it continues to release dopamine onto the MB that signals through the DAMB receptor to cause forgetting of recently acquired labile memories.

Evidence so far suggests that octopamine neurons might function in a similar dual way.

- We showed that octopamine bidirectionally modulates memory performance.
- There are two octopamine receptors, OAMB and octβ1R. The unconditioning stimulus of appetitive learning is mediated through OAMB but not octβ1R[104].
- The subset of octopamine neurons (VAM) which innervate mushroom body does not deliver the US signal[104].

Taken together, it is very plausible that with future experiments we can demonstrate that octopamine might bidirectionally regulates the decay of long term memory.

4.4.2 Octopamine: Ras-dependent memory decay

Ectopic expression of a dominant negative form of RasV12 shows similar performance compared to the parental controls when testing 24-hour after training. In addition, this ectopic expression shows enhanced learning. To explain the enhancement phenotype, future control experiments are needed to evaluate whether this enhancement is due to a starvation phenotype in the ecotopic expression group. Retrieval of the memory correlates only with the degree of starvation, we cannot simply conclude the normal 24-hour performance in the Tdc/V12 group is unaffected LTM. There is a possibility that over-starvation compensates for the memory defects and results in a 'net' performance which is similar to that of the parental controls. Nonetheless, when memory was assessed at 2-day after training, we observe a significant difference of the Tdc/V12 flies from that of parental controls. The steeper curve of memory decay supports the idea that elevated Ras signaling accelerates the decay of memory.

Chapter 5

Discussion

In this thesis I explored the role of neurofibromin on learning and memory. I showed that normal NF1 gene function in the brain is needed for appetitive LTM but not learning. I went on to show that normal NF1 protein is required in octopamine neurons for normal LTM. Mutations in the mushroom body (mainly Kenyon cells) produced no impairment. This the first research to show the involvement of a specific neuron type as playing a role in the cognitive deficits. Manipulating the excitability of octopamine neurons after consolidation time modulated appetitive LTM.

5.1 Octopamine signaling in synaptic plasticity and memory

Auto-regulatory octopamine signaling

Studies from NMJ model have shown that ocpaminergic neurons have auto-regulatory mechanisms for regulating the growth of octopaminergic arbors and octopamine signaling.This auto-regulatory loops include both positive and inhibitory feedback machineries via different auto-receptors.During stress and hunger condition, the octopaminergic neurons have extended arbors and enhanced octopamine signaling both of which are regulated through the octβ2R auto-receptor[118].On the contrary, there is contraction of octopaminergic arbors and reduced octopamine signaling when the flies are sated. This inhibitory mechanism is function through the octβ1R auto-receptor[119].

OAMB is required in the mushroom body for appetitive memory acquisition

In flies, there are three octopamine receptors have been identified. There are octβ1R, octβ2R and OAMB. The former two, locate in many tissues including the octopaminergic neurons themselves. And the positive or inhibitory effect produced by which octopamine receptor depend on the concentration of the octopamine neurotransmitter. Interesting, the predominant octopamine receptor in the mushroom body is neither octβ1R nor octβ2R, but is OAMB instead. Like the octβ2R, OAMB promotes intracellular cAMP and Calcium level[120]. Moreover, in the following study, Han, K et al have found that OAMB expression in the mushroom body is required for normal appetitive memory acquisition indicating the important function of OAMB in the kenyon cell plasticity[121].

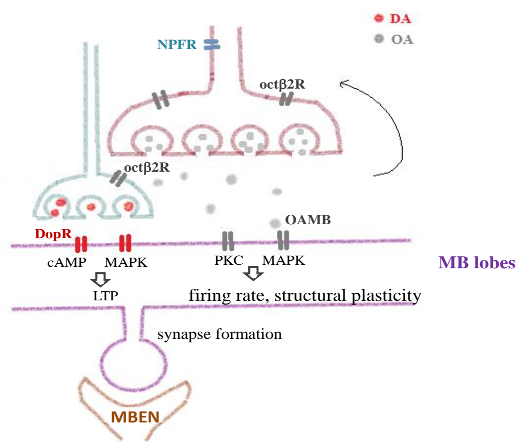
Our working model

It has been shown in many insect systems that the octopamine signaling increases the spontaneous spike rate, the gain of the response to stimuli[122], temperal frequency of turning[123] and the harbor branchings of excitatory neurons[118]. We hypothesize that the general function of octpamine in the nerve system is to boost the sensitivity and the dynamics of the neuron by changing the conductivity and promoting synaptic branching. This general boosting could facilitate the formation of learning-specific synapse, yet also could act as a double-edged sword that it actively decreases the synaptic efficacy or even destabilizes the formed synapses during memory retention.

In figure 5.1, Left: during acquisition, both octopamine signaling and octopaminergic innervation are up-regulated via NPF receptors and octβ2R/auto-regulatory feedback loop. Consequently, octopamine modulates both dopaminegic and kenyon cell firing rate and structural plasticity; upon both dopamine receptor DopR and octopamine receptor OAMB, learning specific synapse is formed in the post-synaptic site of the mushroom body, the MB lobe. Right: during retention, up: in the wild type, octβ1R/auto-regulatory feedback inhibits octopaminegic neuron growth and maintain a low level of octopamine secretion. The low level octopamine signaling keeps promoting structural changes in the MB lobes, and the learning-induced synapse reduces its synaptic efficacy due to synaptic homeostasis mechanism; middle: in the over-expression condition, ectopically expressed human-NF1 inhites ras signaling, and consequently inhibit the basal level octopamine secretion.Without octopamine signaling, the structure in the MB lobes remains stable; bottom: in the NF1 null mutant, the Ras signaling is exacerbated due to the absence of NF1. And consequently

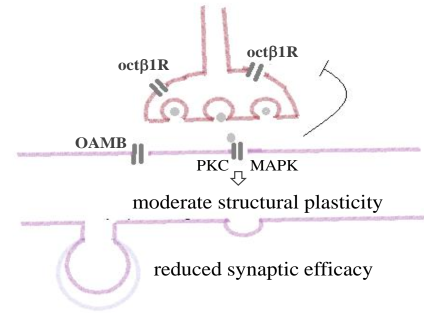
enhanced octopamine signaling and octopaminergic innervation.The elevated structural changes in the MB lobes destabilizes the learning-specific synapse.

Acquisition

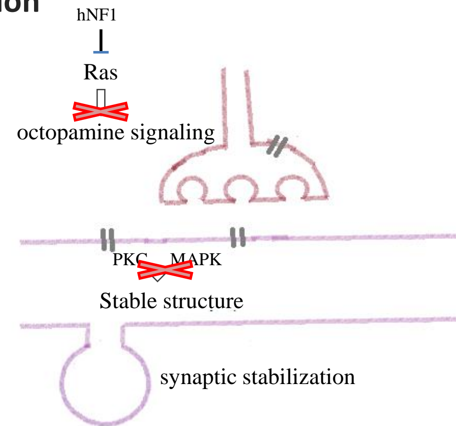


Retention

wt



Over-expression



mutant

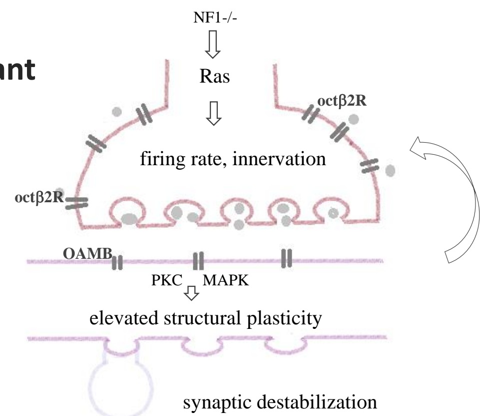


Figure 5.1: Working model for octopamine/NF1 signaling in learning acquistion and memory retention.

5.2 *rutabaga* in these two types of memory

In aversive olfactory learning, the molecular mechanism as well as the neural substrates have been under thorough investigation and a fairly complete picture has been put together:

1. The cAMP/PKA pathway is important for aversive olfactory learning: the very first learning mutants identified with behavior screens are *dunce* and *rutabaga*[73, 74]. Rutabaga encodes an adenylyl cyclase gene whereas dunce is a cAMP phosphodiesterase gene. Adenylyl cyclase (AC) catalyzes the conversion of ATP to 3',5'-cyclic AMP (cAMP), the later of which activates PKA; the dunce cAMP phosphodiesterase inactivates PKA by converting cAMP to AMP. This cAMP/PKA activation pathway has been well known for induction of synaptic facilitation and synaptic plasticity from studies of Aplysia[124, 125], the *Drosophila* NMJ[126] as well as mammalian organic cultures[127].

2. The current model proposes that rutabaga acts as a coincidence detector for associating the US and CS. This is because *rutabaga* can be synergistically activated by calcium and G-protein coupled protein receptors[128, 129]. During the CS odor and US reinforcer pairing in aversive olfactory conditioning, the CS increases intracellular calcium whereas the US activates dopamine receptors, and consequently cAMP as second messenger for synaptic plasticity is elevated by rutabaga. This model has been widely supported by both sequential dissection mushroom body involvement in the memory process as well as anatomical mapping of rutabaga functional site for aversive olfactory learning. The gamma lobe activity as well as dopamine receptor expression in the gamma lobe are required for learning acquisition[83, 130, 131].

3. Interestingly, *rutabaga* mutants show defects in almost all kinds of associative learning[130, 132, 133].

In appetitive olfactory memory,the *rutabaga* mutant has learning defects as well. However, in contrast to the evidence from aversive studies that MB Kenyon cells are required for supporting rutabaga-dependent learning, appetitive learning requires *rutabaga* function in **either** mushroom body **or** projection neurons[134]. Furthermore, the authors showed that rugabaga expression in the projection neurons can also support 3-hour appetitive memory, a phase at which memory is already in consolidated form. This is a very interesting observation and future investigations will be intriguing, such as how long does

this rutabage-dependent memory trace in the PNs last, and if other PN specific GAL4 drivers support the same phenotype. This is important because in work to date inly the GH146-GAL4 driver was used for 3-h rescue, and this is known to label the the large APL neuron that innervates the MB, apparently providing negative feedback.

Nonetheless, work to date supports the idea that other neuronal structures besides the mushroom body intrinsic neurons are involved, if not fully required, in memory stages after acquisition. So far, this idea has been supported by many studies. One of these is the dorsal paired medial (DPM) neuron which innervates the entire MB lobes and is required for the first hour of the 3-hour retention period in aversive memory[135, 136]. Another example is the anterior paired lateral (APL) neuron which also innervates the whole mushroom body, and is required for 3-hour retention in the aversive memory[137]. Interestingly, the octopaminergic property of the APL neuron is essential for its requirement in memory[138]. As for memory at later phases, structures such as the DAL neuron and ellipsoid body have been implicated for long term memory storage[139, 140]. In this thesis, I provided evidence that octopamine neurons are involved with appetitive long term memory in a NF1-dependent manner. Taken all together, memory phases after acquisition require other neurons and structure for support and regulation.

5.3 Developing an automated apparatus

The great advantage of appetitive conditioning is to rapidly study LTM using a naturalistic training regime. The appetitive training procedure takes less than 7 minutes, in contrast, aversive spaced training requires nearly 3 hours. In our hands, the performance of LTM is around 0.5 to 0.6, which is even higher than the performance generated by aversive spaced training. Because of the high performance index we can achieve, defects can be presented more evidently as floor effects in the scores are reduced. Further modifications to the training regime may make it possible to push wild type scores yet higher.

Although appetitive conditioning sounds promising, there are many obstacles which stop the paradigm from gaining widespread popularity. The first is a lack of an automatic apparatus for training. The current way of delivering the US (sucrose) is by pushing flies into tubes coated with sucrose. This switching of vials interrupts the air current. However, Pavlovian conditioning requires fairly precise matching between the onset time of the US and CS+, therefore, after changing tubes, the experimenter has to connect the new tube immediately with the air pathway for flies to receive the CS+ odor. This precises timing is physically challenging and prone to human error as the ex-

periment progresses. An automatic apparatus, to replace can eliminate these issues and turn appetitive LTM into a true high throughput method. The second obstacle, which can be avoid by experience, is the starvation protocol. One first has to acknowledge is that starvation is influenced by genetic background and there is a wide distribution of required starvation time among even different wild type lab strains. Starvation times published by other labs or even colleague can not guarantee a similar effect in one's own hands. Empirical testing is needed to establish one's own starvation regime. Moreover, one common misunderstanding of starvation is simply not to provide food but only water, yet in practice, careless delivery of water can result in stress in addition to the starvation. Crowdedness for instance, is commonly seen in the starving processes. This could affect performance greatly and lead to ambiguous results.

5.4 Future studies

In this thesis, we showed that NF1 is required for appetitive long term memory. And the NF1-dependent long term memory requires octopamine neurons. Furthermore, we have evidence that suggests the involvement of octopamine neurons in regulating appetitive long term memory decay. It will be very interesting in the future, to test truncated forms of the NF1 gene in involvement of long term memory enhancement. Identifying the functional domain involved in memory enhancement will helps us better understand the function of NF1.

Finally, it is clear from our data that octopamine neurons are not the only candidates for the locus of NF1 action in appetitive LTM. For instance, our screen also identified the timeless (clock) neurons as being important. In the future, a larger screen could be conducted using automated apparatus in oder to explore the role of a wider range of cell types.

Appendix A

Sugar preference

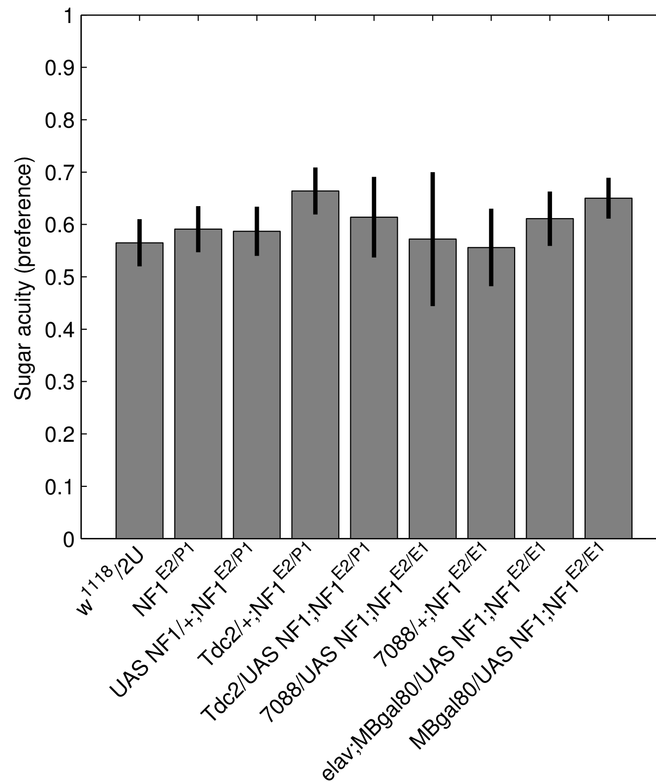


Figure A.1: The preference for sugar (often called the 'sugar acuity') for the different genotypes used in this study. There are no statistically significant differences between groups. Data are organized according to experiment experiment and session of testing. In all cases $n = 8$ and data are means $\pm$ one SEM.

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