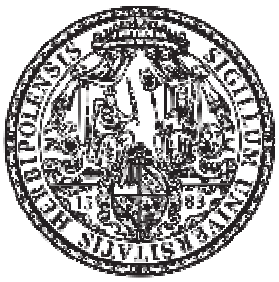

Optogenetic investigation of nervous system functions using walking behavior and genome wide transcript analysis of *Synapsin* and *Sap47* mutants of *Drosophila*

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Index

Part I

1. Introduction

2. Materials and Methods

3. Results

1. Introduction

"The brain is a world consisting of a number of unexplored continents and great stretches of unknown territory."

Santiago Ramón y Cajal

The brain comprises different regions which are attributed to have different functions, and these regions communicate with each other to form a larger functional complex. The wiring between different regions determines the behavioral output of an individual. Although the basic functions of most of the individual regions in the brain have been known for years, the underlying mechanisms and significance of the connections made remain largely unknown. The human brain is estimated to have 100 billion neurons with 100 trillion synapses which pose a daunting task to unravel the mechanisms underlying neuronal communication and functional significance of neuronal subtypes comprising the brain. Thus, model organisms like *Drosophila melanogaster* with smaller neuronal complexity serve as an elementary model to study nervous system functions which could then be applied to vertebrates.

1.1 *Drosophila*: a geneticist's tool

Drosophila has a brain with ~ 100000 neurons which is small compared to the large numbers of nerve cells in vertebrates, but it still displays a huge repertoire of behavioral phenomena. The small genome size, short life cycle and ease of genetic manipulation make *Drosophila* an apt model organism for neurobiological research. The genetic simplicity of *Drosophila* was one of the reasons to select it for sequencing of an entire eukaryotic genome (Adams et al., 2000), which provides a unique resource for genetic information that is also valuable for neurobiological areas of research.

A variety of behaviors have been well investigated in *Drosophila* like olfactory behavior (Carlson, 1996), aggression (Chen et al., 2002), flight behavior (Wolf and Heisenberg, 1991), courtship (Siegel and Hall, 1979), gustatory behavior (Chyb et al.,

2003), learning and memory (Wustmann et al., 1996), or locomotion (Strauss et al., 1992). It is interesting that despite the small brain these behaviors can be rather complex and often resemble in their organization and their principles to behavioral actions exerted by higher vertebrates.

The strongest advantage of the fruit fly as a model organism is the ease by which germ-line cells can be transformed, allowing for a cell-type specific and stable manipulation of the genetic material. Germ-line transformations are fairly simple and can be done by P element or PiggBac vectors (Rubin and Spradling, 1982; Handler and Harrell, 1999). Cell specific gene expression or inhibition is possible by tools like the Gal4-UAS system (Brand and Dormand, 1995) which permit site specific expression of trans-genes with appropriate enhancer trap lines or promoter elements. In addition, the recently described LexA gene- based binary transcriptional system in *Drosophila* (Lai and Lee, 2006) permit simultaneous expression of 2 transgenes in the same animal, which can potentially help to differentially visualize and manipulate pre and postsynaptic partner neurons. Using these different techniques it is possible to monitor neuronal mechanisms (e.g. using calcium reporters like cameleon or G-CaMP (Wang et al., 2003), or chloride sensors like clomeleon (Kuner and Augustine, 2000), or to manipulate neuronal activity (using light activated ion channels like channelrhodopsin-2 (Nagel et al., 2003) or Halorhodopsin (Han and Boyden, 2007), temperature sensitive TRPA1 and TRPM8 channels (Montell, 2005), sodium activated bacterial channel or caged ATP (Lima and Miesenbock, 2005). Also neurons could be selectively silenced by the Gal4-UAS system using Kir 2.1, a potassium inward rectifying channel (Doring et al., 2002), tetanus toxin (Sweeney et al., 1995) or shibire^{ts} (Buzin et al., 1978).

As can be seen from these examples probably the most important contribution in insect-based neurobiological research was the advent of gene expression tools in *Drosophila*.

1.2 Gene expression tools

1.2.1 The Gal4-UAS system

Gal4 is a protein found in *Saccharomyces cerevisiae* as a gene regulator which is responsive to galactose (Laughon et al., 1984). The protein has 2 domains, one functions

as a DNA binding domain and the other as a transcriptional activator domain which is capable of activating any gene that is under the control of a Upstream Activating Sequence (UAS) (Fischer et al., 1988) . This concept has been used to generate a bipartite gene expression system in *Drosophila* where the driver Gal4 construct and the effector UAS construct (Brand and Perrimon, 1993) can be maintained individually as two different fly strains and on crossing these two strains the expression of a desired gene can be achieved. One advantage of such a bipartite system is that neurons could not only be spatially silenced (by expressing tetanus toxin, Kir 2.1 channel, shibire) or activated but also be controlled with temporal resolution. As an example, $Gal80^{ts}$ blocks the Gal4 activity at permissive temperature, but can activate the effects of Gal4 by a temporally controlled shift to restrictive temperatures.

1.2.2 LexA-LexAop

The second type of expression system, which has been described recently (Lai and Lee, 2006), called LexA- LexAop, was isolated from bacteria and functions similar to the Gal4-UAS system. The bacterial promoter LexAop (prokaryotic promoter) with 8 binding sites for LexA replaces UAS. The LexA (Brent and Ptashne, 1985) is a bacterial DNA binding protein which is fused to a transcription activating domain like VP16 (viral transcription factor VP16 acidic activation domain), (Triezenberg et al., 1988) which is Gal80 insensitive or GAD (Gal4 Activating Domain) (Ma and Ptashne, 1987) which is Gal80 sensitive and functions as a transcriptional activator. Importantly, it is possible to drive the LexA-pLOT system and the Gal4-UAS system together in the same animal (Lai and Lee, 2006). This has been applied to visualize and study the interactions between pre and post synaptic partners using MARCM analysis. These two binary expression systems could permit the investigation of neuronal networks by activating neuronal subsets using transgenes like channelrhodopsin-2 (see below) and simultaneously silencing another group of pre-synaptic neurons and study correlation effects. With the development of new cloning vectors like the gateway cloning technology is has become simpler to establish LexAop-LexA: GAD/VP16 fly lines (Diegelmann et al., 2008).

Using these gene expression tools various transgenes can be driven which permit the quantification of neuronal activity along with visualization and manipulation of neurons. Other ways like promoter fusions and enhancer traps can be used for driving

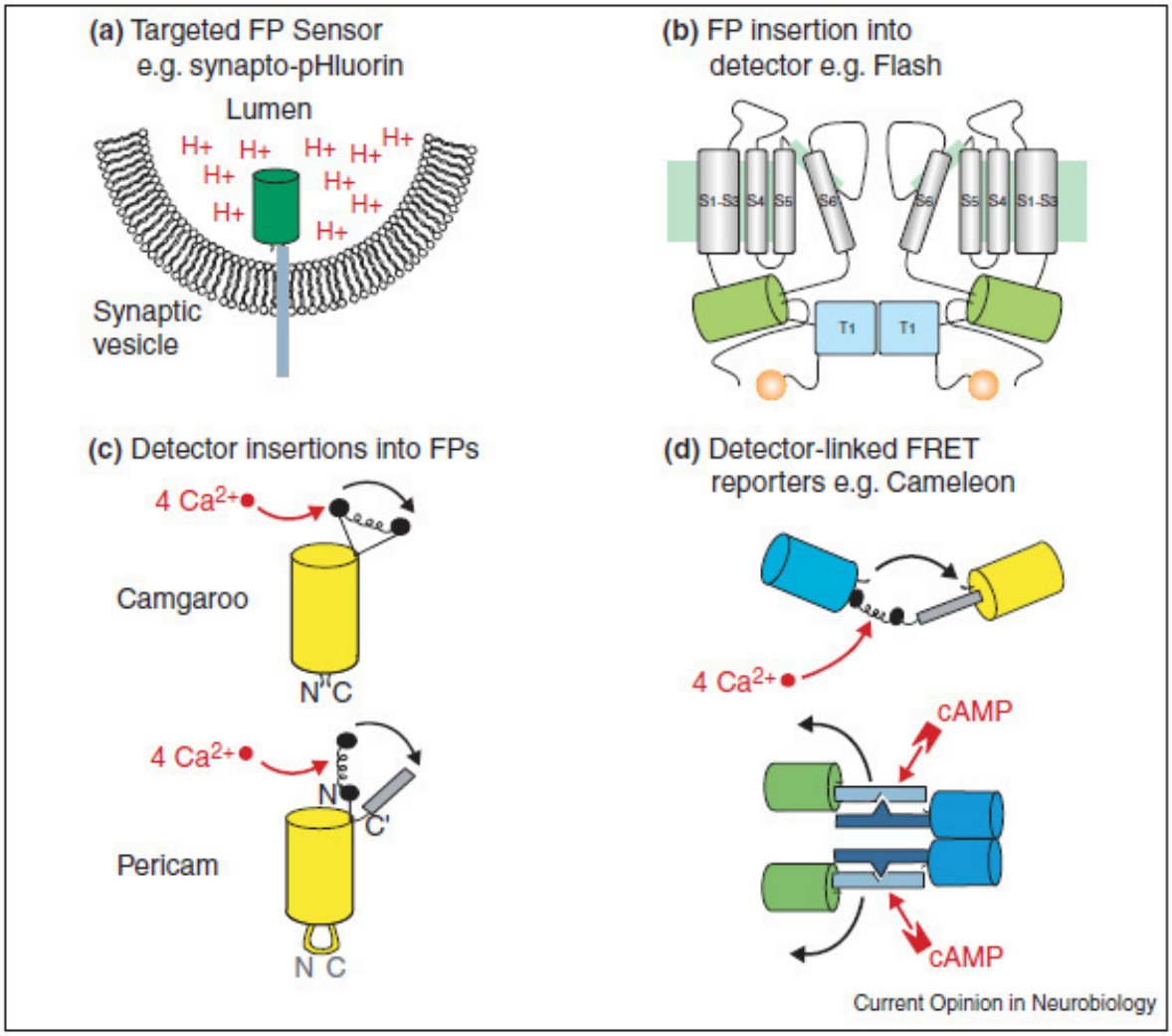
gene expression, but lack the variability by which different transgenes can be expressed in the same neuronal populations.

Scientifically it has always been challenging to prove a proposed neuronal mechanism based on genetic analyses, but with the introduction of optophysiological tools it has become easier to visualize on neuronal level cellular mechanisms and get insights into how signal transmission would work on a molecular level. The tools available are briefly discussed in the next section.

1.3 Optophysiological and optogenetic tools

1.3.1 Tools to visualize and monitor neuronal activity

The advent of a large array of optophysiological tools has made it possible to visualize and manipulate events occurring at a cellular level. The mechanism of information transfer in most of the neurons is mediated by ions and neurotransmitters. Thus, many scientists have exploited this feature to create sensors which have responsiveness or affinity to these ions and neurotransmitters. The most common sensors for correlates of neuronal activity rely on calcium which acts as a secondary messenger and a carrier of charge facilitating many neuronal functions like gene transcription, generation of synaptic potentials and vesicle release (Clapham, 2007). Importantly, intracellular calcium levels rise in correlation with membrane depolarization (Llinas et al., 1972). Therefore, calcium reporters like cameleon (Fiala and Spall, 2003) have found wide applications.



(Guerrero and Isacoff, 2001)

Fig. 1 *Different modifications of fluorescence proteins (FP) for their application as reporters of neuronal activity. FP's can be made sensitive to external ionic environment and targeted to a sub-cellular compartment like synapto-pHluorin (a). FP's could function to detect voltage gated membrane potential changes when integrated with channels like Flash (b). Insertion of detector molecules in FP's could be used to detect ionic changes like in pericam (c). FRET between detector moieties on binding of ions can be used to quantify neuronal activation like in cameleon which detects increase in intracellular calcium (d).*

Typically, fluorescence proteins (FP) have been modified to act as reporters of intracellular activity (Guerrero and Isacoff, 2001). Modifications include mutations in fluorescent proteins which make FP sensitive to its ionic environment. These FP's can be targeted to a specific subcellular component to detect ionic changes as with e.g synapto-

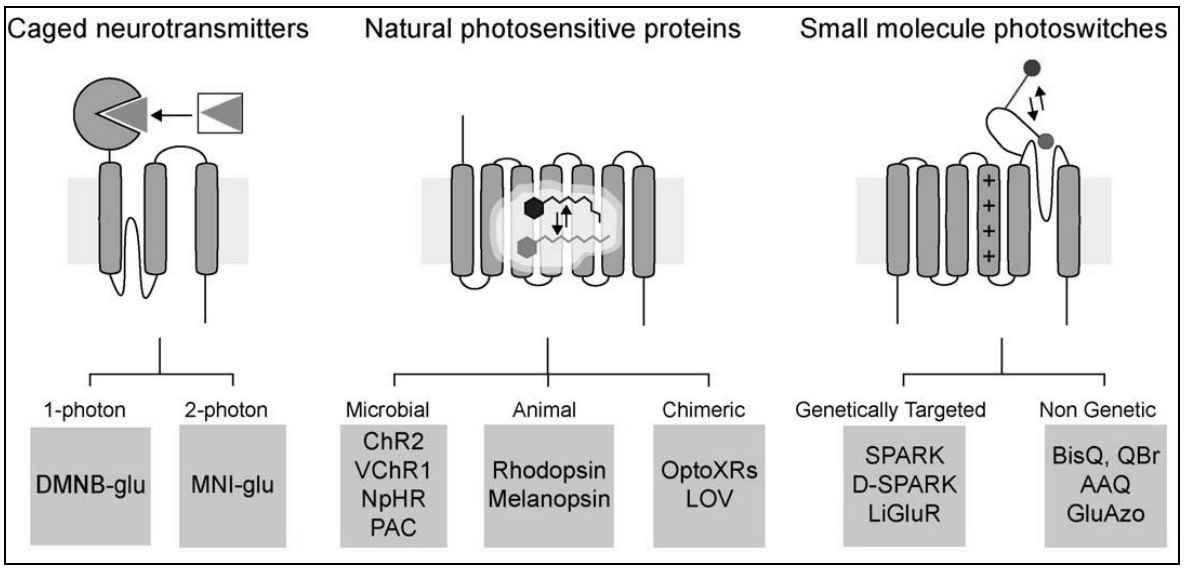
pHluorin (Miesenbock et al., 1998), a sensor for synaptic transmission that is based on changes in pH value within the vesicle that occurs upon vesicle release (Fig.1a). To detect changes in membrane potential FPs have been coupled to channels which are involved in mediating voltage gated change in membrane currents such that any conformational change in the channels would lead to a change in intensity of the fluorescence due to a rearrangement of coupled fluorescing proteins. An example is the FlaSh-insertion of GFP into shaker channel subunits (Siegel and Isacoff, 1997) (Fig. 1b). In a third type of sensors, detectors have been incorporated into the FP's to modify the sensitivity of FP's such that binding of ions would lead to higher fluorescence (Fig. 1c), e.g. in response to calcium (Nagai et al., 2001). Alternatively, such sensors can involve an activation by an ion of a detector which is linked to two different fluorophores at its ends (acceptor and donor moieties, having different activation wavelengths); when these two moieties come in close proximity due to a conformational change in the detector, an increase in Förster Resonance Energy Transfer (FRET) can be observed and hence the fluorescence of the donor moiety decreases whereas fluorescence of the acceptor moiety increases. The calcium sensor cameleon (Fiala and Spall, 2003) is an example for such a sensor (Fig. 1d).

With newly developed microscopy techniques, e.g. two-photon microscopy (Denk and Svoboda, 1997) or STED microscopy (Hell and Wichmann, 1994) scientists are making use of these sensors to look deeper into neuronal tissues with ever increasing spatial resolution and quicker data acquisition and compilation (Dyba et al., 2003). Obviously, the field of neurobiology research has greatly benefited by optophysiological tools, but the recent inclusion of optogenetic and genetic manipulation tools has given a new dimension to this field.

1.3.2 Tools to manipulate neuronal activity

The last decade has seen a dramatic rise in the tools developed to manipulate neuronal activity. Based on the properties of these manipulation tools they can be divided into 3 categories (Fig. 2) : Caged neurotransmitters, natural photosensitive proteins, and small molecule photoswitches (Kramer et al., 2009).

Another categorization of these optical manipulation tools has been based on their molecular properties (Gorostiza and Isacoff, 2007). The two categories are non-reversible phototriggers where the light is involved in breaking a chemical bond which is non-reversible (caged ligands) and photoswitches which are capable of reversibly photo-isomerizing (natural photosensitive proteins- like channelrhodopsin-2 and haloprhodopsin).



(Kramer et al., 2009)

Fig. 2 *Figure illustrates different categories of optical neuronal manipulation tools available. Caged neurotransmitters comprise agonists of neurotransmitters in a photolabile protection which can be broken down by exposure to light of a given wavelength leading to release of the biogenic compound (a). Natural photosensitive proteins which can photo-isomerize in reversible fashion have been isolated from microbes and animals (b). Small molecule photoswitches are optical response elements which can be genetically or non-genetically targeted (c).*

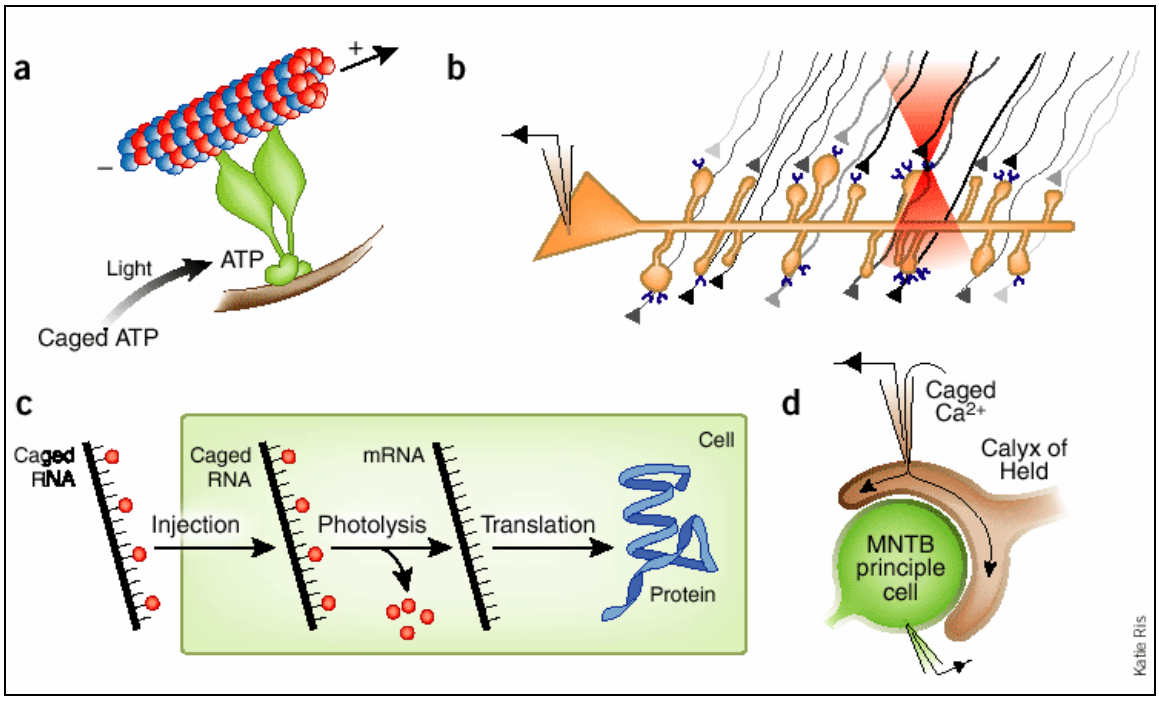
Each tool is described individually in the sections below.

1.3.2.1 Caged biogenic compounds

The concept of caged neurotransmitters involves the packaging of ligand molecules in a photolabile protection group. When exposed to light of a specific wavelength the photolabile protection group is removed leading to the liberation of the

bioactive ligand molecules. The caged bioactive compounds can range from caged nucleotides and small peptides which can have an influence on signal transduction pathways (Fig. 3a), neurotransmitter receptors (Fig. 3b), nucleic acids (Fig. 3c) or calcium (Fig. 3d) (Ellis-Davies, 2007).

The method of uncaging a compound has several advantages when compared to photosensitive proteins, e.g. a high speed of release. Moreover, location and timing of release can be regulated and intracellular regions normally inaccessible by regular techniques can be controlled. An important feature of un-caging compounds is that a quantitative relationship can be established. Most noteworthy was the global uncaging of Ca^{2+} which allowed measurement of precise correlations between presynaptic (Ca^{2+}) and postsynaptic currents by recording at a central synapse for the first time (Bollmann et al., 2000; Schneggenburger and Neher, 2000).



(Ellis-Davies, 2007)

Fig. 3 *Applications of un-caging bioactive compounds. Nucleotides and macromolecular compounds could be un-caged to study cellular functions (a). Neurotransmitters like glutamate could be specifically released to study sensity of AMPA receptors (b). Nucleic acids e.g. RNA could be specifically released to study site specific RNA translation (c).Global unchanging of Ca^{2+} can be used for measurement of precise correlations between presynaptic and postsynaptic currents at central synapses(d).*

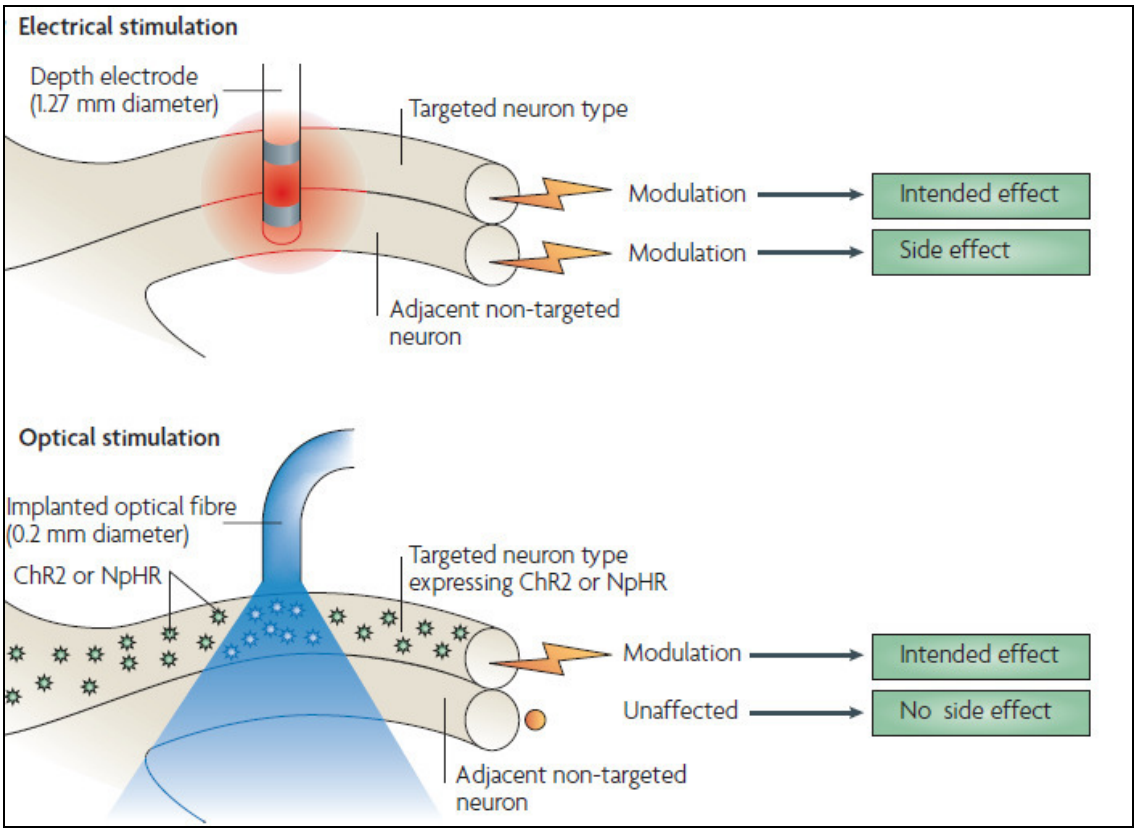
In *Drosophila* the un-caging phenomenon was demonstrated remarkably by photo triggering the release of caged ATP by specifically photostimulating the neurons of the giant fiber system which expressed the ionotropic purinoceptor $P2X_2$, a receptor having a high affinity to ATP) using the Gal4-UAS system (Lima and Miesenbock, 2005).

Although un-caging physiologically active substances has several advantages last decade has seen a rise in the application of naturally occurring photosensitive proteins from microbes as neuronal manipulation tools which have their own, distinct advantages. The next section describes the tools available.

1.3.2.2 Natural photosensitive proteins

The naturally occurring photosensitive proteins have originally been isolated from microbes (Nagel et al., 2003). Some of them belong to the category of membrane ion transport proteins acting as light driven proton pumps, e.g. bacteriorhodopsin, or light gated cation channels, e.g. channelrhodopsin-2 from *Chlamydomonas reinhardtii*, or light driven chloride pumps, e.g. haloprhodopsin from *Natronomonas pharaonis,* or the light-activated adenylyl cyclase (PAC) from *Euglena gracilis*. These photosensitive proteins in natural environment enable microbes to perform phototaxis behavior, but scientists over the last decade have realized their potential as tools to interrogate awaiting questions in the field of neuroscience and several studies have been reported.

The selection of the appropriate tools from the existing repertoire of techniques depends on the type of neurobiological question that is intended to be answered. One of the prime advantages of using optogenetic tools is that neuron specific activation could be achieved without having the side effects from neighboring neurons as would be expected in the case of electrical stimulation. Another advantage of optogenetic tools is that a single type of neuronal population can be activated irrespective of their anatomical position, whereas with extracellular electrical stimulation only activation of specific anatomical brain regions is possible.

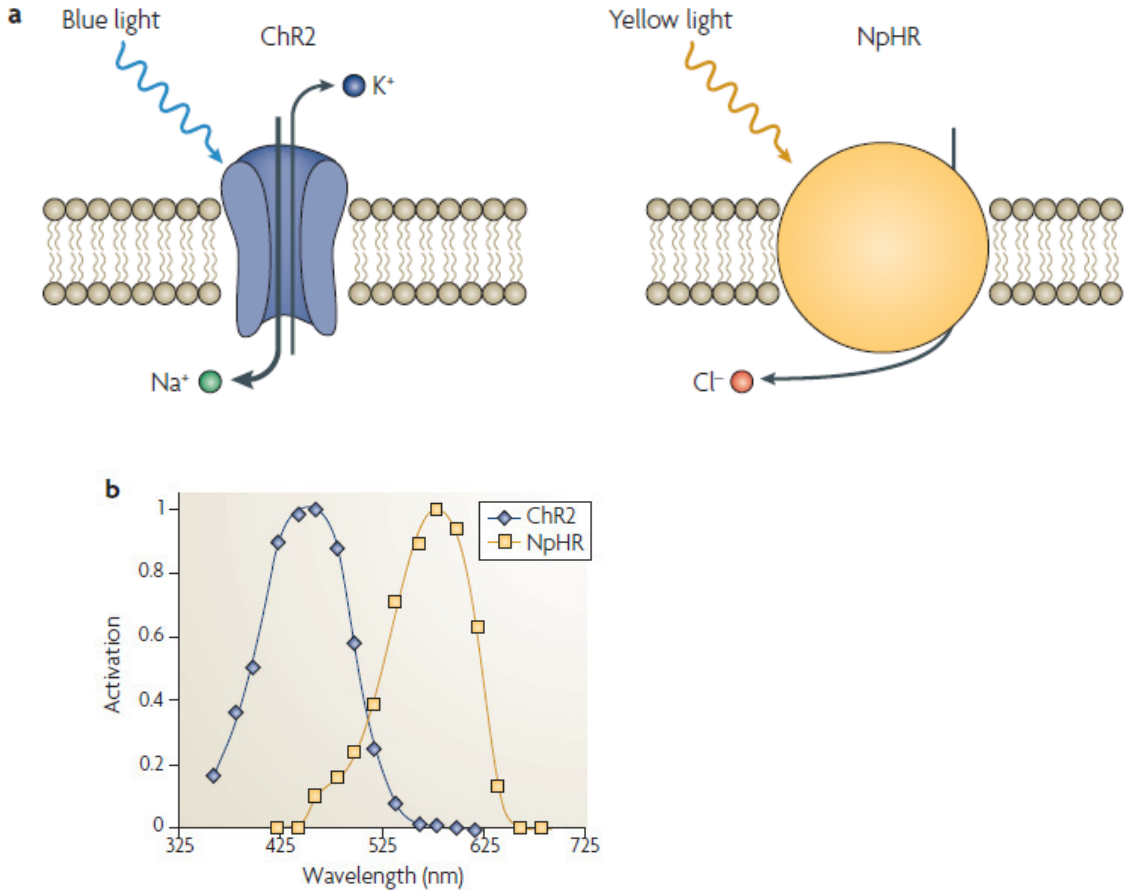


(Zhang et al., 2007a)

Fig. 4 *Advantages of genetic expression of light activated proteins for neuronal manipulation in comparison to electrical stimulation. Electrical stimulation of neurons leads to non specific activation of neighboring non desired neuronal cell types, thereby accounting for the imprecise output. Optical stimulation leads to specific activation of neurons expressing light activated proteins hence the output is precisely from the desired neuronal population.*

Channelrhodopsin-2:

Channelrhodopsin-2 (ChR-2) is a 7 transmembrane light sensitive cation channel which was discovered in 2003 (Nagel et al., 2003). For the channels to function the binding of a chromophore- All Trans Retinal (ATR) is required. In the inactive state of the channel it is bound to ATR and on excitation with blue light there is a conformation change from ATR to 11-cis-retinal. As a consequence the ion channel opens, leading to an influx of cations.



(Zhang et al., 2007a)

Fig. 5 *Physiological and spectral properties of channelrhodopsin-2 and halorhodopsin (natural phosensitive proteins). Blue light with wavelength of 480nm is required for activation of cation channel channelrhodopsin-2. Halorhodopsin (NpHR) a light activated inhibitory chloride channel requires yellow light of 580 nm to open (a). Both channelehodopsin-2 and halorhodopsin could be co-expressed in the same animal and stimulated with temporal coherence as they have different spectral properties (b).*

Certain features of ChR-2 make it an excellent tool for the optical stimulation of neurons. It can be activated within 30 µs in response to light of ~ 480 nm in wavelength and it quickly closes on the offset of light. Short flashes of light can generate neuronal spikes with maximal frequency determined by the natural limit of the neuron. Therefore, it can be used to study synaptic events that are temporally dependent like plasticity changes which require precise spike timing and other temporally coded features in the nervous system. For its effective usage the greatest challenge is to direct the expression of this protein to the membrane of neurons in high quantities as the single channel conductance of ChR2 is 40 fS (Feldbauer et al., 2009) which is very small compared to

that of endogenous voltage gated channels which range from 5-240 pS and control neuronal firing. This problem can be overcome by using a strong promoter to drive the expression of channelrhodopsin-2 (Boyden et al., 2005), and in invertebrates it is necessary to provide the chromophore ATR in sufficient quantities *in vivo* for the functional expression of channelrhodopsin-2 apoprotein to light sensitive holoprotein (Schroll et al., 2006).

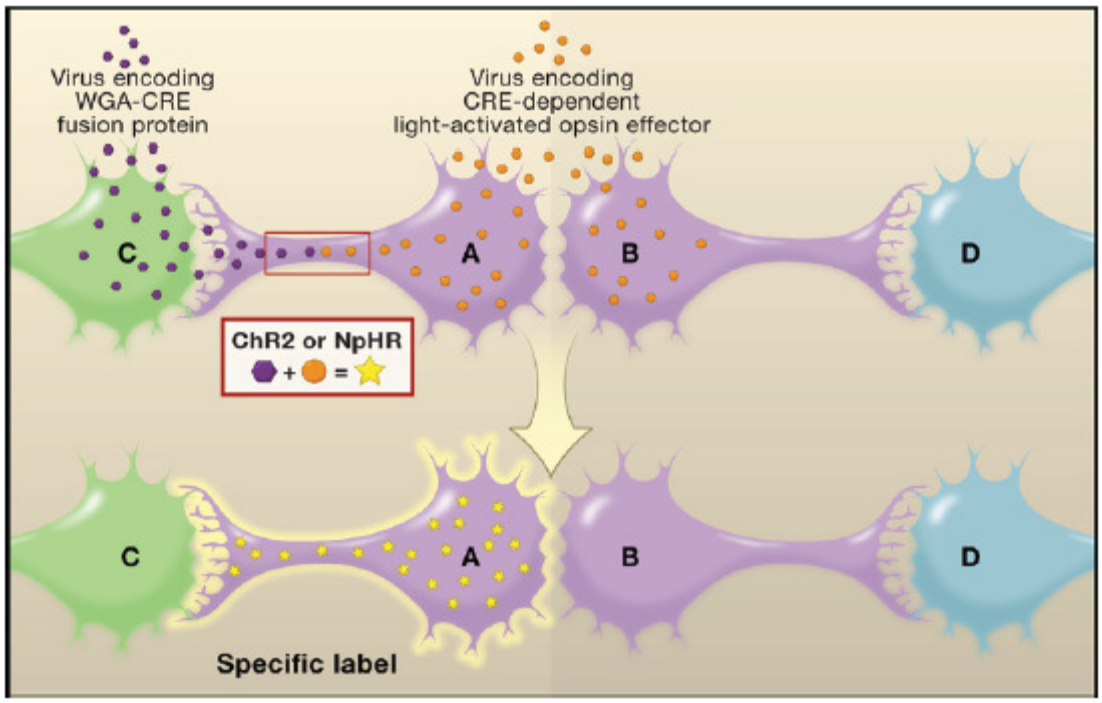
Channelrhodopsin-2 has been modified by many mutations (Lin et al., 2009) to improve its spectral properties, trafficking properties of the protein, and kinetics. Wild type ChR-2 responds quickly to light but prolonged activation leads to partial inactivation of the channel. Thus mutations making ChR-2 into a bi-stable switch have been introduced (Berndt et al., 2009).

In vertebrates where the genetics is complicated it is difficult to drive the expression of an effector in a given subtype of neuronal population. However, deeper insights into functional consequences of activation of a specific type of cell can be achieved in invertebrates using the Gal4-UAS system. To demonstrate this feature, one example is a study published by (Schroll et al., 2006). Here, ChR-2 was selectively expressed in dopaminergic and octopaminergic neurons which were then activated by blue light. The activation of aminergic neurons substituted for natural Unconditioned Stimuli (US), and the pairing of neuronal activation with the neutral stimulus (Conditioned Stimulus, CS) imparted a value to the neutral stimulus. This led to formation of an association between the CS and the US and a memory for the reinforced CS.

Halorhodopsin

Halorhodopsin (NpHR) is a light driven chloride pump derived from halobacterium. It is activated by yellow light of 580 nm wavelength (Fig. 5). It can be driven in cells to prevent action potentials. Since its activation spectrum differs from that of ChR-2 it can be used together with ChR-2 to investigate neuronal functions. This tool has not been widely applied because when the unmodified version of NpHR was expressed at high levels it led to cellular toxicity and produced intracellular swelling of dendrites and bleb formation (Zhao et al., 2008).

In a recently published study Gardinaru et al from the Deisseroth group (Gradinaru et al., 2010; Liu and Tonegawa, 2010) have made a new version of NpHR where they have added a C-terminal trafficking signal of the potassium ion channel Kir 2.1. This improved version, called eNpHR 3.0 (or Arch), can be activated over a broad wavelength spectrum (infrared) as it shows high inhibitory capability (at weak illumination of 3.5mW/mm^2, with current exceeding 1nA at the channel) and is specifically localized at the plasma membrane. They also have used the principle of trans-synaptic trafficking to express the effector in a subpopulation of anatomically and genetically homogeneous population of neurons. They have employed two viruses, one which codes for a CRE recombinase coupled to the transcellular protein Wheat Germ Agglutinin (WGA) and a second virus coding for CRE dependent opsin. These two viruses were delivered to distant but anatomically connected brain regions. As shown in Fig. 6 neurons A and B are neighbors of the same type with their projections ending in different neurons C and D (Fig. 6). A virus coding for a light activated opsin effector which was CRE mediated was taken up by neurons A and B because of their close proximity in space. Another virus coding for WGA-CRE fusion protein was taken up by the soma of neuron C and the resulting coded protein trans-synaptically trafficked in to neuron A. For the activation of the light sensitive opsin the presence of WGA-CRE was essential. Thus, by this method it was possible to excite only neuron A and not B although they belong to the same subpopulation of neurons. This new approach permits the manipulation of a single neuron in a cell population of neurons *in vivo* with high precision.



(from: Gradinaru, Zhang et al. 2010; Liu and Tonegawa 2010)

Fig. 6 *Specialized cell specific activation of a single neuron in a population of neurons of the same type. For the targeted expression of a light activated protein (ChR-2 or NpHR3.0) to a sub population of neurons two viral constructs were employed where one coded for a WGA-CRE fusion protein and another construct coded for a CRE mediated light activated effector. Only in the presence of WGA-CRE fusion protein the expression of the opsin protein was activated. Thus the two neurons with the different viral constructs should be present in close proximity in order to express a functional light activated protein.*

There have been screens (Chow et al., 2010) from a large number of microbes to discover light driven proton pumps which could be targeted genetically with high efficiency to permit neuronal silencing. So far NpHR 3.0 seems to be the best silencing tool.

Photoactivated adenylyl cyclase (PAC):

Photoactivated adenylyl cyclases are blue light sensitive proteins normally found in algae like *Euglena gracilis.* The protein comprises the flavoproteins PACα and PACβ. Each subunit has two photoreceptor domains that bind to flavin adenine nucleotide and two catalytic domains that have adenylyl cyclase activity sensitive to blue light. This has allowed the optical control of intracellular levels of cAMP (Schroder-Lang et al., 2007).

1.3.2.3 Small molecule photoswitches

Currently many scientists are employing synthetic chemistry approaches to design photosensitive tools by combining naturally occurring ion channels or receptors to obtain a photoisomerizable molecule or a "photoswitch". One class called the *optical nanotweezers* involves binding of a photoswitch to an ordinary ion channel such that the isomerization of the photoswitch exerts a force on the channel which produces a conformation change in the ion channel, thereby opening it. The other group is called *optical nanotoggles* where attaching or removing a ligand from the binding site of the channel or receptor regulates its activity (Gorostiza and Isacoff, 2007). In the third group called *optical nanokeys* the ligand binding site is placed very close to the photoisomerizable moiety such that one of the conformations of the isomers permits binding of the ligand and the other conformation prevents ligand binding. Thus using this principle 2 isomers could be selected which have different absorption wavelengths and hence could be used to drive the protein function on and off in the same cell.

Optical tools have high temporal activation advantages, but recently also the potential of other tools is being explored to selectively manipulate neuronal populations. These are discussed in the next section.

1.4 Miscellaneous neuronal manipulation tools

1.4.1 Temperature induced neuronal manipulation

The latest transgenes that have joined the neuronal manipulation tools cluster are temperature sensitive Transient Receptor Potential (TRP) channels. These are naturally expressed in animals and are reported to function in response to temperature changes, e.g. in *Drosophila* (Hamada et al., 2008), mechanical stimuli, e.g. in *C. elegans* (Kindt et al., 2007) and chemical stimuli, e.g. in mice (Macpherson et al., 2007). Some TRP channels like TRPA1 are involved in heat mediated thermal perception, whereas TRPM8 is involved in mediating low temperature sensitivity in mammals.

TRPA1

Like other TRP channels TRPA1 comprises six transmembrane spanning segments (S1-S6) with the N and C terminal located intracellularly. The channel subunits can assemble both as homo- or hetrotetramers thereby forming cation selective ion channels (Clapham, 2003; Nilius, 2007). Calcium is believed to play a central role in functioning of these channels as it acts as a permeant ion and controls the activation (Zurborg et al., 2007) and desensitization (Wang et al., 2008) of the channel. Using the Gal4-UAS system it can be transgenically expressed in desired neuronal subtypes and activated by modulating temperature. The dTRPA1 receptor in *Drosophila* has been reported to be present in a small number of neurons in the brain which mediate temperature preference in *Drosophila* (Hamada et al., 2008).

TRPM8

The TRPM8 protein is activated by cold temperatures and cooling agents, such as menthol. TRPM8 is an ion channel that on activation permits the flow of Na^{+} and Ca^{2+} ions and mediates depolarization of cells to generate an action potential. In flies the expression of this channel in selective neurons has been achieved by the Gal4-UAS system, and exposing the animals to low temperatures (about 18°C) leads to an activation of the neurons expressing this channel (McKemy et al., 2002).

1.4.2 Sodium Activated Bacterial Channel (NaChBac)

Sodium activated bacterial channels (NaChBac) are low threshold voltage gated sodium channels cloned from a halophilic bacterium (Ren et al., 2001). Although it has been cloned from a prokaryote its pore structure resembles eukaryotic channel structure. When transgenically expressed in *Drosophila* neurons its low threshold leads to the spontaneous activation at normal resting membrane potentials, thereby leading to a hyperexcitation of the neurons in a constitutive manner.

The large array of tools available in *Drosophila* makes it an appropriate animal to address fascinating questions in neurobiology. One of the vital fundamental questions that need to be addressed is: what is the basis of neuronal plasticity, and where does the brain undergo structural (Heisenberg et al., 1995) and functional changes? It has been believed for a long time that experiences of the animal lead to plastic changes in the brain (Greenough and Anderson, 1991) which are characterized by the formation of new synapses, increased dendritic length and spine density (Greenough et al., 1985; Horn et al., 1985). These changes on the anatomical level lead to alterations in the behavior of the animal. By combining various genetic tools available in *Drosophila* the inputs and outputs to and from various neuropiles could be manipulated and resulting changes in behavior could be visualized. Consequently manipulation and visualization of neurons along with behavioral measurements would help in unraveling the neuronal mechanisms underlying learning and neuronal plasticity.

In the following section I would like to describe the current state of knowledge of the different types of behaviors studied and the behavioral paradigms that have been used to quantify these behaviors.

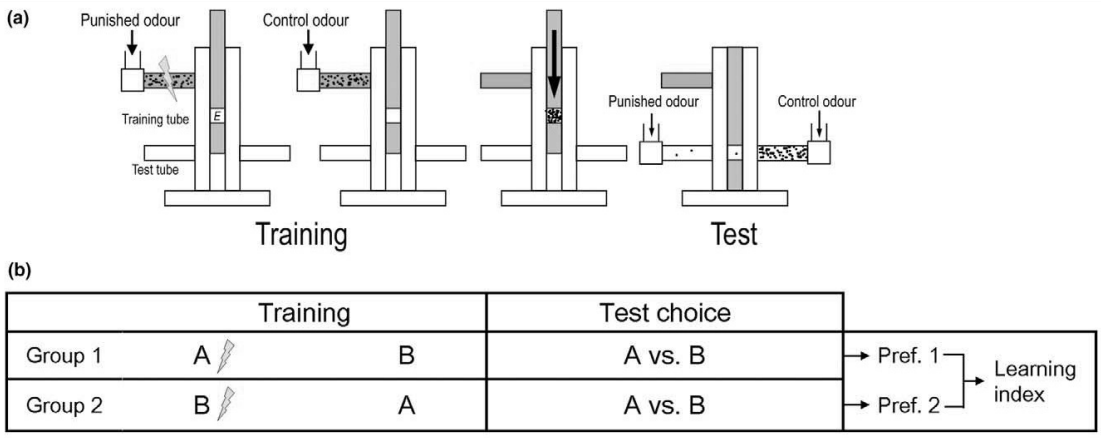
1.5 Experience dependent modification of behavior

Animals need to utilise their discretion power in daily life activities to choose wisely what would prove beneficial to them. Changes in behavior as a result of experience, i.e. learning, could be demarcated into many different forms of which classical conditioning (Pavlov, 1951) and operant conditioning (Thorndike, 1933; Skinner, 1984) represent two well studied and fundamental principles.

1.5.1 Classical conditioning

An animal's environment plays an important role in determining its behavior. In some situations it becomes very important for the animal to evaluate its external environment and to associate neutral cues with coincidently occurring reward (positive significance) like food so that they could later reach or anticipate the reward on encountering the neutral cue. This pairing leads to the acquisition of a memory for the neutral cue (Conditioned Stimulus, CS) and the animal elicits a similar response to CS as how it would respond to the reward (Unconditioned Stimulus, US) even in the absence of US. Thus, this gain in function where a stimulus was previously of no significance to the animal is now of a positive significance has been termed as *classical conditioning or Pavlovian conditioning.* This phenomenon also holds true for a punishment (negative significance) where the animals needs to be aware of negative reinforcers like enemies or predators and associate the related neutral cues to be safe. In some cases the spatial and temporal pairing of the CS and US also plays an important role in assigning value to the CS (Yarali et al., 2009).

A large number of paradigms have been designed to quantify classical associative learning in flies. One of the best established paradigms is the olfactory aversive learning paradigm according to Tully and Quinn (1985). This paradigm involves training of flies with an electric shock. About 100 flies are introduced into the training tube and an odour (CS^+) is paired with 12 electric shocks (ES) each lasting for a second with an inter stimulus interval (ISI) of 5 s. Then a second odour (CS^-) is introduced for 1 min in the absence of ES. Odour memory is tested by introducing the flies to a T maze and allowing them to choose between the two odours each present on one arm of the T maze. Experiments are performed by reciprocal training where with a new group of flies the previously unpunished odour is punished (Gerber et al., 2004). The animals in this paradigm learn to avoid the odour that was paired with the electric shock.



(Gerber et al., 2004)

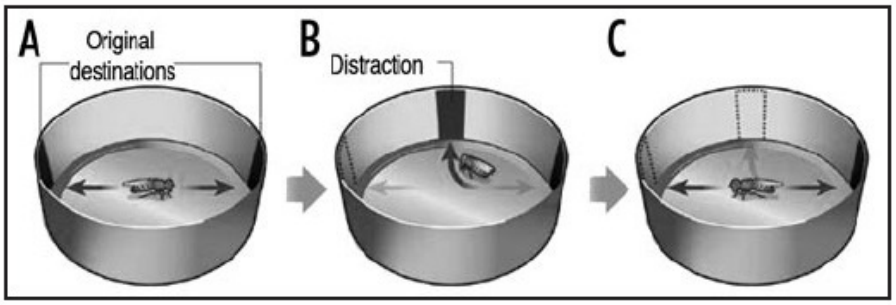
Fig. 7 *Aversive olfactory learning in the Tully and Quinn paradigm. First one odour is paired with an electric shock (12 shocks, 1 s each) in the training tube for 1 min and then a second odour not paired with the electric shock is given for 1 min. Aversive memory is tested in a T maze where the animals have the choice between the two odours (a). Based on the number of flies in each arm the learning index is calculated.*

Aversive olfactory learning experiments have also been done in *Drosophila* larvae where the electric shock in adult aversive learning is replaced by a gustatory aversive material like salt which acts an aversive US. The experiments are performed on petriplates where the tastant is combined into agar and the odor is delivered via small odour cups. The aversive gustatory cue could be replaced by sugar to perform appetitive olfactory learning experiments (Michels et al., 2005). The olfactory associative learning experiments can be considered as stimulus-stimulus learning where one stimulus assigns value to another and thus the response of the animal can be considered as a reflex to a stimulus rather than a self-motivated or internally controlled behavior.

1.5.2 Spatial orientation memory

Spatial orientation learning has been investigated using the Buridan's paradigm in *Drosophila.* In this paradigm a fly with clipped wings is introduced in the middle of a circular arena where it is separated from the walls of the arena by a water moat. When two stripes are introduced on opposite walls of the arena the flies walk between the two landmarks and continue to walk in same manner for 2-8 s even after the landmarks disappear (Strauss and Pichler, 1998). A new variant test for the memory of the landmark

position: When a fly in the same setup walks past the midline the two opposite landmarks disappear and a new landmark appears in a perpendicular direction (distractor). However, as soon as the flies turn towards the newly introduced landmark the new landmark also disappears. On the disappearance of the new landmark most of the animals reorient themselves towards the old landmark and continue walking it that direction although the old landmark is not visible anymore. This indicates that animals form spatial orientation memory for the originally targeted landmark (Neuser et al., 2008).



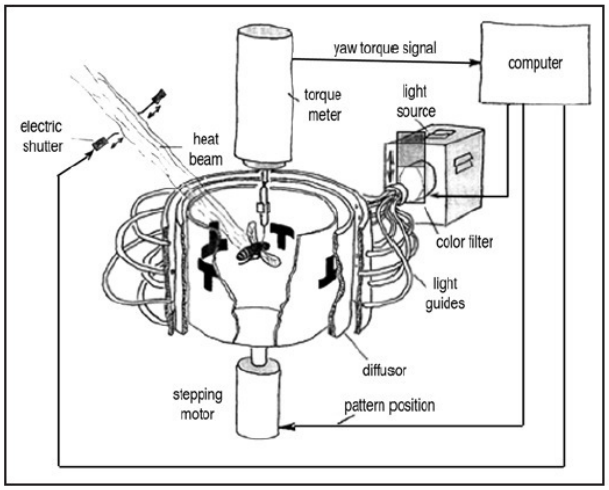
(Tomchik and Davis, 2008)

Fig. 8 *Spatial orientation memory assessment in Buridan's paradigm. Flies are placed in the centre of the arena and two landmarks appear. Once the fly crosses the midline while approaching the landmark both landmarks disappear and another distracting landmark appears. Once the fly starts approaching this distracter it also disappears. The fly again turns and now continues towards its original target showing orientation memory towards it.*

1.5.3 Operant Conditioning

Operant conditioning can be described as a form of learning where the animal learns the consequences of its own behavior. This form of learning differs from classical conditioning by the fact that in classical conditioning the animal has no control over the occurrence of an event or cannot control its immediate environment, whereas in operant conditioning the animals can control its immediate environment by modulating its own behavior.

There have been several paradigms designed to quantify operant behavior. Visual associative learning has been well investigated in *Drosophila* using a flight simulator in the closed loop protocol. In this case the fly controls the position of panorama of the arena depending on its intended direction of flight. The flies are hooked to a torque meter. The yaw torque generated by the fly is measured and accelerates the rotation of the arena proportionally. Thus the fly can choose the position of the panorama just as free flight. One classical example is the presentation of an upright and an inverted T symbol (Fig. 9). Whenever the fly tries to approach the upright T it is punished by heat generated by an infrared Laser. Flight towards the inverted T generates no punishment. The fly visually orients itself to the inverted T to be safe and avoids the punishment in this paradigm. In the test phase where the laser is switched off the animals continue to prefer the inverted T even in the absence of any punishment, thus displaying a visual associative memory in this operant conditioning paradigm (Dill et al., 1995; Brembs and Heisenberg, 2000).



(Brembs and Heisenberg, 2000)

Fig. 9 *Flight simulator operation under closed loop condition. One of the visual cues (CS^+) is paired with punishment (US) and the other with no punishment (CS^-) in the training phase. In the test phase the animals show a memory and prefer to fly towards the visual cue which was unpunished.*

.Another paradigm called the heat box has been used to investigate operant behavior. In this paradigm in the training phase (4 min) the fly can walk back and forth in a box whenever the fly is in one half of the box in the entire box is heated to a dangerous temperature, but the heat is turned off when the fly is in the other half of the box. In the

test phase the memory is tested by monitoring the place preference of the fly in the box (Fig. 10) and quantifying the time spent in the unpunished half of the box (Putz and Heisenberg, 2002).

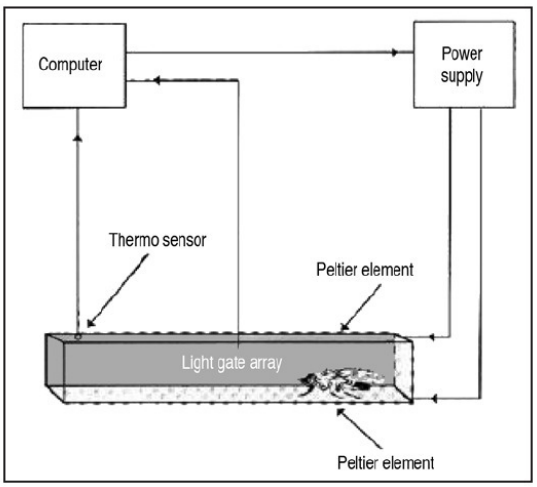
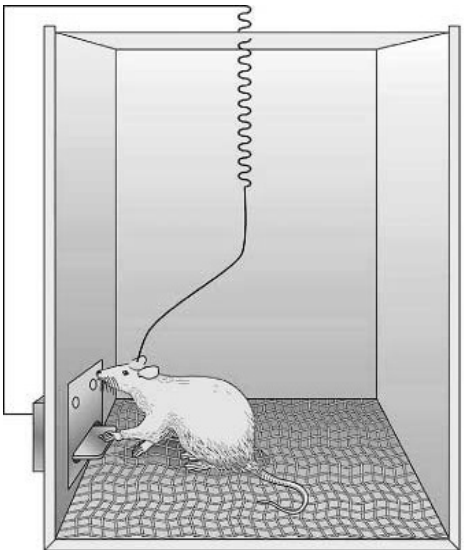


Fig. 10 *Heat box paradigm for operant conditioning. The position of the fly is monitored. During training the presence of the fly in one half of the box leads to an increase in temperature. The fly avoids staying in the punished side of the box and shows a preference for the unpunished side even in the test phase in the absence of punishment.*

Many similar experiments have been done in vertebrates to investigate operant behavior. A key question has been – and still is – which neurons are responsible for mediating the rewarding or aversive value as a consequence of a certain action. In order to unravel this, an experimental design has been established which allows the animal to control the stimulation of a certain brain region. This has been achieved by the coupling of an electrical stimulation of different brain regions and a quantifying whether the stimulation of that region is liked, i.e. repeated, or disliked, i.e, avoided by the animal (Olds and Milner, 1954). In these experiments electrical stimulation acts as an operant reinforcer of a stimulus-releasing action, e.g. pressing a lever. These experiments have permitted an investigation of functions of some anatomical brain regions. Application of some pharmacological substrates at different brain regions also produced a response driving behavior in vertebrates (Stephens and Herberg, 1975). A response contingent stimulation of different brain regions can be used to assign a functional role to different neuronal populations in the brain. Most popular of those experiments were the brain stimulation reward studies.

1.6 Brain Stimulation Reward

In 1954 Olds and Milner accidentally discovered that when they stimulated the septal region of the rat brain in one particular location of the test apparatus the animals preferred that location in the apparatus (Olds and Milner, 1954). This phenomenon has been referred to as brain stimulation reward. In follow-on experiments Olds and Milner placed a lever in a cage and connected a lever to a brain simulator, such that each lever pressing would lead to electrical stimulation. They found that when they stimulated the lateral hypothalamus in such a setting then the animals learned to press the lever frequently, reaching 7000 times in an hour. The animals continued this for hours ignoring thirst and hunger. This demonstrates that activation of brain regions in a response-contingent manner could directly act as an operant reinforcer leading to repetition or avoidance of that action.



(Source: World Wide Web)

Fig. 11 *Rat contingently trained to activate the stimulation of the lateral hypothalamus by pressing a lever.*

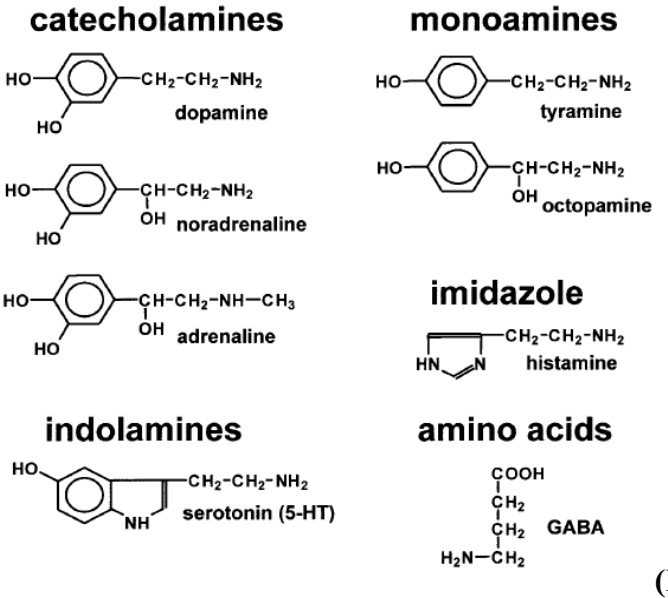
Intracranial deep brain stimulation has also been used to alleviate symptoms of neurodegenerative disorders involving biogenic amines (Bishop et al., 1963; Siegfried and Lippitz, 1994; Pahwa et al., 1997). Some studies in vertebrates have shown that neuronal self stimulation responses can also be modulated by the application of catecholamine agonists and antagonists (Herberg and Stephens, 1975).

Although many studies have been done in vertebrates, little is known about the precise effector roles of activation of neurons in invertebrates. Specifically, it would be interesting to activate aminergic neurons as they are considered to play various roles in modulating behavior of invertebrates.

1.7 Biogenic Amines

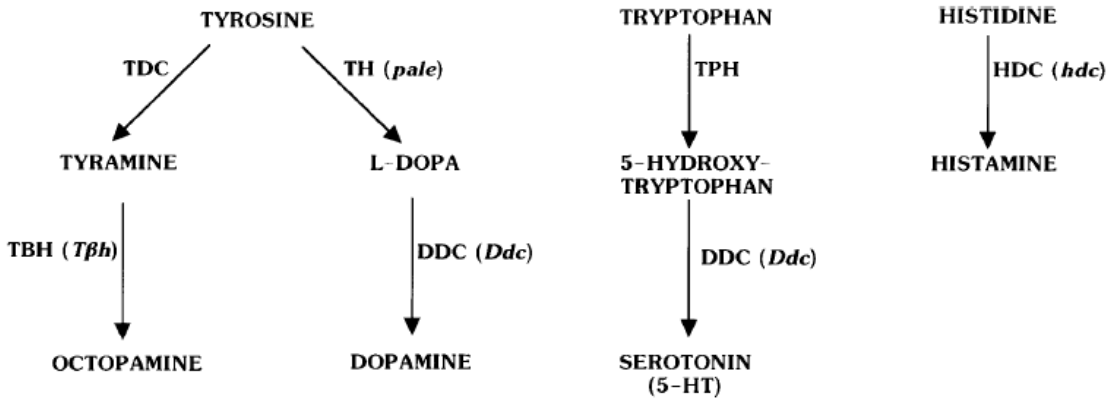
Biogenic amines are required for diverse behaviors in insects ranging from simple locomotion to responses requiring higher order processing in the central nervous system like learning and memory (Schwaerzel et al., 2003). Biogenic amines are bioactive compounds that are formed by a decarboxylation of amino acids. Eight members form the amine group, but only 6 are known to have physiological significance in insects. Noradrenaline and adrenaline are found only in vertebrates, whereas tyramine and octopamine are found only in insects.

The figures below indicate the differentiation of amines into different classes based on their chemical properties (Fig. 12a) and the biosynthesis pathways and the rate controlling enzymes involved in their synthesis (Fig. 12b).



(Roeder, 2002)

Fig. 12 a *Different classes of biogenic amines.*



(Monastirioti, 1999)

Fig. 12b *Synthesis pathways of amines. Tyrosine is converted to tyramine by the tyrosine decarboxylase (Tdc) enzyme, tyramine is further converted to octopamine by the action of tyramine ß hydroxylase (Tβh). Dopamine is also produced from tyrosine in 2 steps. In a first step tyrosine is converted to L-DOPA by tyrosine hydroxylase (Th) and from L-DOPA to dopamine by dopa-decarboxylase (Ddc). Serotonin is synthesized from tryptophan in two steps. In the first step tryptophan hydroxylase (Tph) converts tryptophan to 5-hydroxy tryptophan which is acted upon by dopa-decarboxylase (Ddc) to synthesize serotonin. The neurotransmitter histamine is produced from histidine by the enzyme histidine decarboxylase (hdc)*

Role of biogenic amines have been investigated for a long period of time both in vertebrates and invertebrates. The following sections below I will discuss briefly the known or hypothesized functional roles played by each of these amines in biological systems.

1.7.1 Dopamine

Dopamine plays an important role in maintaining neurological homeostasis both in the central and peripheral nervous system. A loss of dopaminergic neurons in the substantia nigra produces clinical symptoms, e.g. Parkinson's disease in vertebrates (Mosharov et al., 2009), and it is also involved in clinical pathology of schizophrenia. Dopamine is also involved in regulating reward mediating and motivated behaviors in vertebrates (Robinson et al., 1993; Wise, 2004). In addition, dopamine plays a role in neuroendocrine function, emotion, and locomotion behavior (Civelli et al., 1993).

Although the function of this amine has been elaborately studied in vertebrates, relatively little is known in invertebrates.

Tyrosine hydroxylase (TH) is the rate limiting enzyme in the synthesis of L-DOPA which is converted to dopamine by the enzyme dopa decarboxylase (DDC) (Fig. 12). In *Drosophila* the function of dopamine has been investigated by using Gal4 lines with promoter elements of the enzymes involved in the biosynthesis pathway. In insects dopamine is known to play an important role in negative reinforcement learning. The Heisenberg group reported in 2003 (Schwaerzel et al., 2003) that dopamine is required for aversive olfactory learning. On silencing the dopaminergic neurons labeled by TH Gal4 (labeling about 200 dopaminergic neurons) the animals failed to fully form the association between electric shock (US) and a neutral odor (CS), whereas the appetitive reinforced memory remained unaffected. As a target region for dopamine the mushroom body is believed to be involved in the formation of aversive olfactory memories (Riemensperger et al., 2005).

Experiments were performed along similar lines in larvae by Schroll et al., 2006. The US was replaced by direct activation of dopaminergic neurons. This was achieved by selectively expressing channelrhodopsin-2 under UAS control in dopaminergic neurons using TH-Gal4 and blue light activation of ChR-2. The activation was paired with presentation of an odor (neutral conditioned stimulus, CS). Then a second different odor was presented which was not paired with the activation of dopaminergic neurons. On testing the animals for memory the animals avoided the odor that was optically reinforced (coupled to activation of dopaminergic neurons). This was direct evidence that dopamine played a role in negative reinforcement.

A recent study by the Miesenbock's group (Claridge-Chang et al., 2009) has narrowed down the population from about 200 dopaminergic neurons labeled by TH Gal4 to 12 dopaminergic neurons. They report to have traced the origin of negative reinforcement signals to the protocerebral posterior lateral 1 (PPL1) cluster. The projections from this cluster target to regions in the vertical lobes and heel of the mushroom body.

Another recent study from the Waddell group (Krashes et al., 2009) report that a neuropeptide dNPF receptor in 6 dopaminergic neurons which innervate a certain region of the mushroom body regulates appetitive memory in *Drosophila*. The silencing of these 6 dopaminergic neurons leads to an enhancement of appetitive memory recall in satiated flies whereas the stimulation of these dopaminergic neurons in hungry flies suppresses memory performance in flies. Thus this demonstrates that dNPF along with dopamine form a motivational switch that regulates appetitive memory retrieval in *Drosophila*.

Dopamine is known to function via 5 different types of receptors (D1-D5). These receptors have been classified into two groups of D1-like (D1 and D5) and D2-like (D2, D3, D4) receptors, based on their sequence, pharmacological binding and reacting properties (Gingrich and Caron, 1993). There are reports which indicate a role of dopamine in appetitive learning in *Drosophila* as well. These studies have made use of two mutants $dumb^1$ and $dumb^2$ where dopamine receptor dDA1 expression in the mushroom body is affected. In these mutants both appetitive and aversive memories were negligible. The expression of these receptors in the mushroom bodies of the mutants rescued the learning phenotypes (Kim et al., 2007).

Although the function of dopamine is attributed to punishment learning in *Drosophila* there are no reports if its release would guide avoidance behavior in flies.

1.7.2 Tyramine and Octopamine

Tyramine (TA) is a neurotransmitter which is synthesized from tyrosine by the enzyme tyrosine decarboxylase (TDC) and, as a subsequent enzymatic step, octopamine is synthesized from tyramine by the enzyme tyramine beta hydroxylase (TßH) (Fig. 12). Tyramine and octopamine (OA) are believed to have opposing roles in locomotion (Saraswati et al., 2004). Although tyramine is a precursor of octopamine, some tyramine immunoreactive neurons do not contain octopamine, indicating that tyramine also acts as a neuromodulator (Nagaya et al., 2002). Tyramine receptor mutants (honoka) were discovered in an olfactory screen. These mutants showed abnormalities and in response to odorants. Specific electrophysiological effects of tyramine were abolished in hono mutant but the effects of octopamine were intact indicating that tyramine plays a role as a neurotransmitter or neuromodulator in *Drosophila* (Kutsukake et al., 2000).

Octopamine is known to function as a neuromodulator, neurotransmitter, and neurohormone in insect nervous systems. It has been categorized as a neuromodulator as it functions in desensitization of sensory inputs, arousal, initiation and maintenance of rhythmic behaviors and in mediating complex learning and memory tasks. Octopamine is known to play a role in olfactory reward associative learning in *Drosophila* and in honeybees (Menzel et al., 1996; Hammer and Menzel, 1998; Menzel et al., 1999; Schwaerzel et al., 2003). Octopamine is also involved in locomotion and in grooming behavior in *Drosophila (Yellman et al., 1997)*. In addition, octopamine has been reported to play a role in courtship (O'Dell, 1994) and aggression (Hoyer et al., 2008) behavior in *Drosophila*. Lately a small subset of octopaminergic neurons present in the sub-esophageal ganglion has been reported to be important for aggression (Zhou and Rao, 2008). In locust octopaminergic neurons are known to mediate arousal (Bacon et al., 1995) and locomotion.

In conclusion, octopamine is involved in a large array of behaviors and can be superficially considered equivalent to noradrenalin in vertebrates as it shares functional similarities with noradrenalin. Both are involved in fight and flight response, motivational behaviors and aggression.

Octopamine receptors have been characterized in locusts. They can be divided into 2 types, namely Oct_1 and Oct_2 receptors (Evans, 1981). The Oct_1 receptors are involved in rhythmic behaviors and the Oct_2 receptors are involved in neuromuscular functions. The Oct_1 receptors function via mechanisms that lead to an increase in intracellular calcium whereas the Oct_2 receptors function by producing an increase in the levels of cAMP (Evans and Robb, 1993). Perhaps the very diverse roles of octopamine could be attributed to the distribution of different types of receptors in the insect nervous system.

Octopamine has been shown to function in positive reinforcement learning in *Drosophila,* but it remains unknown if its release would directly produce a rewarding sensation in animals.

1.7.3 Serotonin

Serotonin is a neurotransmitter that is produced in 2 steps from tryptophan. In the first step tryptophan is converted to 5-hydroxytryptophan (5-HTP) by the action of tryptophan hydroxylase (TPH). In a second step 5-hydroxytryptophan (5-HTP) is converted to serotonin (5-hydroxytryptamine, 5-HT) by dopa decarboxylase (DDC) (Fig. 12).

Serotonin is a small-molecule neurotransmitter. It is known to have a developmental role in the fly peripheral nervous system (Monastirioti, 1999). In the vertebrate CNS, serotonin is involved in regulating feeding behavior, aggression, mood, perception, pain, and anxiety (Bradley et al., 1986; Roth, 1994). Loss of 5-HT and dopamine in a temperature sensitive *Drosophila* mutant lacking DDC leads to learning abnormalities and to an abnormal pattern of serotonergic neurons (Budnik et al., 1989). In vertebrates 15 types of 5-HT receptors are categorized into 7 families viz. 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6, and 5-HT_7 depending on their signaling pathways. The family including 5-HT_1 and 5-HT_5 receptors interacts negatively with adenylyl cyclase, the 5-HT_2 receptor family mediates activation of phospholipase C (PLC). The family comprising 5-HT_4, 5-HT_6 and 5-HT_7 receptors, activates adenylyl cyclase, whereas the 5-HT_3 receptor is known to function as ligand gated ion channel (Hoyer et al., 1994).

There is convincing evidence that in *Drosophila* cyclic AMP (cAMP) plays an important role for learning. It is strongly believed that the cAMP dependent protein Kinase A II (PKA II) mediates this process. High levels of cAMP have been found in the mushroom body in comparison to other neuropiles and it was reported that the application of serotonin produced higher PKA activity in Kenyon cells of *Drosophila* and honeybees, implicating a role for serotonin in learning (Muller, 1997). From experiments done in the heat box paradigm in *Drosophila* serotonin is known to play also a role in punishment (heat) mediated operant learning (Sitaraman et al., 2008).

Important for this work is that in *Drosophila* it remains unknown if serotonin functions as a positive, negative or neutral reinforcer in classical conditioning and if its release would produce a punishing or rewarding effect in insects.

1.8 Aim of this study

Biogenic amines have a wide variety of functions in an animal. The aim of our study was to investigate the role played by amines in modulating *Drosophila* behavior. The value based reinforcing properties of amines in classical conditioning have already been demonstrated, but it remains unclear if the animals operantly prefer or escape from situations that cause the activation of different populations of aminergic neurons. In vertebrates operant behavior has been investigated using electrical intracranial stimulation, but the disadvantage of this system is that it leads to unspecific activation of undesired populations of neurons and only anatomically demarcated regions could be investigated. Thus, if an anatomical region comprises different types of neurons then the effect observed cannot be attributed to any one of these. In a recent study the Deisseroth group (Tsai et al., 2009) has overcome this shortcoming by expressing channelrhodopsin-2 and thus was able to specifically activate dopaminergic neurons and study the activation effects.

We wanted to work along similar lines and investigate the role of different amines in *Drosophila* in mediating operant behavior. Thus, we want to make use of the ease of genetic manipulation of *Drosophila* and induce neuronal activity using channelrhodopsin-2 as an optogenetic tool. To quantify the effect of such neuronal activity and quantify the resulting behavioral changes we want to design an operant conditioning paradigm which employs walking as a behavioral readout. In our experimental design choosing a direction of walking will be coupled to the occurrence of a reinforcing stimulus. Thus, if the stimulus is evaluated as positive the animal would continue to walk in the direction that leads to the incidence of that stimulus, or if the stimulus was evaluated as negative it would avoid that direction of walking. Using this experimental design we want to investigate the role played by amines in operant behavior of *Drosophila* and determine if direction-contingent activation of aminergic neurons modifies the behavior of the flies. These experiments should enable us to obtain information on the cellular basis of an evaluation system relevant for operant behavior in insects.

2. Materials and methods

2.1 Walking ball paradigm

The aim was to develop a paradigm where the animals could be fixed in space to allow stimulation by laser or light but still perform a natural behavior that could be quantified. Hence, we have used a modified version of the styrofoam ball apparatus, which has been used earlier to study opto-motor responses in flies (Buchner, 1976). In the styrofoam ball apparatus the animals were glued by their head and thorax to a manipulator and could walk on a small styrofoam ball marked with black dots suspended by a gentle stream of air. The movement was detected by a four segment optical waveguide and a sequence detecting electronic device which measured the movement of the black dots on the sphere as the quadrants on the waveguide are sequentially darkened (Fig.13a).

In our version of the walking ball apparatus a fly is glued to a manipulator by its thorax and can walk on a styrofoam ball which is suspended by an air cushion (Fig.13b, c). Hence, again the fly is fixed in space but can walk freely in a given direction by rotating the ball. However, the movements of the ball now are monitored at much higher resolution using the X-Y movement sensor of an optical computer mouse connected to a computer. A threshold for stimulus application in a given direction is set such that all movements above the set threshold would lead to occurrence of a reinforcing stimulus (infrared laser (IR) or blue light stimulation). The computer is programmed to coordinate the input from the mouse chip and control the incidence of the operant reinforcer. Five apparatuses have been constructed in parallel that permit faster data acquisition and operation of yoked control experiments simultaneously with the test group (fig. 13d).

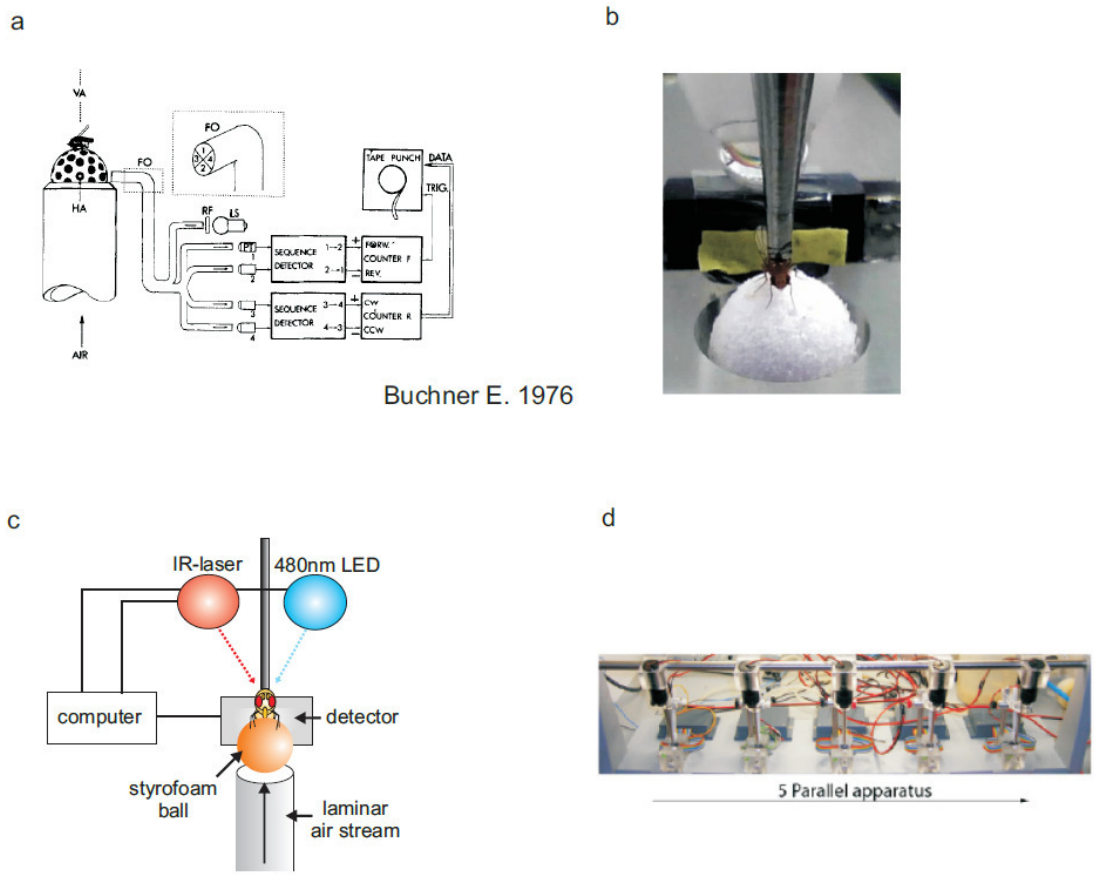


Fig.13 *Description of the walking ball paradigm. The walking ball apparatus is a modification of the styrofoam ball apparatus (a). The fly is glued to a manipulator and can walk on a styrofoam ball suspended by a gentle stream of air (b). The movements of the fly are quantified by measuring the movement of the ball by an electronic chip from an optical mouse. Dependent on the input from the detector to the computer the occurrence of the operant reinforcer (IR laser or blue light) is controlled (c). Five apparatuses enable faster data acquisition and operation of yoked control experiments (d).*

2.1.1 Description of electronics

2.1.1.1Electronic measuring system

The electronics of the machine was custom made by the Biozentrum workshop of the University of Wuerzburg by Konrad Öchsner. The movements of the ball are electronically monitored by a chip (sensor) and the laser illumination system from a mouse. The lens of the sensor from the mouse detects the deflection of an infrared beam

which is incident at an angle of 20° to the surface of the ball when the ball moves. The sensor is based on LaserStream technology and comprises the Image Acquisition System (IAS) which acquires microscopic images of the ball via its lens and the laser illumination system. The sensor sends each image to a digital signal processor (DSP) for analysis which determines the direction and distance of motion in comparison to the previous image. both horizontally (dy) and vertically (dx). Therefore the movements of the 5 flies on the 5 parallel apparatuses can be visualized as traces on the screen. The I/O box receives input from the computer and switches ON and OFF of the stimulus (IR laser or blue light, as specified in the training parameter). For the delivery of pulsed blue light a pulse width modulator was used providing the possibility to alter frequency, on/off cycles and amplitude of the light pulse.

2.1.1.2 Electronic devices and accessories with specifications

Electronic Device	Specifications	Company
Laser mouse	Ultron magic flame laser mouse with ADNS-9500 LaserStream™ gaming Sensor (product of Avago technologies)	Ultron AG, Alsdorf, Germany
Infrared Laser (IR)	808 nm infrared laser. Laser intensity used for most of the training experiments was 20 mW/mm^2 unless specified.	Roither Laser Technik RLDB808-350-3, Vienna, Austria
Blue light emitting diode (LED)	480nm wavelength. Light intensity 6.5mW/ mm^2. 40 Hz light pulses and 2.5 ms illumination time per light pulse. The lens dome of the LED was manually removed and the light fiber adjusted using a micromanipulator to get maximum light output.	Luxeon V LXH-LB5c, Philips Lumileds, San Jose, CA
I/O box	Converts binary logical input into physical output (5V or 0 V)	Meilhaus electronic

Polymethyl methacrylate light guide	1m long and 1mm in diameter. Placed at a distance of 1 mm from fly head.	
Laser intensity monitor	Used to set the intensity of IR lasers and LED's.	LaserCheck, from Coherent Inc.
Pulse Width modulator	Pulsing blue light at different frequency with regulation of on and off time and possibility to vary pulse amplitude.	Custom made workshop Uni. Biozentrum

Table1. List of electronic devices and accessories.

2.1.2 Description of experimental procedure and setup

The flies were anesthetized on ice for 5 minutes and placed on the cold table with the abdomen of the fly facing downwards. Using transparent nail polish (Maybelline, express finish) the flies were glued between their thorax and abdomen on the tip of the manipulator stick (3mm in diameter) which was held by a holder, this holder could be mounted on the ball apparatus. For experiments with blue light illumination the third antennal segment was excised on the cold table and animals were allowed to recover for an hour and then glued. The glued flies were then mounted on the ball apparatus in a way that they could walk freely on the styrofoam ball and were not tilted in a particular direction and their body orientation was in line with the vertical axis. The styrofoam ball was held afloat in moistened laminar air stream (at pressure of 2 Bar) produced by an aquarium pump (Fig.14). The humidified air stream was then bifurcated into 5 tubes to reach 5 parallel apparatuses.

The experiments were performed at 20°C. All the experiments were done in complete darkness. The flies were punished by infrared laser (IR) by focussing the beam on the head which caused the fly's head to heat up. For the experiments done with blue light, separators were constructed that separated each apparatus from the other to prevent the influence of illumination from one of the apparatus to the fly on the nearby apparatus. The increase in temperature on incidence of blue light was ~ 1°C from room temperature

(RT) and increase in temperature on the incidence of IR laser at intensity of 20mW/ mm^2 was ~20°C above RT.

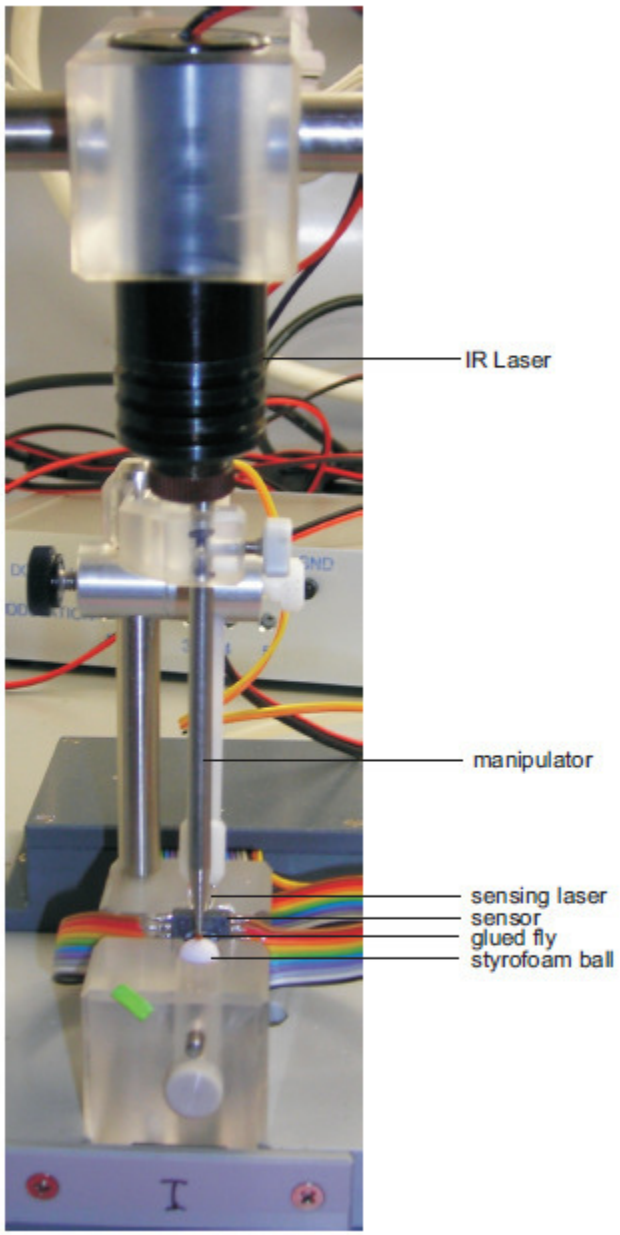


Fig.14 *The fly is glued to the manipulator and can walk freely on the styrofoam ball suspended in air. The movements of the ball are monitored by the electronic sensor placed behind the ball. The sensing laser is inclined at 20° and its deflections are recorded by the sensor chip from laser mouse. The infrared laser used for punishing the fly with heat is focussed on the head of the fly.*

2.1.3 Description of computer software

The computer program was custom made by Jochen Hiemeyer (Diploma thesis, Physics Department, University of Wuerzburg) and upgraded and modified by Benjamin Schmid. The program was written in Java language.

2.1.3.1 Measuring software

Our custom built software module allows us to control the walking ball apparatus from an ordinary laptop. Its tasks are to read out the movements of the five balls, analyze the movements of the fly and apply appropriate actions specified. The user can control the actions of the software via so-called 'journals'. The Journals describe under which circumstances, i.e. upon which fly movement, or at which time point, which action should be taken (which type of stimulus to be triggered-either IR laser or blue light). Most parts of the software are written in Java and thus execute on any operating system which has a recent version of sun's JRE1 installed. If the JRE is not installed by default, it can be downloaded for all common systems freely from sun's homepage (http://java.sun.com). The movements of the five balls are sensed by the hardware of five laser USB mice. The cables of the mice are connected to a USB hub, which delivers their signals as ordinary mice inputs to the PC. A software module written in C, called 'manymouse' (http://a.mx.icculus.org/manymouse) was utilized to separate the 5 signals. Manymouse was incorporated into the Java framework via JNI (http://java.sun.com/j2se/1.5.0/docs/guide/jni). In order to control the stimulation of IR laser or blue light based on fly's performance by the software, a RedLab I/O board was used (RedLab Minilab 1008, Meilhaus Electronics, http://www.meilhaus.de). The drivers of the I/O board are also incorporated into the Java environment via JNI 3.

To install the software, the following steps are necessary:

I. Installation of the I/O board:

The RedLab I/O board ships with the required driver CD and comprehensive documentation. The driver is installed simply by putting in the CD and following the

instructions on the screen. As a second step, it is required to install the board itself. This is done by executing a program called 'InstaCal', which was installed together with the driver. After starting InstaCal, the connected board is detected automatically. No further configuration is necessary.

II. Installation of the Buchnerball software:

Create a new folder on an arbitrary place. For the rest of the instructions, we will refer to this folder as the 'Buchnerball' folder. In this folder, create two more folders called 'Daten' and 'Journals'. 'Daten' is used by the software to store the recorded data, and 'Journals' should be used to save user-created journals. Install Buchnerball.jar custom built software and copy it to the 'Buchnerball' folder. On a windows PC, the software can now be started by double-clicking on 'Buchnerball.jar' (located in the Buchnerball folder).

2.1.3.2 Features of measuring and evaluation software

The measuring program was programmed to simultaneously measure activities on 5 parallel ball machines. The experimenter could set the criteria for training by creating journals. The journals comprised training start point (column1), flies to be trained (master) (column 2), mode of action (column 3), and the training criteria (column 4)- directionality (dx), threshold for stimulus presentation (> <, negative value would indicate movements in left direction and positive values indicate movements to right), type of stimulus to be presented (laser or blue light), stimulus presentation on reaching threshold (turned on or off), duration of training (number of bins, 1 bin size 50ms), and flies serving as internal control (slave) (Fig. 15).

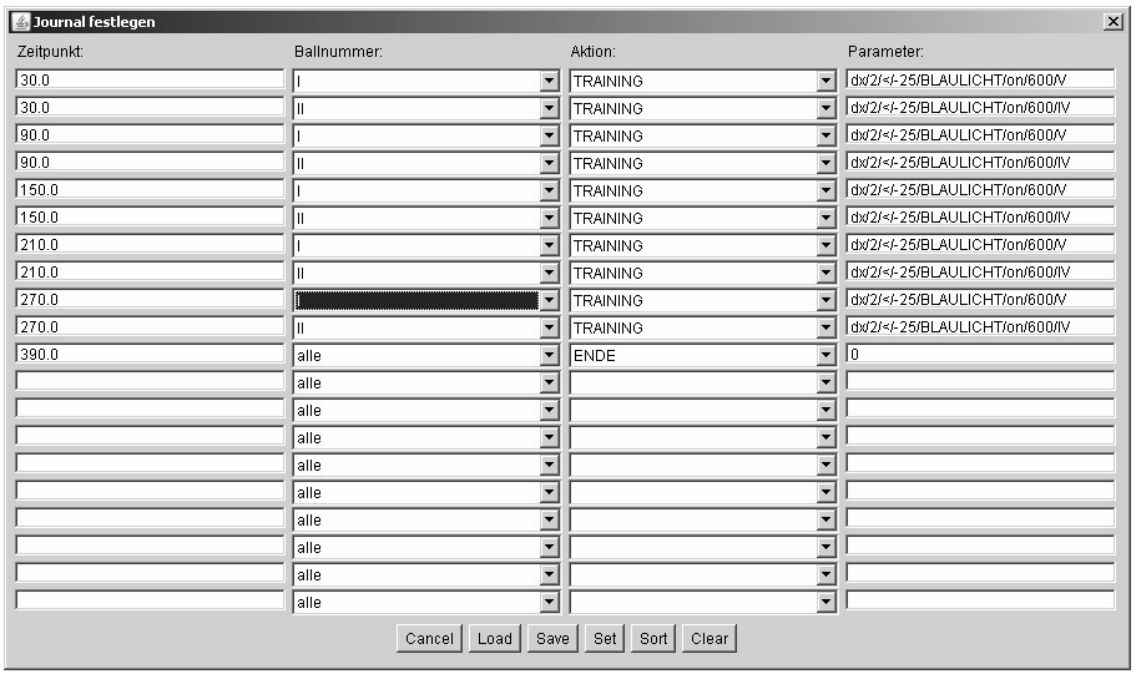


Fig.15 *The journal enables the user to set parameters for training. First column indicates various time points in the training phase. The second column indicates the ball numbers included in training. In case of master slave protocol the ball to be specified as master is indicated here. The third column indicates the action the program has to execute. The fourth column indicates the parameters for training. Each measuring bin is 50ms long and dx/2 indicates that relative rotational movement in 2 measuring bins should be considered for the execution of threshold, which is followed by the magnitude of threshold (negative indicates left and positive indicates movements to right, in this case all movements exceeding 25 pixels in the left would lead to switching on of the blue light. This would continue for 30 sec (each second would have 20 measuring bins each 50ms in size, thus the action would continue for 600 measuring bins). The ball apparatus that would act as the respective slave is indicated towards the end of the training parameter.*

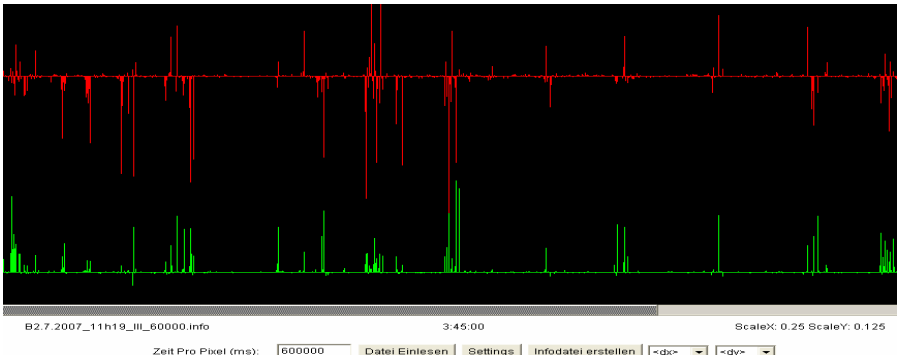


Fig.16a *Visualization of fly movement traces. The red trace indicates movements on x axis (rotational movements), movements above baseline indicate movements to right and movements below baseline indicate movements to left. The green trace indicates the movements in the y axis (forward and backward).*

Fig.16b *Custom made software for acquiring and analyzing data.*

The overall locomotion activity could be determined by pooling in absolute rotational and forward movement (by using the auswerten option). The traces could be visualized by spuren auslesen option (Fig.16 a and b).

The data from the measurements was automatically stored in the output directory as info and dat files. The info files displayed the details of the experiment (type of journal used, overall activity-forward and rotational, duration of measurement and end point). For evaluations the dat files were used which comprised measurements over 50 ms time bins.

The *histogram generating* (histogramm anzeigen) option enabled quantification of number of movements above threshold (movements were displayed as 4 files indicating movements between 0-25, 25-50, 50-100 and above 100 pixels, thus to quantify the movements above 25 pixels data from three files was pooled) in both directions for the desired time bin window. Using these data preference index was calculated using the formula:

Preference index: (Movements above threshold in punished direction - Movements above threshold in punished direction)/ total movements

Positive values would indicate higher movements to punished direction
Negative values would indicate higher movements to unpunished direction

2.1.3.3 Data analysis

For data analysis the custom-written software described above was used. The distances walked by the animals are reflected by the distances of the rotations of the ball's surface. Ball rotations around the horizontal axis perpendicular to the animals' body axis (Y-axis of chip) reflected forward movements (backward movements were negligible), whereas rotations around the vertical axis (X-axis of chip) to either side reflected turning movements. The distances in Y or X directions were averaged over time bins of either 5 min or 30 s, dependent on the duration of the experiments. The numbers of rotational movements exceeding a rotational angle of 3.6° in either direction were counted in 50ms time windows. Preference indices were determined by subtracting the number of rotational movements in one direction from the number of movements in the opposite direction divided by the total number of movements. In order to avoid counting small rotational movements caused by fluctuations of the balls floating on the air stream deviations below an angle of 3.6° to either direction were not included into the analysis. For statistical analysis of preference indices the one-sample Wilcoxon's ranked sign test was used and the values in either direction were tested against 0 using the MicroCal Origin software (OriginLab Corp., Northampton, MA). For a comparison of the number of movements in both directions the paired sample Wilcoxon's signed rank test was used. For a comparison of activity change between an experimental and a control group the Mann-Whitney-U test was used.

2.2 Light-elicitation of proboscis extension reflex

Flies were starved for ~ 20 h under humid conditions and restrained in a Heisenberg collar so that the proboscis could be observed under a stereomicroscope. Blue light (480 nm) of 6.5 mW/mm^2 was provided by a LED (Luxeon V LXH-LB5c, Philips Lumileds, San Jose, CA) to which a plastic (polymethyl methacrylate) light guide (1 mm diameter) was coupled. The light guide was placed ~ 1mm in front of the fly's head and proboscis extension was observed in response to a 1s blue light pulse. The blue light stimulus was pulsed at 40 Hz (2.5 ms on, 22.5 ms off) using a custom-built pulse generator. Real-time movies of light-evoked proboscis extension were recorded using a widefield microscope (Leica DMR, Leica Microsystems, Wetzlar, Germany) and a digital camera (Spot RT-SE, Diagnostic Instruments, Sterling Heights, MI).

2.3 Molecular techniques used

Inverse PCR:

Inverse PCR was done to verify the presence of two copies of UAS-ChR2 on the second and third chromosome. The crossings were done for several generations to bring the homozygous UAS-ChR2 (II; III Chr) flies into NorpA$^-$ background. The protocol followed is a combination of the protocol from Jay Rehm and Roger Hoskins.

DNA Extraction:

1. 30 flies were collected in a tube and frozen at -80°C.
2. Flies were homogenised in 200µl of Buffer A (100mM Tris-Hcl pH7.5, 100mM EDTA, 100mM Nacl and 0.5% SDS) in an eppendorf tube. Another 200 µl was added and ground further.
3. The tube was incubated at 65°C for 30 min.
4. 800µl of Li/KAc (1 part of 5M Potassium Acetate, KAc and 2.5 parts of 6M Lithium Chloride, LiCl stock) solution was added and incubated on ice for 10 min.
5. The tube was spun at 13200 rpm for 15 min at room temperature.
6. 1ml of the supernatant was transferred into a new tube, avoiding the crud layer.
7. 600 µl of isopropanol was added and mixed gently. The mixture was spun at 13200 rpm at room temperature for 15 min.

8. Supernatant was aspirated and washed with 70% ethanol and then and the ethanol was aspirated and pellet was dried.
9. The DNA was re-suspended in 150 µl of Tris-EDTA (TE) buffer. DNA was stored at -20°C.

Restriction digestion:

1. A restriction digestion reaction was set up with the following:

Genomic DNA	12 µl
10X buffer	2.5 µl
dd H_2O	8 µl
Restriction enzyme	2.5 µl
Total volume	25 µl

Restriction enzymes *Msp*I and *Hin* P1 I (New England Biolabs Inc., Arundel, Queensland, Australia) were used for digestion of DNA.

2. The mixture was incubated at 37°C for 2.5 hrs
3. The restriction enzyme was heat inactivated at 75 °C for 20 min.

Ligation

1. Ligation reaction was set up with the following:

Digested DNA	10 µl
10X ligation buffer	40 µl
ddH_2O	350 µl
T4 DNA ligase (Fermentas Inc.)	2.0 µl
Total volume	402 µl

2. The reaction was setup and the tube was inverted to mix the contents and was spun briefly. The tube was incubated at 4°C overnight.
3. 40 µl of NaAc was added and 1.2 ml of 100% ethanol was added and inverted and mixed.
4. The tube was kept at -80°C for 30 min.
5. The tube was centrifuged at 13200 rpm for 45min-1 hr at 4° C (the pellet side was marked before starting).. The supernatant was carefully aspirated without disturbing the pellet.
6. DNA was dissolved in 80 µl of TE buffer.

PCR

1. PCR reaction was setup with following components:

Ligated genomic DNA	5.0 µl
2.5 mM dNTP mixture (Invitrogen Inc.)	2.0 µl
25 mM $MgCl_2$ (Invitrogen Inc.)	2.5 µl
10 µM Forward primer (EY.3.F)	0.5 µl
10 µM Reverse primer (EY.3.R)	0.5 µl
10X PCR buffer (Invitrogen Inc.)	2.5 µl
Taq polymerase (Invitrogen Inc.)	0.5 µl
dH_2O	11.5 µl
Total volume	25 µl

Primer sequence: **EY.3.F**: CCT TTC ACT CGC ACT TAT TG

EY.3.R: GTG AGA CAG CGA TAT GAT TGT

2. PCR temperature cycle: 95°C -15min

35X cycles- 95°C (30s)/55°C (1min)/72°C (2min)

72°C -10 min

3. Visualize on 1.5 % agarose gel.

2.4 Fly Strains used

Fly strain	**Reference**
[w^{1118}/w^{1118}; +/+; P{Gal4}TH/P{Gal4}TH]	(Friggi-Grelin et al., 2003)
[w^{1118}/w^{1118}; P{Tdc2-Gal4.C}2/P{Tdc2-Gal4.c}2; +/+]	(Cole et al., 2005)
{Ddc-GAL4.L} [w^{1118}/w^{1118}; P4.36/P{Ddc-GAL4.L}4.36; +/+]	From Hirsch J. 2006**,** cited in (Sitaraman et al., 2008)
TPH-2 Gal4	Jaeson Kim, Korea Advanced Institute of Science and Technology cited by (Borue et al., 2009)
[w^{1118}/w^{1118} ; Gr5a Gal4/ Gr5a Gal4; Gr5a Gal4/	Carlson J. laboratory

Gr5a Gal4]	
[w^{1118}/w^{1118}; UAS-ChR2/UAS-ChR-2; UAS-ChR2/UAS-ChR-2]	(Schroll et al., 2006)
[norpA^{-}/ norpA^{-}; UAS-ChR2/UAS-ChR-2; UAS-ChR2/UAS-ChR-2]	Generated and verified by iPCR by Nuwal N
[norpA^{-}/ norpA^{-}; UAS-ChR2-eYFP/UAS-ChR-2 -eYFP; UAS-ChR2-eYFP /UAS-ChR-2 eYFP]	Generated and verified by iPCR by Nuwal N
[norpA^{-}/ norpA^{-}; UAS-Pacα/ UAS-Pacα; +/+] and [norpA^{-}/ norpA^{-}; +/+; UAS-Pacα/ UAS-Pacα;]	Generated by Nuwal N
[w^{1118}/w^{1118}; UAS-Kir2.1/UAS-Kir2.1;tub gal80^{ts}/tub gal80^{ts}]	Biozentrum, Wü
rut2080	Biozentrum, Wü
Syn^{97CS}	Buchner E. group Biozentrum, Wü
TβH^{M18}	Scholz H, Uni of Köln
W+ control	Scholz H, Uni of Köln
TDC-2 mutant	Hoyer S, Biozentrum, Wü
[FM7a/FM7a; +/+;+/+]	Biozentrum, Wü
[+/+;CyO/Sp;TM3ser/Sb]	Bloomington
CS wild type	Biozentrum, Wü
norpA P24 on X	Biozentrum, Wü

3. Results

3.1 Standardization of the walking ball paradigm

3.1.1 Verification of the effect of the lasers

To establish the new operant conditioning paradigm we intended to use an unconditioned stimulus which innately had strong effects on the behavior of the animal and thus would make initial quantification on the new setup simpler. We chose a heat-producing infrared (IR) laser as the unconditioned stimulus to punish the flies. The beam was focused on the head of the fly which caused an increase in temperature. The aim was to punish the flies every time they made a turn beyond the set threshold of 25 pixels (3.43°) in one particular direction while walking on the ball. Thus the animals in principle could realize the contingency of punishment with their movements and could avoid making turns in the direction coupled to infrared punishment.

Since locomotion of flies formed the prime criterion in quantifying the behavior in the walking ball paradigm, it was essential to quantify amount and the time course of the changes that took place when the animals received IR laser stimulation. To determine the changes, 5 IR stimulations of 1s duration were given at Inter-Stimulus Interval (ISI) of 1 min to wild type CS flies (Fig. 17a) and activity over 300 s was plotted in 1 s bins. The resultant change in absolute rotational (irrespective of direction) and forward activity was calculated by pooling the 5 pulses activity together into a single pulse (Fig. 17b). To determine the change in activity on stimulation to the basal activity, 5 s prior to stimulation and 5 s during stimulation and post-stimulation were pooled together and compared for both rotational and forward activity (Fig. 17c). Animals showed an increase in rotational (Wilcoxon signed paired rank test, $p= 7.821E^{-11}$; Z=-5.369) and forward activity (Wilcoxon signed paired rank test, $p= 4.602E^{-10}$; Z=-5.262) with IR in post stimulation (5s) period when compared to pre-stimulation period (5s).

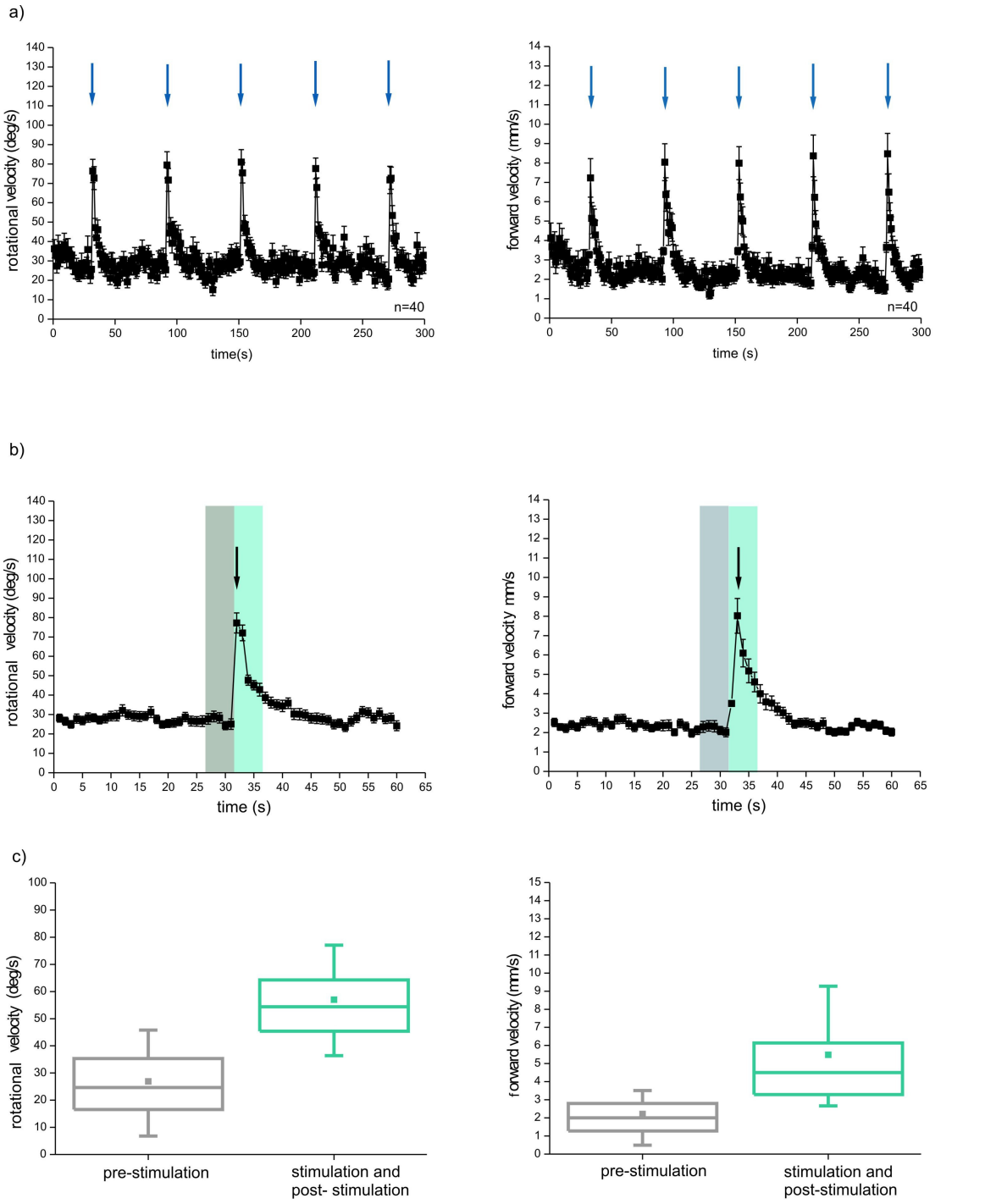


Fig. 17 *Effect of laser stimulation on overall locomotion activity. Five IR laser stimulations were given at ISI of 1 min (a). Arrow indicates IR stimulation, increase in rotation and forward activity is observed (b). On pooling the 5 sec before stimulation and post stimulation increase in locomotion both rotational and forward in response to IR laser was observed (c).*

After determining the effect of laser on animals we wanted to determine the appropriate laser intensity for training and time periods for which animals could be trained on the ball apparatus.

3.1.2 Choosing the appropriate laser intensity for training

As one of the initial steps it was required that an appropriate intensity of the laser was chosen such that it was punishing to the fly but did not kill it. Thus behavioral experiments were performed with different laser intensities. The experimental design consisted of 30 s of *pretest* followed by 5 trials each comprising 30 s of *experimental phase* where the animals received a punishment whenever they turned in the punished direction and 30 s of *test phase* where the animals were just walking on the ball with no laser stimulation (punishment). Three laser intensities were chosen - 40 mW/mm^2, 20 mW/ mm^{2}and 10 mW/ mm^2 (Fig.18 a, b, c). From these experiments it was ascertained that laser intensity of 20 mW/ mm^2 (One sample Wilcoxon signed ranked test for training p=2.45E^{-10}; Z=6.32 and test p=0.00202; Z=3.087; n=100) was appropriate to investigate avoidance behavior on the walking ball apparatus, although the animals also avoided the punished direction at other laser intensities of 40 mW/ mm^2 (One sample Wilcoxon signed ranked test for training p=0.00153; Z=3.049 and test p=0.915; Z=0.108; n=30) and 10 mW/ mm^2 (One sample Wilcoxon signed ranked test for training p=5.973E^{-6}; Z=4.093and test p=0.855; Z=0.185; n=30).

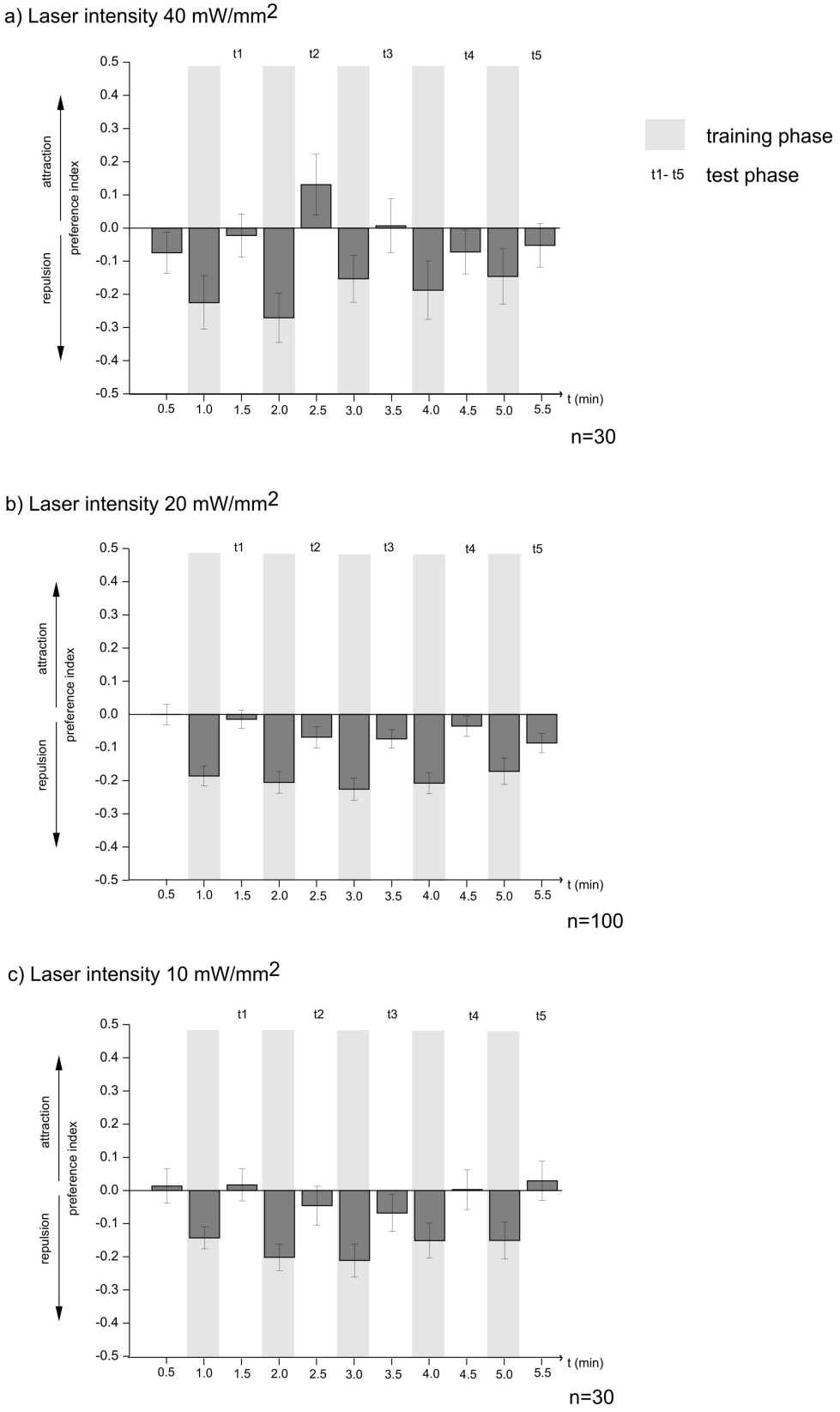


Fig. 18: *Selection of appropriate laser intensity for avoidance behavior in the walking ball paradigm. All three different laser intensities used for analysis of avoidance (a, b, c) behavior. All intensities induced avoidance (a, b, c), but 20mW/mm^2 was chosen for further experiments (b).*

The preference index was calculated by counting all the movements in both directions (using histogram calculation option from the walking ball evaluation software) that exceeded the threshold of 25 pixels (3.43° of rotation).

Preference index = (No. of punished movements- No. of unpunished movements) / total number of movements

- Positive value indicated movements toward laser punishment.
- Negative values indicated movements away from laser punishment.

On determining the appropriate laser intensity for training and changes in laser induced locomotion activity, we wanted to determine different time periods for which animals could be subjected to laser-avoidance behavior on the walking ball apparatus.

3.1.3 Choosing time window best suited for training

We tried longer protocols for a time range from 24 hours to one hour. The 24 hour protocols comprised of 5 min pretest, followed by 24 hours of directional laser punishment. 80% of the animals survived during the training for 24 hours. During the 24 hour experiments high locomotion was observed in the first hour and from 18-24 hours (data not shown). Since the animals did not walk enough the behavioral avoidance effects were not pronounced and hence no direction preferences were observed.

Next, we tested the animals for an hour with a protocol comprising 5 min of pretest followed by 60 min of direction induced laser stimulation and 5 min of test where the animals were freely walking without any laser stimulation. The purpose of the test was to determine if the animals continued to avoid the previously punished direction.

The avoidance test was done reciprocally in both directions. It was observed that the animals showed no directional preference in the pre-test. In the avoidance test phase when the animals were subjected to laser stimulation when turning in the left direction animals showed preference for right turns; n=15 (Fig.19 a), and vice-versa; n=15 (Fig.19 b). Both directionally trained groups were pooled (Fig. 19c). On pooling all the training bins (One sample Wilcoxon signed rank test, $p=9.76E^{-4}$; Z= -2.88; n=30) it was observed

that the overall movements in the unpunished direction exceeded movements in the punished direction (Fig.19 d)

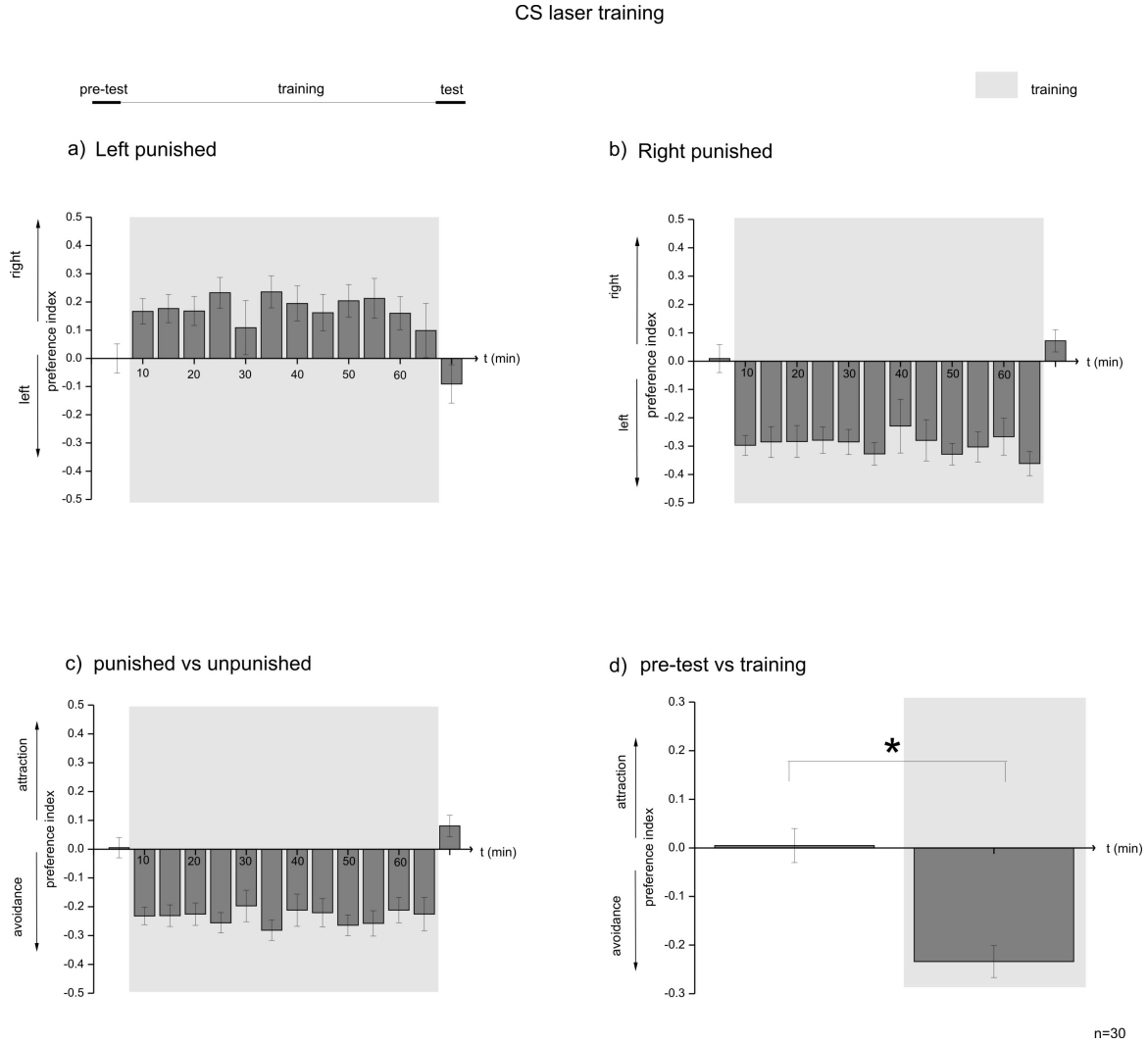


Fig. 19 *Directional laser training for 1 hour. Animals were induced to avoid the laser for an hour in the left direction. Thus they show a positive index indicating movements to right (a). The animals were reciprocally tested in opposite direction viz. right and thereby show a negative preference index indicating movements to left (b). On pooling the two groups the overall preference can be ascertained (c). On pooling all the time bins a clear preference for the unpunished direction is observed.*

The reciprocal training indicated that the animals could avoid both directions and that they did not show any spontaneous preference for any particular direction. Although the reciprocally designed experiments indicated the specificity for directional heat avoidance, we wanted to monitor the behavior of a freely walking animal when it

received heat stimulations similar to our training experiments but irrespective of its directional choice.

3.1.4 Avoidance effect specific to laser stimulation

To confirm that the observed avoidance was only due to the punishing effects of the laser and not due to any kind of artifact, we designed master-slave (yoked control) experiments. An animal on one of the ball apparatuses was considered as "master" who received punishment contingent to its own movement and another animal on a parallel setup acted as a yoked "slave" control receiving the punishment in temporal coherence with the master irrespective of its own movements.

The master showed higher number of movements to the unpunished direction (one sample Wilcoxon signed rank test, in training $p= 8.175E^{-9}$; $Z= -5.764$, in test $p=0.0824$; $Z= -0.2219$; $n=78$) in the laser training phase (Fig. 20 a) whereas the slave control showed equal number of movements (one sample Wilcoxon signed rank test, in training $p= 0.739$; $Z= -0.333$, in test $p=0.404$; $Z= 0.836$; $n=78$) in both directions (Fig. 20 b). Since both the groups received same number of laser stimulations with temporal paring we compared the locomotion changes in both the groups. It was observed that the animals showed similar increase in both rotational irrespective of direction (Fig. 20 c) and forward activity (Fig. 20 d).

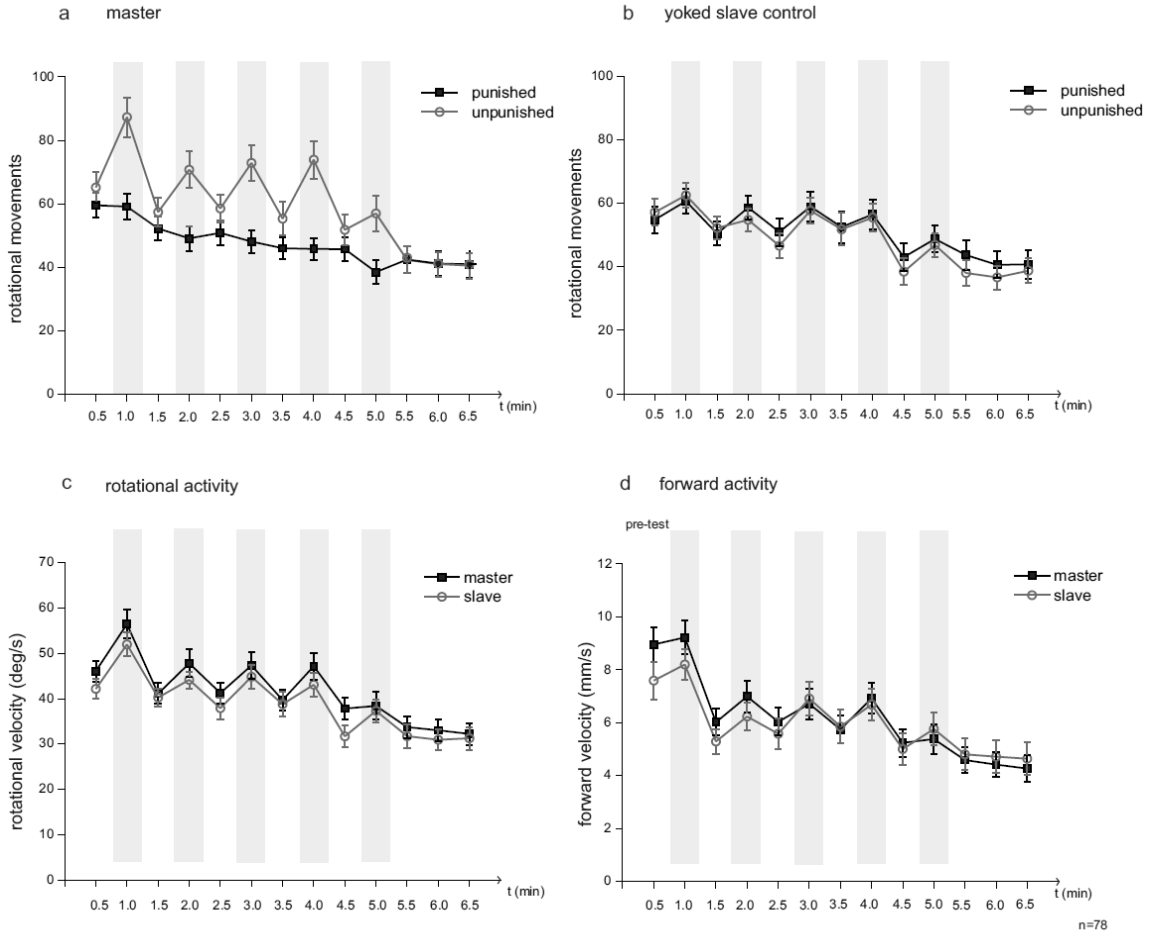


Fig. 20 *Master-slave control experiments to prove that the specificity of avoidance is due to the punishing effect of the laser. Animals were trained for 6.5 min which comprised 5 "punished" and 5 unpunished phases each lasting for 30s. Movements made above threshold quantified separately in the two directions show higher number of movements in the unpunished direction (a). The corresponding slave control which received quantitatively and temporally the same punishments shows equal number of movements in both directions; n=78 (b). The two groups differ only in their directional preference as the overall locomotion i.e. absolute rotational activity irrespective of direction (Fig. c) and the forward activity (Fig. d) of both the groups remains the same.*

This experiment proved that the preference towards the unpunished side was indeed only due to the punishing effects of laser and the animals could indeed realize the contingency of their movements with occurrence of the heat punishment.

Our next aim was to determine the mode of avoidance chosen by the animals. Was the avoidance only a choice between the two directions or did the animals also consider the amplitudes of their walking movements in order to avoid punishment, as the punishment occurred only when the animals exceeded threshold in a given direction?

3.2 Threshold dependent avoidance

We designed 3 experimental groups where 3 different thresholds were set (25, 50, 100 pixels where 1°= 7.3 pixels) and the animals received the punishment only if their movements exceeded the threshold in the punished direction.

On punishing the animals at turns above 25 pixels as threshold in a given direction it was observed that when only the movements above 25 pixels were considered for the calculation of preference indices, then animals significantly avoided the punished turns (one sample Wilcoxon signed rank test, for left training $p=1.5288E^{-5}$; Z= -3.5977; median =+0.33837; n=15, for punishment in the right direction $p==1.5288E^{-5}$; Z= -3.5977; median=-0.35377; n=15) (Fig. 21 a). When the same raw data were evaluated such that only movements exceeding 50 pixels (Fig. 21b) or 100 pixels (Fig. 21c) considered for the calculation of preference indices essentially the same results were obtained with a small increase in the p values (50 pixels:$p= 3.662E^{-4}$; Z= 3.138 ; 100 pixels: $p=3.6E^{-4}$; Z= 3.1).

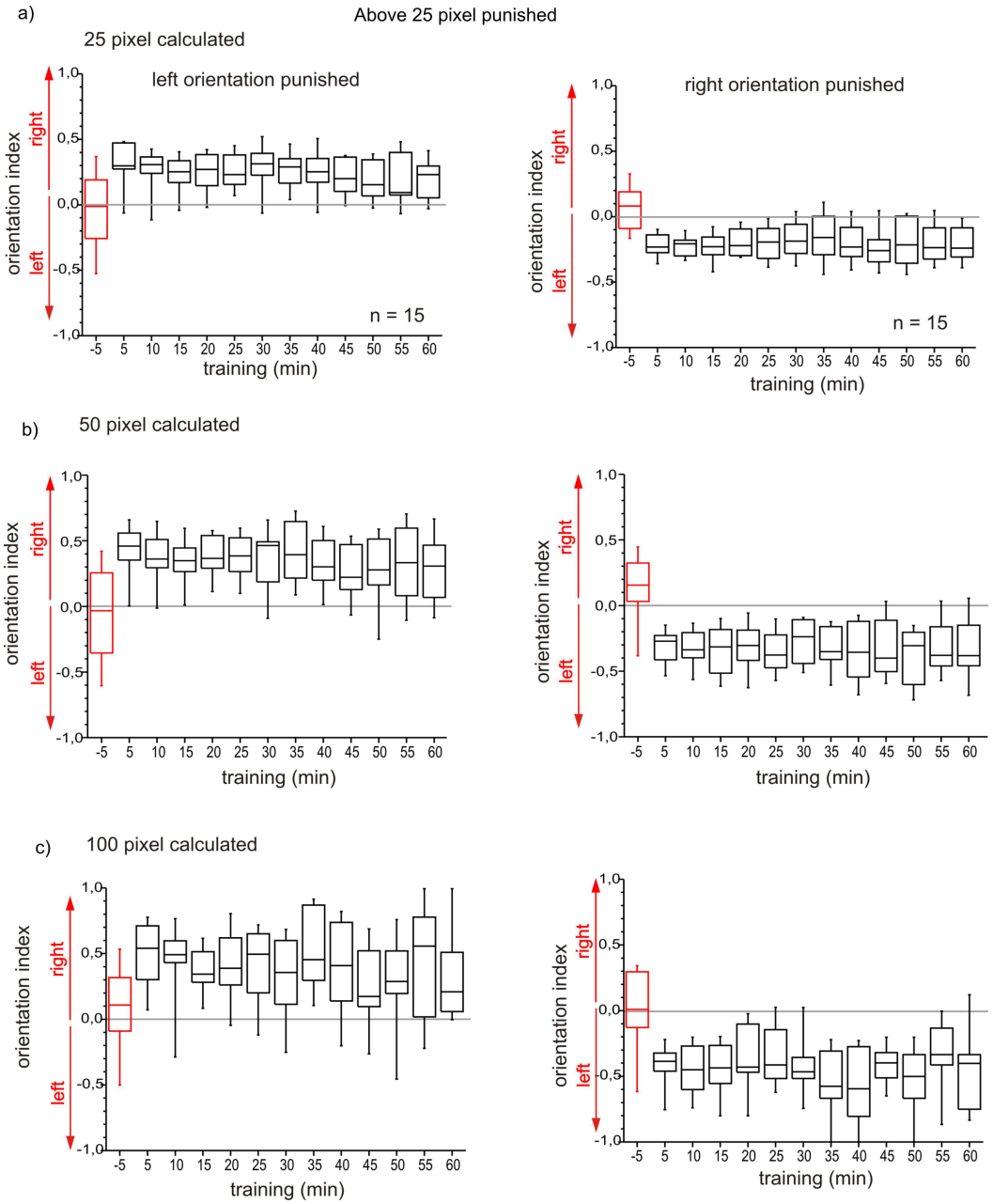


Fig. 21 *Animals punished at turns above 25 pixel threshold in a given direction show higher number of movements exceeding 25, 50 or 100 pixels in unpunished direction when compared to the punished direction, n=15 (a, b, c).*

On training the animals using a punishment threshold of 50 pixels for turning in a given direction it was noted that when movements above 25 pixels were considered for the calculation of preference indices no significant preference for the unpunished direction was observed in both the reciprocally treated groups (Fig. 22 a). When only movements above 50 pixels in both the directions were considered for the calculation of preference indices then a preference for the unpunished direction was observed in both

the groups (Fig. 22 b). (One sample Wilcoxon signed rank test, for left training $p=1.5288E^{-5}$; Z= -3.5977; median =+0.21943; n=15, for right training $p==1.5288E^{-5}$; Z= -3.5977; median= -0.26725; n=15) When only movements above 100 pixels were considered for calculation of preference index, again a significant preference for unpunished direction was observed in both groups (Fig. 22 c)

Preference index: *(No. of movements to right above threshold- No. of movements to left above threshold) / total number of movements above threshold*

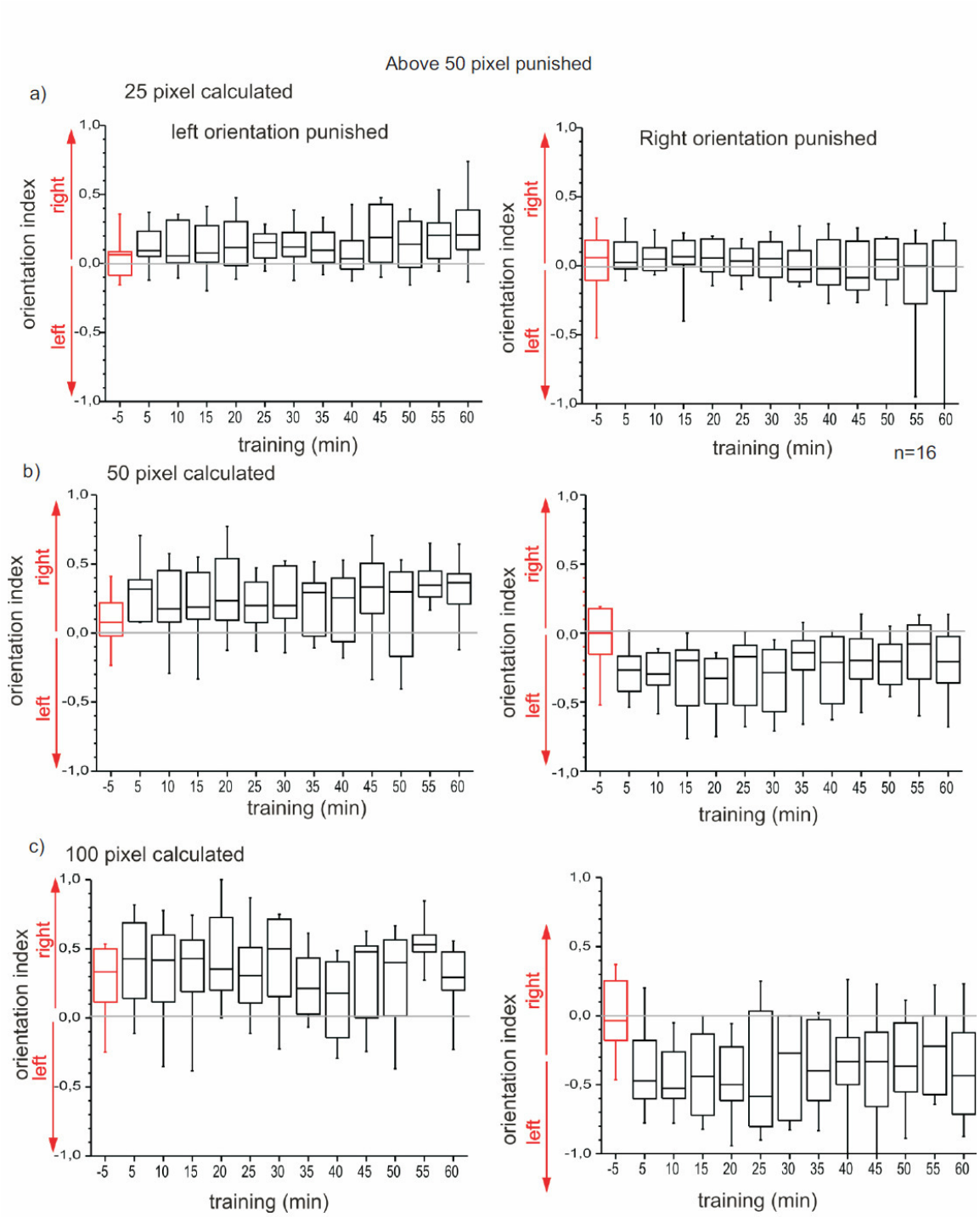


Fig. 22 *Animals punished at turns above 50 pixel threshold in a given direction show higher number of movements exceeding 50 or 100 pixels in unpunished direction when compared to the punished direction, n=15 (Fig. b, c). When movements above 25 pixels are considered no directional preference is observed.*

When movements above 100 pixels were punished then no preference for the unpunished direction was observed in all three groups, i.e. with a threshold exceeding 25

pixels, 50 pixels and 100 pixels (One sample Wilcoxon signed rank test, for left training p=0.61122; Z= -0.52072; n=16, for right training p=0.45857; Z= -0.75741; n=16) preference index calculation groups (Fig. 23 a, b, c). This could be probably because animals exceeded the threshold of 100 pixels only a few times which was not sufficient to establish a preference for the unpunished direction.

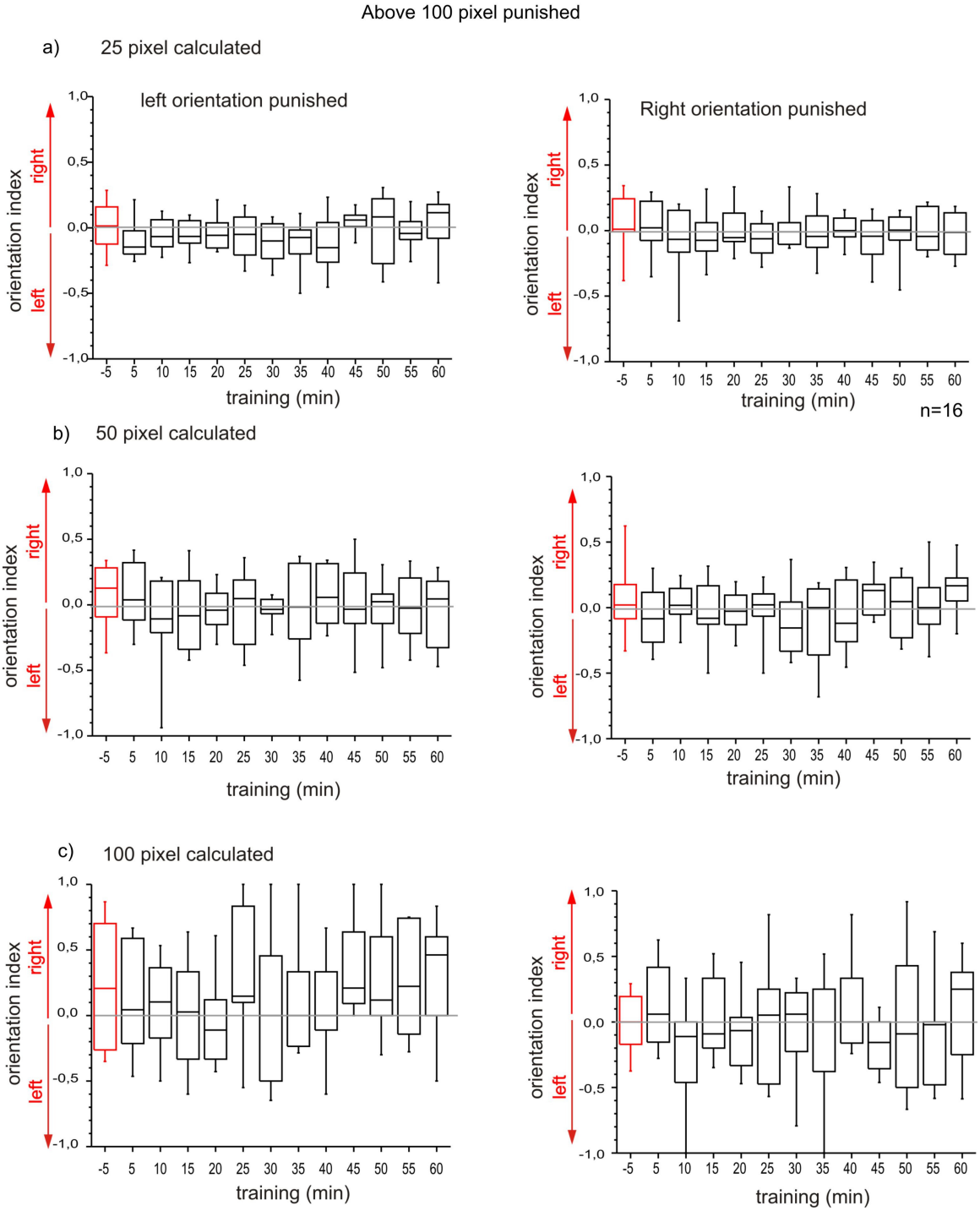


Fig. 23 *Animals punished at turns above 100 pixel threshold in a given direction do not show higher number of movements exceeding 25, 50 or 100 pixels in unpunished direction when compared to the punished direction, n=15 (Fig. a, b, c).*

These experiments with punishing different thresholds of movement indicate that the animals realize the amplitude of movements at which they are punished and therefore make movements that are below the punishing threshold to be safe.

The test phase after the avoidance training phase in the laser punishment experiments were designed to visualize if memory was developed during the training and if the animals continued to move in the unpunished direction even when there was no laser stimulation present. We did not observe any consistent memory effect for the safe direction from our previous experiments (3.1 and 3.2) and thus we questioned if the animals could follow a reversal of direction once the direction of training was reversed. Thus in order to clarify whether the avoidance produces a memory that perhaps cannot be seen we indirectly measured a potential memory effect for the preference of the unpunished direction. We devised experiments where we wanted to see the behavior of the flies if the direction causing the punishment reversed: would the animals follow the reversal in direction immediately or would they take some time to realize the reversal and hence show a poorer or a delayed reversal in their avoidance behavior?

3.3 Dual training of animals to analyze memory for the laser training

To test for directional memory in our paradigm we designed three experimental groups. The first group we called *dual trained group* which received punishment upon turning in one direction (30s each) for first three phases of punishments and upon turning in the other direction in the next three phases (Fig. 24 a). The second group was called *single trained group* in which animals received punishments randomly in the first 3 phases of punishment (sessions) and in the next three training sessions the animals received laser training contingent to their movement in a given direction (Fig. 24 b). The third group was called *naive single trained group* where the animals were not exposed to laser stimulations in the time period equivalent to first three trainings for the other 2 groups and then received training for the first time for the time period equivalent to three trainings (Fig. 24 c).

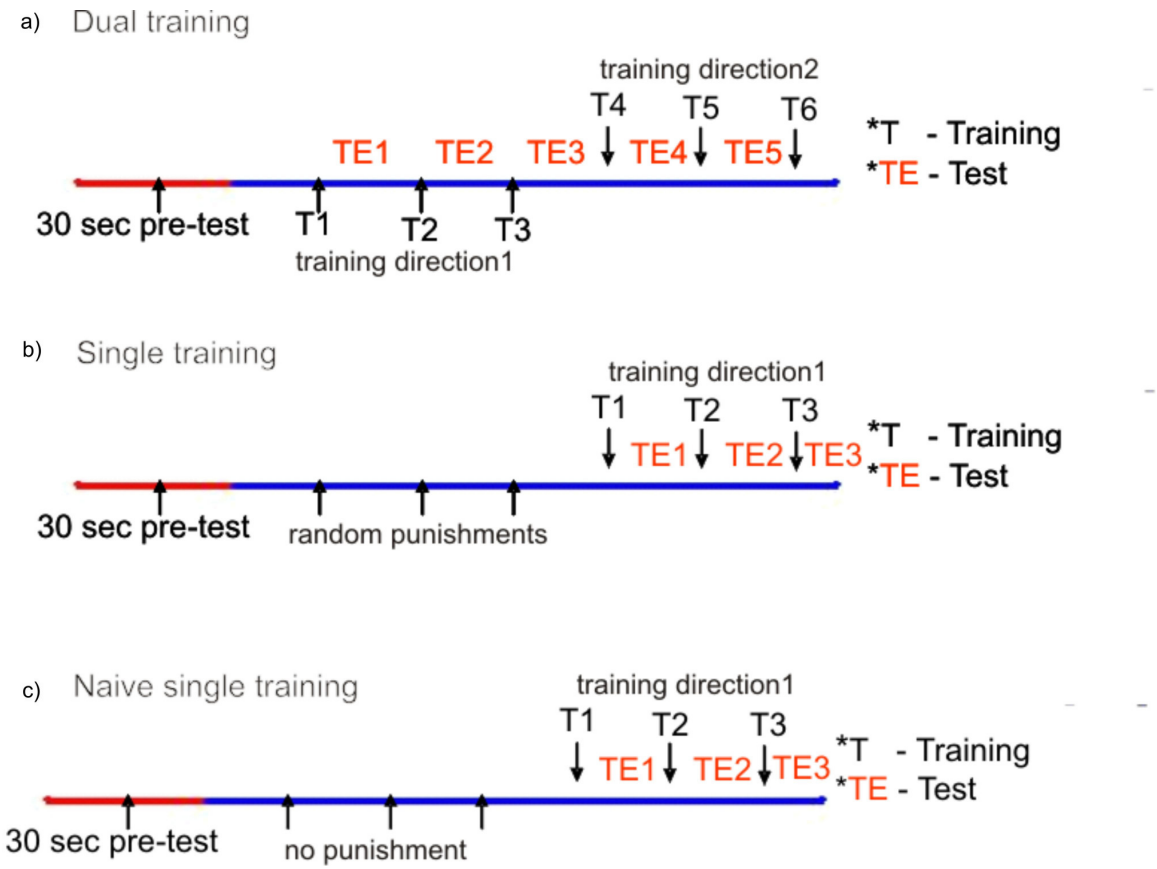


Fig. 24 *Dual training of animals to determine possible memory effects of laser training. In dual training animals were trained for the first three trainings in one direction and during the next three trainings the direction of training was reversed (a). In single training animals were randomly punished for the first three trainings with no directional contingency and in the next three trainings they were directionally trained with laser punishment (b). In naïve single training group animals were not trained in the time period equivalent to 3 trainings and for the next three trainings trained to a given direction (c).*

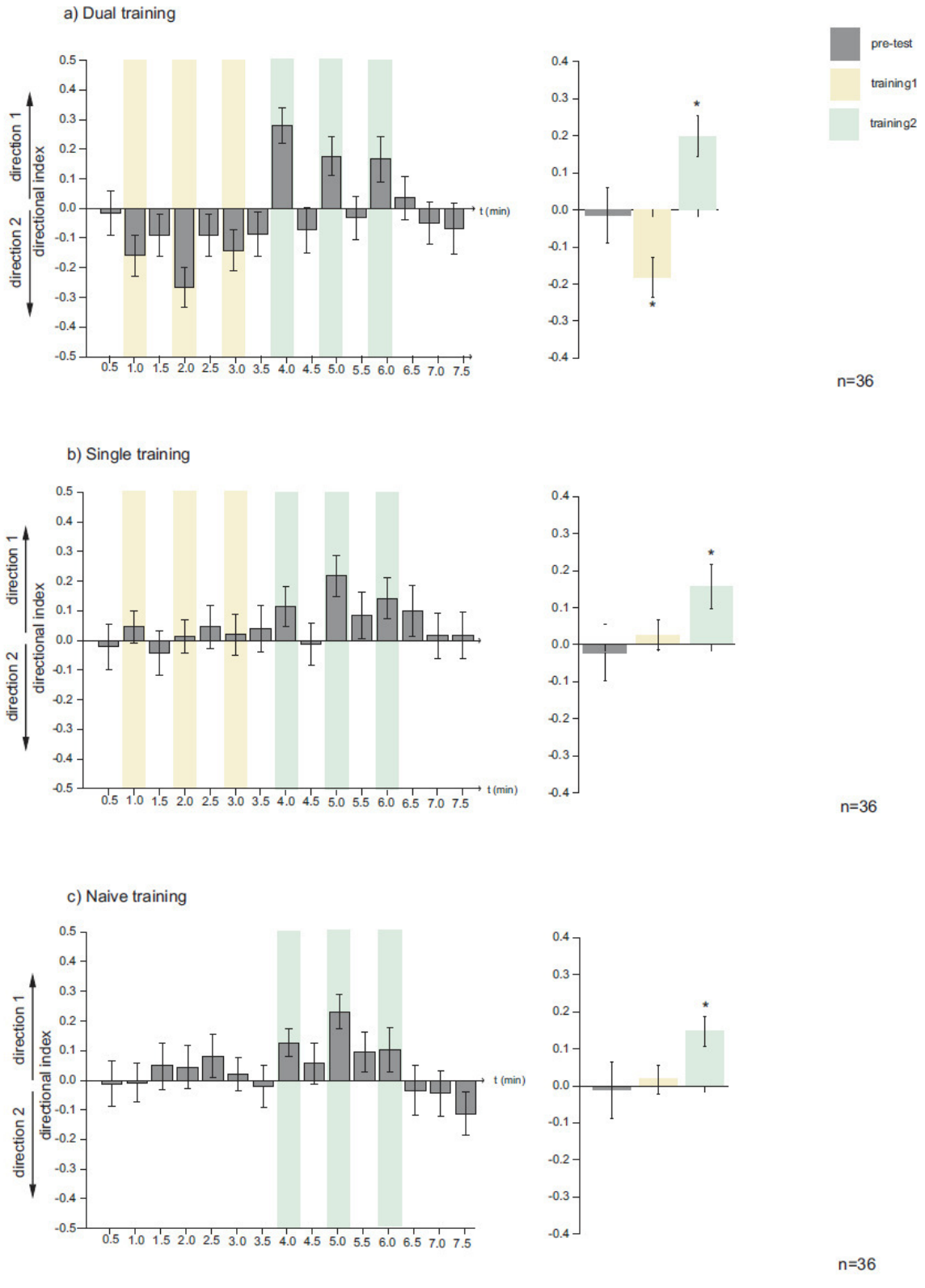

a) Dual training
pre-test
training1
training2
direction 1
directional index
direction 2
t (min)
n=36
b) Single training
direction 1
directional index
direction 2
t (min)
n=36
c) Naive training
direction 1
directional index
direction 2
t (min)
n=36

Fig. 25 *Dual training of animals to determine possible memory effects of laser training. In Dual training the animals were trained for the first three trainings in one direction where they showed preference to the unpunished direction. During the next three trainings the direction of training was reversed, the animals quickly realized the reversal and turned in the new unpunished direction (a). In single training animals were randomly punished for the first three trainings with no directional contingency and hence did not show any directional preference in the next three trainings they were directionally trained with laser punishment and showed directional preference to unpunished direction (b). In the naive single training group animals were not trained in the time period equivalent to 3 trainings and showed no directional preference and for the next three trainings on being trained to a given direction they showed preference for the unpunished direction (c).*

In the dual trained group during the first training session for a given direction the animals showed preference in the unpunished direction (one sample Wilcoxon signed rank test, p=0.001; Z= -3.084; n=36) on reversal of training direction the animals quickly realized the change and showed preference for the new unpunished direction (one sample Wilcoxon signed rank test, p=0.001; Z= 3.118; n=36) (Fig.25 a), the animals realized this change within the first 10 s of reversal of direction (seen on splitting the 30s bins to 10s bins, not shown). In the single trained group the animals did not show any preference in the first three trainings where they randomly received punishments (one sample Wilcoxon signed rank test, p=0.548; Z= 0.607; n=36) (Fig. 25 b), in the next three trainings where they received directional training they showed a preference for the unpunished direction though the preference index in the first training bin was slightly lower than the index observed in the dual training equivalent temporal group (one sample Wilcoxon signed rank test, p=0.011; Z= 2.477; n=36). In the naive trained group animals received no punishment in the time period equivalent to three trainings (one sample Wilcoxon signed rank test, p=0.988; Z= 0.015; n=36), in the next three training bins they received directional punishment and hence showed a preference to the unpunished direction (one sample Wilcoxon signed rank test, $p=4.950E^{-4}$; Z= 3.349; n=36) (Fig. 25 c).

These experiments showed that animals did not stick to the previously trained direction indicating that they could quickly adapt to the new environmental conditions when the direction of training reversed. This argues that the avoidance behavior does not induce any robust memory to avoid a particular direction. To further confirm that our paradigm induces simple avoidance behavior rather than operant learning that involves a memory formation we next wanted to evaluate the performances of classical learning mutants and mutants for synaptic proteins believed to play a key role in associative learning.

3.4 Laser training effect on mutants

3.4.1 *rutabaga* mutant

The *rutabaga* gene encodes an adenylate cyclase which is believed to be involved in the coincidence detection of the unconditioned stimulus (US) and the conditioned stimulus (CS) at the neuronal level. Thus, in mutants for this gene the animals are usually

impaired in forming an association between the US and the CS and are defective in Pavlovian learning (Davis et al., 1995; Blum et al., 2009). In our experiments we wanted to investigate if the absence of this protein led to any changes in response of the animals to stimuli, locomotion changes or defects in the operant behavior.

To first quantify the pure locomotion and responsiveness to stimuli the animals were subjected to 5 laser stimulations at an ISI of 1 min, responses to 5 individual pulses were pooled together into a single pulse for both rotational (Fig. 26 a) and forward activity (Fig. 26 b). The resulting changes in forward and rotational activity were compared to the wild type control (Fig. 26 c). It was observed that the animals had a slightly higher basal locomotion and showed higher responses to the laser stimulation in comparison to the wild type Canton-S (CS) control flies (Wilcoxon paired signed rank test, $p=1.73E^{-18}$; $Z=6.732$; $n=40$ each).

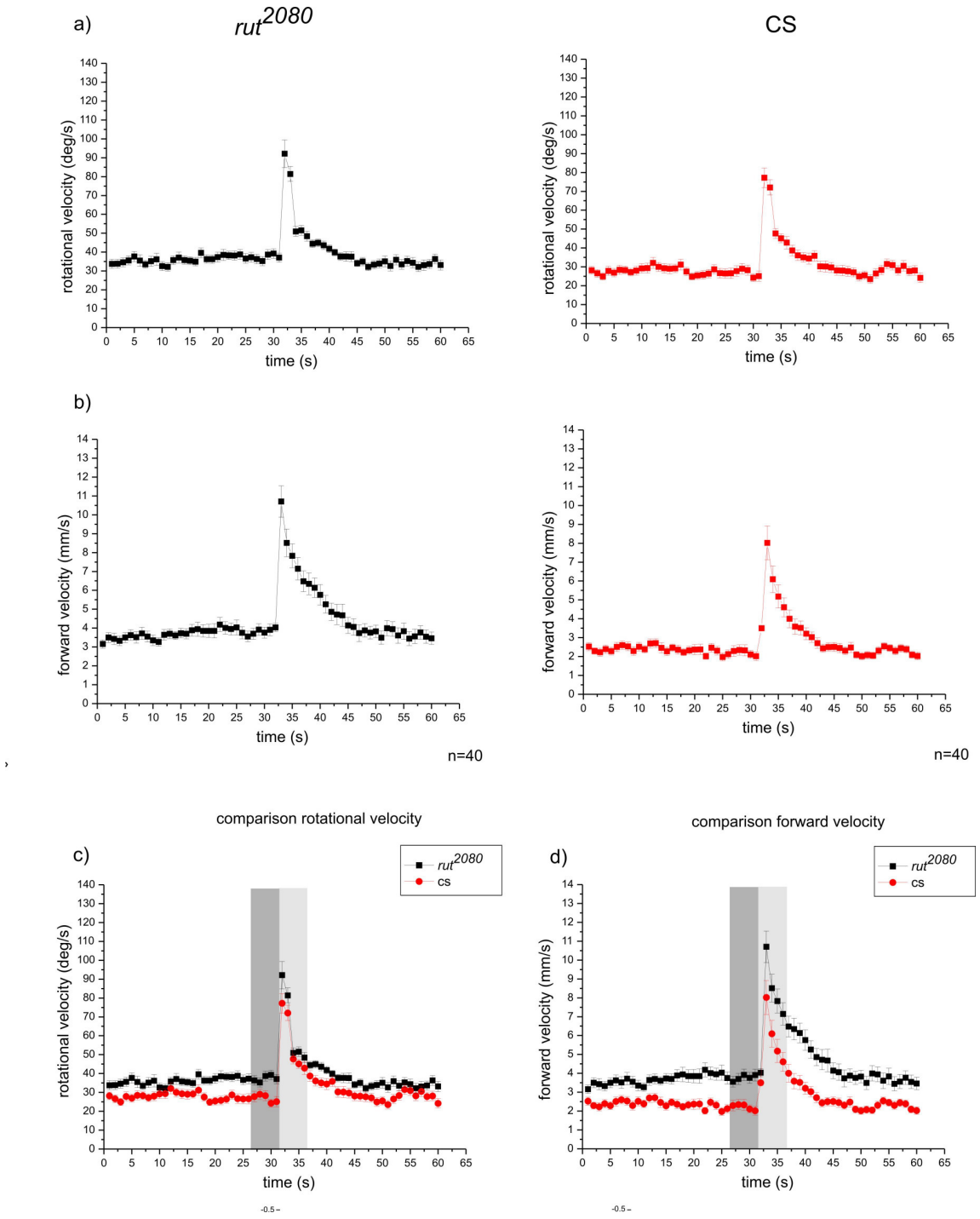


Fig. 26 *rut^{2080} mutant animals are impaired in locomotion and in responding to laser punishment in comparison to the controls. The rotational activity (irrespective of direction) of rut^{2080} mutant and CS (a) and forward activity (b) for both groups were quantified. rut^{2080} mutant had high basal rotational activity(c) and high forward activity (d), they showed higher response to laser stimulation when compared to CS control.*

Next the animals were directionally subjected to the laser induced directional avoidance paradigm. It was observed that *rut*^{2080} animals could perfectly correlate the directional turning on the ball with the laser punishment and showed more movements in the unpunished direction (Fig. 27 a) (one sample Wilcoxon signed rank test, p= 0.009; Z=-2.57; n=40) like the wild type CS control (one sample Wilcoxon signed rank test, p= 0.001; Z=-3.074; n=38) (Fig. 27 c).

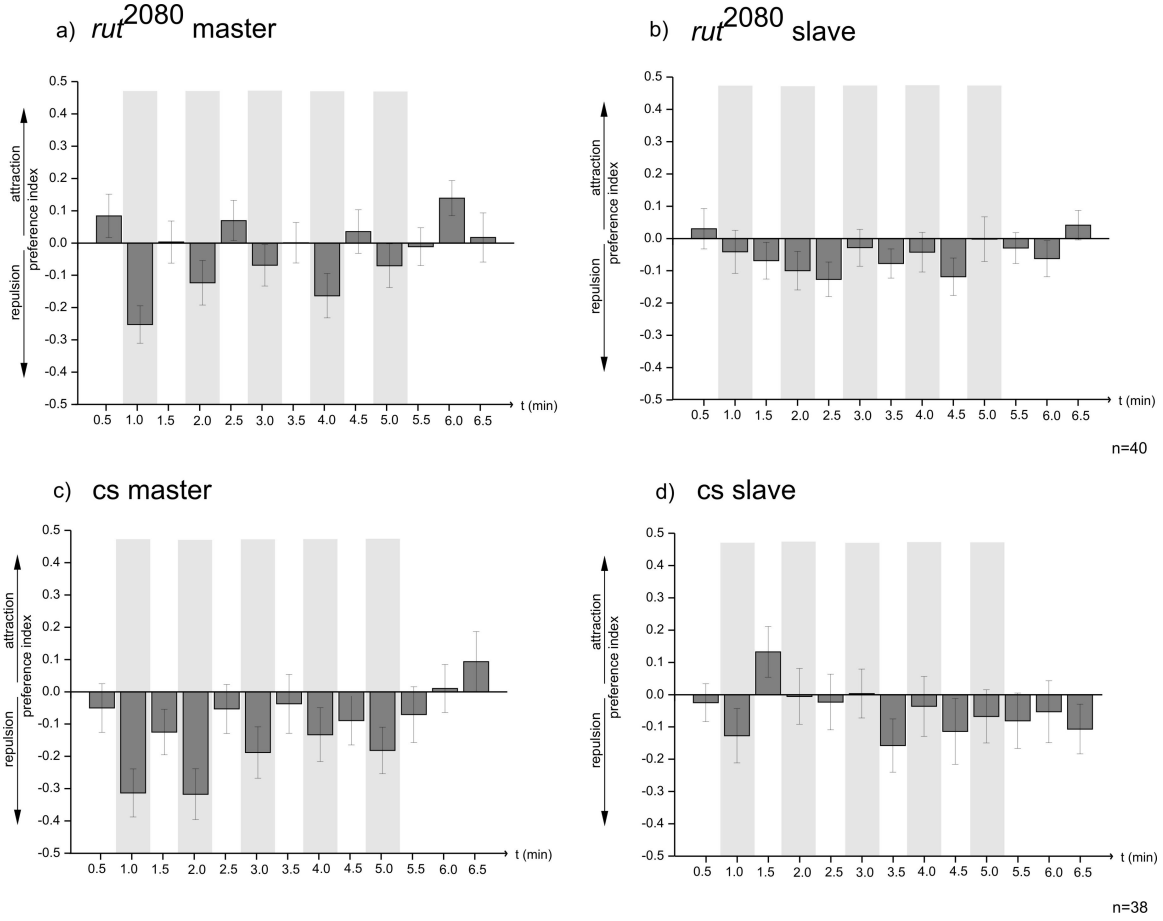


Fig. 27 *rut*^{2080} *mutant animals can be operantly trained on the walking ball apparatus. The rut*^{2080} *mutant and CS master group could operantly choose the unpunished direction when trained on the walking ball apparatus (a, c). The slaves for both groups which received punishment in temporal coherence with their masters did not show any directional preference (b, d).*

The internal slave controls for *rut*2080 mutant (one sample Wilcoxon signed rank test, p=0.412; Z=-0.826; n=40) and CS (one sample Wilcoxon signed rank test, p=0.135; Z=-1.498; n=38) both did not show any directional preference (Fig. 27 b, d).

3.4.2 *Synapsin* mutant

Synapsin is a synaptic protein which is involved in the vesicle docking and release at the pre-synapse. The adenylate cyclase coded by the *rutabaga* gene produces cAMP which is believed to activate Protein Kinase A (PKA). PKA is predicted to be involved in phosphorylation of synapsin. The phosphorylation of synapsin is believed to be an essential step in the release of vesicles from their cytoskeleton mesh making them available for release at synapse. The current hypothesis predicts that synapsin protein could be involved in short term learning via a PKA mediated pathway. We wanted to test if a mutation in the *Synapsin* gene would have an effect on the operant behavior of the animals.

As an initial step to test if a mutation in this gene produces any changes in walking on the ball or response of the animals to laser stimulation, the animals were subjected to 5 laser stimulations at an ISI of 1 min. The rotational (Fig. 28 a) and forward movements (Fig. 28 b) were quantified. The Syn mutants did not show any impairment in rotational activity (irrespective of direction) (Mann Whitney U test, rotational activity before stimulation $p=0.802$; $Z=-0.249$ and post stimulation $p=0.183$, $Z=-1.330$, $n=40$ for Syn , $n=37$ for CS) and forward locomotion (Mann Whitney U test, rotational activity before stimulation $p=0.504$; $Z=-0.667$ and post stimulation $p=0.922$, $Z= 0.096$, $n=40$ for Syn , $n=37$ for CS) when compared to CS flies (Fig. 28 c).

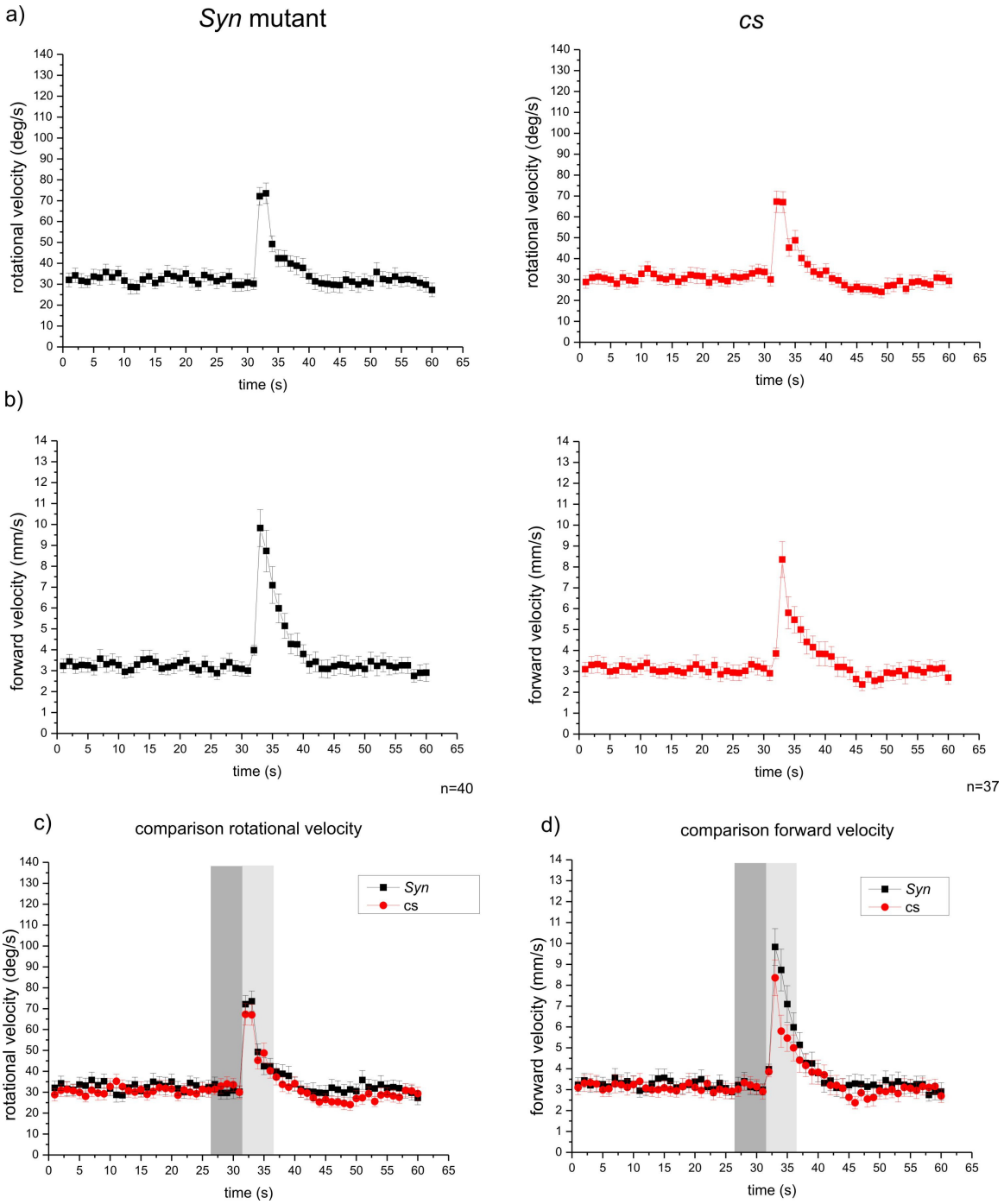


Fig. 28 *Syn mutant animals are comparable to the controls in locomotion and in responding to laser punishment. The rotational activity (irrespective of direction) of Syn mutant and CS (a) and forward activity (b) for both groups were quantified. Syn mutant had rotational (c) and forward activity (d) similar to the CS control.*

The *Syn* mutant master group on directional laser training on the walking ball apparatus preferred the unpunished direction (one sample Wilcoxon signed rank test, in training p= $3.50E^{-5}$; Z=3.900, test p=0.257; Z=-1.137; n=38) (Fig. 29a) similar to the CS

control master group (one sample Wilcoxon signed rank test, in training p= 0.005; Z=-2.70, test p=0.669; Z=-0.432; n=28) (Fig. 29 c). The slave groups for *Syn* mutants (one sample Wilcoxon signed rank test, in training p= 0.868; Z=-0.168; n=38) (Fig. 29 b) and CS (one sample Wilcoxon signed rank test, in training p= 0.454; Z=-0.756; n=28) (Fig. 29 d) which received punishment irrespective of their movements did not show and directional preference.

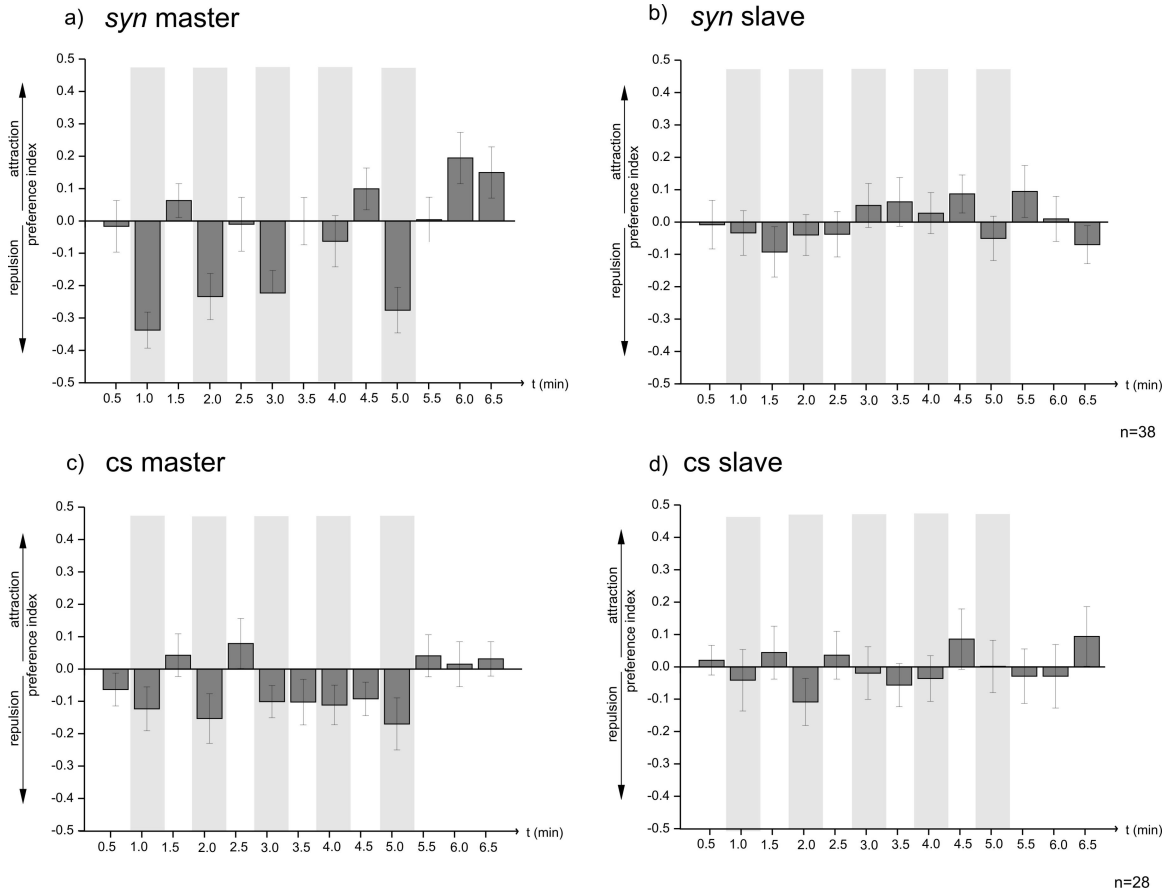


Fig. 29 *Syn mutant animals subjected to the laser avoidance regime on the walking ball apparatus. The Syn mutant and CS master groups could operantly choose the unpunished direction when trained on the walking ball apparatus (Fig. a, c). The slaves for both groups which received punishment in temporal coherence with their masters did not show any directional preference (Fig. b, d).*

These data show that the avoidance of an IR laser through a directed locomotion is not affected by the mutation in the *synapsin* gene. Next, we wanted to evaluate the behavior of animals when their aminergic system was silenced or affected as amines are known to play a role in arousal and motivated behaviors in animals. Also, the aim was to

quantify their performance and observe if they had a defect in aversive operant conditioning.

3.4.3 Tßh and TDC-2 mutant

Tyrosine Decarboxylase **(**TDC) is an enzyme participating in the synthesis of tyramine and, whereas the enzyme tyramine ß hydroxylase (Tßh) is involved in the conversion of tyramine to octopamine. Tyramine is the precursor of octopamine. Both tyramine and octopamine function as neurotransmitters, neurohormones and neuromodulators and have so far been observed only in invertebrates. Since octopamine is involved in motivational behavior we wanted to clarify if this amine was involved in the punishment avoidance in the walking ball paradigm.

Therefore, *Tβh* mutants were subjected to 5 laser stimulations, the 5 pulses were pooled together for quantification. It was observed that *Tβh* mutants basal activity before stimulation was similar to its genetic control (Wilcoxon paired signed rank test, rotational activity p=0.589; Z=-0.544, forward activity p=0.857; z=0.181; n=40) but on stimulation with laser they showed lower response in comparison to their controls (Wilcoxon paired signed rank test, rotational activity p=0.038; Z=-2.063, forward activity $p=1.6007E^{-10}$; z=-5.329; n=40) (Fig 30 a, b, c) that could be due to accumulation of excess of tyramine, as tyramine is believed to play a role in locomotion (Saraswati et al., 2004). The *TDC-2* mutants show an overall decrease in their basal locomotion (Wilcoxon paired signed rank test, pre and post-stimulation rotational activity and forward activity $p=1.734E^{-18}$; Z=6.732; n=40) (Fig 31 a, b, c).

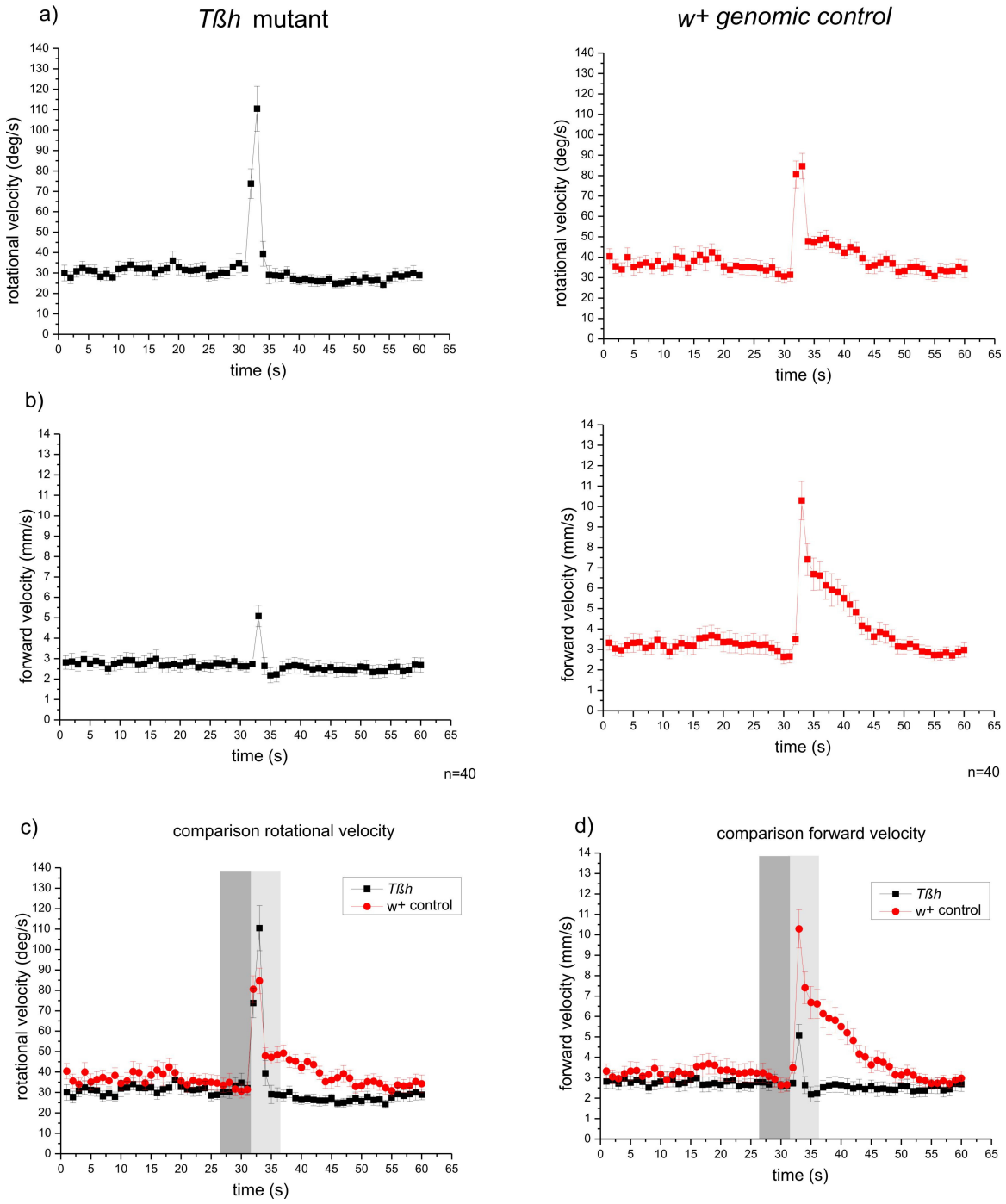


Fig. 30 *Tβh mutants show lower response to the laser stimulation compared to their controls. The basal rotational activity before stimulation of Tβh mutants is comparable to its control (a, c) but during and after stimulation the response is lower compared to the control.The basal forward activity of the Tβh mutant before stimulation is comparable to its control but lower response is observed on stimulation with laser (b, d)*

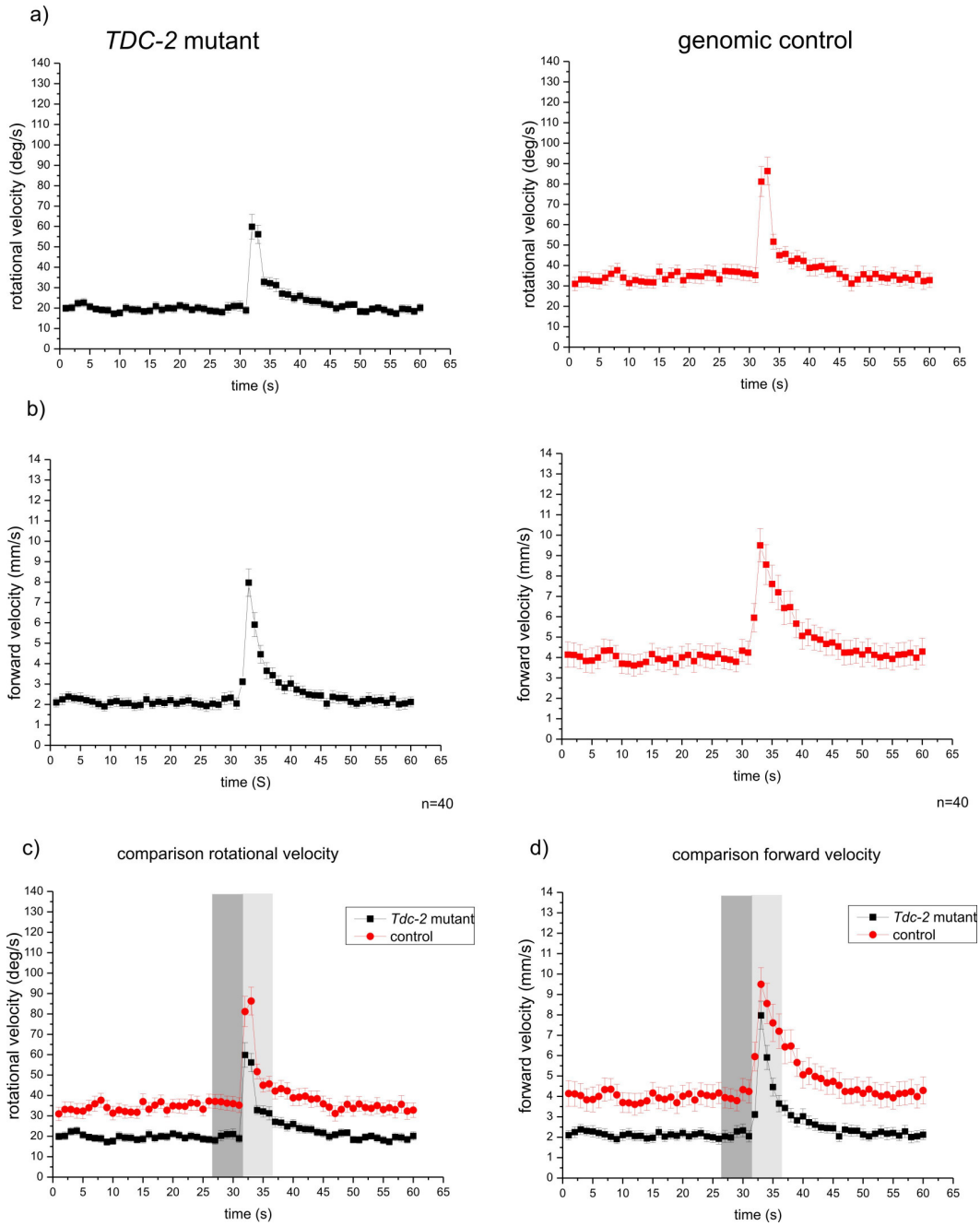


Fig. 31 *TDC-2 mutants show lower basal locomotion and lower response to the laser stimulation compared to their controls. The overall rotational activity and response to laser stimulation of TDC-2 mutant is lower when compared to the control (a, c).The overall forward activity of the TDC-2 mutant and the response to IR laser is reduced when compared to the control (b, d)*

The *Tβh* mutant master group on laser training preferred the unpunished direction (One sample Wilcoxon signed rank test, in training p=0.046, Z=-1.98; n=22) similar to the master of the W^+ control group (One sample Wilcoxon signed rank test, in training

p=3.218E^{-4}, Z= -3.453; n=40). The slave of *Tβh* mutant (One sample Wilcoxon signed rank test, in training p=0.848, Z= 0.194; n=22) and W^{+} control group (One sample Wilcoxon signed rank test, in training p=0.289, Z=1.067; n=40) showed no directional preference (Fig.32).

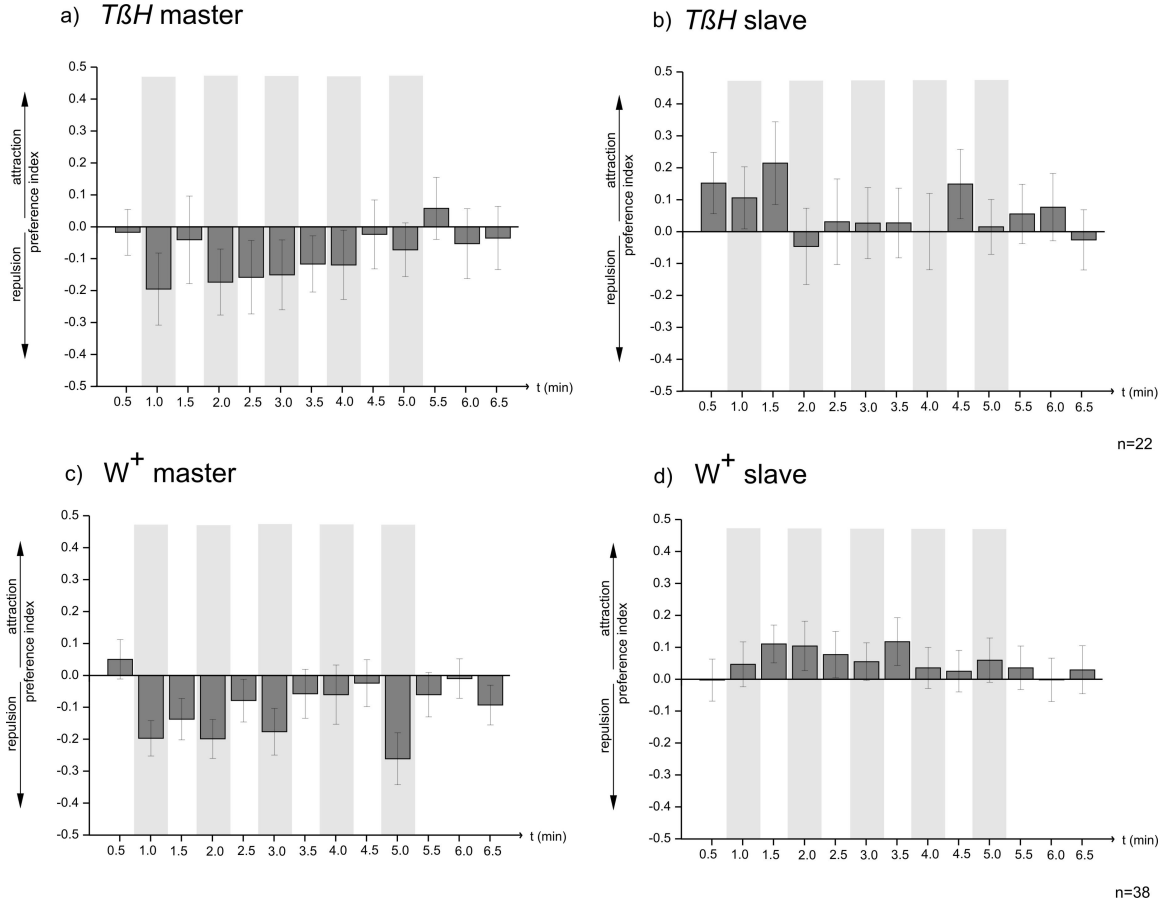


Fig. 32 *Tβh mutant animals can be operantly trained on the walking ball apparatus. The Tβh mutant and CS master group could operantly choose the unpunished direction when trained on the walking ball apparatus (a, c). The slaves for both groups which received punishment in temporal coherence with their masters did not show any directional preference (b, d)*

In conclusion, Tβh mutants that are affected in the synthesis of octopamine and may have altered levels of tyramine did not show any defect in the operant behavior tested here.

3.5 Silencing of aminergic neurons

Amines have functions as neurotransmitters, neuromodulators and neurohormones. Any disruption in the signaling of these amines could lead to a large variety of behavioral changes. Most of the biogenic amines have been proposed to play a role in value based evaluation of the environment and response to the environment. We wanted to investigate if silencing of aminergic neurons had an effect on heat mediated avoidance in the walking ball paradigm.

3.5.1 Silencing of dopaminergic neurons

Dopaminergic neurons labeled by TH Gal4 were temporally silenced by expressing UAS-Kir (Potassium inward rectifier) in conjunction with tubulin gal-80^{ts} which permited temporal activation of Gal4 on heat induction for 24 hours at 31°C The temporal silencing of dopaminergic neurons led to an overall decrease in locomotion (Wilcoxon paired signed rank test, rotational activity $p=0.019$; $Z=-2.318$, forward activity $p=0.007$; $Z=-2.627$; $n=40$) when compared to its control, but had no effect (Wilcoxon paired signed rank test, rotational activity $p=0.291$; $Z=-1.055$, forward activity $p=0.276$; $Z=-1.095$; $n=40$) on the heat induced response elicited by these animals in comparison to the control animals (Fig. 33 a, b, c).

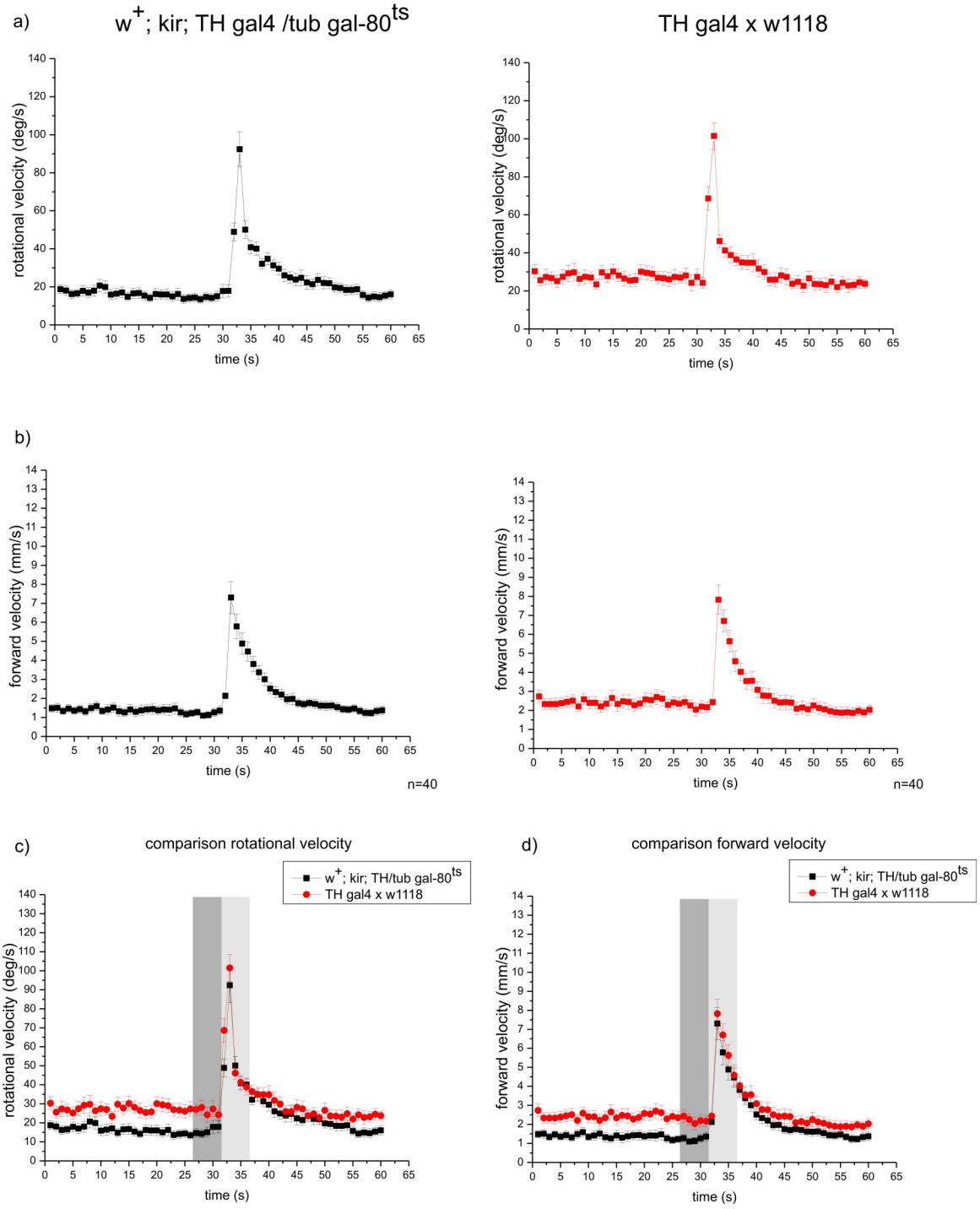


Fig. 33 *Silencing of dopaminergic neurons labeled by TH Gal4 leads to an overall decrease in locomotion when compared to the controls. The silencing of dopaminergic neurons leads to a decrease in basal rotational activity (a, c). A decrease in forward activity is also observed on silencing dopaminergic neurons when compared to controls (b, d), but the response to heat is similar to controls for both parameters.*

The silencing of dopaminergic neurons did not have any noticeable effect on directional heat induced laser avoidance on the walking ball apparatus (Fig. 34 a) (one sample Wilcoxon signed rank test, in training p=5.517E-4; Z=-3.239, in test p=0.213; Z=1.247; n=20). The animals preferred to move in the unpunished direction. The yoked slave control did not show any directional preference (one sample Wilcoxon signed rank test, in training p=0.247; Z=1.168, in test p=0.185; Z=1.327; n=20) (Fig. 34 b).

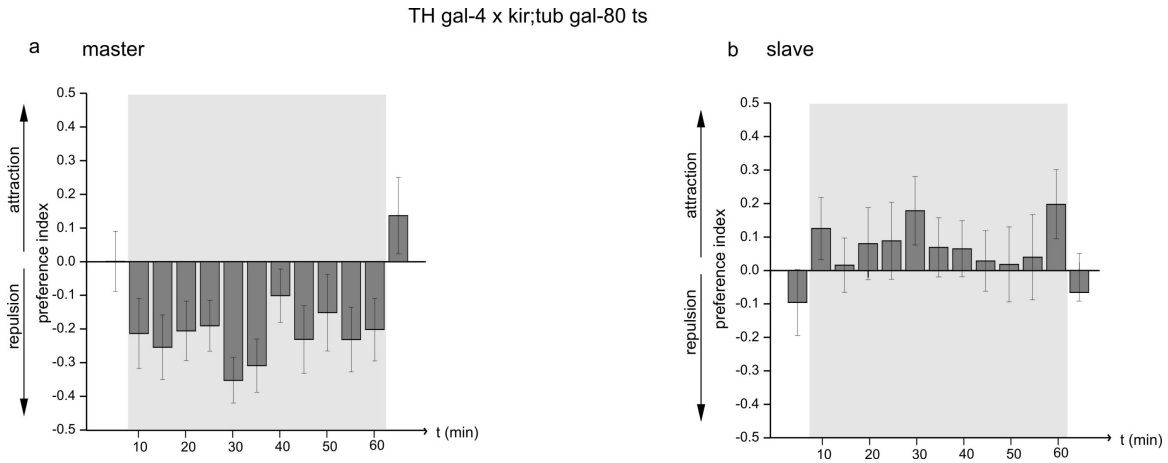


Fig. 34 *Silencing of dopaminergic neurons labeled by TH Gal4 did not have an effect on laser induced avoidance. The master group of flies whose dopaminergic neurons had been silenced could operantly choose the unpunished direction when trained on the walking ball apparatus (a). Their corresponding slave control which received punishment in temporal coherence with their masters did not show any directional preference (b)*

3.5.2 Silencing of dopaminergic and serotonergic neurons

Dopaminergic and serotonergic neurons labeled by DDC Gal4 were temporally silenced by expressing UAS-Kir; tub gal-80^{ts} and the resulting locomotion and response to laser stimulation was quantified. The pre-stimulation locomotion (Wilcoxon paired signed rank test, rotational activity p=0.685; Z=-0.409, forward activity p=0.389; Z=-0.866; n=40) and immediate response (Wilcoxon paired signed rank test, rotational activity p=0.80571; Z=-0.248, forward activity p=0.435; Z= 0.786; n=40) to stimulation remained unaltered in comparison to the controls. However, the animals showed a prolonged decay of the response to heat in comparison to the controls (Fig. 35 a, b, c).

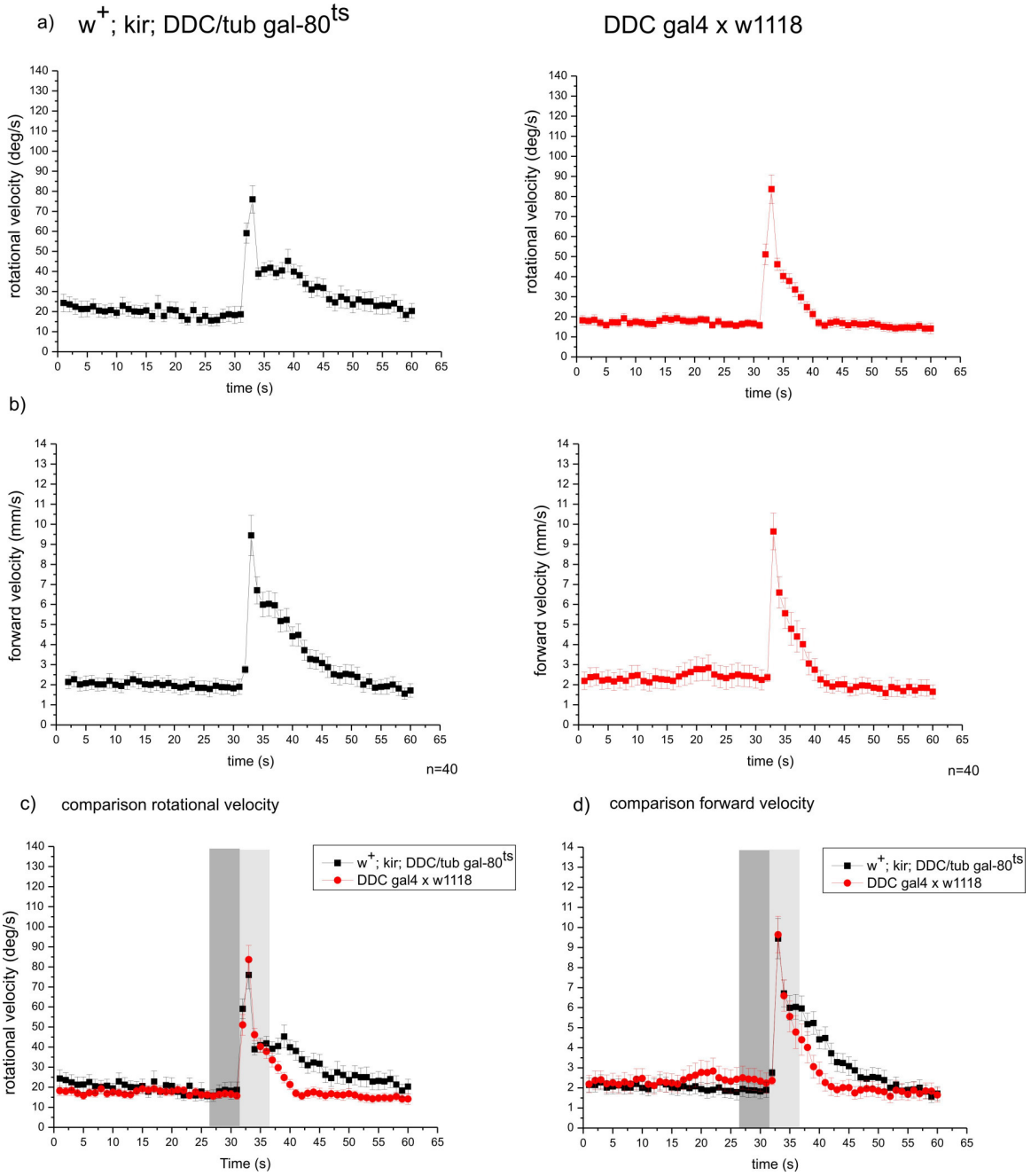


Fig. 35 *Silencing of dopaminergic and serotonergic neurons labeled by DDC Gal4 changes the animal's response to heat. The silencing of DDC labeled neurons leads to a slower decay of rotational activity (a, c) and forward activity (b,d) in response to heat.*

The animals with silenced dopaminergic and serotonergic neurons labeled by DDC Gal4 could operantly choose the unpunished direction (one sample Wilcoxon signed rank test, in training $p=2.136E^{-4}$; Z=-3.283, in test p=0.743; Z=-0.336; n=16) (Fig. 36 a). The slaves did not show a directional preference (one sample Wilcoxon signed rank test, in training p=0.211; Z=-1.266, in test p=0.556; Z=0.611; n=16) (Fig. 36 b)

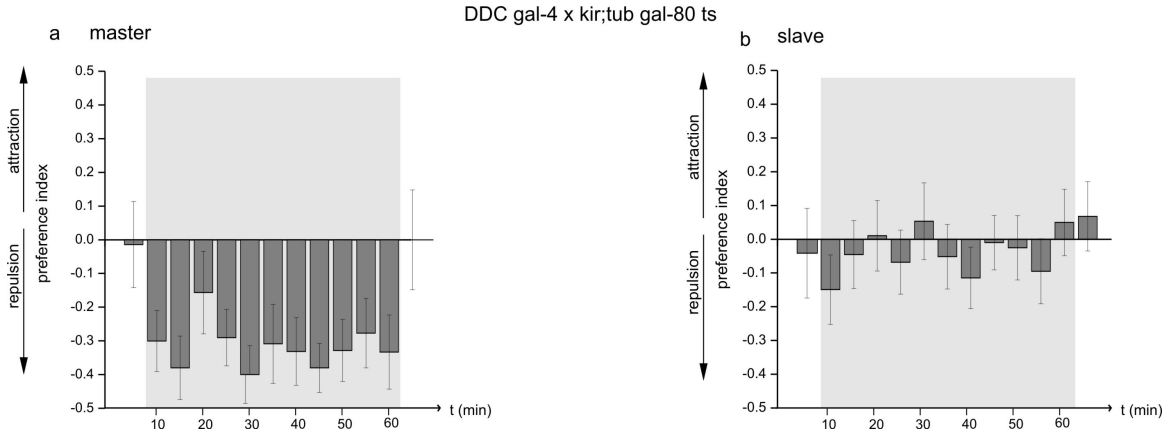


Fig. 36 *Silencing of dopaminergic and serotonergic neurons labeled by DDC Gal4 did not have a noticeable effect on laser induced avoidance. The master group comprising animals with silenced DDC labeled neurons could operantly choose the unpunished direction when trained on the walking ball apparatus (a). Their corresponding slave control which received punishment in temporal coherence with their masters did not show any directional preference (b)*

3.5.3 Silencing of tyraminergic and octopaminergic neurons

Tyraminergic and octopaminergic neurons labeled by TDC-2 Gal4 were silenced by expressing the potassium channel Kir 2.1. Silencing these neurons led to lowered pre-stimulation rotational activity (Fig. 37 a) (Wilcoxon paired signed rank test, $p=4.022E^{-4}$; Z=-3.407; n=40) but forward activity (Fig. 37 b) remained unaltered (Wilcoxon paired signed rank test, p=0.100; Z=-1.646; n=40) in comparison to controls. The post IR laser stimulation rotational and forward response (Wilcoxon paired signed rank test, rotational activity p=0.003; Z=-2.869, forward activity $p=4.527E^{-6}$; Z= -4.254; n=40) was lowered in comparison to the control (Fig. 37 c, d).

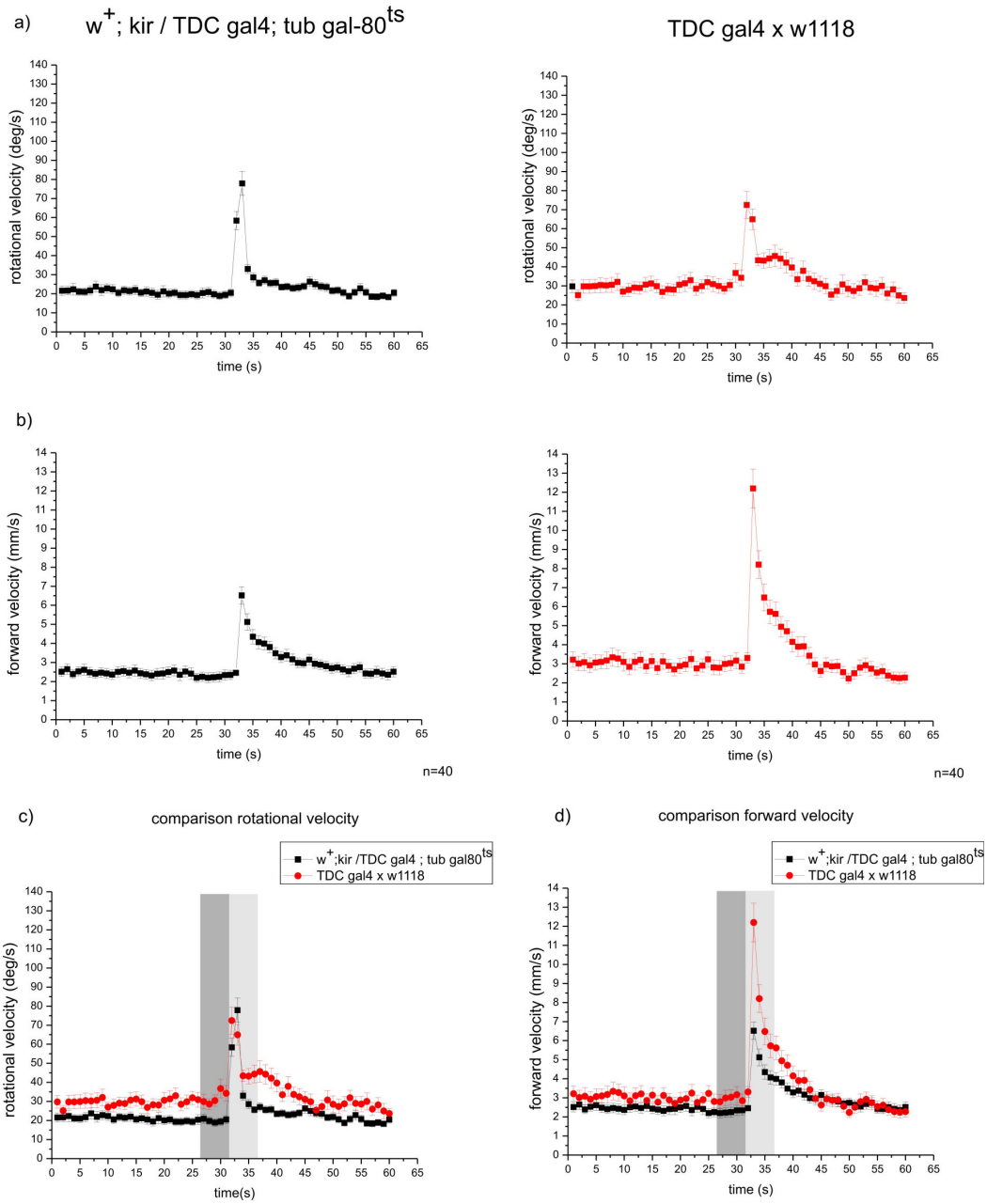


Fig. 37 *Silencing of dopaminergic neurons labeled by TDC-2 Gal4 leads to an overall decrease in rotational locomotion and response to heat stimulation when compared to the controls. The silencing of tyraminergic and octopaminergic neurons leads to a decrease in basal rotational activity and response to laser stimulation (a, c). A decrease in the response to heat is observed when forward activity of the group whose TDC-2 labeled neurons were silenced is compared to their controls (b, d).*

Silencing of tyraminergic and octopaminergic neurons did not impair the operant avoidance to the heat stimulus. The animals continued to move in the unpunished direction (one sample Wilcoxon signed rank test, in training p=5.67E-4; Z=-3.242; n=24)

(Fig. 38 a) whereas the yoked slave control (one sample Wilcoxon signed rank test, in training p=0.811; Z=0.242; n=24) did not show any directional preference (Fig.38 b).

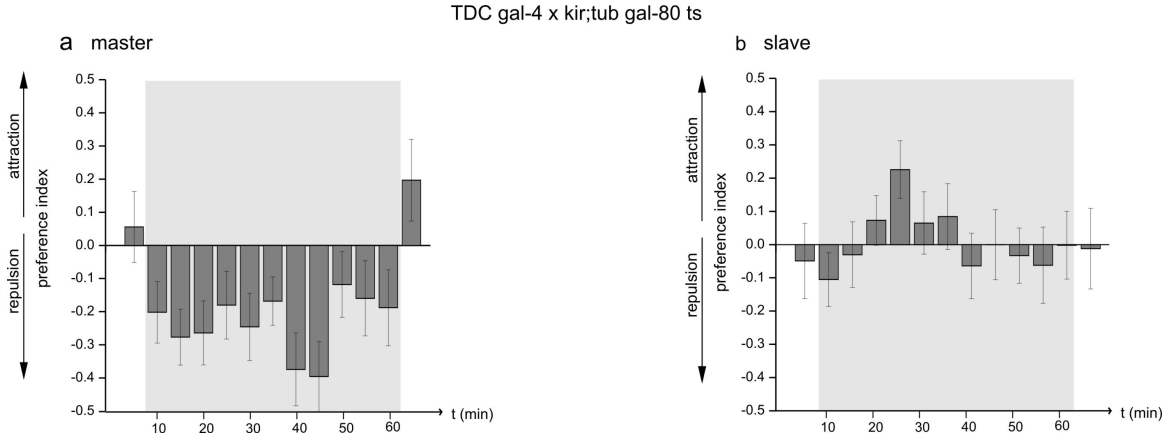


Fig. 38 *Silencing of tyraminergic and octopaminergic neurons labeled by TDC-2 Gal4 did not have an effect on laser induced avoidance. The master group comprising animals with silenced TDC-2 labeled neurons could operantly choose the unpunished direction when trained on the walking ball apparatus (a). Their corresponding slave control which received punishment in temporal coherence with their masters did not show any directional preference (b)*

3.5.4 Silencing of serotonergic neurons labeled by TPH Gal4

Which neurons are actually labeled by the Gal4 line TPH-Gal4. The presumed promoter region of the gene encoding the enzyme tryptophan hydroxylase (TPH) has been used to generate these flies (Borue et al., 2009), and therefore it is assumed that at least a fraction of serotonergic neurons are affected. These presumably serotonergic neurons labeled by TPH Gal4 were temporally silenced by expressing UAS-Kir; tub gal-80^{ts}. The silencing of serotonergic neurons did not affect the operant avoidance behavior of the animals at all. In fact, these animals showed a higher number of movements to the unpunished direction (one sample Wilcoxon signed rank test, in training p=0.003; Z=-2.762; n=14) (Fig. 39 a). The slave control flies did not show a directional preference (one sample Wilcoxon signed rank test, in training p=1; Z= 0; n=14) (Fig. 39 b)

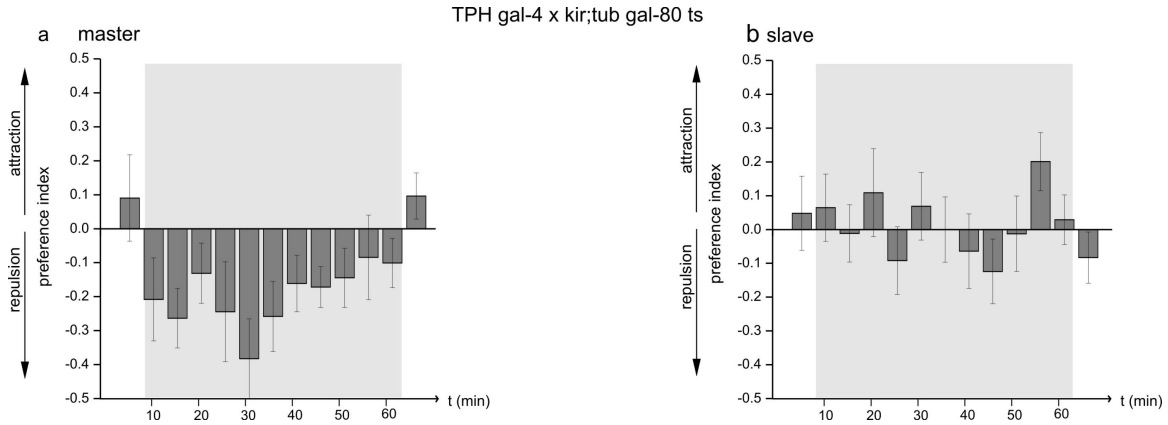


Fig. 39 *Silencing of serotonergic neurons labeled by TPH Gal4 did not have an effect on laser induced avoidance. The master group comprising animals with silenced TPH labeled neurons could operantly choose the unpunished direction when tested on the walking ball apparatus (a). Their corresponding slave control which received punishment in temporal coherence with their masters did not show any directional preference (b)*

Overall, the silencing of selected aminergic neurons used for our study did not have any noticeable effect (statistical test values in individual evaluations) on the operant avoidance behavior of the flies (Fig. 40 a, b) in our experimental setup, although locomotion changes were observed. We conclude that the operant behavior observed represents a pure reflexive avoidance behavior induced by the heat stimulus. That means the behavioral paradigm is not confounded by any experience-dependent change in behavior, which makes it easier to address the main question of this thesis: Do the animals try to avoid or try to achieve the self-activation of aminergic neurons? The avoidance behavior can serve as a behavioral readout to address this question.

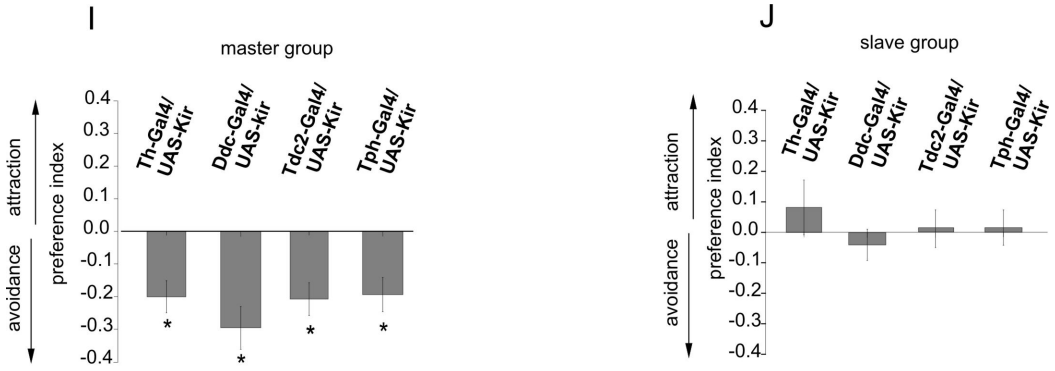


Fig. 40 *Silencing of aminergic neurons did not have an effect on operant avoidance behavior (a). The yoked controls did not show any directional preference (b)*

In conclusion,, after having studied the neuronal silencing effects on animals in locomotion behavior, we wanted to investigate if the activation of aminergic neurons played a role in guiding operant behavior in animals.

3.6 Neuronal activation studies

The aim of the neuronal activation studies was to investigate if the activation of aminergic neurons would cause a behaviorally defined rewarding or punishing sensation. To address this question we intended to operantly activate selected populations of aminergic neurons by expressing channelrhodopsin-2 (a light activated protein) on the ball apparatus and evaluate the preference of the animals. Thus, if animals approached an illuminated direction then the inference would be that the activation of those neurons causes a rewarding sensation and avoidance of the illuminated direction would mean that they dislike the activation of those neurons as it produces a punishing sensation.

First, preliminary experiments investigating how control animals respond to blue light showed us that several problems with this approach had to be solved first. It was observed that control animals which did not express channelrhodopsin-2 avoided the illuminated direction. This was due to two factors: The blue light used for activation of channelrhodopsin-2 was too bright and thus animals avoided it. Secondly the blue light produced an increase in temperature of 1~2 °C which could be easily sensed by the animals. Thus the next goal was to get rid of these secondary effects of light.

3.6.1 Standardization of parameters for neuronal activation studies

3.6.1.1 Bringing transgenic flies genetically into a “blind background”

To get rid of these secondary factors we decided to use all transgenic animals in combination with the mutation *norpA*$^{-}$. The X-chromosome linked *norpA* (No Receptor Potential A) gene codes for a phospholipase C (Hardie et al., 2003), which plays an important role in visual signal transduction pathway. Thus crossings were done for several generations to bring the UAS line into *norpA*$^{-}$ background (Fig. 41)

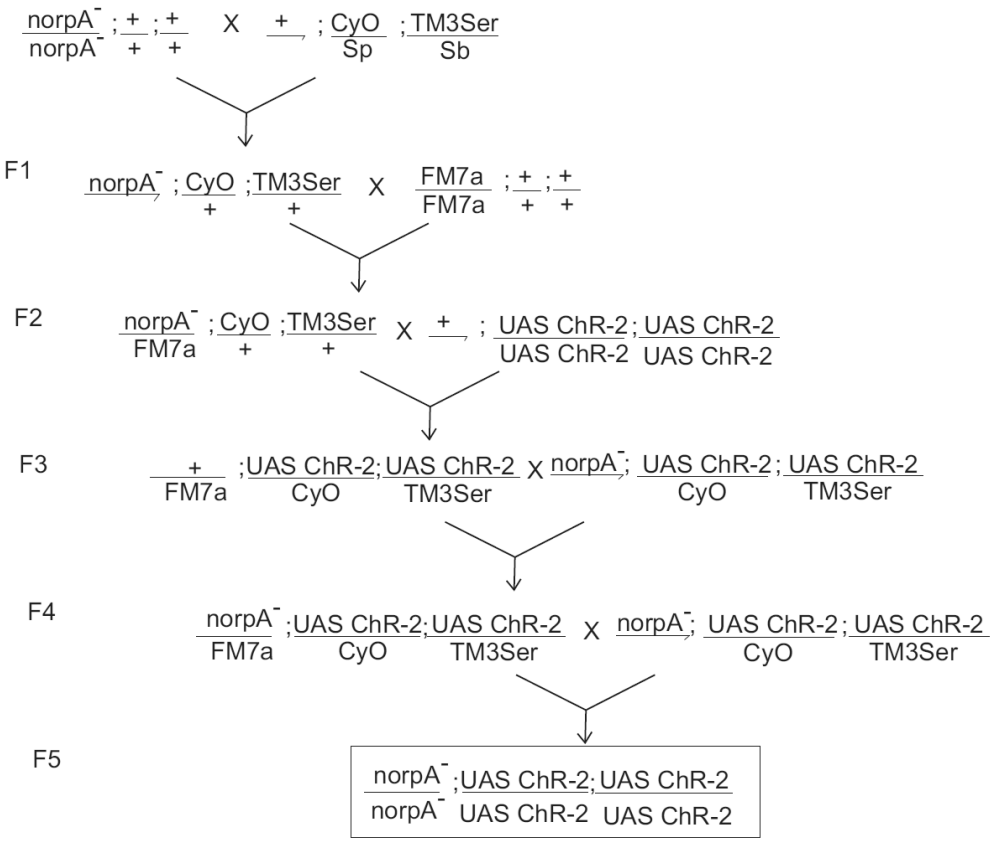


Fig. 41 *Crossing scheme to bring the UAS- channelrhodopsin-2 (ChR-2) in norpA mutant background to make flies blind.*

In a similar crossing scheme as above the norpA$^-$;UAS ChR-2-eYFP; UAS ChR-2-eYFP was generated.

3.6.1.2 Verification of genotype of flies by inverse PCR

Since the flies were crossed for several generations it was important to determine if the genotype of the flies was right. Thus, inverse PCR was done to verify the presence of UAS- ChR2 construct. By sequencing the PCR product UAS-ChR2-eYFP construct was mapped to chromosome 2L (3478271-3478403) and 3R (17122272-17122337) and the UAS-ChR2 construct was also found to have two insertions at 2L (7576522-7576638) and 3L (9638317-9638652).

3.6.1.3 Quantification of secondary effects of blue light

Animals bearing the mutation in the *norpA* gene were compared to CS flies for their response to 5 blue light stimulations. If the visual activation was the only effect of blue light then the animals with disabled vision would not show any change in activity, but if heat was also sensed by the animals then they would show a change in activity. Thus two groups of animals were tested, viz. a group with antennae intact and a group in which antennae had been excised. This was done with both norpA mutants and CS animals. The third segment of antenna is known to play a role in sensing temperature changes (Hamada et al., 2008), because the majority of thermoreceptors is located there. Thus the excision of antennae should minimize the heat effects. It was observed that *norpA*$^{-}$ animals with intact antenna showed an increase in locomotion in response to blue light stimulation but much lower than CS flies (Mann Whitney U test, rotational activity pre-stimulation p=0.175; Z=-1.356, forward activity pre-stimulation p=0.748; Z=-1.356, rotational activity post stimulation p=0.240; Z=1.173, forward activity post-stimulation p=0.006; Z=2.697; n=20 each), whereas norpA^{-} animals with antenna excised did not show a significant increase in locomotion to blue light stimulation (Mann Whitney U test, rotational activity pre-stimulation p=0.123; Z=1.539, forward activity pre-stimulation p=0.074; Z=1.782, rotational activity post stimulation p=0.004; Z=2.849, forward activity post-stimulation p=0.001; Z=3.139; n=20 each) (Fig. 42 a, c) in comparison to CS flies. The comparison of pre and post blue light stimulation period of norpA^{-} animals with excised antennae showed no change in absolute rotational (irrespective of direction) and forward activity (Paired Sample Wilcoxon Signed Rank test, rotational activity p=0.621, Z= 0.503;forward activity p=0.784, Z=-0.28) The control CS animals with normal vision and antenna intact showed an increase in locomotion in response to blue light (higher than norpA^{-} animals), the CS animals with antenna excised showed a lower increase in activity in comparison to CS animals with intact antenna (Fig. 42 b, d).

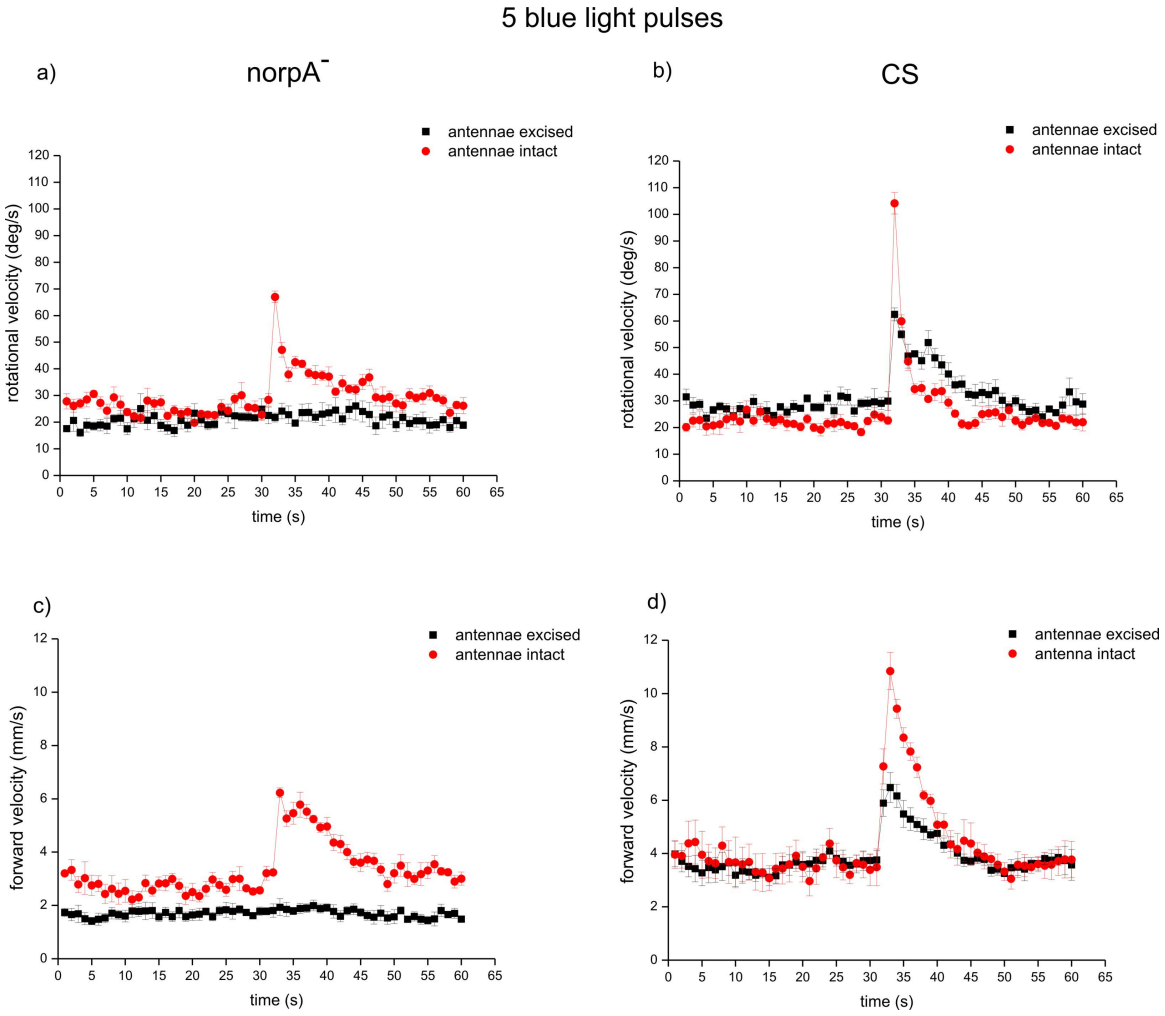


Fig. 42 *The norpA- blind flies show a lower increase in locomotion in response to light, the excision of the antennae further minimizes the secondary effect of light. The norpA- blind flies with antennae intact show higher rotational and forward activity compared to antennae excised group (a, c). CS flies with antenna intact show higher rotational and forward activity compared to norpA- blind flies. The excision of antennae reduces this effect (b, d)*

Also to verify the involvement of the third antennal segment in the detection of temperature, experiments were performed with infrared (IR) laser stimulation to quantify the temperature dependent locomotion changes in norpA- animals. IR laser stimulations were given to both the antennae intact and the antennae excised group of norpA- and CS animals. The norpA- animals without antennae showed a lower increase in locomotion in comparison to antennae intact animals (Wilcoxon paired signed rank test, rotational and forward activity $p=1.734E^{-18}$; Z=-6.732) (Fig. 43 a, c). The CS flies without antenna also showed lower increase in locomotion in comparison to the antennae intact animals

(Wilcoxon paired signed rank test, rotational and forward activity $p=5.67E^{-4}$; Z=-3.242) (Fig. 43 b, d).

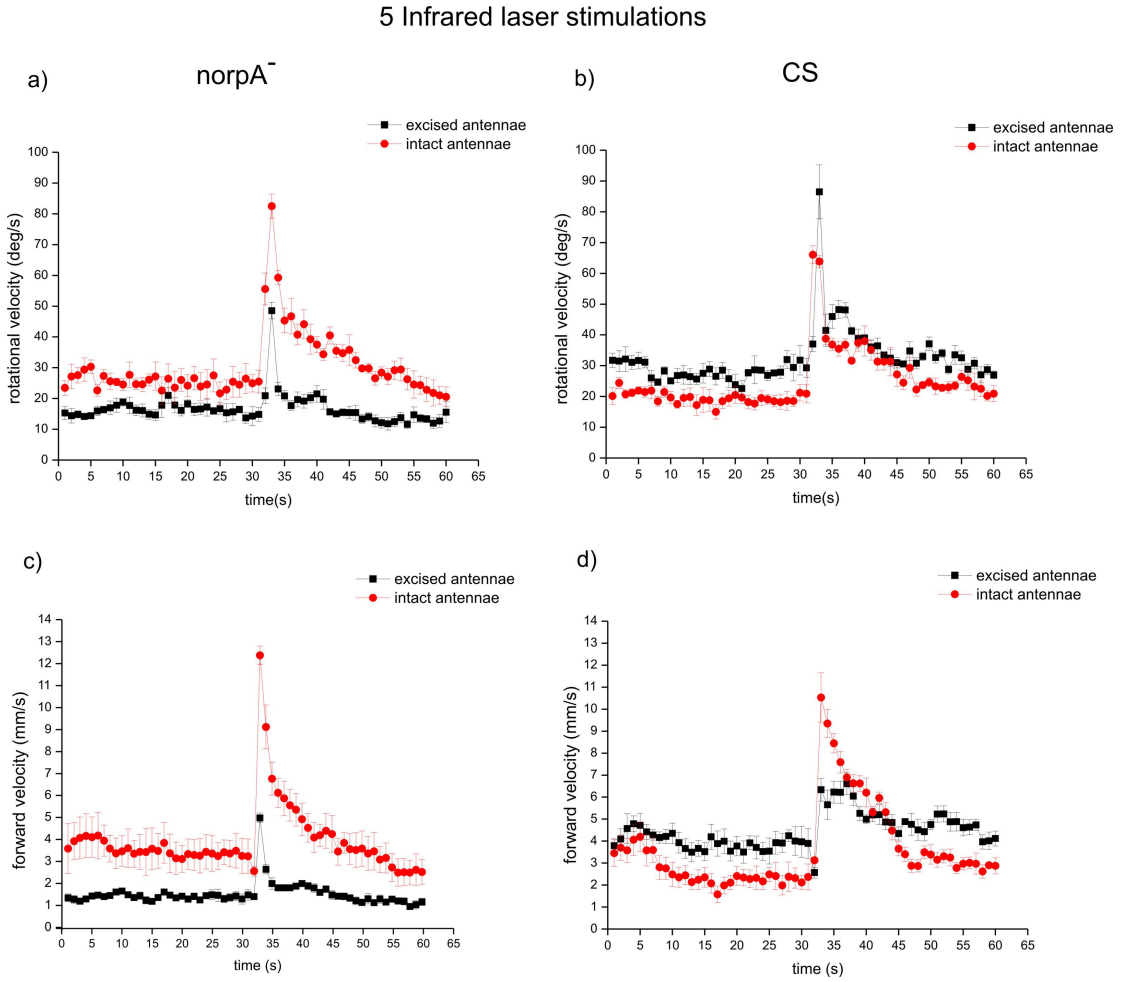


Fig. 43 *The norpA- flies with excised antennae show a lower increase in locomotion in response to infrared laser stimulation. The norpA- blind flies with antennae intact show higher rotational and forward activity compared to the antennae excised group (a, c). CS flies with intact antennae show higher rotational and forward activity compared to CS antennae excised flies (b, d).*

3.6.2 Proof of principle: Training animals by selective activation of neurons using Channelrhosopsin-2

After having ascertained that the flies could operantly avoid the heat on the walking ball apparatus we wanted to test if the selective activation of a subset of neurons expressing channelrhodopsin-2 with blue light was possible and if neurons could be activated. As a first test we chose an optogenetic activation of gustatory receptor neurons. Gustatory receptors are G protein coupled receptors that are normally activated on the

binding of a taste ligand (Amrein and Thorne, 2005). Using computer algorithms many gustatory receptors have been identified. We first demonstrate that it is possible to stimulate neurons that express one such well characterised gustatory receptor, namely Gr5a (Thorne et al., 2004; Thorne et al., 2005), which is activated by binding of sugars, by expressing channelrhodospin-2 using the GAL4-UAS system. The activation of these neurons by blue light was visualised and quantified by measuring the *Proboscis Extension Reflex* (PER, Fig. 44 a, b, c, d, movies on enclosed CD). Next we attempted to activate these neurons by placing a blue light fibre in front of the proboscis and pulsing the light at 40 Hz (22.5 ms off and 2.5 ms on) and simultaneously test them under the condition that light pulsing was activated when the fly turned to one direction by more than 3.6°on the walking ball apparatus. The animals showed approach behavior to the direction that caused an optogenetic activation of gustatory neurons (Fig. 44 e). The animals not only approached the illuminated "rewarded" direction but also kept up with the previously rewarded direction once the illumination was switched off in the test phase after the experiment (one sample Wilcoxon signed rank test, training phase $p=0.011$; $Z=2.494$, test phase $p=0.005$; $Z=2.711$; $n=38$). The corresponding slave control which received blue light stimulation in temporal coherence with its master irrespective of its own turning direction did not show any directional preference (one sample Wilcoxon signed rank test, training phase $p=0.187$; $Z=-1.319$, test phase $p=0.771$; $Z=0.290$; $n=38$) (Fig. 44 f). Of course, to minimise the secondary effects of the blue light we used animals which were brought in norpA^{-} mutant background. The 3rd antennal segment which detects temperature changes was also excised to further minimise these effects.

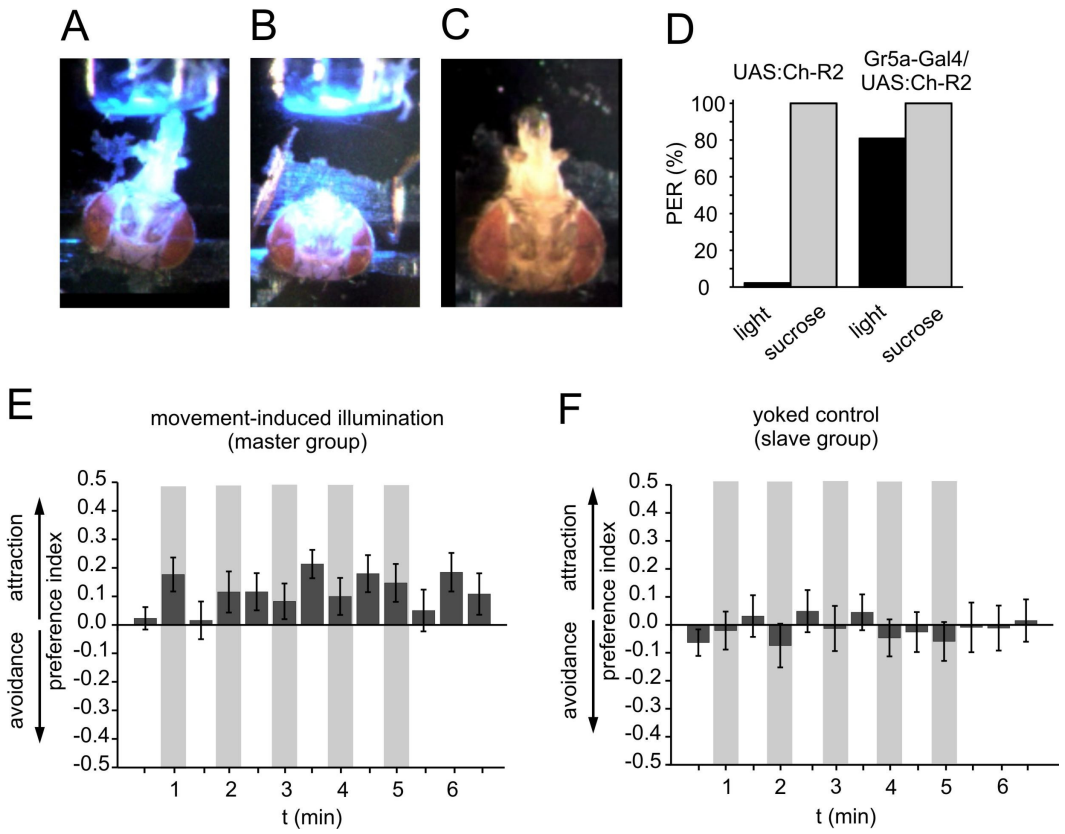


Fig. 44 *The optogenetic activation of sugar sensitive gustatory neurons leads to proboscis extension reflex (PER) and operant approach of the illuminated direction. The control animals with no expression of channelrhodopsin-2 (no Gal4 driver) show no PER (a), the expression of channelrhodopsin-2 in sugar sensitive gustatory receptor (GR5a) leads to PER in response to blue light(b), which is equivalent to responses elicited by sucrose (c).The quantification of PER with sucrose and blue light stimulation is compared for both the control group and the experimental group expressing channelrhodopsin-2 in GR5a neurons (d).Direction contingent activation of GR5a labeled neurons expressing channelrhodopsin-2 on the walking ball leads to preference of the illuminated direction (e). The yoked slave control does not show any directional preference (f). The training phase is indicated by grey bar and test phase with t.*

In conclusion, we have observed that optogenetic activation of gustatory neurons is sufficient to elicit a behavioral response (PER). In addition, the animals can operantly approach those directions that cause the activation of sugar-sensitive neurons on the ball apparatus. After this proof of principle we wanted to investigate whether response driven behavior can also be guided by activation of different aminergic neurons on the ball apparatus.

3.6.3 Activation of modulatory aminergic neurons

3.6.3.1 Activation of dopaminergic neurons labeled by Tyrosine Hydroxylase Gal4

Dopaminergic neurons labeled by TH-Gal4 were selectively activated by expressing channelrhodopsin-2 using the GAL4-UAS system. Tyrosine Hydroxylase (TH) is the rate limiting enzyme in the synthesis of L-DOPA (precursor for dopamine). The activation was again achieved by a blue light fibre which was placed directly above the head capsule but did not make any physical contact with the head. To avoid the inactivation of channels due to continuous and extended illumination, pulsed light at a frequency of 40 Hz (2.5 ms on 22.5 ms off) was used.

The animals showed a slight increase in locomotion on activation of dopaminergic neurons when compared to the controls after pooling both rotation and forward activity (Mann Whitney U test, $p= 4.725E^{-4}$; $Z=3.495$; $n=38$ each) (Fig. 45 a, b). This result complemented the inactivation experiments performed in the same neuronal subsets by expressing Kir (potassium inward rectifier) which inhibits the depolarisation of these neurons (see Fig. 33). However, a slight effect of the blue light on the control animals remains as they show a small increase in locomotor activity as well.

a)

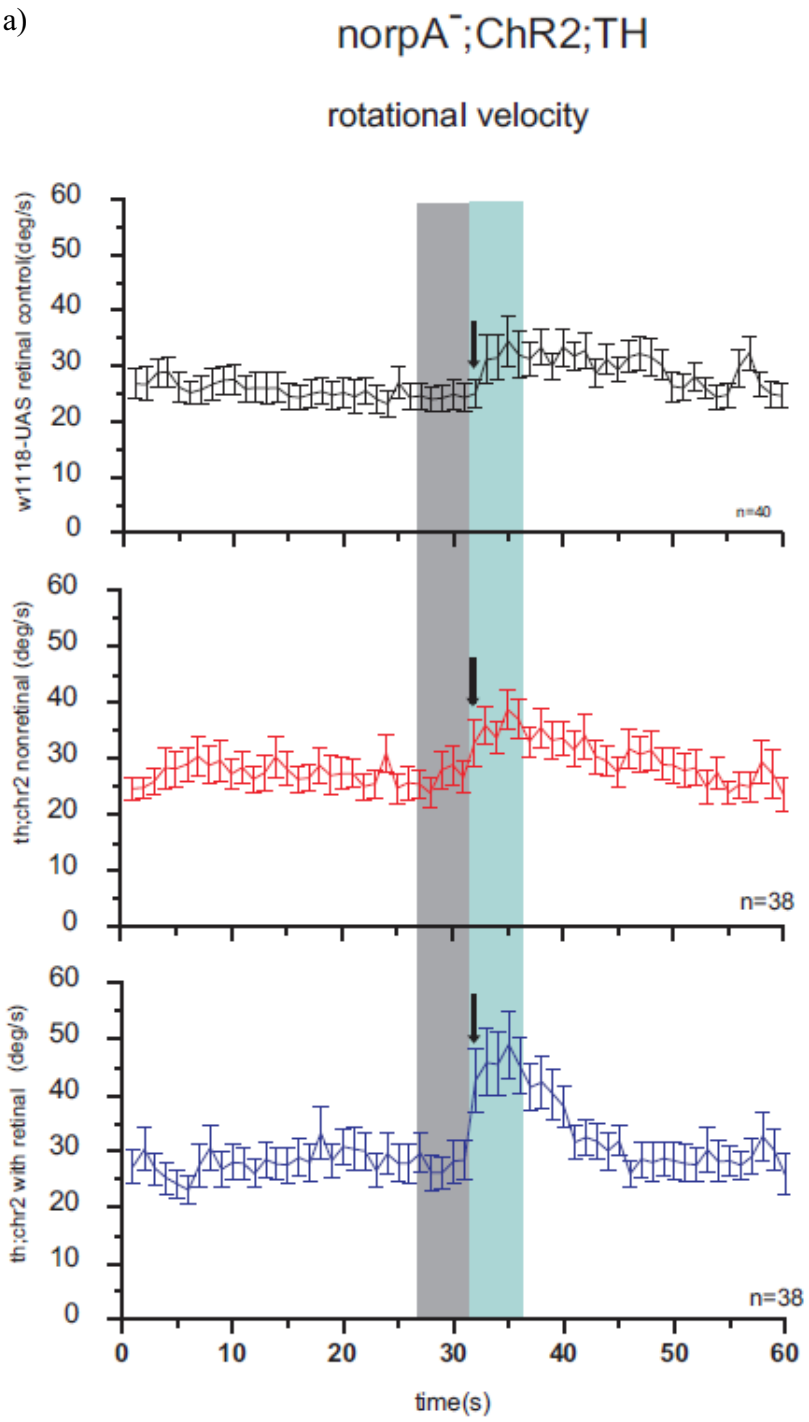


Fig. 45 a *The activation of dopaminergic neurons labeled by TH Gal4 leads to an increase in rotational activity. The norpA⁻;UAS-ChR2 X W^{1118} control flies and the norpA⁻;ChR-2;TH flies without all-trans retinal (non-ATR flies) flies showed lower change in rotational activity response to blue light stimulation (blue shaded region) when compared to norpA⁻;ChR-2;TH flies raised on ATR (bottom panel).*

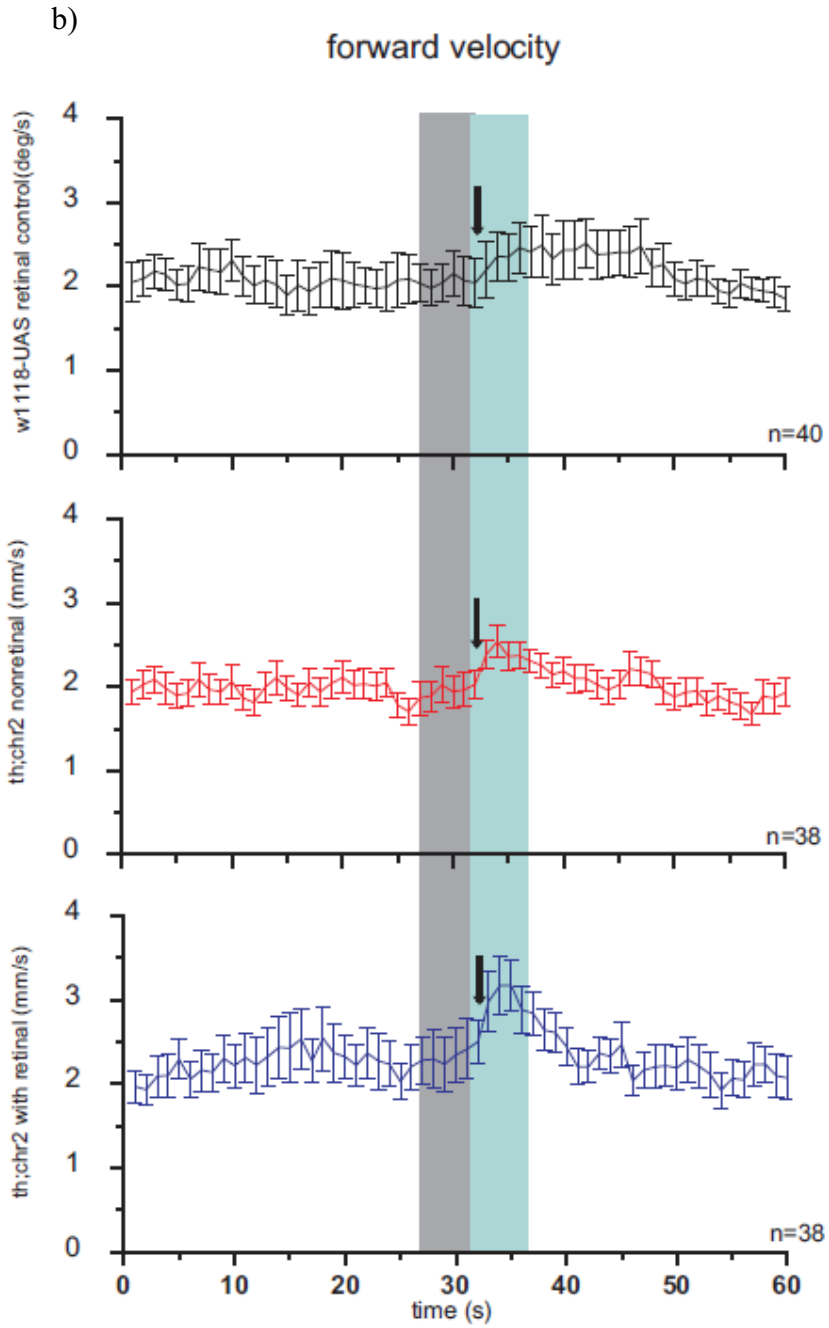


Fig. 45 b *The activation of dopaminergic neurons labeled by TH Gal4 leads to an increase in forward movement activity. The norpA⁻;UAS-ChR2 X W^{1118} control flies and the norpA⁻;ChR-2;TH non ATR flies showed a slightly lower change in forward activity response to blue light stimulation (blue shaded region) when compared to norpA⁻;ChR-2;TH flies raised on ATR (bottom panel).*

In the next experiment we subjected the animals to the walking ball paradigm and designed it so that the activation of dopaminergic neurons was dependent on the direction which animals chose; only one of the two directions was associated with blue light

stimulation and thereby an optogenetic activation of neurons. Interestingly, it was observed that animals avoided the situations which lead to activation of dopaminergic neurons labeled by TH Gal4 and preferred the non illuminated direction for walking (One sample Wilcoxon signed rank test, in training p= 0.001; Z=-3.042, in test p=0.495; Z=-0.696; n=20) (Fig. 46). The yoked slave controls did not show any directional preference (One sample Wilcoxon signed rank test, in training p= 0.701; Z=0.391; n=20).

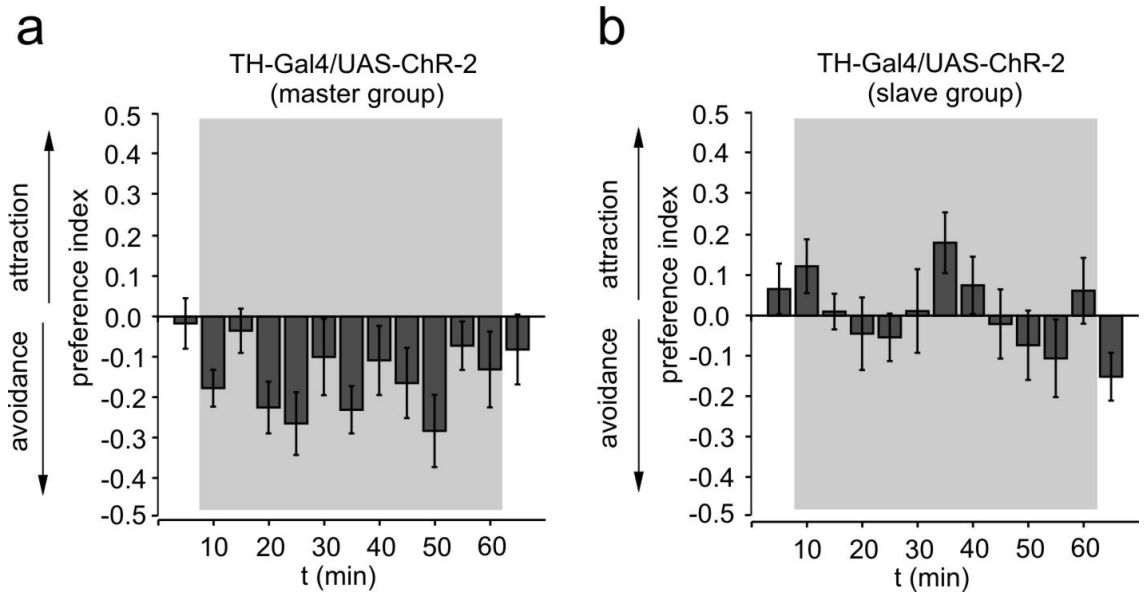


Fig. 46 *The direction specific activation of dopaminergic neurons labeled by TH Gal4 leads to avoidance of the direction that is coupled to their activation. The master shows higher number of movements to the non-illuminated direction (a). The yoked slaved control did not show any directional preference (b)*

This experiment proved that the animals avoid those turning actions that cause an activation of dopaminergic neurons labeled by TH Gal4. Next we wanted to investigate if the same was observed when a different subset set of dopaminergic neurons labeled by DDC Gal4 was activated. This Gal4 line encompasses a different population of dopaminergic neurons and, in addition, a subset of serotonergic neurons.

3.6.3.2 Activation of dopaminergic and serotonergic neurons labeled by Dopa-Decarboxylase Gal4

Dopa-decarboxylase (ddc) is an enzyme for the conversion of L-DOPA to dopamine and for the conversion of 5 hydroxytryptophan to serotonin. On activation of neurons expressing channelrhodopsin-2 under the control of DDC Gal4 by optical stimulation the animals showed an increase in overall locomotion (Mann Whitney U test, p= 0.019; Z=2.342; n=34 for experimental group, n=27 for control) (Fig. 47 a, b). Surprisingly, the inactivation of these neurons using Kir also increases the basal locomotion and responsiveness to laser stimulation (See Fig. 35).

a)

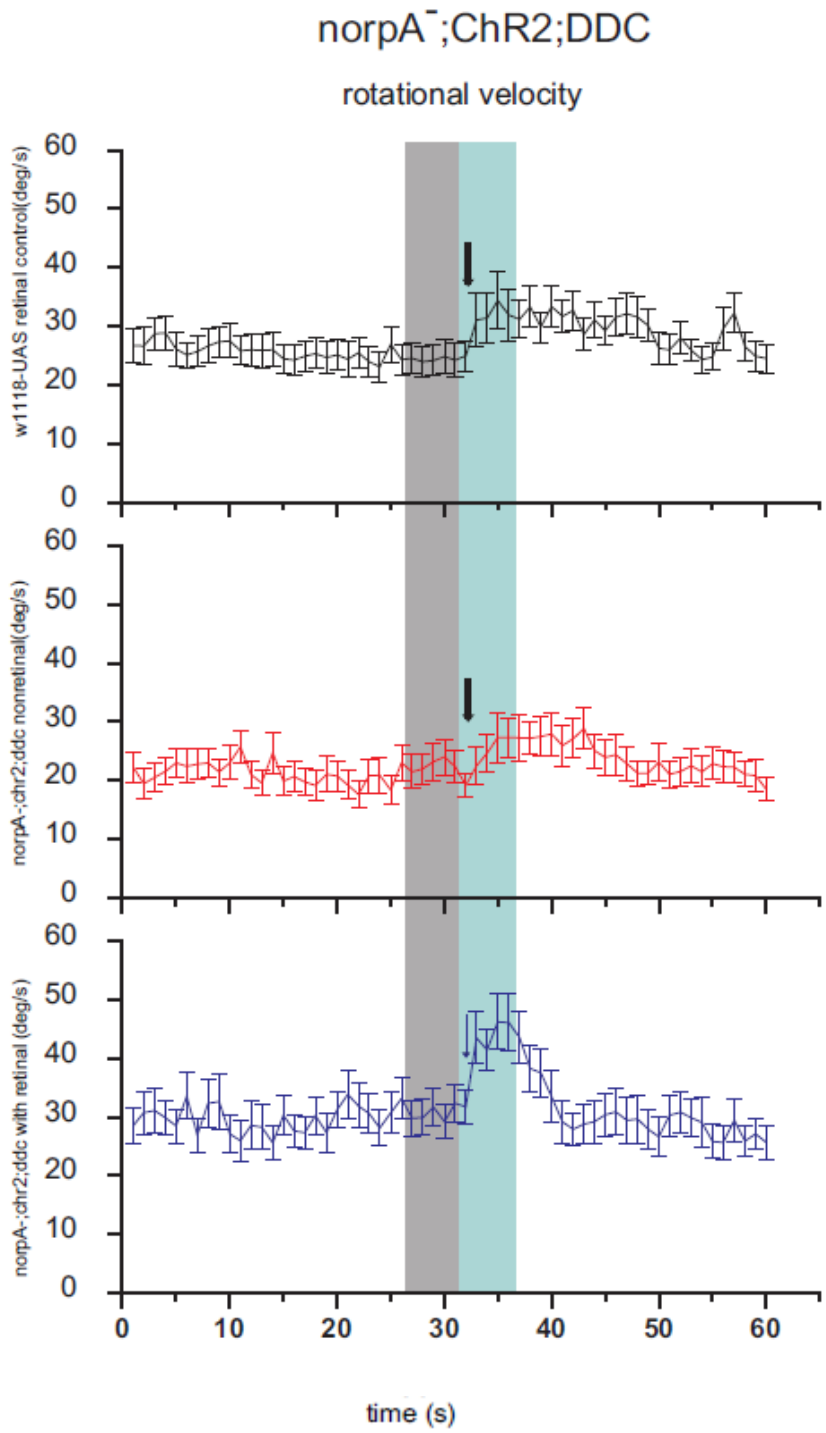


Fig. 47 a *The activation of dopaminergic and serotonergic neurons labeled by DDC Gal4 leads to an increase in rotational activity. The norpA-;UAS-ChR2 X W^{1118} control flies and the norpA-;ChR-2;DDC non ATR flies showed lower change in rotational activity in response to blue light stimulation (blue shaded region) when compared to norpA-;ChR-2;DDC flies raised on ATR (bottom panel) .*

b)

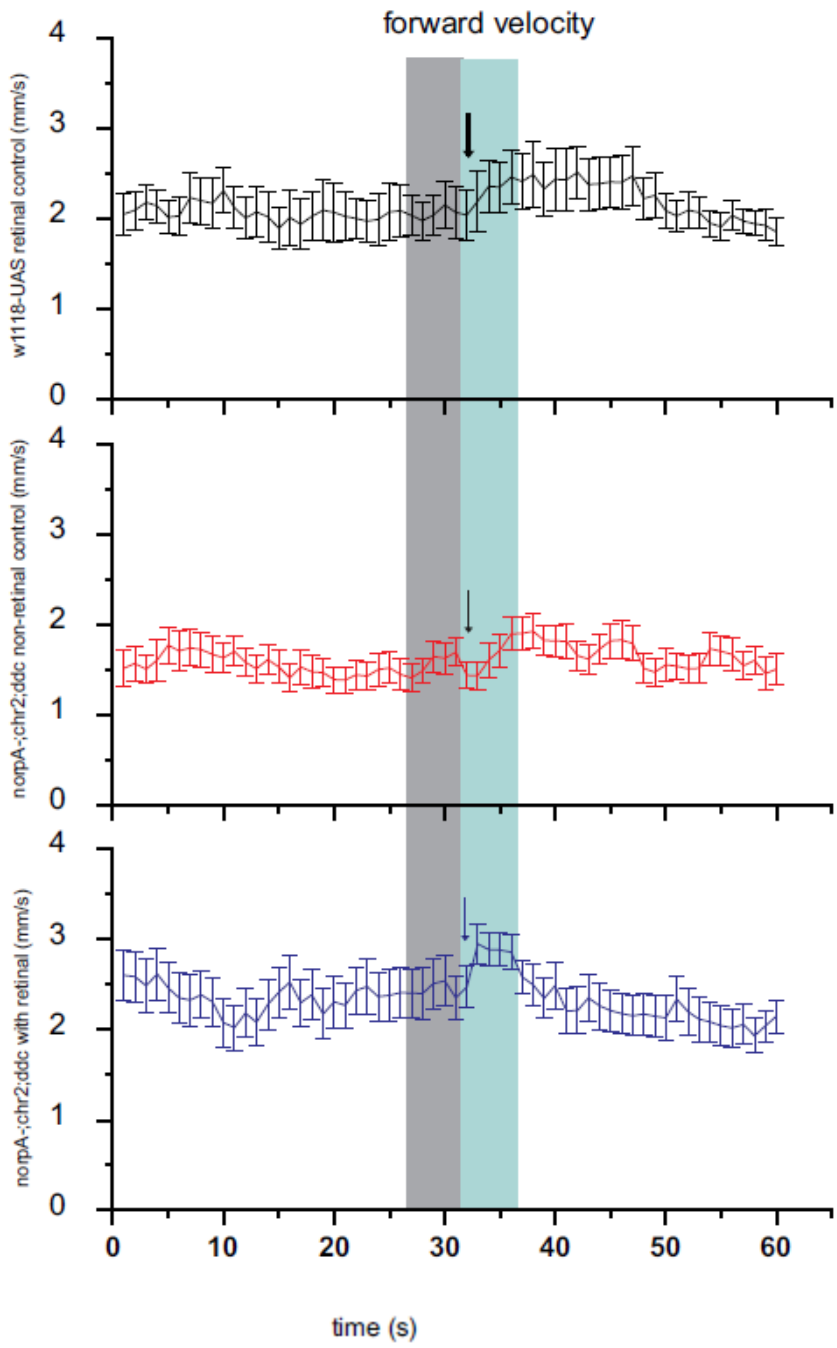


Fig. 47 b *The activation of dopaminergic and serotonergic neurons labeled by DDC Gal4 leads to an increase in forward movement activity. The norpA⁻;UAS-ChR2 X W^{1118} control flies and the norpA⁻;ChR-2;DDC non ATR flies showed lower change in forward activity in response to blue light stimulation (blue shaded region) when compared to norpA⁻;ChR-2;DDC flies raised on ATR (bottom panel).*

When we tested whether the animals operantly avoid or approach those directions that caused an activation of neurons, we found that the animals avoided the situations

which lead to activation of neurons labeled by DDC Gal4, as observed with TH Gal4 (One sample Wilcoxon signed rank test, in training $p=4.183E^{-4}$; Z=-3.352, in test p=0.169; Z= -1.38; n=30) (Fig.48), whereas the yoked slave control showed no preference (One sample Wilcoxon signed rank test, in training p=0.823; Z=0.226; n=30).

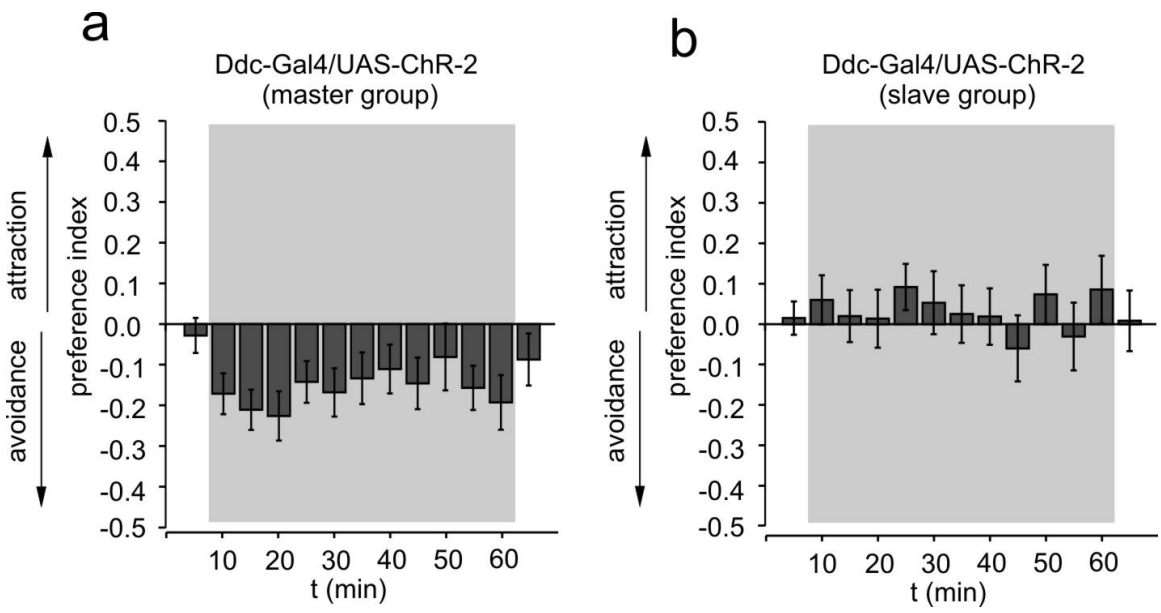


Fig. 48 *The direction specific activation of dopaminergic neurons labeled by DDC Gal4 leads to avoidance of the direction coupled to their activation. The master shows a higher number of movements to the non-illuminated direction (a). The yoked slaved control did not show any directional preference (b)*

Next we wanted to evaluate response of animals when tyraminergic and octopaminergic neurons were activated. From neuronal silencing studies they have been shown to mediate reinforcing effects of a positive stimulus in classical conditioning (Schwaerzel et al., 2003) and thus it is interesting to see whether or not the animals operantly approach situations that lead to activation of these neurons on the ball apparatus.

3.6.3.3 Activation of tyraminergic and octopaminergic neurons

Tyrosine decarboxylase (TDC) is an enzyme responsible for the conversion of tyrosine to tyramine which is the precursor for octopamine. The optical activation of these

neurons by expressing channelrhodopsin-2 caused an increase in locomotion (Mann Whitney U test, p=0.031; Z=2.145; n=33 for experimental group, n=32 for control) (Fig.49 a, b). This result complemented the inactivation experiments which were performed using Kir (see Fig.37).

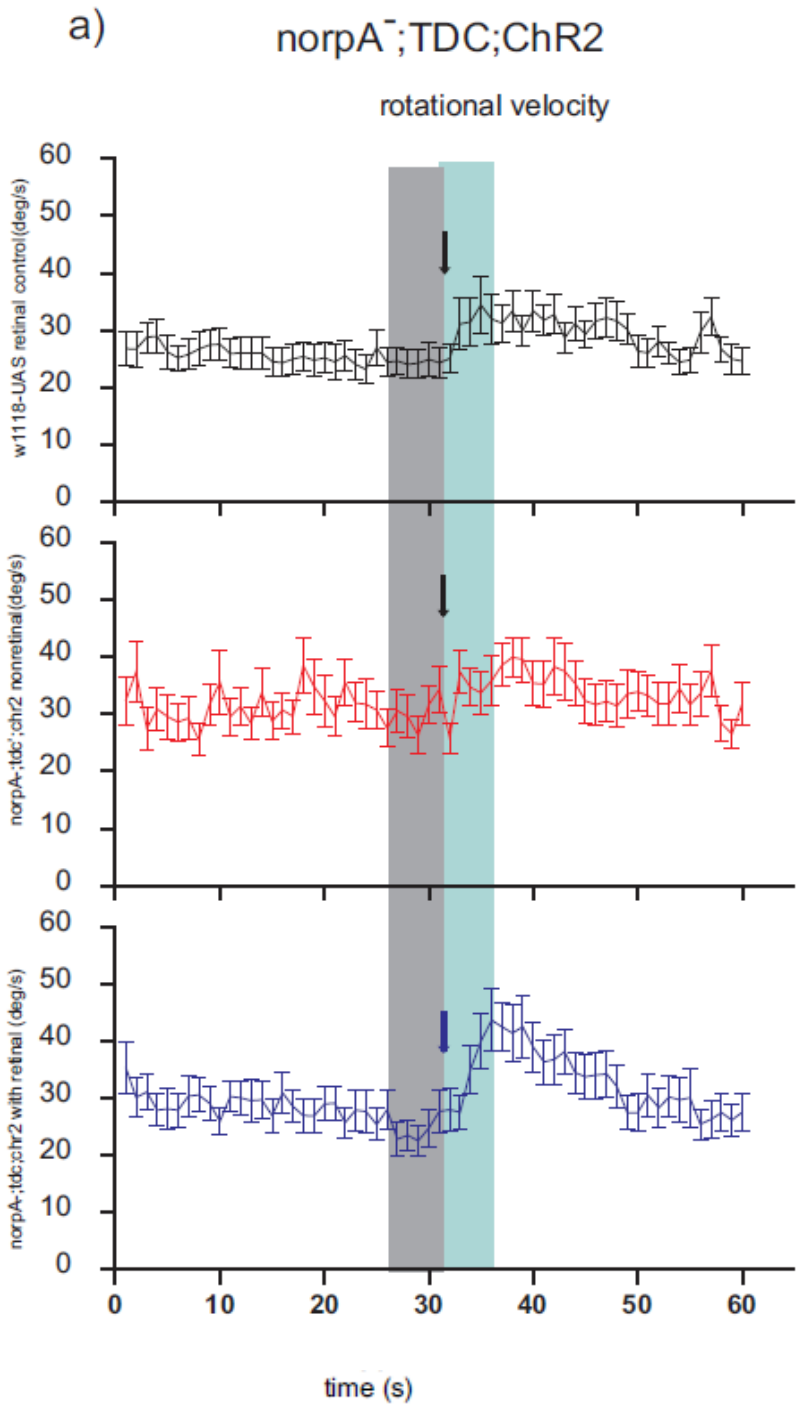


Fig. 49 a *The activation of tyraminergic and octopaminergic neurons labeled by TDC Gal4 leads to an increase in rotational activity. The norpA⁻;UAS-ChR2 X W^{1118} control flies and the norpA⁻;ChR-2;TDC non ATR flies showed lower change in rotational*

activity in response to blue light stimulation (blue shaded region) when compared to norpA⁻;ChR-2;TDC flies raised on ATR (bottom panel) .

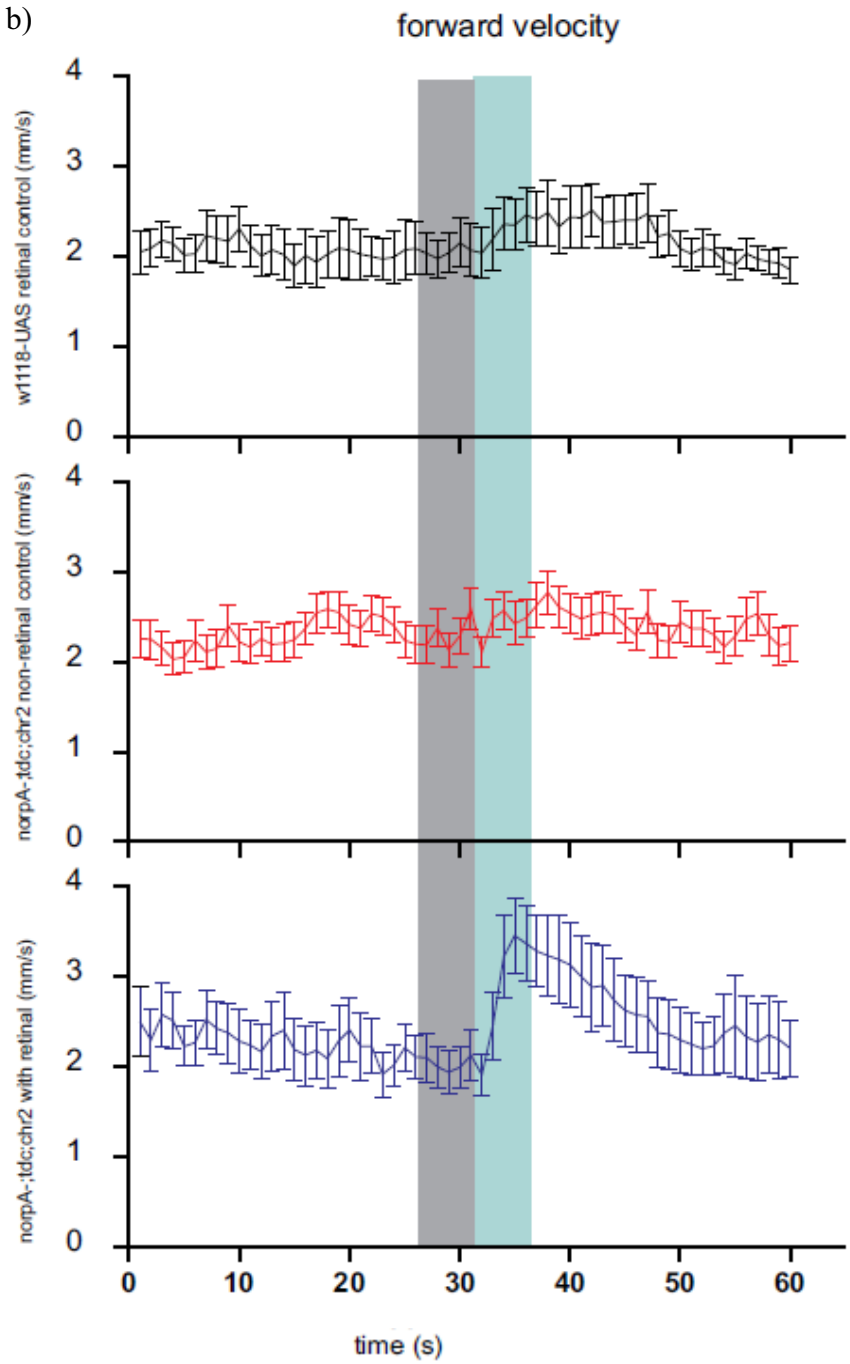


Fig. 49 b *The activation of tyraminergic and octopaminergic neurons labeled by TDC Gal4 leads to an increase in forward movement activity. The norpA⁻;UAS-ChR2 X W^{1118} control flies and the norpA⁻;ChR-2;TDC non ATR flies showed lower change in forward activity in response to blue light stimulation (blue shaded region) when compared to norpA⁻;ChR-2;TDC flies raised on ATR (bottom panel) .*

Next, we wanted to investigate if the activation of these neurons only leads to arousal in these flies or also to an approach to the situations which lead to their activation. We observed that the animals did not show a significant preference to a given direction under these conditions (One sample Wilcoxon signed rank test, in training p=0.583; Z=-0.564, in test p=0.807; Z=0.251; n=14) (Fig. 50 a), the yoked controls also did not show any preference (One sample Wilcoxon signed rank test, in training p=1; Z=0, test p=0.807; Z=0.807; n=14) (Fig. 50 b). We conclude that octopamine/tyramine release does not provide any rewarding or punishing signal to the animal in itself the animal is trying to achieve or to avoid.

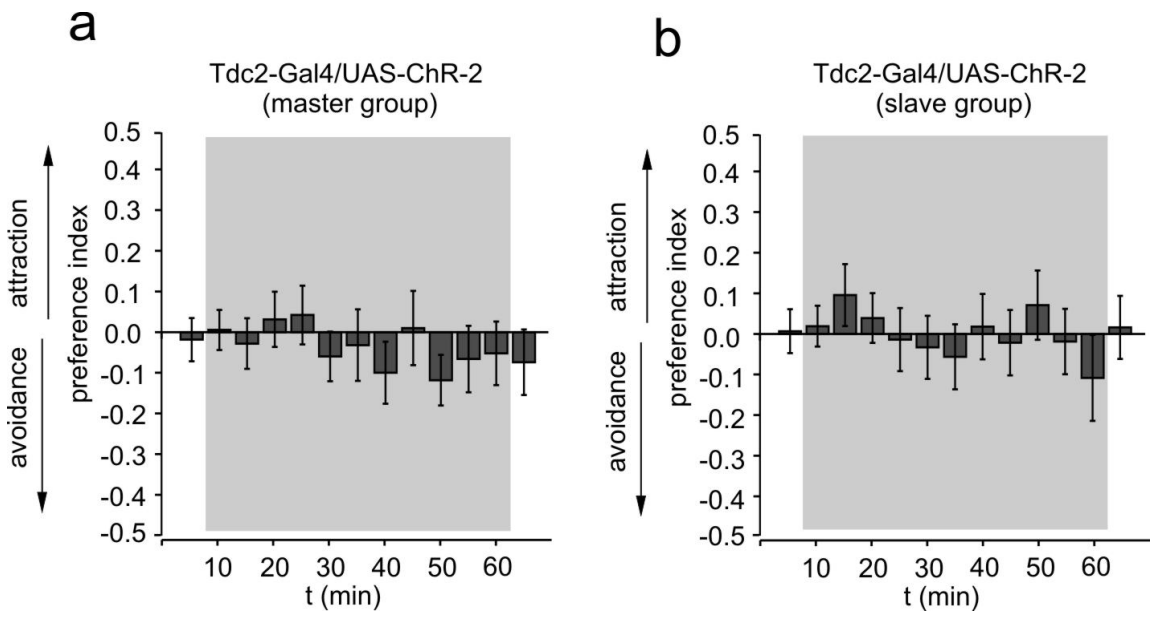


Fig. 50 *The direction specific activation of tyraminergic and octopaminergic neurons labeled by TDC Gal4 has no effect on operant approach or avoidance behavior. The master shows equal number of movements in both illuminated and the non-illuminated direction (a). The yoked slaved control did not show any directional preference either (b).*

3.6.3.4 Activation of putative serotonergic neurons

Tryptophan hydroxylase is an enzyme that is involved in the production of serotonin from tryptophan. Not much is known about neuro-modulatory functions of serotonin in *Drosophila*. We wanted to investigate if activation of serotonergic neurons was involved in value based evaluation in animals where their activation would lead to an approach or avoidance behavior. The Gal4-line Tph-Gal4 has not been described

thoroughly with respect to the neuronal populations that express the Gal4 protein. Therefore, the data achieved using this particular Gal4 line represent only a first step towards the detailed investigation of possible rewarding or punishing effects of serotonin release.

Firstly we wanted to quantify if the activation of the neurons labeled by Tph-Gal4 led to any changes in locomotion behavior. Thus, the animals were subjected to 5 blue light pulses and their rotational and forward activity was quantified. It was observed that activation of these neurons did not produce any significant change in rotational (Fig. 51 a) or forward movement activity (Fig. 51 b) (Mann Whitney U test, $p= 0.990$; $Z=0.011$; $n=40$ for experimental group, $n=31$ for control).

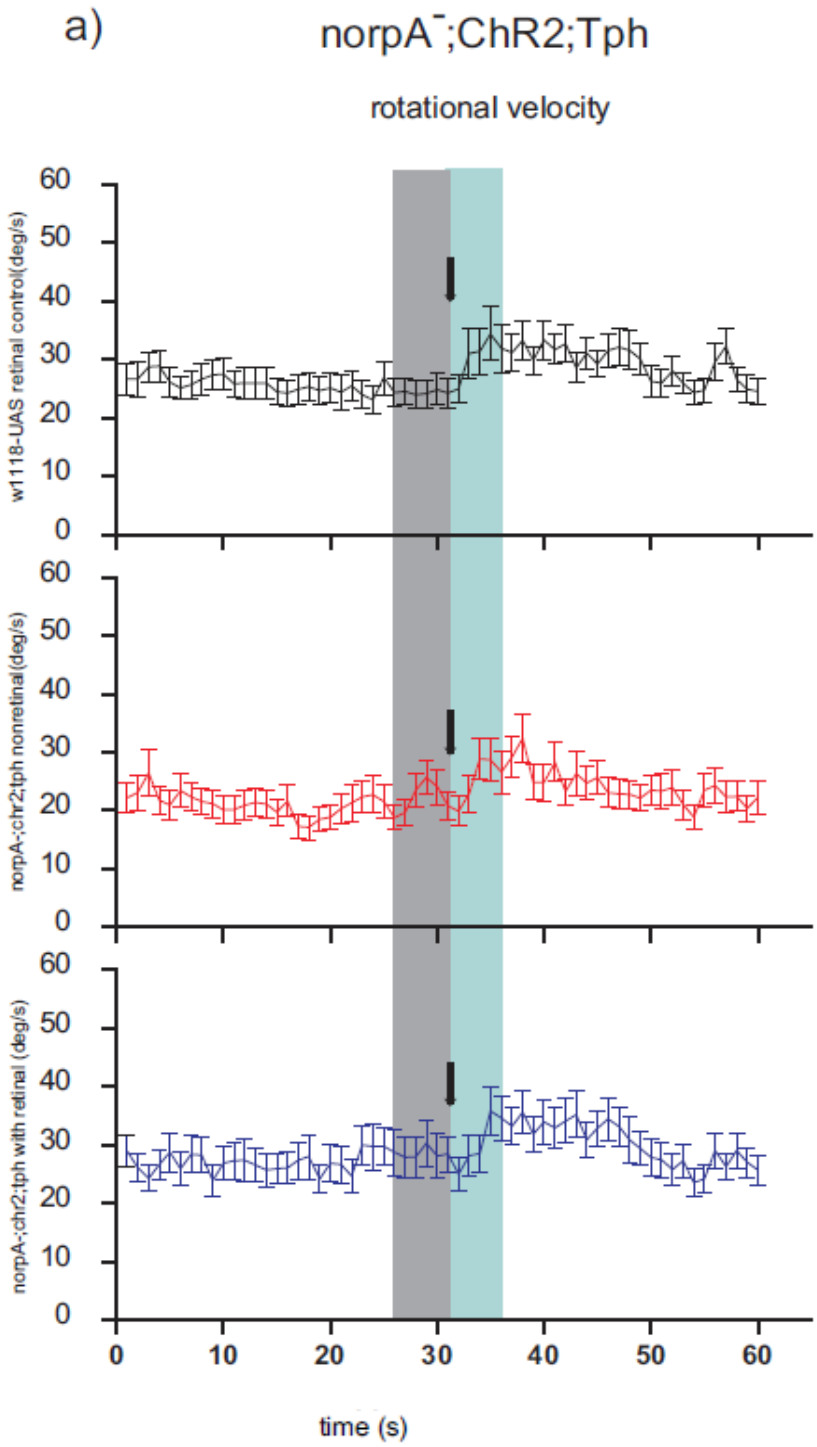


Fig. 51 a *The activation of serotonergic neurons labeled by TPH Gal4 did not have any effect on rotational activity. The norpA-;UAS-ChR2 X W^{1118} control flies and the norpA-;ChR-2;TPH non ATR flies showed changes in rotational activity similar to norpA-;ChR-2;TPH flies raised on ATR in response to blue light stimulation (blue shaded region) .*

b)

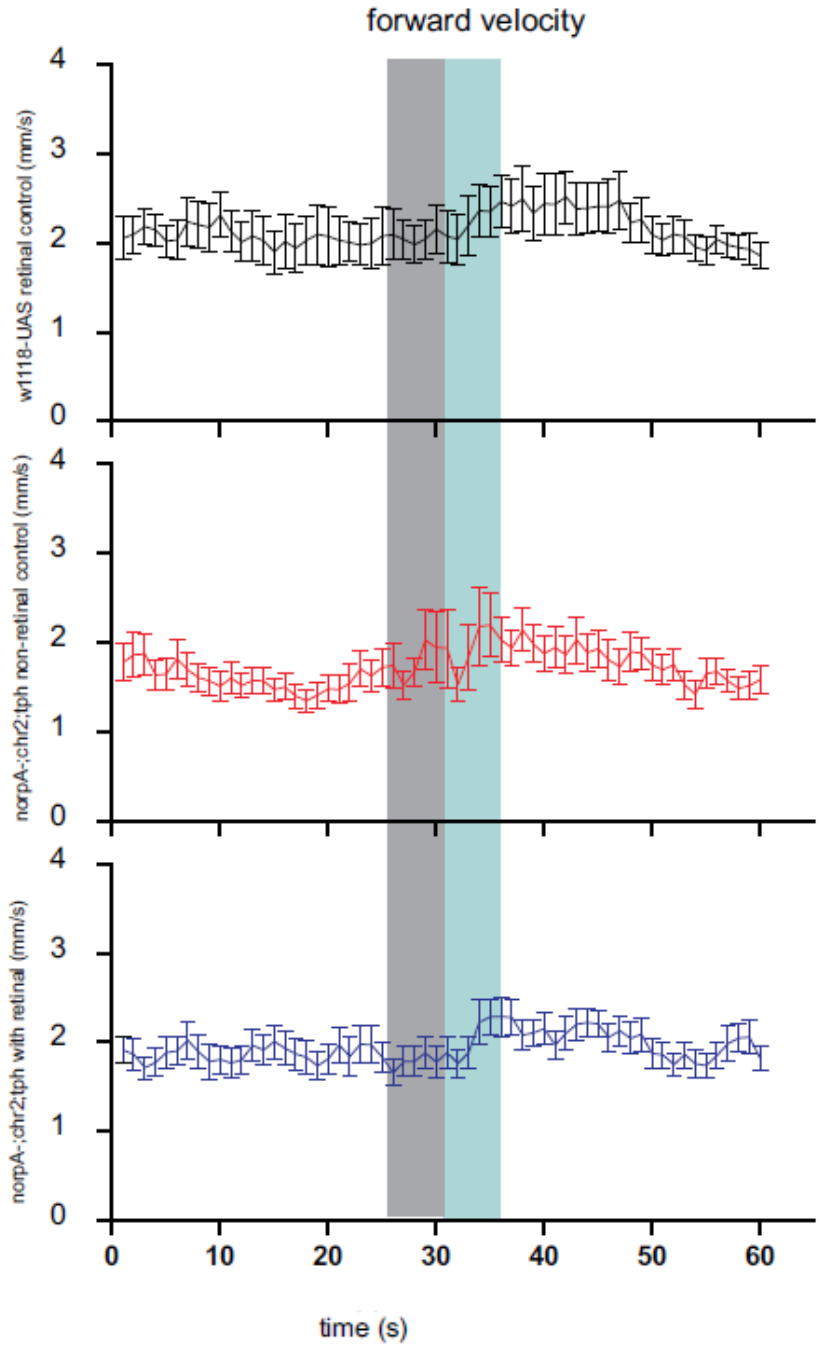


Fig. 51 b *The activation of serotonergic neurons labeled by TPH Gal4 did not have any effect on forward movement activity. The norpA⁻;UAS-ChR2 X W^{1118} control flies and the norpA⁻;ChR-2;TPH non ATR flies showed changes in forward activity similar to norpA⁻;ChR-2;TPH flies raised on ATR in response to blue light stimulation (blue shaded region) .*

Next the animals were subjected to the approach/avoidance paradigm in which the walking direction caused the light-activation of Tph-positive neurons on the ball apparatus. It was observed that animals moved equally in both directions and did not

prefer any direction (One sample Wilcoxon signed rank test, in training p=0.31179; Z=-1.026, in test p=0.822; Z=-0.228; n=20) (Fig. 52a). The yoked slave control did not show any directional preference either (One sample Wilcoxon ranked test, in training p=0.388; Z=0.877; n=20) (Fig. 52 b).

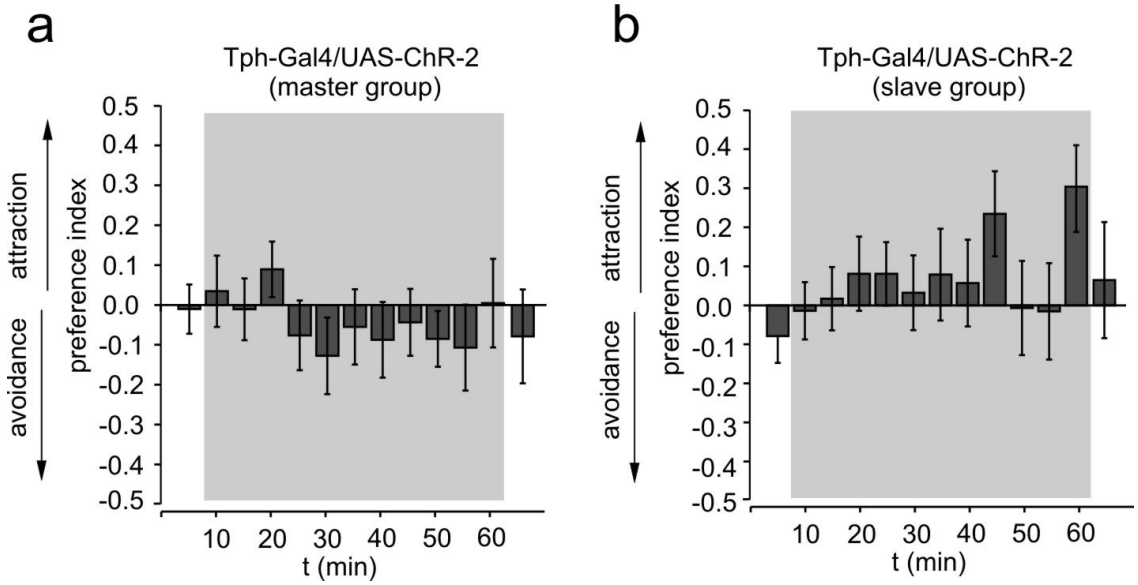


Fig. 52 *The direction specific activation of serotonergic neurons labeled by TPH Gal4 had no effect on operant approach or avoidance behavior. The master shows non-significant difference in the number of movements in both illuminated and the non-illuminated direction (a). The yoked slaved control did not show any directional preference as well (b).*

3.6.4 Summary of master slave neuronal activation studies

Direction contingent activation of dopaminergic neurons labeled by TH Gal4 or DDC Gal4 led to an avoidance of situations that caused their activation group. The activation of neurons labeled by TDC and Tph Gal4 did not have an effect on directional preference of the animals on the ball apparatuses. The yoked control group did not show any directional preference (Fig.53) (for statistical tests refer to the individual sections above).

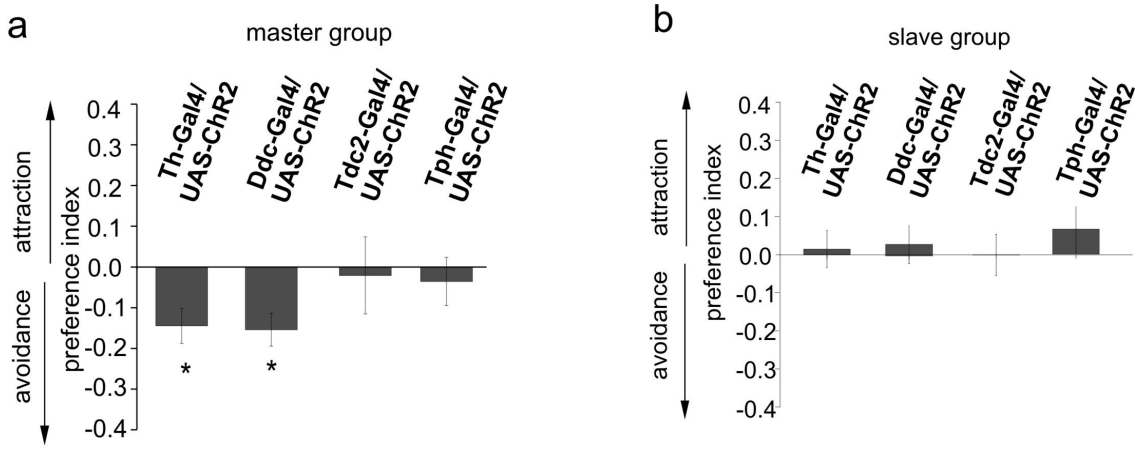


Fig.53 *Summary of master slave experiments for neuronal activation studies.*

The experiments were repeated with their respective non-All Trans Retinal (ATR) controls where control animals were also subjected to walking direction contingent blue light stimulation but they did not show any directional preference (Fig. 54 a), confirming that indeed the effect was because of activation of the dopaminergic neurons as all trans retinal is required for the functionality of the channels.

Fig. 54 a statistics:
One Sample Wilcoxon sign ranked test for
TH;ChR-2 (with ATR) p=0.01208; Z=-2.44539; n=20
DDC;ChR-2 (with ATR) p=0.00583; Z=-2.69804; n=32
TDC;ChR-2(with ATR) p= 0.20785; Z=-1.2699; n=26
TPH;ChR-2(with ATR) p=0.31179; Z=-1.02665; n=20

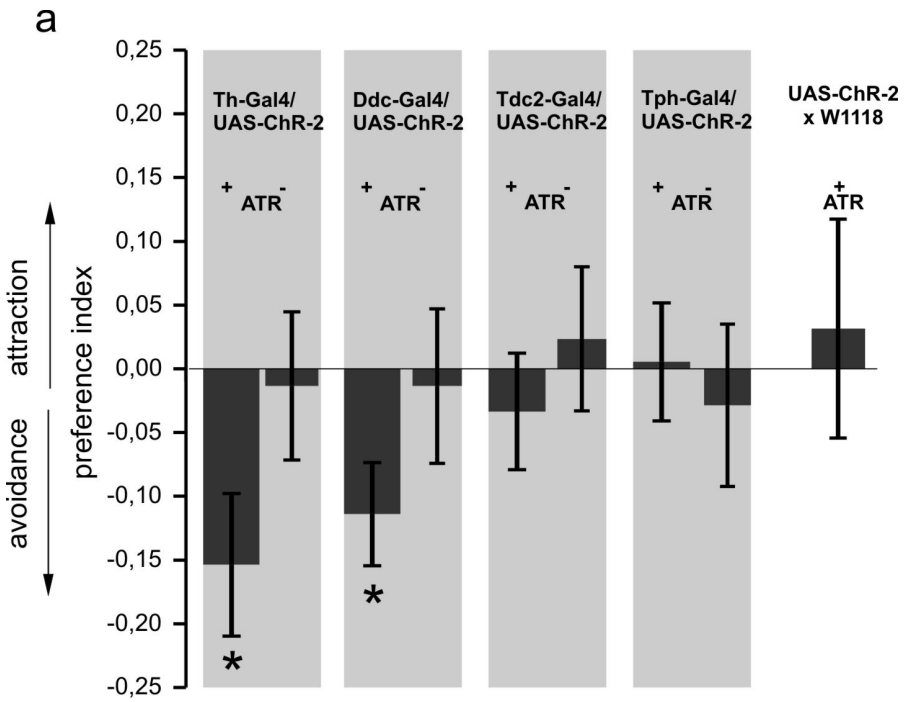


Fig.54 a *Summary of experiments for neuronal activation studies with their corresponding non retinal controls.*

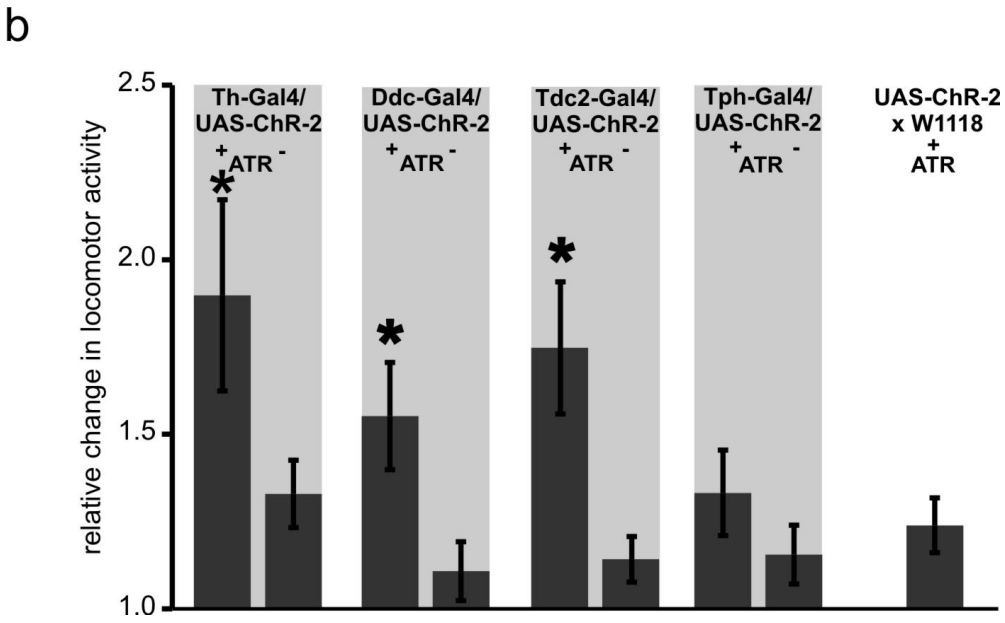


Fig.54 b *Summary of locomotion changes on activation of different neuronal subsets by expressing channelrhodopsin-2.*

The relative change in activity was calculated by considering the total activity (rotational and forward) in time bin of 5 sec before stimulation and comparing it with the next time bin of 5 sec comprising stimulation and post stimulation period .Optogenetic

activation of aminergic neurons after feeding of all-trans retinal (+ATR) leads to an increased locomotion in comparison to controls not fed with all-trans retinal (-ATR) when ChR2 is expressed under the control of Th-Gal4 (Mann Whitney U test $p < 0.001$; $n = 38$ + ATR, $n = 38$ - ATR), Ddc-Gal4 (Mann Whitney U test $p < 0.02$; $n = 34$ + ATR; $n = 27$ – ATR) or Tdc-2-Gal4 (Mann Whitney U test $p < 0.04$; $n = 33$ + ATR; $n = 32$ - ATR) . The activation of neurons labeled by Tph Gal4 has no significant effect on locomotion behavior (Mann Whitney U test $p > 0.9$; $n = 40$ + ATR; $n = 31$- ATR). The blue light induced increase in locomotion of –ATR controls is not significantly different from that of flies lacking any Gal4 driver, also feeding ATR in the absence of the Gal4 drivers does not have any other side effects on locomotion (right most bar) (Fig.54 b).

In summary we can conclude from our experiments that insect's have a designated punishment signaling system mediated by dopamine in the brain. However, our data do not support a potential role of any of the biogenic amines with respect to reward signaling in insects. Therefore nothing could be concluded about the reward mediating aminergic system in flies.

The summary of neurons labeled by the Gal4 used in our experiments are indicated in figure 54 c

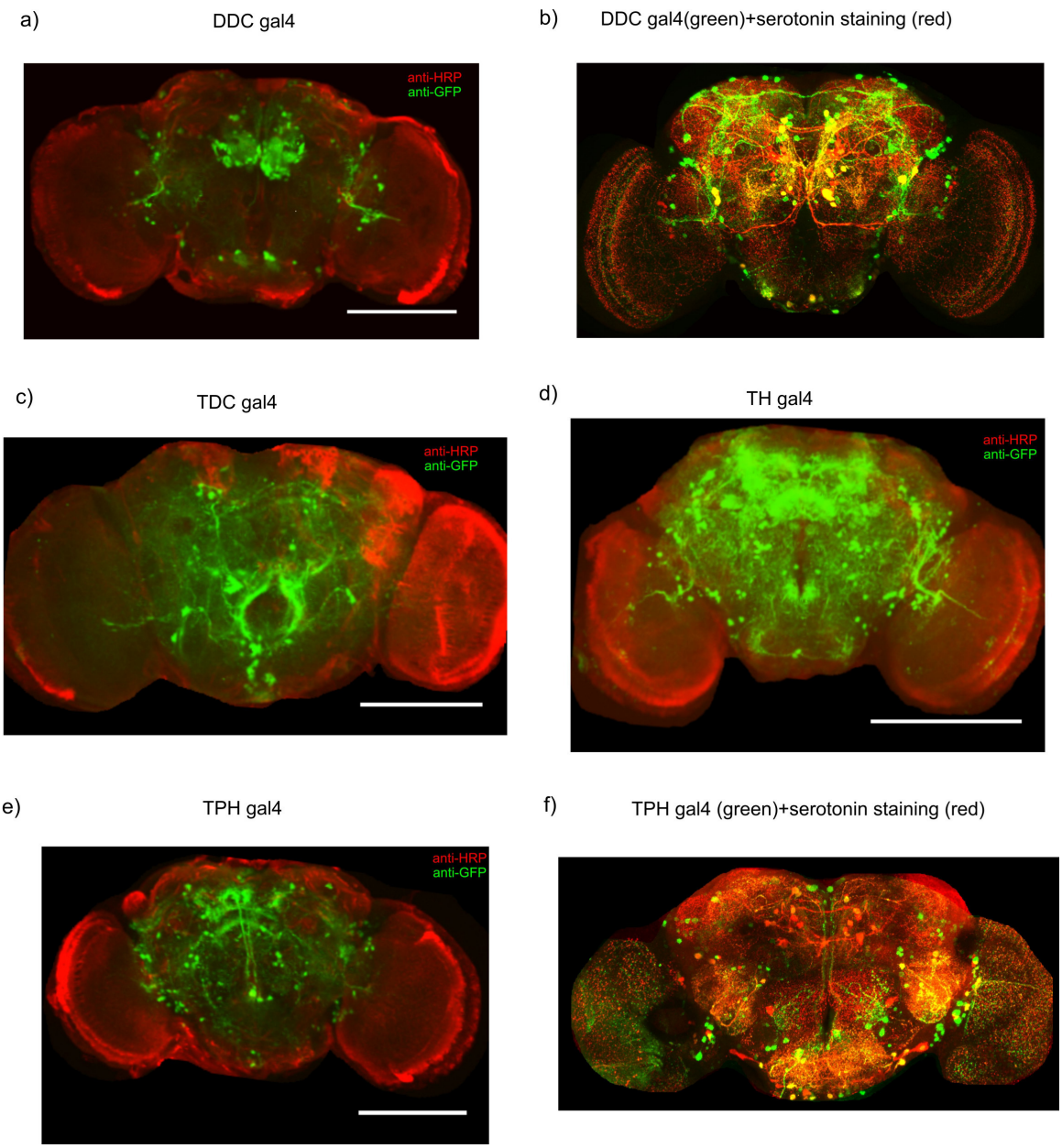


Fig. 54c The neurons labeled by the different Gal4 lines used in walking ball paradigm are shown above. The neurons labeled by Gal4 driver are seen in green (GFP) and HRP staining (a, c, d, e) and serotonin staining (b, f) are seen in red. Figure b and f provided kindly by Prof. A. Fiala.

4. Discussion

4.1 Establishment of new paradigm

Experience-dependent behavioral plasticity has been extensively examined in *Drosophila* using classical conditioning paradigms in which a neutral conditioned stimulus (CS), e.g. an odor, is temporally paired with a punitive or a rewarding unconditioned stimulus (US) (see section 1.5.1). In a classical conditioning experiment the outcome of the animal's behavior is dependent on the external cues which are controlled by the experimenter. The current concepts of how aversive or appetitive signals are mediated in the course of such an associative learning process proposes that biogenic amines are differentially involved. Whereas dopamine is believed to mediate the reinforcing properties of an aversive unconditioned stimulus, octopamine is hypothesized to mediate the reinforcing properties of food-related, rewarding stimuli. However, it is unclear whether or not the release of these amines represents punitive or rewarding sensations the animals are trying to achieve or to avoid through their own behavior. Exactly this question we attempted to clarify in the course of this work.

The robust walking activity of *Drosophila* was chosen to function as a behavioral readout. At the beginning of this project we had no indication if this paradigm was appropriate to quantify behavior and evaluation methods by which we could quantify behavior had to be developed. Therefore, we decided to use a simple stimulus like heat which we knew would be innately punishing to the animal. The paradigm and evaluation procedure was standardised with heat punishment (infrared laser). We observed that animals could survive in such a setup for about 24 hours and be trained to avoid IR laser effectively for periods ranging from 30s to 1 hr (Results section 3.1).

One interesting phenomenon we observed was that when animals were punished contingent to different thresholds of their movement the animals could not only avoid the punished direction but they could also estimate the amplitude of their movements at which the punitive stimulus occurred. Consequently, they constrained the amplitude of their movements below the threshold to avoid the incidence of IR laser activation (Results section 3.2).

In all IR laser training experiments we did not observe reliably any memory for the safe direction in the test phase where the stimulus was absent (Results section 3.1 and 3.2) therefore in order to assess possible memory effects we designed dual training experiments where one group received punishment in one direction in the first three training phases and there was a reversal in training direction in the next three training phases (Results section 3.3). It was observed that animals could quickly adapt to reversal in training direction, this indicated that there is either a very quick experience dependent change in the performance of animals that cannot be resolved using our method or that the behavior observed represents a pure avoidance behavior that is independent of any learning process. One possible explanation of why we fail to see a memory effect in the absence of the IR laser punishment is that animals do not find the necessity to use the information gained in the training period as the reinforcer is not present anymore. This phenomenon is similar to observations made from classical conditioning experiments done in *Drosophila* larvae where the animals are trained to a negative reinforcer and when tested for memory in the test phase in the absence of the negative reinforcer no memory is recalled, whereas in the presence of the negative reinforcer in the test phase memory effects are observed (Gerber and Hendel, 2006).

4.2 Molecular players in operant conditioning

From previous classical conditioning experiments many molecules are known to play a role in development of the association between the CS and the US (Waddell and Quinn, 2001). We wanted to find the molecules involved in operant conditioning. Therefore we have tested several mutants. The rut^{2080} mutants did not show any defect in heat avoidance thereby showing that the *rutabaga* coded adenylate cyclase is not required in our operant paradigm (see results section 3.4.1), although its absence impairs classical associative conditioning as it is believed to be involved in coincidence detection of the CS with US. These mutants however show higher responses to the reinforcing stimulus.

We did not find any molecules necessary to mediate operant avoidance behavior in our paradigm. This argues for a learning-independent process mediating the avoidance of the heat stimulus. However, this interpretation must be taken with caution because the involvement of the investigated mutations in operant learning paradigms is not clear. In

fact, it appears that operant learning is independent of the rutabaga mutation also in another paradigm (Wustmann et al., 1996)

4.3 Neuronal activation studies

4.3.1 Activation of gustatory neurons

It has been shown that frequently an output from certain interneurons which are activated by a sensory stimulus is required for behavioral responses to the sensory functions which involve their activation by a sensory stimulus. However it remains unknown if behavioral actions can be subjected to decision making by spatially activating a distinct subset of neurons (aminergic and sensory neurons), without the contribution from a specific sensory stimulus which is in principle required for their activation. Therefore to test this possibility of driving behavior only by spatial activation of neurons without the actual physical input of the sensory stimulus, we drove expression of channelrhodopsin-2 in sugar sensitive gustatory neurons (Gr5a) and observed that activation of GR5a neurons elicited the proboscis extension reflex. This proved that for a sensory reflex the presence of the sensory cue (sugar) was not necessary and a sugar-elicited reflex can be substituted by an optogenetic activation of the respective sensory neurons. Next we tested the possibility of guiding behavior towards decision making by optically driving the activation of Gr5a neurons contingent to directional choice on the walking ball, and indeed we could drive the behavior of animals towards the direction which was optically reinforced. This demonstrates that apart from a sensory reflex, the goal driven behavior could also be elicited by driving a neuronal population.

Contrary to the heat punishment training experiments we observed that when animals were trained on the ball apparatus by directional choice contingent activation of sugar sensitive neurons, the animals showed a preference for the previously optically "rewarded" direction in the test phase (Fig.44). This shows that when positive reinforcement is involved then animals exhibit memory for the previously reinforced direction in the test phase in the absence of the reinforcing stimulus. This indicates that animals also in operant conditioning are motivated to recall negatively valued memory only in the presence of punishments but utilize positive memory only when rewarding

stimulus is lacking. Overall, the feasibility of optogenetically influencing the animals' behavior on the walking balls has been demonstrated.

4.3.2 Activation of aminergic neurons

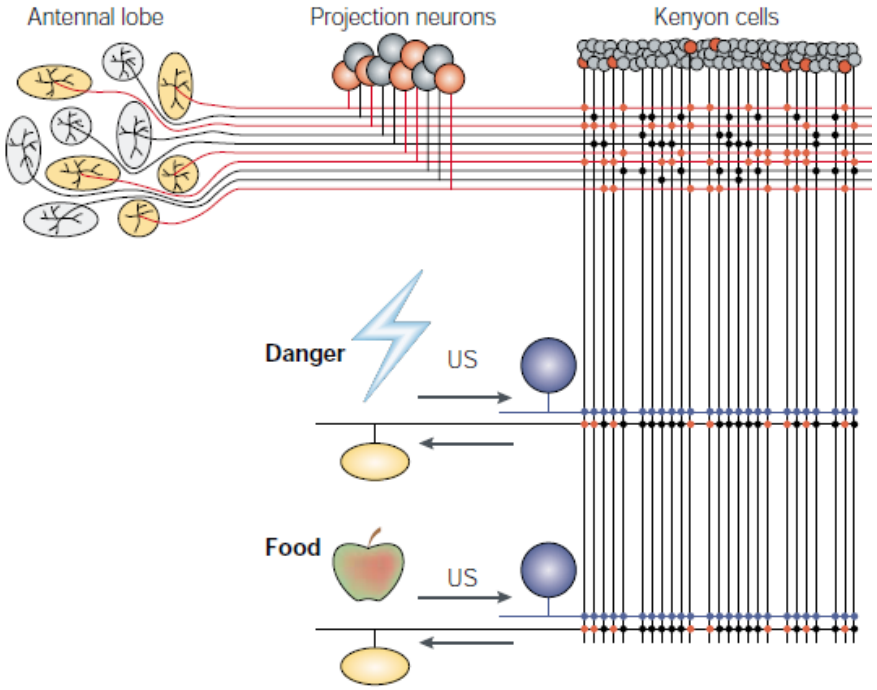
One of the important goals of our studies was to ascertain if the release of a particular amine would induce an arousal state which is preferred or avoided by the animal. In other words we wanted to test if release of amines modulates intrinsic parameters that advance the occurrence of a behavior in an operant context. The release of an amine was facilitated on the ball apparatus by expressing channelrhodopsin-2 in aminergic neurons and activating the channels by bright blue light.

Most of these amines are known to play a role in arousal and motivational behavior which has been inferred from neuronal silencing studies and pharmacological studies(Herberg and Stephens, 1975). The problem with silencing studies is that since aminergic neurons are known to function in a large array of behaviors apart from arousal behavior, any effect of neuronal silencing cannot be concluded to be directly caused by a loss of amines. Our neuronal activation studies indicate that amine release (dopamine, octopamine and serotonin) indeed functions in producing an arousal state which is indicated by an increased locomotion of animals. What could be concluded from our studies on amine functions is discussed below:

4.3.2.1 Dopamine

From classical conditioning experiments dopamine is assumed to function as a neuromodulator strengthening certain synapse when the signals from the CS and US arrive simultaneously. Thus, when dopamine release is paired with a neutral cue then the neutral cue is assigned a negative value, indicating dopamine's role as a "neuromodulator" (Fig.55) (Heisenberg, 2003) which would support the theory of incentive salience hypothesized from mammalian studies (Berridge and Aldridge, 2008). Also, Riemensperger et al., (Riemensperger et al., 2005) show that electric shock activates dopaminergic neurons innervating the mushroom body, indicating that punishment activates dopaminergic neurons. However if activation of dopaminergic neurons is sufficient to be interpreted as punishing remained unknown.

From our experiments we can hypothesize that the release of dopamine produces a punishing sensation such that on the ball apparatus the animals avoid situations that leads to release of dopamine, indicating that dopamine might function as "neuro-effector", where its release brings about a result or an event accomplishing a purpose. This essentially would mean that function of dopamine release on a behavioral level in insects is comparable to mammals where there are reports that stimulation of dopaminergic neurons interacts with reward perception signals during learning of behavioral reactions for positive stimuli (Schultz, 2001). Another study by Deisseroth's group indicates that channelrhodopsin-2 mediated activation of dopaminergic neurons is sufficient for place learning in mice, then supporting earlier studies (Tsai et al., 2009).



(Heisenberg, 2003)

Fig. 55 *Model proposed for classical olfactory learning. The presentation of an odour (CS) leads to activation of a subset of intrinsic mushroom body neurons (Kenyon cells). A negative US like electric shock leads to activation of dopaminergic neurons (positive US leads to activation of octopaminergic neurons). When the CS and the US arrive simultaneously then the aminergic neurons are assumed to strengthen synapses between the particular subset of Kenyon cells and output neurons which drive the escape behavior.*

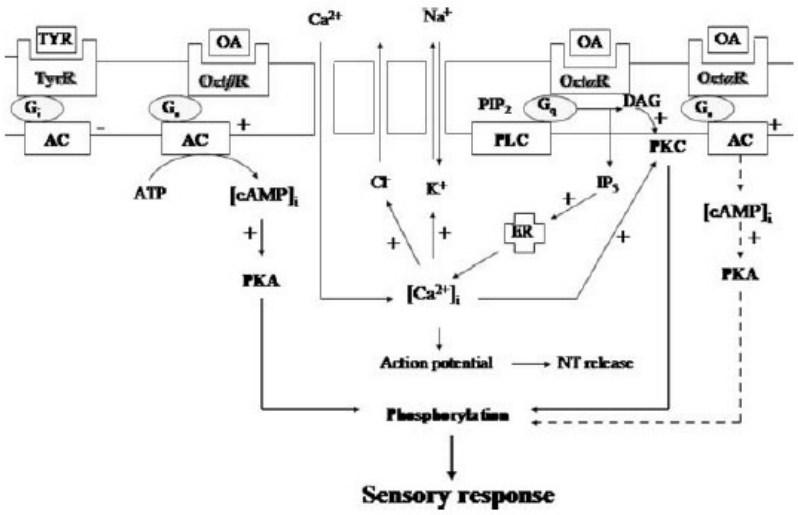
However, another study points toward a role of the D1 dopamine receptor in Kenyon cells also for appetitive Pavlovian learning, indicating that dopamine might function in appetitive learning (Kim et al., 2007). Probably different functions of dopamine can be accredited to different types of receptors present in different neuropiles. Therefore, a localized release of dopamine at one neuropile would produce an action dependent on the dopamine receptors located in the neuropile. Also, distinct dopaminergic neurons innervating different neuropiles might contribute to distinct effector roles.

Different firing rates of neurons could also contribute to the kind of behavior they are involved in. Possibly spiking at different frequencies could account for their involvement in appetitive or aversive behaviors.

There is a possibility that these differential effects were masked in our experiments as we drive dopamine release using TH and DDC Gal4 (covering most of the dopaminergic neurons in the fly) which produces a wave of dopamine release in the brain leading to uncoordinated release at different sites. In conclusion, our data supports that dopamine cumulatively is more prevalent as an aversive effector signal than possibly an appetitive one.

4.3.2.2 Octopamine

Octopamine is known to be involved in appetitive classical conditioning and plays a role in large of mixed behaviors in insects like aggression, starvation, flight and flight behavior, desensitization etc (see introduction for details). Thereby its general role as a positive or negative reinforcer remains unclear. From our experiments we observe that animals do get aroused on activation of octopaminergic neurons which is depicted by increase in locomotion on octopamine release. However the flies do not show any directional preference on the ball apparatus when octopamine release is coupled to turning in a given direction. This could indicate that octopamine does not play a role in reward generating reinforcement in operant locomotion behavior, but might function as an intrinsic cue evaluator promoting the actions that would be beneficial to the animal dependent on its current state and requirements.



(**Farooqui**, **2007**)

Fig. 56 *Figure representing the activation pathway of octopamine receptors. PKC and PKA pathway regulate the sensory responses. The pathway mediated by OctR receptor is via phospholipase C pathway, which is the same pathway used in visual transduction via norpA.*

Another less likely possibility is that octopamine might function pre-dominantly via the phospholipase C (PLC) pathway, and the *norpA* mutation masks the effect as it affects the PLC pathway. This seems less likely as there are other receptors that do not function via the PLC pathway and indeed, we see an increase in locomotion on octopamine release. However this effect may play a role in goal targeted behavior.

In our case we have used TDC Gal4 which in combination with UAS ChR-2 and light effects both tyramine and octopamine release. The TyrR receptor has high affinity to tyramine and its activation causes lowed adenylate cyclase activity (low cAMP) whereas OctßR and other receptors in Fig.2 show high affinity to octopamine and their activation produce high adenylate cyclase activity (high cAMP), this could indicate that tyramine and octopamine act in an opposing manner thereby nullifying the overall effect.

The activation of octopaminergic and tyraminergic neurons labeled by TDC Gal4 leads to an increased locomotion which indicates that their release produces an arousal effect in animals but when those neurons were activated contingent to their walking

direction on the ball we do not observe any directional preference. This points towards the possibility that octopamine and tyramine might function as motivational modulators (both amplifying and dampening of the response) in animals depending on the current situation of the animal rather than as response generators.

4.4 Outlook

From our experiments we did not find any amine that functions in producing an arousal state that is "liked" by the animal as determined through directed locomotion. This can be attributed to difference in the mechanisms that exist between mammals and insects or to the fact that our study has not included other possible amines and neuro-peptides which could be involved in reward signalling. Also the limitation of this approach is that possibly we were unable to activate neurons which might be shielded from the blue light e.g dense tracheal network deep in the brain. However by using channelrhodopsin-2 we could achieve high temporal precision of neuronal activation. Hence, it would be interesting to continue this study at higher resolution using more sophisticated genetic tools.

It would be interesting by using more selective Gal4 lines to narrow down the number of dopaminergic neurons that are necessary or sufficient to induce an arousal state that is avoided by the animal. Possible candidate neurons could be the neurons innervating the mushroom bodies as this brain structure is believed to be an important neuropile involved in saliency based decision making in visually based flight behavior (Zhang et al., 2007b). The electric shock based activation of dopaminergic neurons also refered to the region of mushroom body lobes (Riemensperger et al., 2005).

Alternatively other brain structures that are known to mediate goal directed locomotion can be targeted with the question whether they have an influence on the animal's walking direction. Therefore, the central complex would be a candidate brain structure as it is known to be involved in co-ordinating and balancing body movements between the two halves of the body. Also, recently it has been shown that dopaminergic neurons independently regulate two different forms of arousal in opposite directions (Lebestky et al., 2009).

Using the genetic tools together with quantification of the neuronal self stimulation approach or escape behaviors can serve to generate important links to compute the animal's behavioral preference and to identify the neuronal target structures involved in mediating those functions. Our study reports for the first time in insects that the animal's voluntary behavior can be controlled by manipulating neuronal circuits, paving the way for further investigations of neuronal functions and circuits.

4.4.1 Our proposed model

Based on data from our experiments and other studies we propose (Fig.57) that activation of dopaminergic neurons (either by sensory cues or direct neuronal activation) produces a negative arousal state in the animal which activates the decision making circuit of the animal and produces a motor output which leads to escape of the situations that cause the activation

From our experiments we observe that activation of octopaminergic neurons labeled by TDC Gal4 produces an increase in locomotion indicating that the animals are aroused, but when the activation of these neurons is made contingent to direction of walking on the ball apparatus they show no directional preference indicating that octopamine release in a neutral environment does not have an effect on operant conditioning. Therefore, we hypothesize that in the case of octopamine their release produces an arousal state (inferred from increased locomotion) which instructs the animal to make a goal directed choice dependent on its current state (e.g. if confronted by an enemy it motivates aggression, if the animal is starved it induces the animals to feed) thereby the animal is directed to benefit from its behavior in its environment. In order to achieve the benefiting behavior the animal has to produce a motor output and this could be regulated by the central complex.

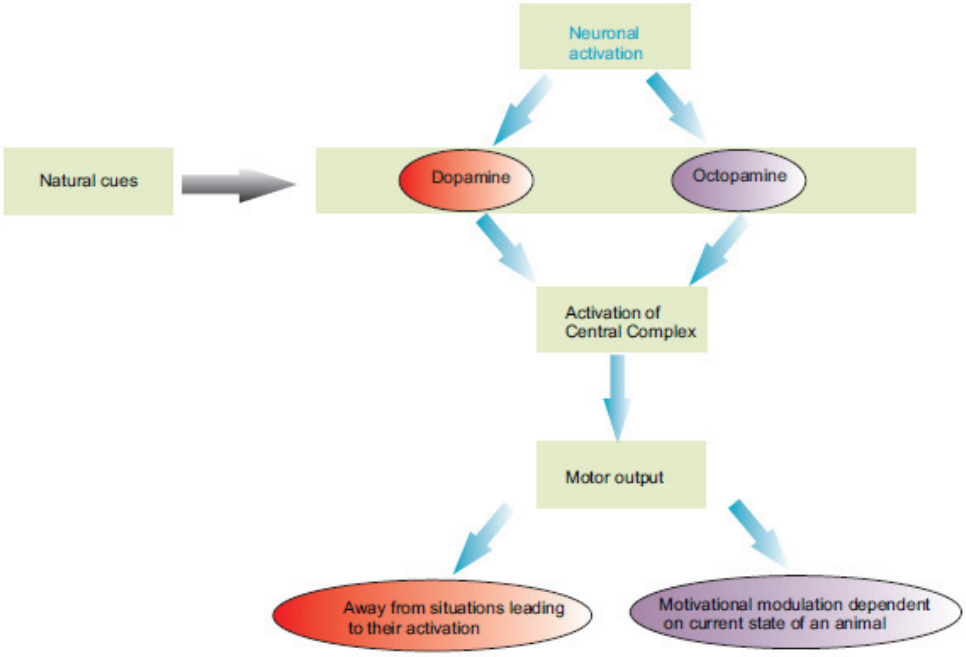


Fig. 57 *Hypothesis from our experiments. Activation of aminergic neurons by natural cues or direct neuronal activation leads to an arousal state which is liked or dislike by the animal. In order to maintain the aversion or approach of the arousal state the animal has to make a decision which could be regulated by the central complex which leads to the appropriate motor output. In case of dopamine release the animals avoid the situations that produce the release and in case of octopamine release the animals perform an action that corresponds to the internal state of the animal which is adjusted such that the animal is likely to benefit from in its current environment.*

Alternatively, the lack of a designated reward system might represent a difference between insects and mammals, where rewarding functions of dopamine have been clearly shown (Schultz, 2001). Thus to verify amine functions context based paradigms (starved animals, competition of mating partner or food etc.) would help in giving an insight.

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PART II

Genome wide transcript analysis of *Synapsin* and *Sap47* mutants

(In collaboration with S. Kneitz and T. Nuwal)

1. Introduction

In this study our aim was to perform genome-wide expression analysis for two pre-synaptic mutants viz. *Synapsin* (*Syn*^{97}) and *Synapse associated protein of 47 kDa* (*Sap47*^{156}) as well as the *Syn*^{97}, *Sap47*^{156} double mutant. The Synapsin protein is believed to be involved in vesicle clustering and tethering of vesicles to actin and micro filaments to form the reserve pool at the pre-synapse. Phosphorylation of synapsin is considered as an event that is involved in vesicle docking and release (Cesca et al., 2010; Benfenati et al., 1989; Rosahl et al., 1995; Hosaka et al., 1999). From studies done in *Drosophila* the mutation in the *Synapsin* gene does not produce any drastic phenotypes but causes defects in learning and memory (Knapek et al., 2010; Godenschwege et al., 2004; Michels et al., 2005). The synapsin protein has been postulated to interact with another pre-synaptic protein called SAP47, as the level of synapsin protein is up-regulated in *Sap47* mutants. To verify this semi-quantitative PCR (qPCR) experiments were done to quantify *Synapsin* transcript levels in *Sap47* mutants, but no changes were observed in transcript levels (refer to Ph.D thesis of T. Nuwal, 2010). Absence of SAP47 protein in flies does not affect their viability and fertility (Funk et al., 2004).

Since these two mutants do not show any drastic phenotypes, we wanted to investigate at the transcriptome level the changes brought about by these two mutations. Thus the aim was to study expression levels of all other *Drosophila* genes and determine by comparison with wild type (*CS*) whether they are affected by these mutations (Fig.1). If these two genes interacted on the molecular level, there could be common candidate genes between the two individual mutants which would lead to a stronger alteration of expression levels when mutations in both genes occurred in a single fly. Conversely, another possibility could be that occurrence of both mutations together might nullify expression differences of individual mutants if their functions were compensating each other.

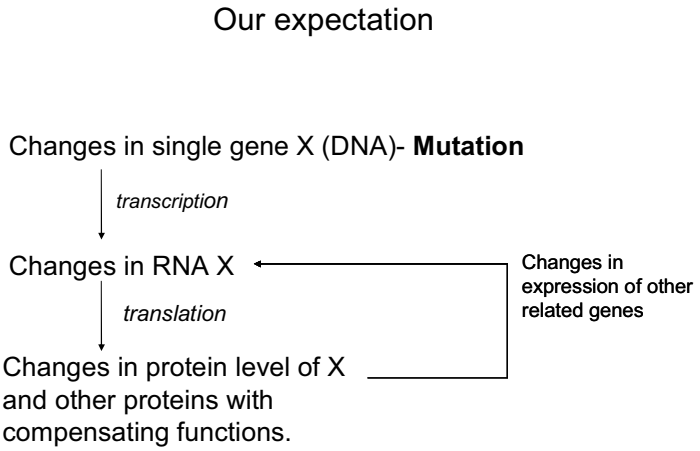


Fig. 1 *Schematic describing the rationale behind performing gene expression studies. Any changes in the genome (gene X) would affect the RNA levels (X), which would bring about a change at the protein level of X. The absence of protein X might lead to alterations of other proteins that would compensate for the absence of protein X which should be reflected in their expression level at the transcript level (RNA).*

1.1 Background about fly lines

Synapsin-Sap47 double mutants were generated from the single mutants four times independently by homologous recombination. In all four cases the recombinants were homozygous lethal but the lethality of each recombinant chromosome was complemented by each of the other three recombinant chromosomes. Our speculation is that, genetic background polymorphisms exist for several genes which permit viability of the double mutant. One of the recombinant double mutant lines (from V. Albertowa) was outcrossed to Canton-S (wild type, CS^{V}) for 7 generations, the recombinant chromosome was traced by PCR. In the 8^{th} generation the flies were crossed to a cantonized balancer line for the third chromosome and single balanced flies were paired and after egg laying the parents were analyzed by PCR. Among 23 single fly pairs with both parents containing the double mutant chromosome, three produced viable and fertile homozygous double mutant offspring which could be established as three stocks *V1*, *V2*, and *V3*. For transcriptome investigation in this study we have used the double mutant stocks *V2* and *V3*.

1.2 Questions addressed in this part of thesis

- Which genes are up- or down-regulated in *Syn-Sap47* double mutants compared to wild type and the individual mutants?
- How important is the "founder" effect of independently established lines for the same mutant?
- How large is the variability between the wild types?
- How reliably reproducible are data from independent populations of samples from the same genotype (to verify the robustness of the technique and consider variability between different generations). The following samples were tested:

6 RNA samples (6 chips):

- Canton S^{NF} (wild-type control), from Natalja Funk
- Canton S^{V} (wild-type control), from Viera
- *Sap47* mutant outcrossed to CS^{NF}
- *Synapsin* mutant outcrossed to *CS*
- *Syn*apsin and Sap double mutant *V2*, low larval density outcrossed to CS^{V}
- *Syn*apsin and Sap double mutant *V3*, low larval density outcrossed to CS^{V}

2. Materials and methods

2.1 Fly samples

Fruit flies from all the experimental groups were raised on standard cornmeal medium from the same food batch at 25 °C, 12 h light-dark cycle and 60 % humidity. The samples used for the transcriptome analysis were collected 2-3 days after eclosion and frozen at -80°C for future use.

2.1.1 Primers

Gene name	Sense primer	Anti-sense primer
Synapsin 5' end	CTTAACGTTCATCGGCCATT - **1f**	AGGGGTTCGCTTCGTTACTA - **1r**
Synapsin 3' end	CTGAACAATCCAAGCGATCT - **2f**	TACATCCAGATCCGAGTGCT.- **2r**
Sap 5' end	TAAAAGTTGGAGAGCCAGGA - **3f**	GGTGGCTTCGGATACTAATG.- **3r**
Sap 3' end	ACCAAGCCCTAACCAATCC - **4f**	ATACCGACGACTCCCTCTAC.- **4r**

Table 1: *Primer sequences for verification of the 5' and 3' end of Synapsin and Sap47 mutants (see Fig.10 for positions)*

Synapsin **candidates**

Gene name	Sense primer	Anti-sense primer	Product size
CG4073	TCATGGCCTACGTGTACCTG	GACACCAGTCATCCAGAGCA	134bp
Cirl	GGATGATGCTCATGGATTG	AAAGCCCCGTAGTCAAGAG	135bp
fru	GCCGCATAAGGTCAAATAAA	GAAAAGGGCGTATGGATTG	84bp
mthl8	GTCGTTTGCATATGCTCCTT	TCACAGGTATCTTCGCCATT	179bp
nAChRbeta 64B	AGCCATGTCCCTGGAGTAAG	GATTACATAACGGCCCTTGC	171bp
Shaker	ACACTAGCGACTGTCGTTGC	CCAAGGACATAGCCTGATTC	100bp
Syn	CTGAACAATCCAAGCGATCT	TACATCCAGATCCGAGTGCT	90bp
to	GCCAAGCACGAAGTGAAAAT	TTGCCCTGACGTTAACCATT	133bp
CG14305	AAGGCCACTGTTTCAGTGCT	CAGCCATGCACAGTTAAGGA	81bp
Syn 5' primer	CTTAACGTTCATCGGCCATT	AGGGGTTCGCTTCGTTACTA	133bp

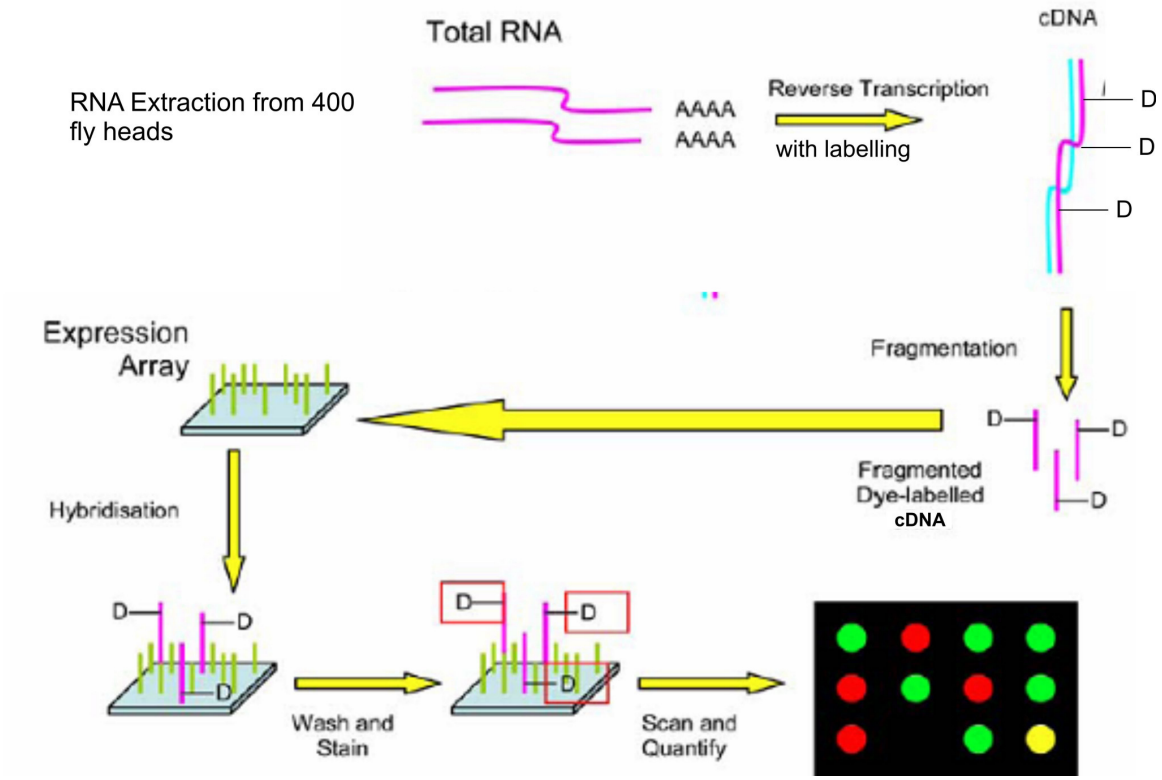
Table 2: *Primer list of Synapsin candidates chosen for qPCR experiments.*

Sap47 **Candidates**

Gene name	Sense primer	Anti-sense primer	Product size
CG9279	AAAAGTTTCCCACGGCTTGT	GCATCAAGATCACTGCGGTA	199bp
zeelin-1	CAAAGCGACGCTATACAAATCA	AAAATTGAGCGGTGAGCAAT	108bp
frizzled2	GAAGCGCGTTTGTTCTACCT	GCTCTACGTGGCGGTTTATT	150bp
CG31097	ATGCATCTGCCAGTGAAGC	CCCGACCTTCGATTACGTT	121bp

Table 3: *Primer list of sap mutant candidates chosen for qPCR experiments.*

2.2 Overview of gene chip hybridization procedure



Source: World Wide Web

Fig. 2 *Overview of microarray technique procedure.*

The steps involved in gene chip hybridization experiments are

1. Extraction of the total RNA
2. Conversion from RNA to cDNA and transcription in the presence of labeled nucleotides.
3. Purification of the samples.
4. Hybridization to gene chip.
5. Read-out of the dye signals for each probe.
6. Data analysis

2.3 RNA extraction

- 400 flies frozen in liquid nitrogen, heads (30 mg of brain tissue) were isolated by vortexing.
- **RNeasy mini kit** from QIAGEN was used for isolation of total RNA. The place was cleaned to by RNase Zap from sigma to make the workplace RNase free. Sterile filter tips were used at all steps.

- **Steps:**
  - Fly heads were homogenised in 600 µl of RLT buffer and 6 µl of ß-mercaptoethanol with glass homogenisers.
  - The homogenized sample was centrifuged for 3 min at 13200 rpm.
  - Pellet was discarded and supernatant was transferred into a new tube.
  - 600 µl of 100% ethanol was added and gently mixed.
  - Take 600 µl of the mixture and add it to the columns of the kit.
  - 600 µl of the sample was added to 2 ml RNeasy columns and centrifuged at 12500 rpm for 15s.
  - The flow through was discarded and 700 µl of RW1 buffer was added to the column and centrifuged at 12500 rpm for 15s.
  - The columns were transferred to new collection tubes.
  - 500 µl of RPE buffer was added and centrifuged for 15s at 12500 rpm. Once again 500 µl was added to the columns and centrifuged for 2 min at 12500 rpm.
  - The flow through was discarded and the column was centrifuged for 1 min.
  - The column was transferred to new collection tube and 30 µl of RNase free H_2O was added to the columns and centrifuged at 12500 rpm for 1 min to elute the RNA. Then the columns were discarded.
  - 1 µl of RNase-free DNase was added to the eluted RNA.
  - RNA was incubated at 37°C for 45 min to activate DNase function.
  - To inactivate DNase tube was placed in 95°C water bath for 10 min.
  - RNA concentration was measured using a spectrophotometer.

2.4 Verification of RNA quality on a gel

When working with RNA it is important to make sure that working space and all the apparatuses and reagents used are RNase free. Therefore water was made RNase free by dissolving DEPC (Di-Ethyl Pyro Carbonate) with final concentration being 0.1% and left overnight for dissolving before autoclaving it. The gel running chamber and the rigs were treated with 3% hydrogen peroxide and left overnight. They were rinsed with DEPC treated water.

Steps for preparing Formaldehyde Agarose gel for RNA visualisation:

Gel preparation

- **Preparation of 10X MOPS buffer:** 200 mM of 3-[morpholino] propanesulphonic acid (MOPS) with 50 mM sodium acetate, 10mM EDTA and pH was set to 7.0 with NaOH
- 0.5 g of agarose was boiled in 50 ml of DEPC water and 6ml of 10X MOPS buffer
- The mixture was cooled to 65 °C and to it 2ml of 37% (12.3M) of formaldehyde was added under the hood.
- The mixture was poured into the casting tray and allowed to solidify.

RNA sample preparation

- To 4.5 µl of RNA sample 2 µl of 5X MOPS buffer, 3.5 µl formaldehyde (37%), and 10 µl of formamide.
- The mixture was heated to 65°C for 15 min and cooled on ice.
- 2 µl of 6X sample loading dye and 1 µl of ethidium bromide were added to the sample.
- The gel was run in 1X MOPS buffer at 60V for 1 hour.

2.5 Reverse transcription

Reverse transcription was performed to verify the presence/absence of transcripts in the *Sap47*^{156} and *Syn*^{97} mutants. The RNA was extracted using the protocol mentioned in section 2.3. The downstream procedure was as follows:

- 11 µl of RNA was taken.
- 1 µl of oligo-dT (Invitrogen) primer and 1 µl of dNTPs (Invitrogen) were added.
- The mixture was incubated for 5 min at 65°C
- At the end of 5 min sample was immediately placed on ice and centrifuged.
- 5 µl of 5x first strand buffer (Invitrogen) was added.
- 2 µl of 0.1M DTT (Invitrogen) was added.
- Sample was incubated for 2 min at 42°C.
- 1 µl of Superscript (Invitrogen) was added and incubated for 1.5 hours at 42°C.

- Sample at the end of 1.5 hours was incubated at 72°C for 10 min.
- 1 µl of RNase was added and incubated at 37°C for 30 min.
- The sample was frozen.

2.5.1 Quantitative real time PCR

The reverse transcribed cDNA was used as a template to quantify gene expression. The following components were added to setup a reaction

Component	Quantity
Template	~100 ng in 1 µl
Master mix (Rotor-Gene Q SYBR Green)	10 µl
Primers	1 µl each sense and antisense
Total	H_2O to 20 µl

The following PCR conditions were used on a Rotor Gene-Q machine (Qiagen)

Step	Temperature	Time	Cycles
Hot start	95 °C	5 minutes	1
Denaturation	95 °C	10 seconds	45
Annealing	60 °C	15 seconds	45
Extension	72 °C	20 seconds	45
Melt	72 °C-95°C ramp		

2.6 Processing steps for gene chip hybridization (In collaboration with S. Kneitz, IZKF Wuerzburg)

2.6.1 Extraction of RNA and quality verification

Once the RNA is extracted from the biological tissue it is very important to analyse the RNA quality as it is a critical factor in downstream steps of hybridization when using Affymetrix arrays as they are highly sensitive to RNA quality. The RNA quality and the extraction procedure should be optimised so that results each time are comparable to the previous trials. The RNA quality is usually measured before

commencement of the processing steps for hybridization of labeled cDNA by capillary electrophoresis system called BioAnalyser StdSens (Agilent Inc.). A good quality RNA should have Optical Density (OD) ratio 260/280 at least 1.8 and 260/230 at 2.0. There should also be no degradation visible on the RNA gel. The RNA Integrity Number (RIN) from the BioAnalyzer should be high and comparable. On visualization of the gel 2 bands are observed at 2 kb, one band corresponds to 18S RNA which should give a signal at 2 kb and the other band corresponds to 28S RNA which should ideally give signal at 4 kb, but in *Drosophila* the 28S RNA is cleaved into two fragments which cannot be distinguished from each other on the gel. After verification of the quality of extracted RNA, it is reverse transcribed to cDNA and labeled.

2.6.2 Reverse transcription (done in S. Kneitz laboratory)

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- 9 µl (>5 µg) of RNA, 1 µl of random primer and 1 µl of anchored oligo dT was mixed and spun down at 4700 rpm.
- The mixture was incubated at 70°C for 5 min.
- The reactions were cooled at room temperature for 10 min (primer annealing) and the mixture was spun down at 4700 rpm.
- **Master mix** was prepared, with all components from Amersham Inc.

Components	Volume (µl)
5X CyScript buffer	4
0.1 M DTT	2
CyDye	1
Nucleotide mix	1
Reverse transcriptase	1
Total volume	9

- 9 µl of master mix was added to each sample.
- The mixture was spun down for 15 s.
- The mixture was incubated at 42°C for 1.5 hours.

2.6.3 Stopping the reaction and RNA degradation (done in S. Kneitz laboratory)

Next step was to stop the reverse transcription reaction and degrade the RNA to obtain single stranded cDNA

- 2 µl of 2.5 M NaOH was added to each probe, mixed and spun down.
- The mixture was incubated at 37°C for 15 min.
- 10 µl of 2M HEPES free acid was added, mixed and spun down.
- The cDNA's were purified.

2.6.4 Purification with GFX columns (done in S. Kneitz laboratory)

For the purification of cDNA GFX columns and reagents from amersham pharmacia were used, the procedure was as follows:

- The mixture was centrifuged at 13200 rpm.
- 500 µl of capture buffer was added to each sample and mixed.
- The mixture was transferred to column and centrifuged at 13200 rpm for 30 s.
- The flow through was discarded and 600 µl of wash buffer was added and centrifuged at 13200 rpm for 30 s.
- The washing step was repeated two more times.
- The flow through was discarded, and the column was spun for additional 10 s.
- The collection tube was discarded and transferred to a fresh collection tube.
- 60 µl of elution buffer was added directly to the top of matrix column.
- It was incubated at room temperature for 5 min.
- The column was spun at 13200 rpm for 1 min.

2.6.5 Hybridization (done in S. Kneitz laboratory)

- 60 µl of sample was heated to 95°C for 2 min and on completion of 2 min it was immediately placed on ice.
- The sample was incubated at 75°C for 5 min.
- 430 µl of Hybridization buffer (Amersham Inc., warmed to 42°C) was added, mixed and spun down. The total volume of hybchamber is 490 µl.
- Gasket slide was placed in hybchamber.

- The sample was pipetted onto the gasket slide.
- The array (Affymetrix Inc.) was kept facing downwards to the gasket slide.
- The hybridization chamber was closed.
- The hybridization reaction took place for 16 hours at 42°C at 6-7 rpm.

The arrays were scanned using GeneChip® Scanner 3000 (Affymetrix Inc.).

2.7 Analysis of gene chip expression data

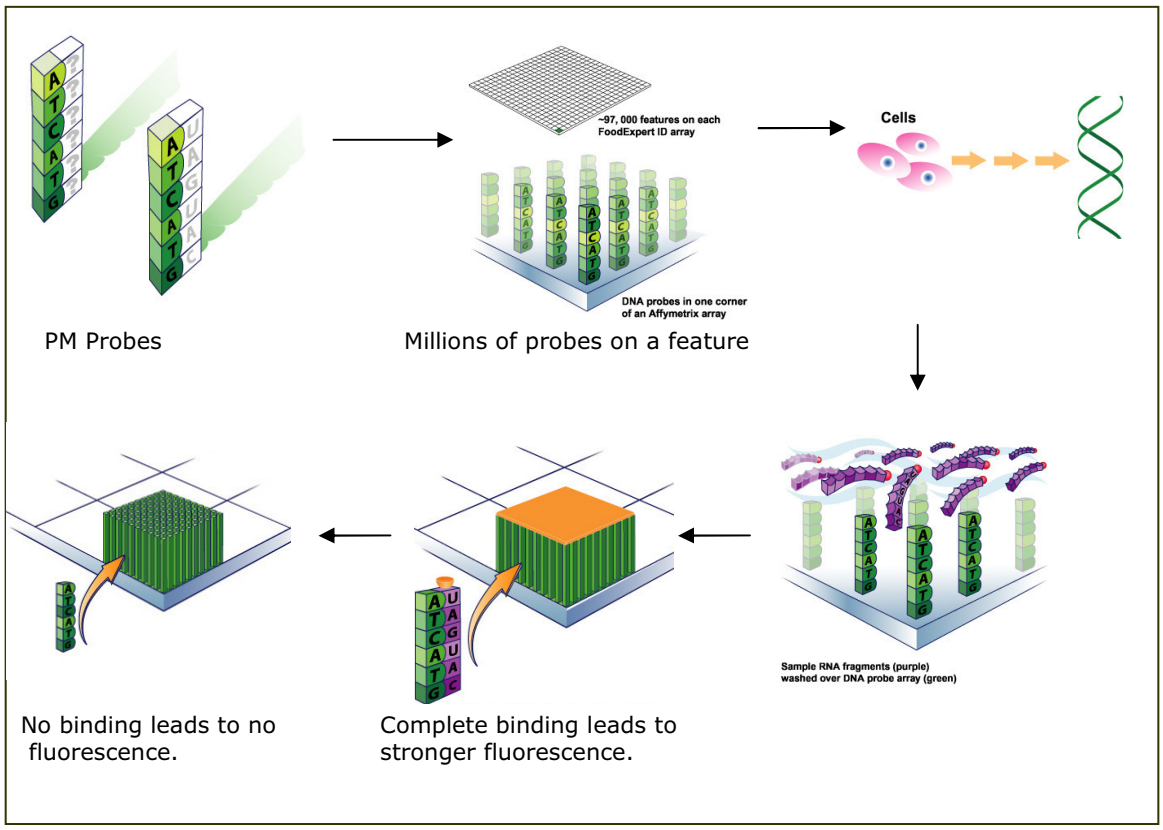
The gene expression chip from Affymetrix contains transcript-specific sequences usually complementary to the 3' end of the transcripts. For each transcript there is a *probe set* which comprises 14 *probe pairs*, each 25 nucleotides long. All probe pairs consist of a *Perfect Match probe* (PM, perfectly complementary to target sequence of the transcript) and a *Mismatch probe* (MM, One of the bases from the target sequence replaced by a non specific base) (Fig.3, 4 a, b). The MM probe acts as an internal control and the fluorescence observed by non specific binding at the MM probe will be subtracted from the fluorescence of the PM probe. All probe pairs of a similar kind are arranged in a single square called a *feature*. For quantification of transcript copy number the normalized fluorescence (generated by comparison of fluorescence from PM and MM probe) from all the 14 individual probe pairs (a probe set) are pooled together.

The GeneChip Drosophila Genome 2.0 Array was used for hybridization. The characteristics of the gene expression chip for *Drosophila* are described below (Fig.3):

Critical Specifications	
Number of arrays in set	One
Number of transcripts	~18,500
Number of probe sets	18,880
Feature size	11 µm
Oligonucleotide probe length	25-mer
Probe pairs/sequence	14

Source: www.affymetrix.com

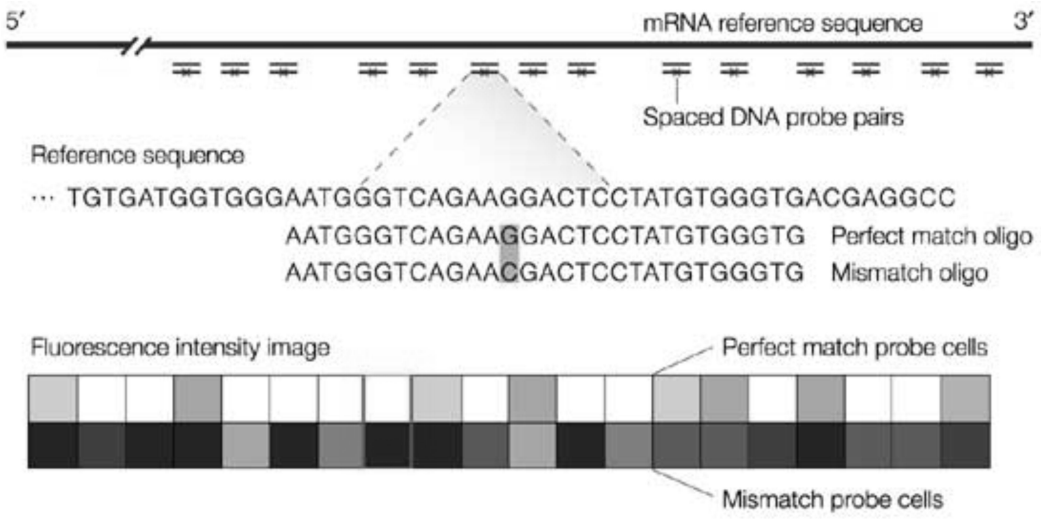
Fig. 3 *Characteristics of a Drosophila Genome 2.0 Array GeneChip. There are 18500 transcripts specific sequences spotted on the chip. For all the transcripts and their variants around 18880 probe sets are designed. Each probe set has 14 probe pairs (Perfect Match probes, PM, and Mismatch probes, MM) which are arranged in 11µm squares called features. The length of each probe is 25 nucleotides.*



Modified from www.affymetrix.com

Fig. 4a *Probe binding and quantification of gene transcript levels. The feature on a gene chip comprises millions of copies of PM probe and MM probe enclosed in it. On hybridization if the sample contains high copy number of labeled transcripts then a high difference in binding to PM vs MM would be observed which would be proportional to high difference in PM vs MM fluorescence intensity of the feature. If the copy number of labeled transcript is low or absent then low or no PM vs MM fluorescence difference would be observed.*

Signal intensity (measure of gene expression) = (∑ PM- MM)/ # probe pairs used.



Source: world wide web

Fig. 4b *Probe design in an Affymetrix gene chip. The mRNA reference sequence is taken as the target sequence and for each transcript two types of probes are designed. One type of probe is called the perfect match (PM) which is perfectly complementary to the target sequence, whereas the other probe called the mismatch probe (MM) has one of the bases replaced by a non complementary base. For quantification of transcript number the average of fluorescence difference between the PM and MM for all probe pairs of a probe set are considered.*

2.7.1 Normalization of intensities between different arrays (done in collaboration with S. Kneitz)

The aim was to calculate which of the gene transcripts showed a significant difference in *Sap47*, *Syn*^{97} and the *Sap47-Syn*^{97} double mutant in comparison to the wild type. For analysis the statistical program *R* was used. The bioconductor software was run in R program mode and the limma (Linear Models for Microarray Data) package (Smyth, 2004) was installed for evaluation of data. The limma package in our study would be used for differential expression analysis.

For installing this package, R was started and the following was entered:

```
source("http://bioconductor.org/biocLite.R")
biocLite("limma")
```

Ideally the simplest way to quantify would be to compare the intensities of fluorescence for each probe set, but differences in RNA quality, quantity and hybridization efficiency could lead to non specific differences in intensities. Therefore it

is essential to make the data within an array and between arrays comparable by normalizing their fluorescence intensities. There are different ways of normalization:

1. Normalization by multiplying each array by a constant to make the mean (median) intensity the same for all chips. This procedure can be applied within the same array (when dual colour labeling is done on the same chip) or for normalizing intensities between arrays.

2 *Variance Stabilization and Normalization* (VSN, this method transforms data such that they have equal variance for all intensities) can be applied. This normalization procedure makes use of the fact that for higher means the variance is higher, therefore data points at lower intensities are linearly transformed and points at higher intensities are logarithmically transformed (Huber et al., 2002).Normalization by adjusting the arrays using some control or housekeeping genes that would be expected to remain unchanged and have the same intensity across all of the samples.

3. Match the percentiles of each array, not only the medians are matched but all quantiles are considered and all data points are scaled by a factor to match the respective quantiles on the reference array- *quantile normalization*. Thereby with this procedure the distribution of probe intensities for each array in a set of arrays is the same.

4. Adjustment by non linear smoothening - *Loess normalization* is applied (this assumes that at least half the genes in a chip are not differentially expressed and smoothens the data scatter by applying non linear regression).

The limma package was used for analysis and to normalise data by VSN and quantile normalization method.The Robust Multichip Average (RMA) approach (Irizarry et al., 2003a; Irizarry et al., 2003b) does not consider the MM probes but only the PM for VSN and quantile coupled normalization.

Method	Background Adjustment	Normalization	Mismatch adjustment	Probeset Summary	Reference
MAS5	regional adjustment*	scaling by a constant*	subtract idealized mismatch*	Tukey biweight average*	[17]
gcRMA	by GC content of probe*	quantile normalization*	PM only*	medianpolish* (robust fit of linear model)	[9]
RMA	whole array adjustment	quantile normalization*	PM only*	medianpolish* (robust fit of linear model)	[2]
VSN	none*	variance stabilizing transformation	PM only*	medianpolish* (robust fit of linear model)	[18]
dChip	none*	invariant set*	PM only*	Li-Wong multiplicative model*	[19]
dChip.mm	none*	invariant set*	subtract mismatch*		

(Qin et al., 2006)

Fig. 5 *Summary of different evaluation methods and the actions performed by them.*

MA-plots are used for visualization of differences in transcriptome expression. In our experiments it has been used to visualize the difference between two arrays (control and experimental group). They represent a scatter plot of the log-ratios called M-values (minus, y axis) against the log-intensities called A-values (average, x axis) for arrays. MA-plots are also called RI-plots (ratio-intensity).

Log-ratios for each spot: $M = \log_2 A - \log_2 B = \log_2 (A_i / B_i)$
log-intensity of each spot: $A = (\log_2 A + \log_2 B)/2 = \log_2 \sqrt{} (Ax B)$

The conversion to log scale is appropriate as fold changes in both directions (negative or positive) are transformed equally by normalization software.

2.7.2 Selection of candidate genes based on fold change

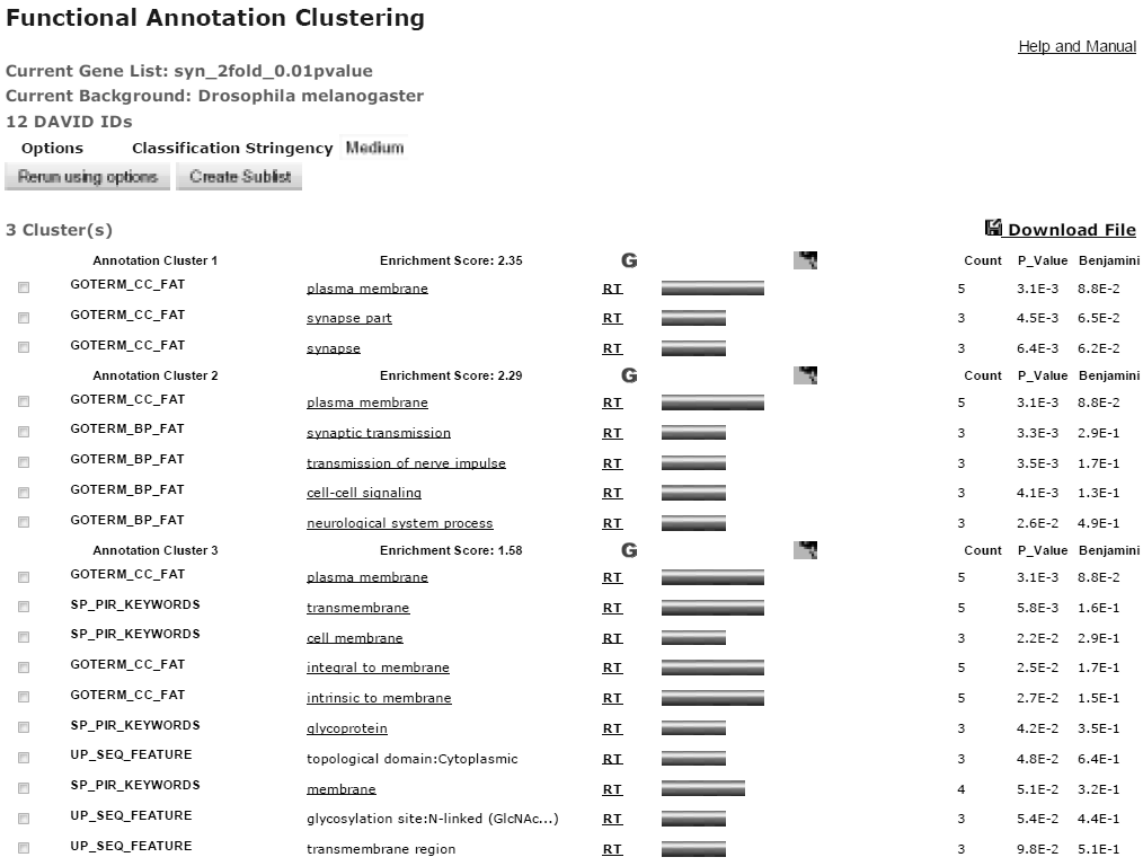
On normalization of intensities of different arrays, the next step is to find the differences in expression patterns between different samples (arrays). The direct way of determining the difference in expression is the calculation of difference in intensities for a given gene (as errors due to experimental procedures has already been corrected by normalization) between arrays. The difference in intensities indicates the *signal log ratio* of gene expression between the arrays compared. To find the *fold change* the absolute signal log ratio can be converted from log scale to linear scale.

For microarray experiments normally a threshold of >1.5 fold and significance of $p < 0.05$ from *t test* is considered while selecting probable candidates. The t test considers the signal (difference between means) to noise ratio (variability within groups) for analysis, this permits the selection of genes that are up- or down-regulated by a factor higher than 1.5 fold and with a 95% confidence level. Since the array comprises about

18800~ transcripts about 700-800 gene transcripts could come up as probable candidates at significance value of $p < 0.05$ just due to random variations of the technique. Therefore multiple testing methods like False Discovery Rate (FDR) and Family Wide Error Rates (FWER) (Benjamini et al., 2001) could be applied. In our experiments we have considered Bonferroni-Holm and Benjamini-Hochberg adjusted p values (<0.01) to select for appropriate candidates. According to Bonferroni a p value of 2.6×10^{-6} should be attained.

2.7.3 Selection of candidate genes according to functional clustering by the DAVID software

The DAVID software permitted the *functional annotation clustering* of the genes that were altered in a given mutant with selection criterion of a fold change >2 and adjusted $p < 0.01$. Using this tool one can generate a gene to gene similarity matrix based on functional annotation which comprises 75000 terms from 14 different functional annotation sources. The enrichment score indicates the number of terms clustered together in a group and the number of genes grouped in each term. Thus a high enrichment score indicates a likely involvement of the mutated gene in the indicated functional context (Fig.6).



Functional Annotation Clustering

Help and Manual

Current Gene List: syn_2fold_0.01pvalue
Current Background: Drosophila melanogaster
12 DAVID IDs

Options Classification Stringency Medium

Rerun using options | Create Sublist

3 Cluster(s)

Download File

Annotation Cluster 1	Enrichment Score: 2.35			Count	P_Value	Benjamini
GOTERM_CC_FAT	plasma membrane	RT		5	3.1E-3	8.8E-2
GOTERM_CC_FAT	synapse part	RT		3	4.5E-3	6.5E-2
GOTERM_CC_FAT	synapse	RT		3	6.4E-3	6.2E-2
Annotation Cluster 2	Enrichment Score: 2.29			Count	P_Value	Benjamini
GOTERM_CC_FAT	plasma membrane	RT		5	3.1E-3	8.8E-2
GOTERM_BP_FAT	synaptic transmission	RT		3	3.3E-3	2.9E-1
GOTERM_BP_FAT	transmission of nerve impulse	RT		3	3.5E-3	1.7E-1
GOTERM_BP_FAT	cell-cell signaling	RT		3	4.1E-3	1.3E-1
GOTERM_BP_FAT	neurological system process	RT		3	2.6E-2	4.9E-1
Annotation Cluster 3	Enrichment Score: 1.58			Count	P_Value	Benjamini
GOTERM_CC_FAT	plasma membrane	RT		5	3.1E-3	8.8E-2
SP_PIR_KEYWORDS	transmembrane	RT		5	5.8E-3	1.6E-1
SP_PIR_KEYWORDS	cell membrane	RT		3	2.2E-2	2.9E-1
GOTERM_CC_FAT	integral to membrane	RT		5	2.5E-2	1.7E-1
GOTERM_CC_FAT	intrinsic to membrane	RT		5	2.7E-2	1.5E-1
SP_PIR_KEYWORDS	glycoprotein	RT		3	4.2E-2	3.5E-1
UP_SEQ_FEATURE	topological domain:Cytoplasmic	RT		3	4.8E-2	6.4E-1
SP_PIR_KEYWORDS	membrane	RT		4	5.1E-2	3.2E-1
UP_SEQ_FEATURE	glycosylation site:N-linked (GlcNAc...)	RT		3	5.4E-2	4.4E-1
UP_SEQ_FEATURE	transmembrane region	RT		3	9.8E-2	5.1E-1

Fig. 6 *Functional annotation clustering for genes expressed differently in Synapsin mutants compared to WT.*

The heat plots based for each cluster from Fig. 6 can be used to see how many terms in the cluster are shared by individual genes present in the cluster (Fig.7). The *functional annotation table* compiled known functions associated with each gene in the list Candidates whose expression is altered in *Synapsin* mutants are considered here to illustrate the examples.

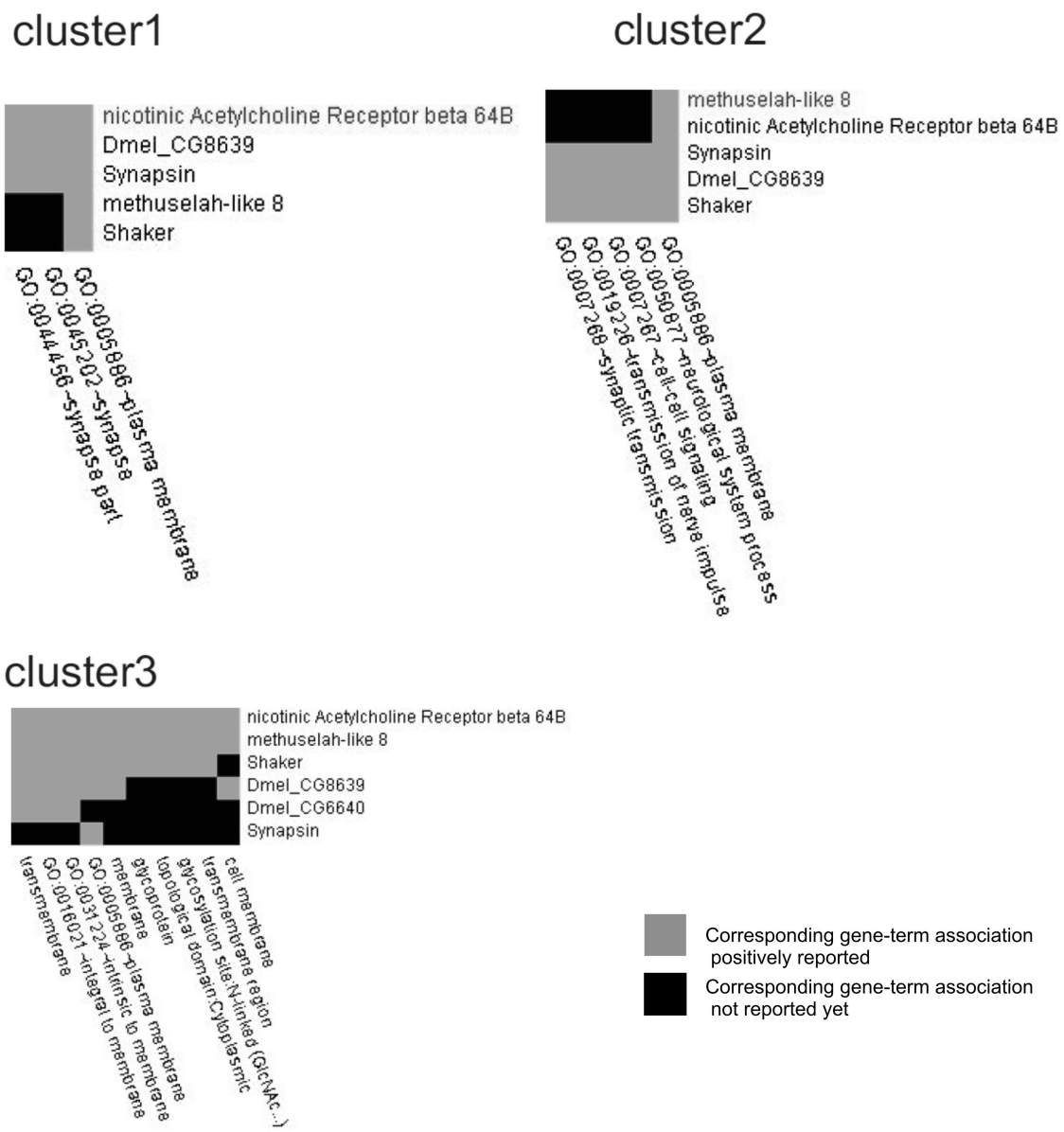


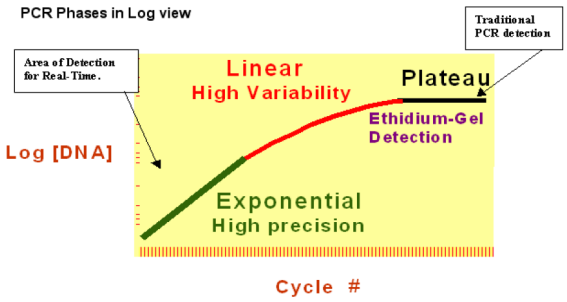
Fig. 7 *Grouping of data into different functional groups based on their enrichment score using functional annotation clustering feature of David software.*

The generation of functional tables in DAVID software required the Affymetrix probe-set id's of the candidate genes that fulfilled the selection criteria. Once the list was fed into DAVID the grouping of data on different features was possible. The *functional annotation clustering* served as an important tool in determining if the candidate genes were related functionally or structurally. It segregated the data into clusters of genes that were closely related (Fig.26 and 27). The *functional annotation table* helped to visualize at a glance the predicted function of the genes which helped us to select the genes for further analysis by qPCR (description of genes given in results section 3.3).

Similar analysis was done for all the groups viz. *Sap47*, *Synapsin* and *V2* and *V3* double mutants and this allowed us to select interesting genes based on their functions.

2.7.4 Verification of candidates from chip experiments

The chip experiments form a platform for screening transcript levels of a large number of genes at once. For establishment of the results from microarray experiments and narrowing down the candidates by removing false positives it becomes essential to perform quantification of transcript levels individually in the experimental and control genotypes. Real Time PCR (RT PCR) or qPCR approach has been used to quantify transcript levels. The advantages of a real time PCR is that the transcript copy number is measured in the logarithmic phase in contrast to traditional semi quantitative PCR where the quantification may occur in the variable or even in the plateau phase (Fig. 8). In the plateau phase the differential expression cannot be quantified as the saturation level reached is independent of the original transcript number, whereas in the logarithmic phase the number of cycles required to produce a given amount of PCR product is proportional to $\log_2$ of the number of transcripts.

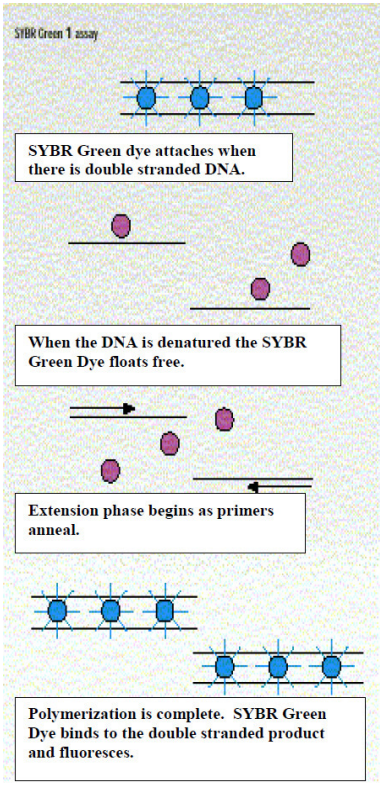


Source: world wide web

Fig. 8 *Difference between RT PCR and a traditional semi-quantitative PCR. Traditional semi-quantitative PCR detection may occur in the variable or plateau phase whereas in RT PCR the detection is in the logarithmic phase.*

There are two methods by which real time PCR experiments could be performed. One is by taqman chemistry and the other method is SYBR green based quantification. We have used the SYBR green chemistry to quantify transcript number. SYBR green dye has the property that it can bind only to the minor groove of double stranded DNA. As the

PCR reaction proceeds increasing amounts of double stranded DNA are produced and proportional to these amounts the SYBR green dye gets incorporated. Therefore a direct measure of transcript copy number can be obtained by quantifying the fluorescence of the dye. Care should be taken that the primers designed do not generate a product of more than 200 base pairs, and the product sizes generated by the housekeeping control primers and the primers specific for the gene under investigation are roughly to avoid additional errors in quantification.



Source: world wide web

Fig. 9 *SYBR green based detection of transcript copy number. SYBR green dye is incorporated into newly formed double stranded DNA in a PCR reaction, thereby quantification of fluorescence of the dye represents a direct measure of transcript copy number.*

3. Results (In collaboration with Kneitz, S. and Nuwal, T.)

3.1 Standardization experiments prior to microarray experiments

For the microarray analysis we wanted to use *Syn*^{97}, *Sap47*^{156} and *Syn-Sap* double mutants. These mutants had deletions in the 5' region of the gene and were null mutants for protein expression (Funk et al., 2004; Godenschwege et al., 2004) but it remained to be tested if a truncated transcript was being produced. The probes on the microarray chip comprise sequences from the 3' end of the transcript and hence it was essential to know if the 3' transcript was still being made. Therefore, primers specific for 5' and 3' end of the S*ynapsin* and *Sap47* genes were designed (Fig. 10).

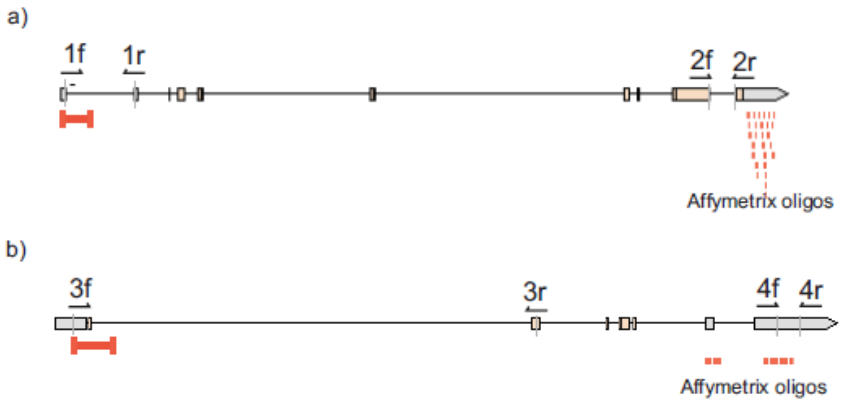


Fig. 10 *Position of primers for RT- PCR analysis of transcripts at 5' and 3' end of Synapsin (a) and Sap47 (b) genes in CS and Syn* ^{97} *and Sap47*^{156}*deletion mutants. The deletions are indicated by red bold bar.*

For *Syn*^{97} mutant it was observed that the 5' transcript was not present, however a 3' truncated transcript was formed which could be amplified with primers 2f/2r specific for 3' end of *Synapsin* gene (Fig. 11).

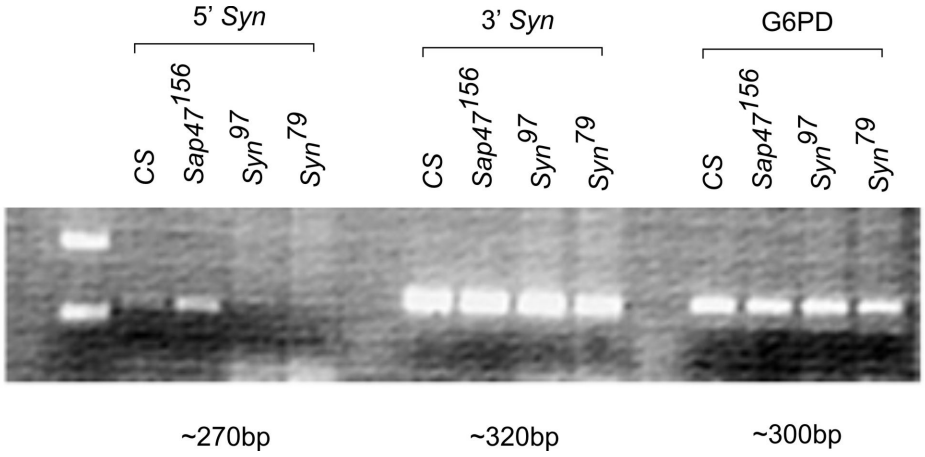


Fig. 11 *Examination of the presence of Synapsin transcript in Syn^{97} and $Sap47^{156}$ mutants. No signal is observed for Syn^{97} when primers specific for 5' end are used, however a truncated transcript containing the 3' end is formed. The transcript level for the gene G6PD housekeeping control does not show any difference between genotypes.*

The *$Sap47^{156}$* mutant did not show a signal for the 5'end of the *Sap47* transcript, but again the 3' end was intact. This indicated that a truncated transcript was being formed, similar to the *Syn* mutants (Fig. 12).

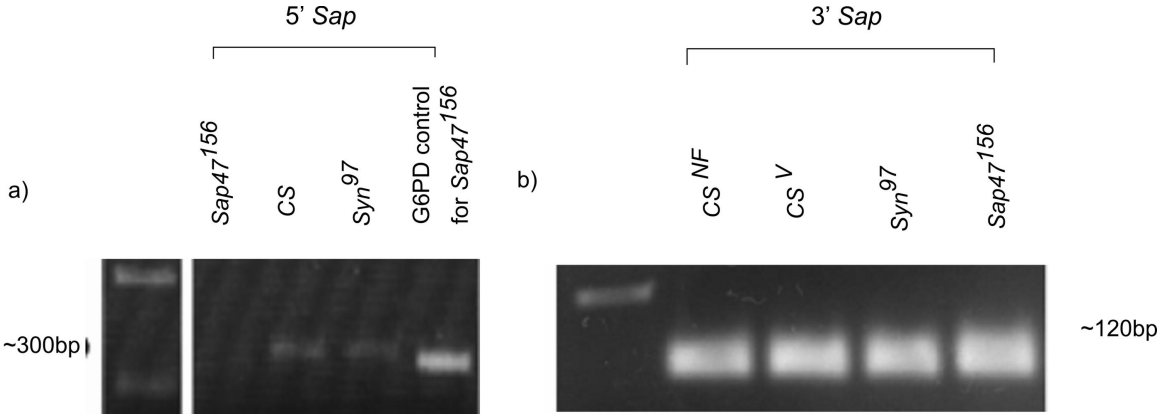


Fig. 12 *Examination of the presence of Sap47 transcript in Syn^{97} and $Sap47^{156}$ mutants. No signal is observed in $Sap47^{156}$ when primers specific for 5' end are used, however a truncated transcript at the 3' end is formed. The housekeeping control does not show any difference between genotypes (not shown).*

These preliminary standardization experiments proved that in both the mutant used in this study although they were null mutants for protein expression, a truncated transcript of the mutated gene was being formed. Hence, in the microarray experiments a

signal should be observed as the microarray probes bind only to 3' regions of the transcripts.

3.2 Microarray experiments

For the microarray experiments we have used 6 samples viz. CS^{NF} (wildtype used for cantonising the *Syn* and *Sap47* mutants), CS^{V} (wildtype used for cantonising the double mutants), Syn^{97}, $Sap47^{156}$, *Syn Sap* double mutants *V2* and *V3*. The RNA extraction was carried out with the procedure mentioned in section 2.3 and the quality of RNA was verified by the procedure mentioned in section 2.4 and 2.6.1.

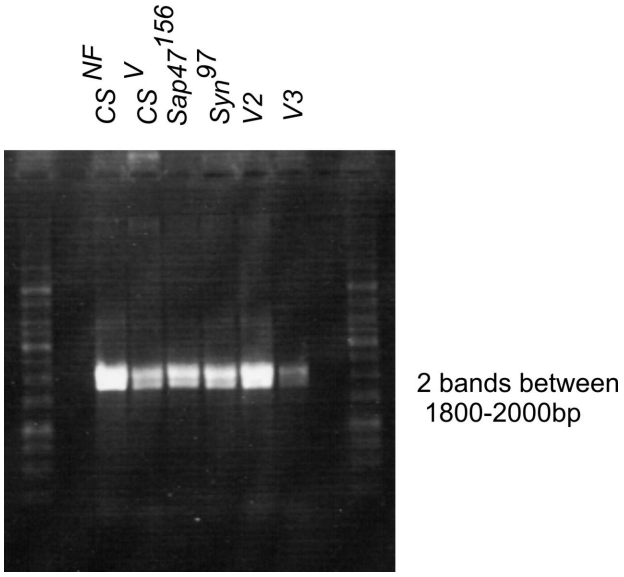


Fig. 13 Verification of RNA quality prior to hybridization. Two bands were observed between 1500 and 2000 bp. The 18S RNA gives a band of 2000bp and 28S RNA which normally gives a signal at 4000bp is cleaved in Drosophila into two bands.

On verification of the quality of RNA the samples were hybridized by the microarray core unit of the IZKF (Interdisziplinären Zentrum für Klinische Forschung at the University of Würzburg) in collaboration between our group and the group of Dr. Susanne Kneitz (IZKF). The analysis of the raw data was done by Dr. Kneitz in R using the bioconductor software with the limma package (see section 2.7). The data was normalized (see section 2.7.1) by quantile normalization for comparison between arrays (Fig.14 and 15).

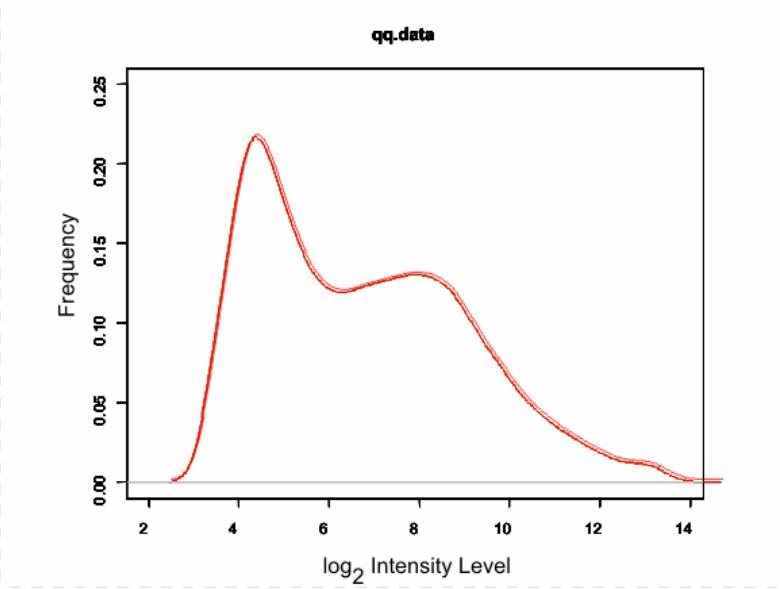


Fig. 14 *Normalization between arrays to match all the quantiles by quantile normalization.*

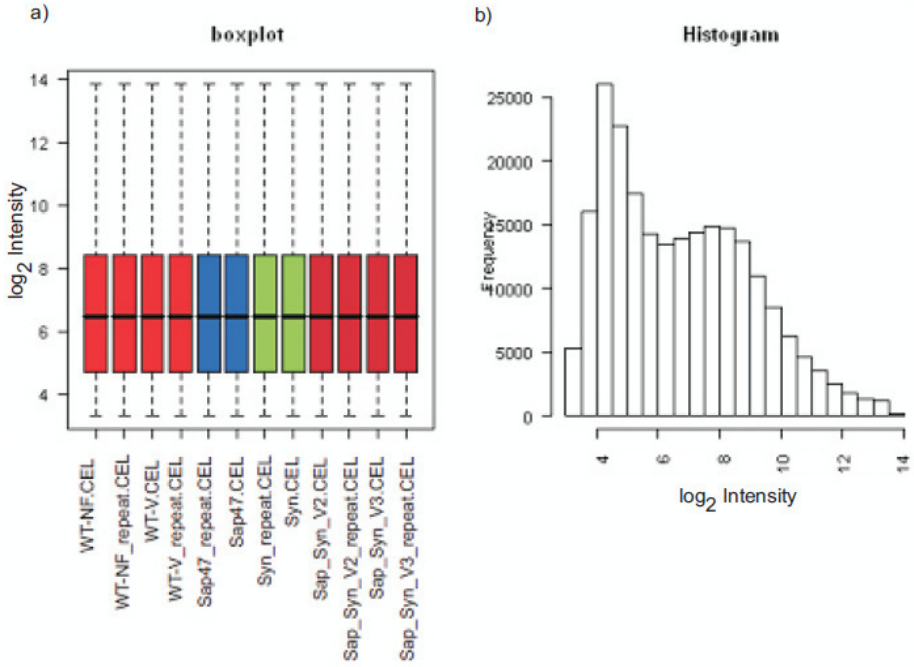


Fig. 15 *Normalization of all arrays such that the median intensity and other quantiles are same in all chips (a). The frequency of occurrence of data points are a given intensity is plotted (b).*

Analysis of all mutants was done individually by comparing gene expression in mutants to

wildtypes.

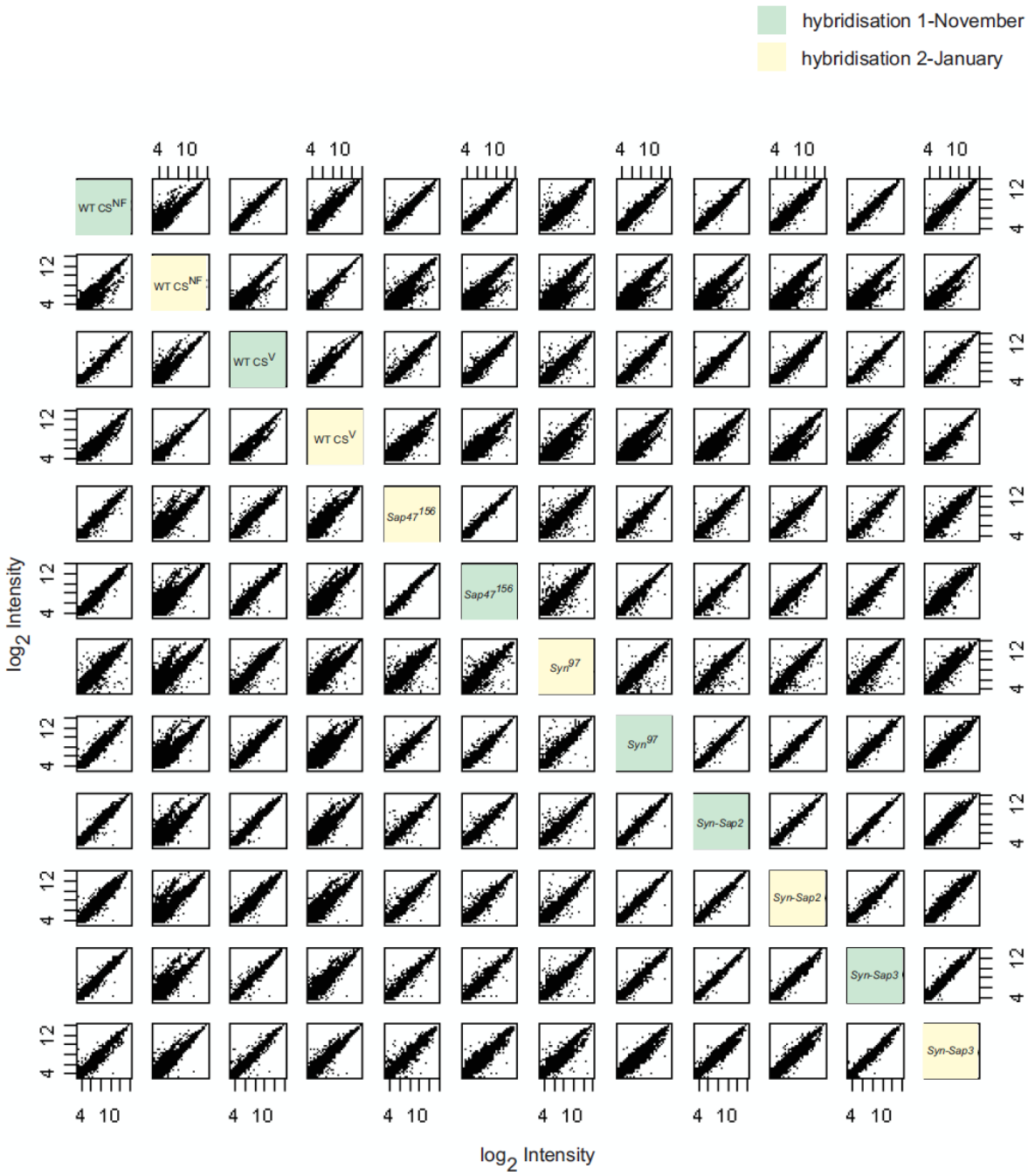


Fig. 16 *Figure shows scatter plots of pair wise comparison for all chips. The two hybridization procedures are demarcated, green represents samples from first hybridization and yellow represents samples from second hybridization.*

3.2.1 *Sap47* mutant

After normalization of the arrays all the samples became comparable in intensities. Hence a direct measure of difference in expression between experimental groups and controls is the difference in their fluorescence intensities. To compare the expression level between two genotypes the ma-plot was used (see section 2.7.1 for description of ma plot).

When for all genes the $\log_2$ value of the ratios of expression in the wild type over expression in *Sap47* mutants are presented by the ma plot, genes with a higher expression in wild type in comparison to the mutant were seen above the zero line in the plot indicating down-regulation of those genes in the mutants (e.g. *Sap47* gene is seen above zero), whereas genes that were up-regulated in the mutant are seen below the zero line (Fig.13).

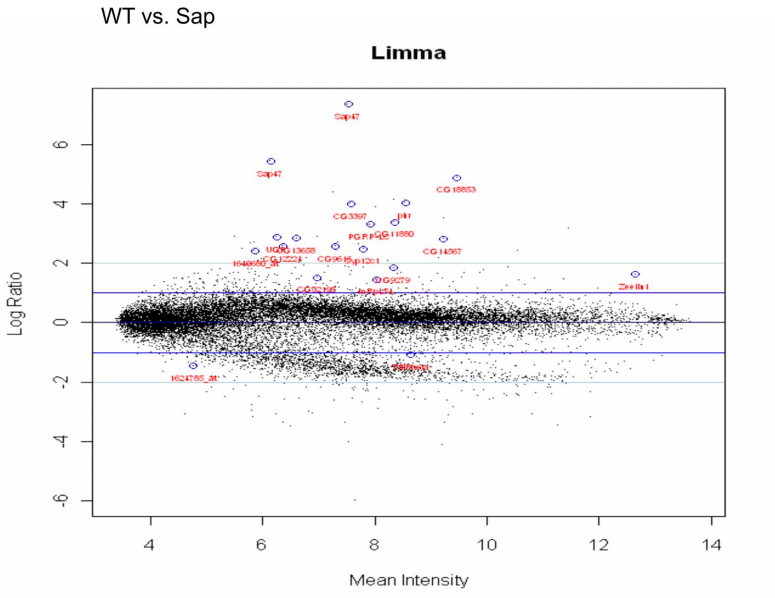


Fig. 17 *ma-plot with comparison of gene expression between wild type (WT) flies and Sap47 mutants. Genes down-regulated in Sap47 mutants in comparison to WT are seen above the zero line. X axis indicates $\log_2$ of average intensity (I) of spots and Y axis indicates $\log_2$ of the ratio I_{WT}/I_{Sap} of the normalised fluorescence intensities I for each gene. Encircled spots fulfil the selection criterion of fold change>2 and adjusted p value <0.01.*

For further analysis the genes showing more than three fold change and adjusted p value <0.01 were considered potential members of a functional network involving *Sap47*and functionally interesting genes from the list were selected by DAVID analysing software for further investigation (Table 4), the complete list is given in Appendix A. If the ratio in this table is less than 1 the corresponding gene is up-regulated in the *Sap47* mutant in comparison to wild type, a ratio higher than one indicates down-regulation in *Sap47* mutants in comparison to wild type.

Query1						
ID	**GENENAME**	**SYMBOL**	**Ratio**	**fold-change**	**P#Value**	**adj#P#Val**
1630169_a_at	*Syn*apse-associated protein 47kD	Sap47	162.624	162.624	1.15988620357476E-12	2.19821633301488E-08
1637387_s_at	*Syn*apse-associated protein 47kD	Sap47	43.273	43.273	3.77110573541679E-10	3.57349979488095E-06
1636758_s_at	CG18853	CG18853	29.442	29.442	1.59531644045744E-08	7.55860929488736E-05
1630728_a_at	photorepair	phr	16.432	16.432	7.8113332729091E-08	2.20160579138796E-04
1631805_at	CG3397	CG3397	16.078	16.078	2.39889816573516E-07	5.68298975462 66E-04
1638021_at	CG4757	CG4757	14.835	14.835	1.15895469659803E-05	6.65591194240179E-03
1628536_s_at	CG11880	CG11880	10.469	10.469	7.09305564534018E-07	1.06282750505285E-03
1631312_at	CG4073	CG4073	0.096	10.403	2.04209361542557E-05	9.43945321940 13E-03
1641723_a_at	Peptidoglycan recognition protein LC	PGRP-LC	9.897	9.897	1.73330734463181E-09	1.09498802650 154E-05
1624543_s_at	NA	NA	9.092	9.092	8.86879096315086E-06	5.25254144790 261E-03
1633218_a_at	UGP	UGP	7.359	7.359	7.29039550743298E-07	1.06282750505285E-03
1638080_at	CG13658	CG13658	7.163	7.163	2.24874979755 22E-08	8.52366123264186E-05
1640978_at	CG14567	CG14567	7.030	7.030	5.02300831361459E-07	9.51960535596236E-04
1632537_at	CdGAPr	CdGAPr	6.456	6.456	1.40492766670832E-05	7.57478960993962E-03
1631693_at	CG9616	CG9616	5.946	5.946	2.53674577460477E-06	2.82485028209627E-03
1639971_at	CG12224	CG12224	5.944	5.944	3.44252410474887E-06	3.11537431734844E-03
1624953_at	Cyp12c1	Cyp12c1	5.592	5.592	4.24706799713238E-07	8.94338140907255E-04
1640680_at	NA	NA	5.347	5.347	9.6151966429	1.3016229055

Query1						
ID	**GENENAME**	**SYMBOL**	**Ratio**	**fold-change**	**P#Value**	**adj#P#Val**
					1963E-07	4 723E-03
1626257_at	CG13656	CG13656	0.220	4.541	4.1396360701 0718E-06	3.4110601217 6832E-03
1641650_at	alpha-Esterase-5	alpha-Est5	4.474	4.474	4.6313013225 0248E-06	3.4949706565 2172E-03
1637197_at	nebbish	neb	4.311	4.311	3.4520293723 2573E-06	3.1153743173 4844E-03
1637055_s_at	NA	NA	4.229	4.229	4.9791160682 8231E-06	3.4949706565 2172E-03
1624075_at	CG31495	CG31495	3.848	3.848	7.7380352546 8749E-06	4.7306852950 5927E-03
1635802_s_at	CG9279	CG9279	3.598	3.598	6.5760356948 8911E-07	1.0628275050 5285E-03
1628327_at	NA	NA	3.518	3.518	1.5422488902 3155E-05	7.5747896099 3962E-03
1635770_at	CG31097	CG31097	3.474	3.474	1.6895629243 1714E-05	8.0051491354 1459E-03
1626999_at	NA	NA	3.429	3.429	1.5574257264 5924E-05	7.5747896099 3962E-03
1624631_x_at	NA	NA	3.325	3.325	6.3501200721 0957E-06	4.2981241288 0788E-03
1637006_at	CG33174	CG33174	3.219	3.219	7.6638287603 4272E-06	4.7306852950 5927E-03
1628076_at	Lip3	Lip3	3.143	3.143	4.8970095189 7904E-06	3.4949706565 2172E-03
1635372_a_at	Zeelin1	Zeelin1	3.070	3.070	8.1317225304 5363E-08	2.2016057913 8796E-04

Table 4: *List of selected genes showing more than 3 fold change with a significance of p<0.01(adjusted p value) in Sap47 mutants, NA corresponds to non-annotated genes. Probe id 1630169_a_at indicates probe sequence specific for shorter iso-form (Sap47 RB and RF), whereas probe id 1637387_s_at refers to probe sequence specific for longer isoforms.*

Some of the genes from the list are graphically represented below. The transcript levels in *Sap47* mutants are compared with those in wild type (WT), *Syn* mutants and double mutants (*V2* and *V3*) (Fig.18).

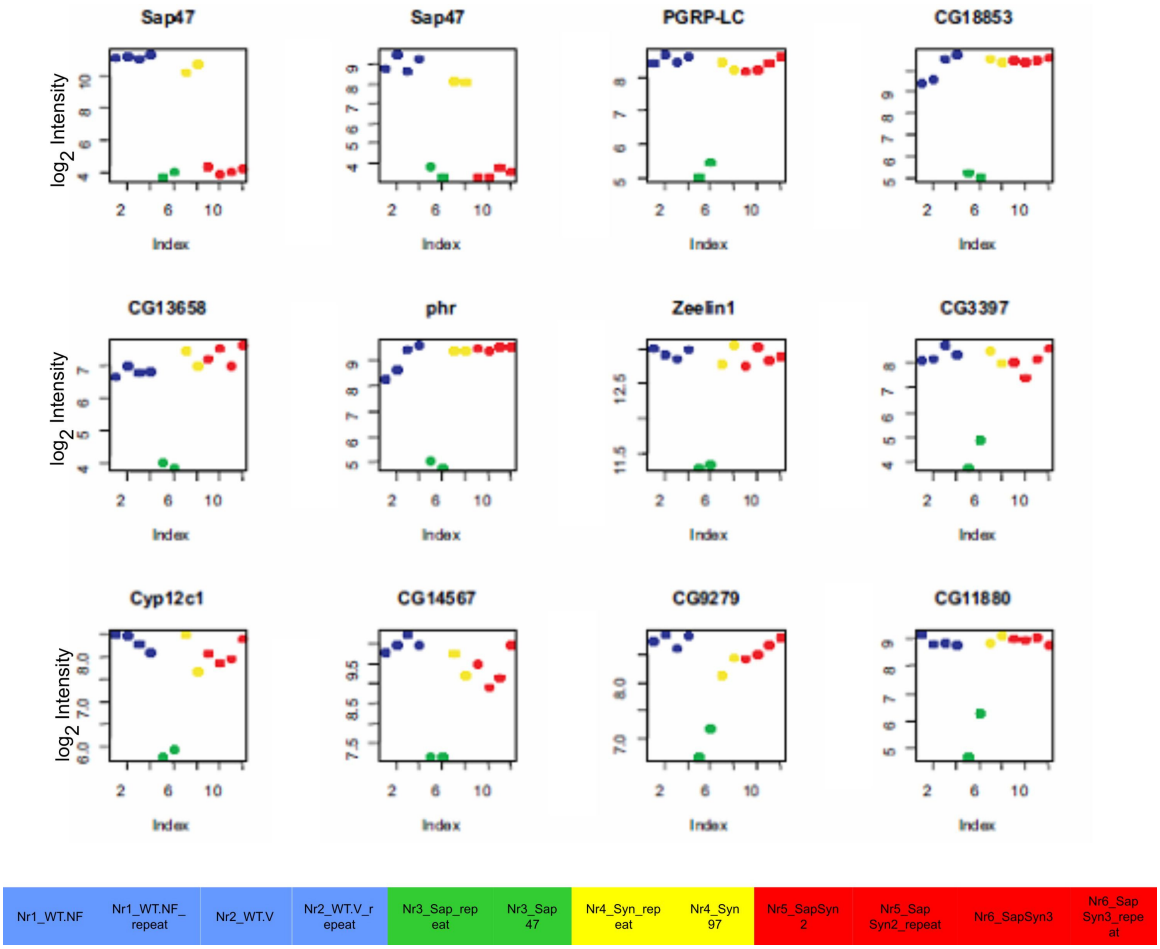


Fig. 18 E*xpression of selected genes showing significant fold change in Sap47 mutants in comparison to their expression in WT, Syn mutants and Sap-Syn double mutants (V2 and V3)*

Interestingly only the *Sap47* transcript is strongly reduced both in the single *Sap47* mutant and in *Sap-Syn* double mutants. All other transcripts that are down-regulated in the *Sap47* mutant appear to have wild type expression levels in the *Sap-Syn* double mutants (see discussion).

3.2.2 *Synapsin* mutant

To find the genes that showed a modified expression levels in the *Synapsin* mutant, the normalised gene expression values of the *Synapsin* mutant were compared to WT. When *Synapsin* mutant expression was deducted from gene expression in wild type (WT, *CS*) flies and compared by ma-plot, genes with a higher expression in wild type in comparison to the mutant are seen above the zero line in the plot indicating down-

regulation of those genes, in the mutant, whereas genes that were up-regulated in the mutant are seen below the zero line (Fig.19).

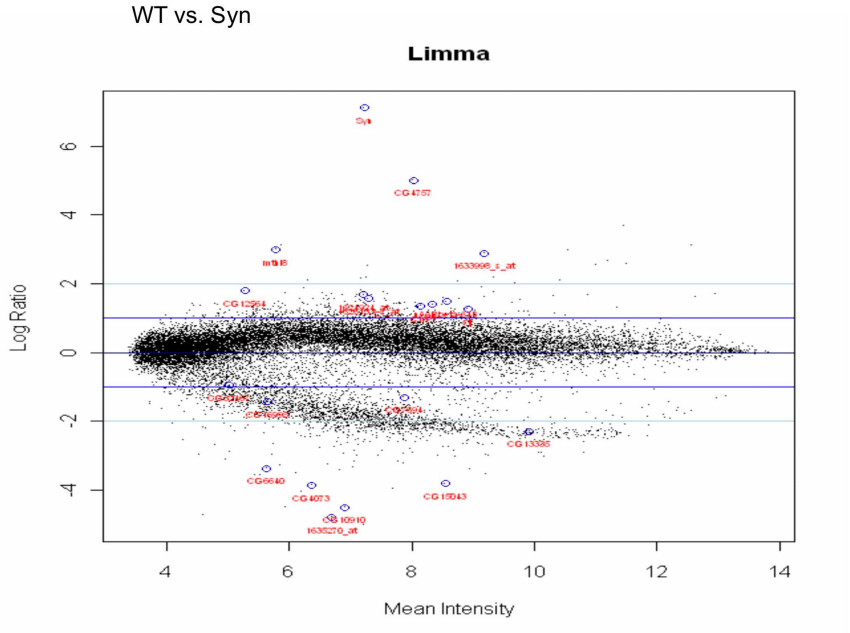


Fig. 19 *ma-plot with comparison of gene expression between wild type (WT) flies and Synapsin mutants. Genes down-regulated in Synapsin mutants in comparison to WT are seen above the zero line. X axis indicates* log_2 *of average intensity (I) of spots and Y axis indicates* log_2 *of the ratio* I_{WT}/I_{Syn} *of the normalised fluorescence intensities I for each gene. Encircled spots fulfil the selection criterion of fold change>2 and p value <0.05.*

For further analysis the genes showing more than two fold change and adjusted p value <0.01 were considered potential members of a functional network involving *Synapsin* and functionally interesting genes were selected from the list by DAVID analysing software for further investigation (Table 5), the complete list is given in Appendix B. If the ratio in this table is less than 1 the corresponding gene is up-regulated in the *Synapsin* mutant in comparison to wild type, a ratio higher than one indicates down-regulation in *Synapsin* mutants in comparison to wild type.

Query2						
ID	**GENENAME**	**SYMBOL**	**Ratio**	**fold-change**	**P#Value**	**adj#P#Val**
1636378_a_at	*Syn*apsin	*Syn*	138.505	138.505	6.11671967776339E-12	1.15924071332972E-07
1638021_at	CG4757	CG4757	32.259	32.259	1.04746063303899E-06	8.93367455860935E-03
1635270_at	NA	NA	0.036	27.694	1.41415278998671E-06	8.93367455860935E-03
1633998_s_at	NA	NA	7.407	7.407	2.15630584964007E-06	9.77382565160971E-03
1633009_a_at	CG6640	CG6640	0.097	10.320	3.06148510642 39E-06	9.77382565160971E-03
1626802_a_at	Shaker	Sh	2.623	2.623	3.11147941428636E-06	9.77382565160971E-03
1640640_at	Cirl	Cirl	2.555	2.555	3.74215237402623E-06	9.77382565160971E-03
1626133_s_at	NA	NA	2.957	2.957	5.58854722142157E-06	9.77382565160971E-03
1631312_at	CG4073	CG4073	0.069	14.477	6.05631234167673E-06	9.77382565160971E-03
1627219_at	nicotinic Acetylcholine Receptor beta 64B	nAcRbeta-64B	2.783	2.783	6.14316766644704E-06	9.77382565160971E-03
1629362_at	methuselah-like 8	mthl8	7.947	7.947	6.17855434032129E-06	9.77382565160971E-03
1637275_a_at	CG13335	CG13335	0.204	4.893	6.18857681613 11E-06	9.77382565160971E-03

Table 5: *List of selected genes showing more than 2 fold change with a significance of p<0.01(adjusted p value) in Synapsin mutants, NA corresponds to non-annotated genes.*

The transcript levels in *Synapsin* mutants are compared with those in wild type (WT), *Sap47* mutant and double mutants (*V2* and *V3*) (Fig. 16).

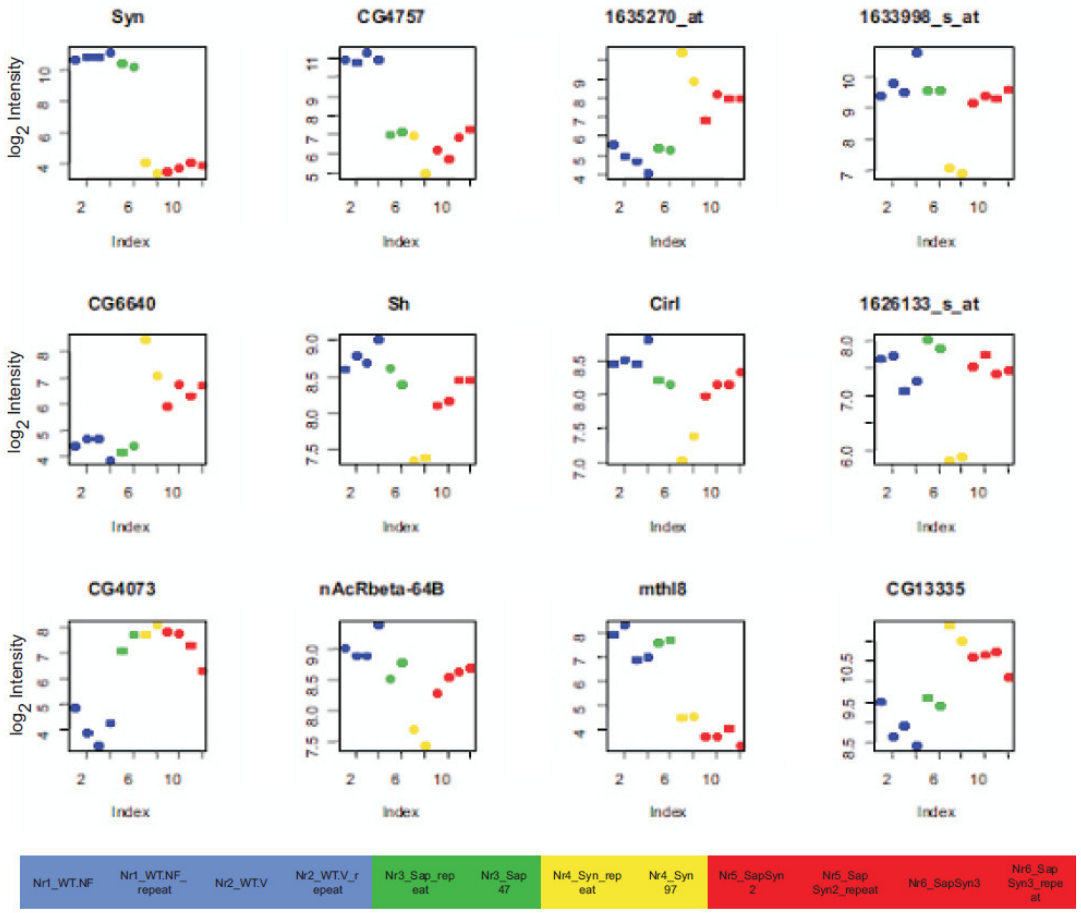


Fig.20 *Expression of selected genes showing significant fold change in Synapsin mutants in comparison to their expression in WT, Sap47 mutants and Sap-Syn double mutants (V2 and V3)*

Again, the pattern expected if the *Syn* mutation influences expression of other genes irrespective of the *Sap47* mutation is observed only for the *Syn* transcript (see discussion).

3.2.3 *Sap-Synapsin* double mutant

The normalised expression intensities *Sap-Synapsin* double mutants (*V2* and *V3*) were compared to WT to find the genes affected by the concerted effect of the two mutations. The ma- plot for this comparison shows the genes that are down-regulated in double mutants in comparison to WT above the zero line (*Sap47* and *Synapsin* gene seen above zero) and the up-regulated genes below zero line (Fig.21).

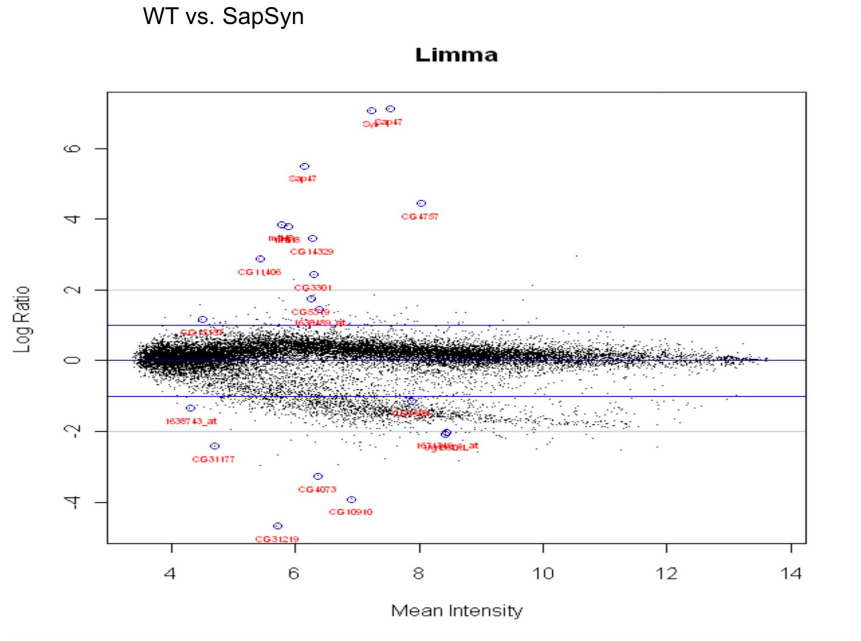


Fig. 21 *ma-plot with comparison of gene expression between wild type (WT) flies and Sap-Synapsin double mutants. Genes down-regulated in double mutants in comparison to WT are seen above the zero line. X axis indicates log_2 of average intensity (I) of spots and Y axis indicates log_2 of the ratio $I_{WT}/I_{Sap\text{-}Syn}$ of the normalised fluorescence intensities I for each gene. Encircled spots fulfil the selection criterion of fold change>2 and p value <0.05.*

For further analysis the genes showing more than two fold change and adjusted p value <0.01 were considered potential members of a functional network involving *Sap*47 and *Synapsin*. Functionally interesting genes were selected from the list by DAVID analysing software for further investigation (Table 6), the complete list is given in Appendix C. If the ratio in this table is less than 1 the corresponding gene is up-regulated in the *Sap-Synapsin* mutant in comparison to wild type, a ratio higher than one indicates down-regulation in *Sap-Synapsin* mutants in comparison to wild type.

Query3						
ID	**GENENAME**	**SYMBOL**	**Ratio**	**fold-change**	**P#Value**	**adj#P#Val**
1630169_a_at	*Syn*apse-associated protein 47kD	Sap47	138.668	138.668	1.86892693768421E-13	3.54199033229912E-09
1636378_a_at	*Syn*apsin	*Syn*	133.332	133.332	7.65615930847621E-13	7.25497656071206E-09

Query3						
ID	**GENENAME**	**SYMBOL**	**Ratio**	**fold-change**	**P#Value**	**adj#P#Val**
1637387_s_at	*Syn*apse-associated protein 47kD	Sap47	45.603	45.603	3.83083867720532E-11	2.42006848701318E-07
1627572_at	CG31219	CG31219	0.039	25.755	1.20957738755286E-10	5.73097766222547E-07
1638021_at	CG4757	CG4757	21.853	21.853	4.59586898921618E-07	8.72438833059516E-04
1624945_a_at	CG10910	CG10910	0.066	15.117	4.79574193661497E-06	4.86712998659665E-03
1629362_at	methuselah-like 8	mthl8	14.343	14.343	7.25472179438902E-08	1.96416410638944E-04
1633386_s_at	methuselah-like 8	mthl8	13.828	13.828	1.30811723006688E-06	2.06595314535229E-03
1626024_at	CG14329	CG14329	10.923	10.923	4.60341300685688E-07	8.72438833059516E-04
1631312_at	CG4073	CG4073	0.104	9.573	4.3712669231319E-06	4.86712998659665E-03
1626791_at	CG11406	CG11406	7.307	7.307	4.52502624080967E-08	1.42930495526375E-04
1637721_at	CG3301	CG3301	5.418	5.418	1.43120476303603E-08	5.42483853381178E-05
1634477_at	CG31177	CG31177	0.186	5.378	4.59318182865741E-06	4.86712998659665E-03
1641481_at	Ugt86Dd	Ugt86Dd	0.236	4.240	1.60704589865078E-06	2.34282568240227E-03
1631349_s_at	NA	NA	0.244	4.098	2.15020916567912E-06	2.91076886485363E-03
1641678_at	CG5319	CG5319	3.384	3.384	2.65968765446583E-07	6.30080005342956E-04

Table 6 *List of selected genes showing more than 2 fold change with a significance of p<0.01(adjusted p value) in Sap-Synapsin mutants, NA corresponds to non-annotated genes.*

The transcript levels in *Sap-Synapsin* mutants (*V2* and *V3*) are compared with those in wild type (WT), *Sap47* and *Synapsin* mutants (Fig. 22)

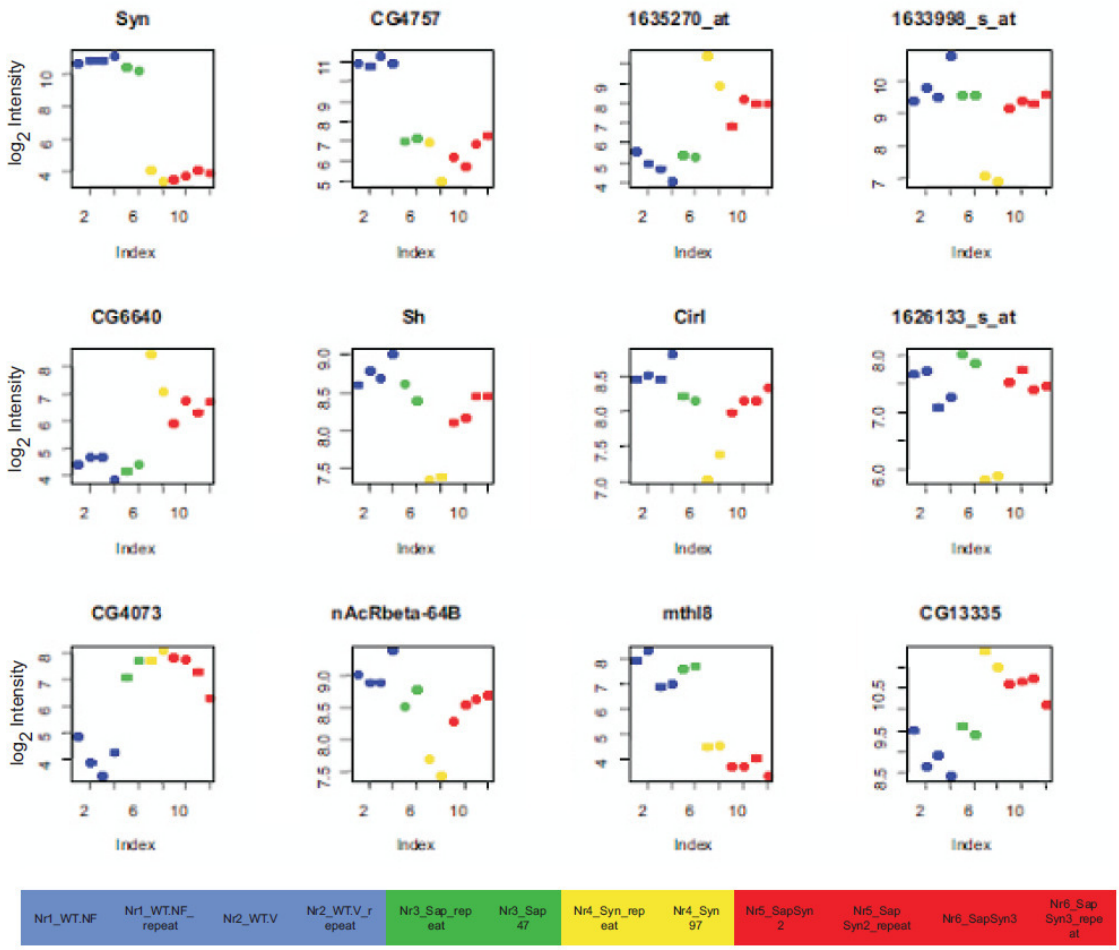


Fig. 22 E*xpression of selected genes showing significant fold change in Sap-Synapsin (V2 and V3) double mutants in comparison to their expression in WT, Sap and Syn mutants.*

We also compared the two pairs of wild type stocks to look for genes that are differentially expressed. The two wild types used for the first experiment viz. CS^{NF} and CS^{V} were essentially the same strain which had been maintained separately for about 10 years (about 120 generations) (Fig. 23). The experiment was performed simultaneously with both stocks in November. All microarray experiments were repeated two months later. Surprisingly a large number of genes appear to be up-regulated within the same WT stock in January compared to November (Fig. 16), but on pooling both hybridizations from January and November not much of difference is observed between the two WT stocks (Fig. 23). This phenomenon is difficult to interpret, in particular as it is only observed for the two WT stocks but not for the mutant stocks (see Fig. 16 and discussion).

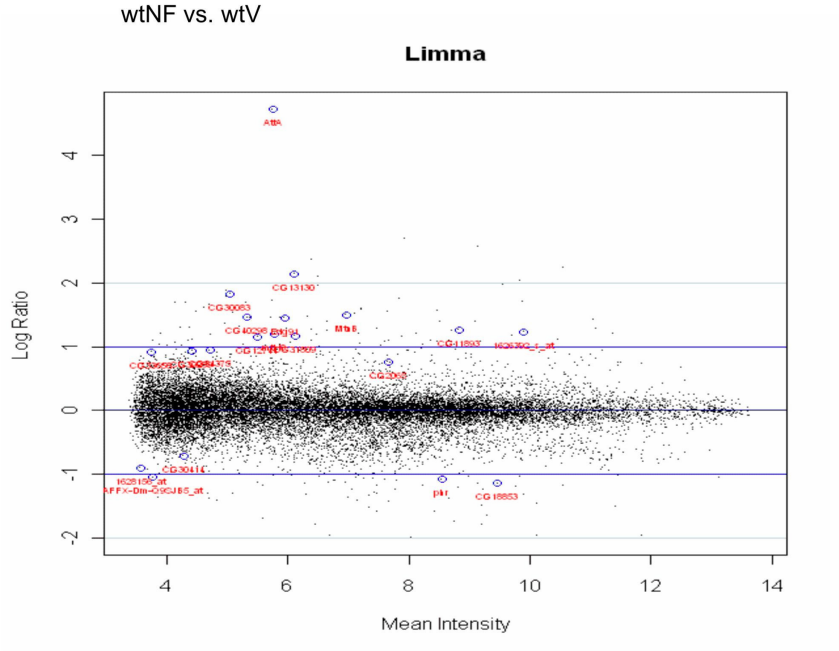


Fig. 23 *ma-plot with comparison of gene expression between the two wild types* CS^{NF} *and* CS^{V} *.Genes down-regulated in* CS^{V} *in comparison to* CS^{NF} *are seen above the zero line. X axis indicates* log_2 *of average intensity (I) of spots and Y axis indicates* log_2 *of the ratio* I_{CS}^{NF} / I_{CS}^{V} *of the normalised fluorescence intensities I for each gene. Encircled spots fulfil the selection criterion of fold change>2 and p value <0.05.*

Using similar parameters for analysis of gene expression as before (fold change by more than a factor of 2 and adjusted p value <0.01) we did not find any genes that could be potentially altered in expression between the two wild types. The top few genes that showed up irrespective of these thresholds are summarised in the Fig. 24

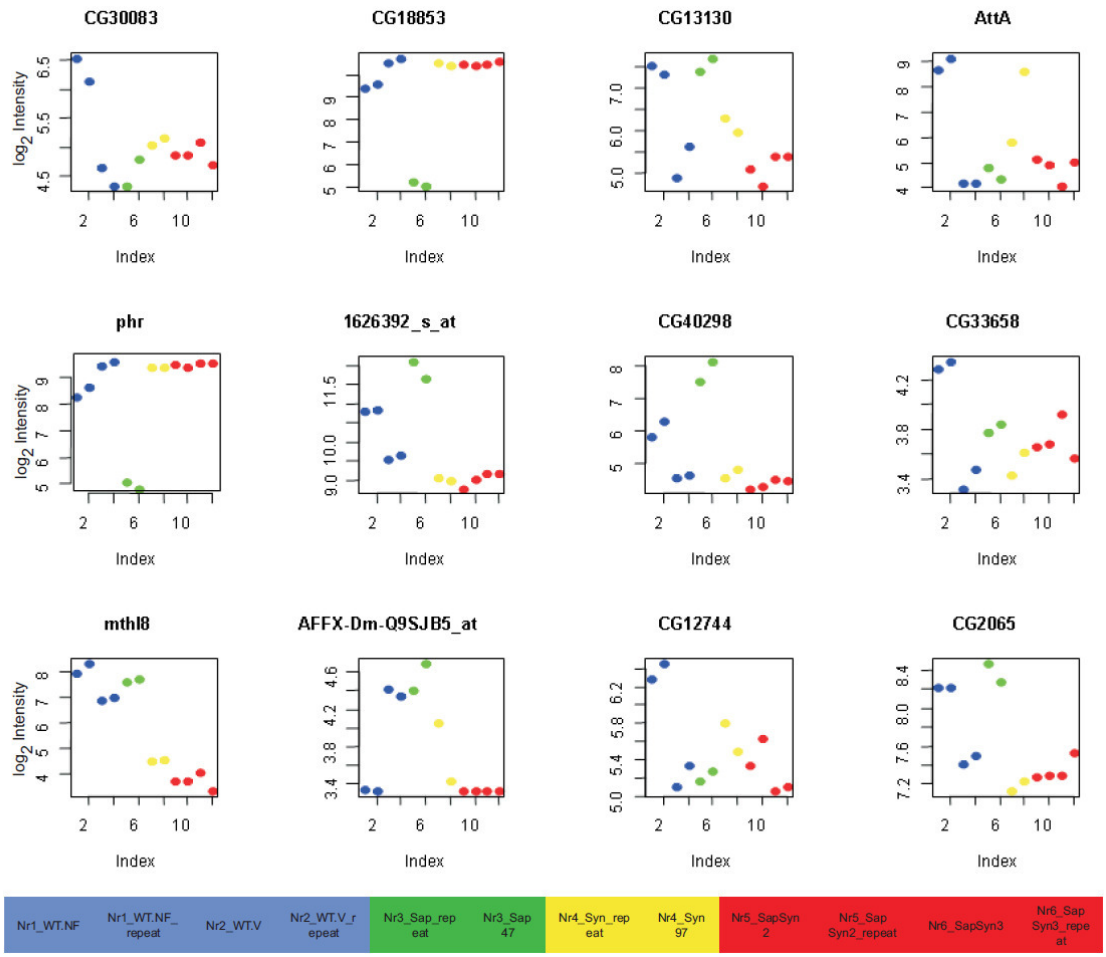


Fig. 24 E*xpression of selected genes showing significant fold change in WT (CS^{NF} and CS^{V})double mutants in comparison to their expression in Sap47, Syn and Sap-Syn double mutants* .

The microarray experiments provided the platform to screen a large number of genes in our experimental groups. In the statistical analysis, therefore random fluctuations in gene expression may produce false positives as probable candidates for specifically altered expression. Hence it is necessary to verify the expression of selected candidates of the microarray analysis by quantitative real time PCR experiments.

3.3 Verification of microarray data by quantitative Polymerase Chain Reaction (qPCR)

To verify the microarray results and exclude the possibility that candidates from those experiments could be false positives we performed real-time PCR (RT-PCR)

experiments. The aim was to look for transcript levels of individual genes in the mutants and compare them to WT.

RT-PCR experiments were performed using the SYBR green based quantification method (see section 2.7.3). As an internal control RpLP0 house keeping gene was selected, it was assumed that expression of this gene was unaffected in the mutants and was comparable to the controls. A threshold in the linear region of the logarithmic phase of the PCR was chosen to quantify the # of cycles required to obtain the same amount of transcript of gene of interest (GOI) in different genotypes. Transcript of *mthl-8* gene was not in the detectable range in *Sap-Syn* double mutants (Fig. 25).

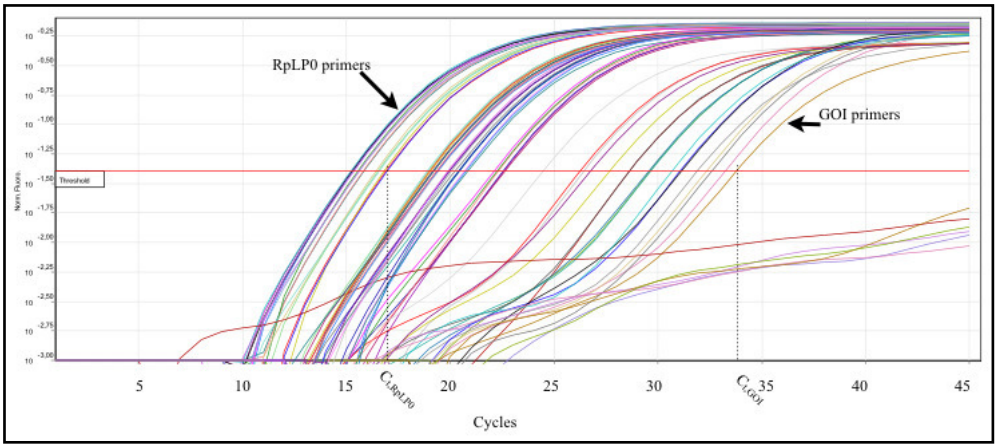


Fig.25 *SYBR green based quantification of transcript copy number. RpLP0 housekeeping gene is chosen as internal control for all genotypes. A threshold chosen in the logarithmic phase (red line) of PCR helps in determining the number of cycles required to accumulate the same amount of transcript in different genotypes. Therefore the difference in # of cycles between the WT and the mutant helps in ascertaining the log fold change in the mutant.*

As an elementary control step it was essential to compare the transcript levels of *Sap47* in *Sap47* mutants, WT, *Synapsin* mutant and *Sap-Synapsin* double mutants (*V2* and *V3*) and similarly compare *Syn*apsin transcript levels in *Sap47* mutants, WT, *Synapsin* mutant and *Sap-Synapsin* double mutants (*V2* and *V3*).These comparisons of microarray data and qPCR results were done in collaboration with T. Nuwal (see Ph.D thesis T. Nuwal, 2010).

We observed that *Syn*apsin transcript was reduced about ~ 2^8 fold in *Synapsin* mutants and *Sap-Synapsin* double mutants, whereas their levels were not significantly changed in *Sap47* mutants. The *Sap47* transcript in *Sap47* mutants and *Sap-Syn* double mutants was reduced ~2^9 fold, but the level of *Sap47* transcript remain unchanged in *Synapsin* mutants (Fig.26).

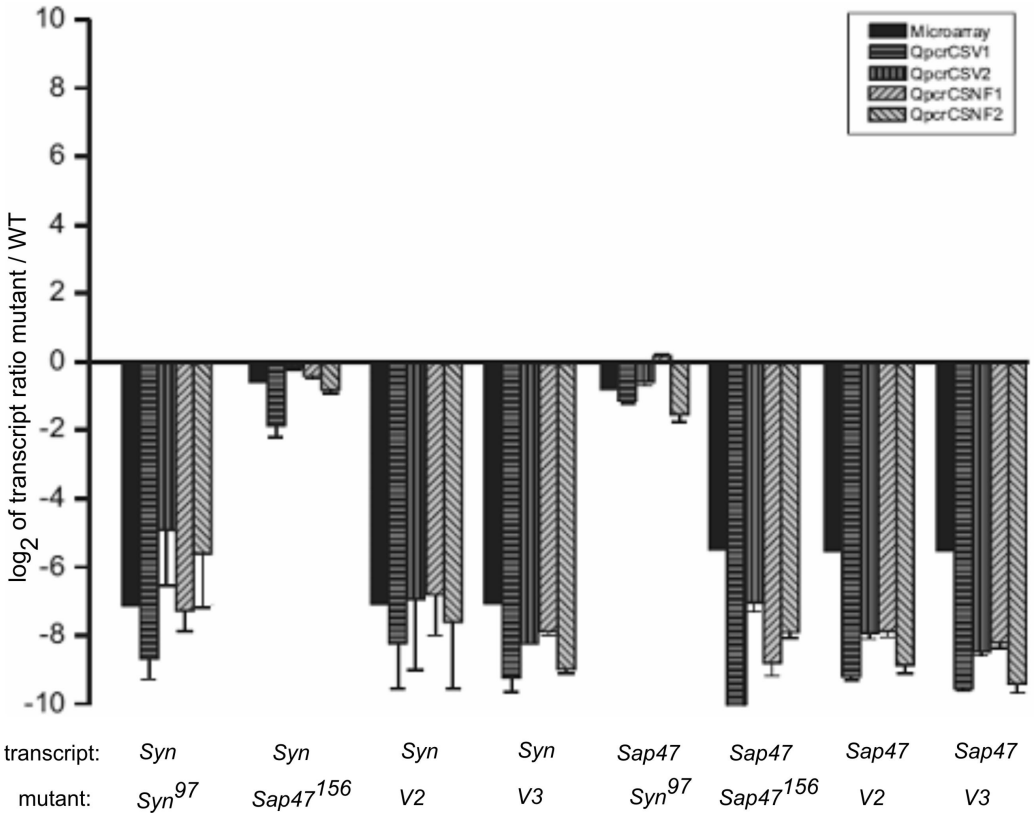


Fig. 26 *Summary of microarray and qPCR results for Sap and Syn transcripts in comparison to WT for Syn^{97} and $Sap47^{156}$ mutants and the double mutants V2 and V3.Syn transcript is down-regulated in Synapsin mutants and double mutants (V2 and V3), but no change in Syn transcript level is observed in $Sap47^{156}$ mutants. Similarly Sap47 transcript is down-regulated in $Sap47^{156}$ mutants and double mutants (V2 and V3), but no change in Sap47 transcript level is observed in Syn^{97} mutants. Note that the error bars reflect only variability of 3 repetitions of qPCR with same template.*

Thus we can conclude that there is a very good agreement between the microarray data and the qPCR for the "control" genes *Sap47* and *Syn*. Next other genes which in the microarray analysis showed significantly altered expression in mutants (Table 4-6) and had predicted neuronal function were selected for further analysis by qPCR. The following genes were selected:

Cirl (calcium independent receptor for α-latrotoxin) came up as a candidate which is 2.5 fold down-regulated in *Synapsin* mutants (Table 3). It encodes a GPCR which is believed to mediate the toxicity produced by the venom α-latrotoxin produced by the black widow spider (Frontali et al., 1976). α-latrotoxin induces neurotransmitter release at neuro-muscular junctions (NMJ's), by Ca^{2+} dependent and Ca^{2+} independent mechanisms (Ceccarelli and Hurlbut, 1980). The Ca^{2+} independent exocytosis is known to be mediated by CIRL (Kiyatkin et al., 1993). A recent study indicates that Cirl induced toxicity of α-latrotoxin is mediated by SNARE complex Synaptobrevin/VAMP, SNAP-25 and partly by Munc 13-1 (Deak et al., 2009). The quantification by qPCR suggests that this change in expression in *Synapsin* mutants is significant (Fig. 27).

The **Shaker** gene also came up as a candidate in microarray experiments because it was 2.6 fold down-regulated in Syn^{97}mutants (Table 5). It codes for a potassium channel. These channels belong to 'A type channels' and open when the membrane is sufficiently depolarised. After activation they enter a long lived non-conducting state. A mutation in this gene leads to prolonged depolarisation of neurons (Hoshi et al., 1990) and delay in repolarisation. Mutations also lead to prolonged release of neurotransmitter at NMJ's. The mutants are characterised by rapid shaking of the appendages and twitching when anesthetized by ether (Trout and Kaplan, 1973; Ganetzky and Wu, 1982). The quantification by qPCR does not show a reliable change in expression in *Synapsin* mutants (Fig. 27).

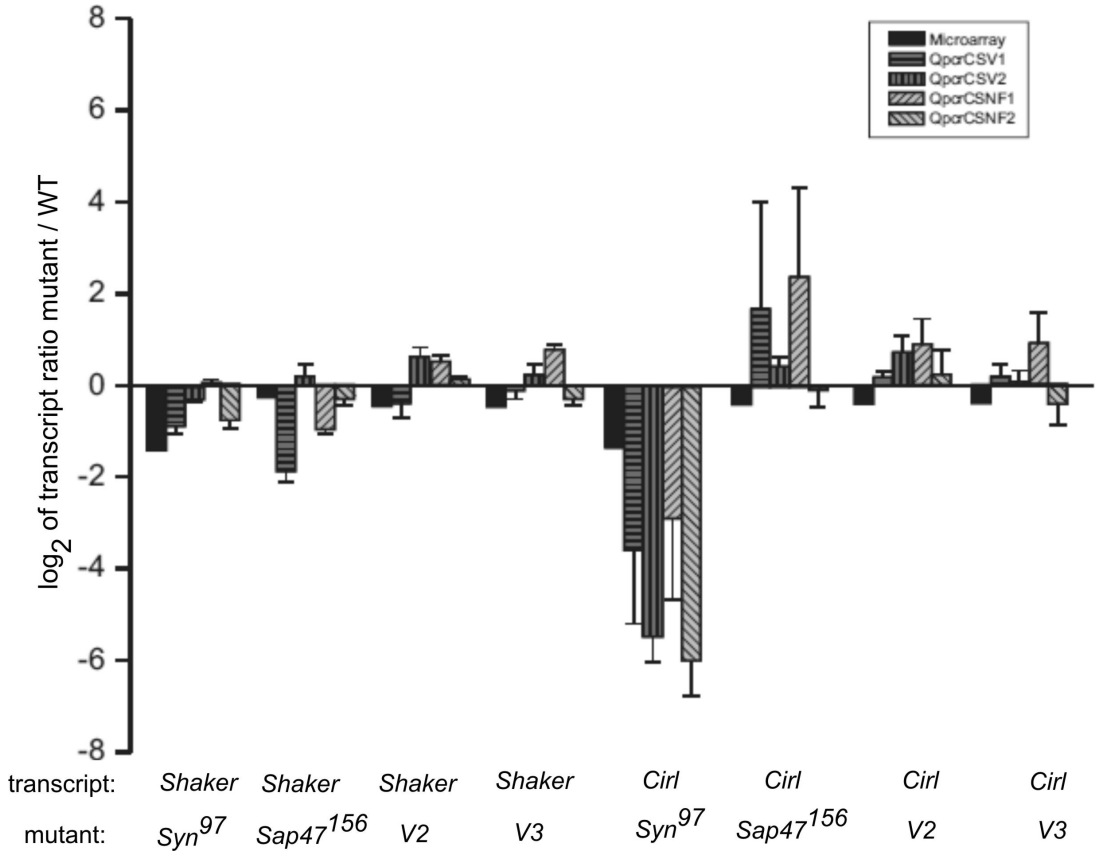


Fig.27 *Shaker transcript levels are not significantly down-regulated in Syn^{97}, Sap47^{156}, V2 and V3 mutants. Cirl transcript is significantly down-regulated in Syn^{97}, but Sap47^{156}, V2 and V3 mutants do not show any reliable change.*

Expression of **methuselah like-8 (mthl-8)** was found to be ~8 fold down-regulated in *Synapsin* mutants (Table 5) and 14 fold in the double mutants (*V2* and *V3*) (Table 6). In the qPCR experiments down regulation of *mthl-8* gene was so severe that it was not in the detection range of the technique (see discussion) (Fig. 28). Methuselah like genes code for proteins that form G protein coupled receptors. While not much is known about role of *methuselah like-8* gene, but some studies with *mthl-8* gene indicate that it is involved in regulating aging (Alic and Partridge, 2007). Another study indicates involvement of the *methuselah* gene in regulating exocytosis in larval NMJ'S (Song et al., 2002b).

Gene **CG4073** is uncharacterized and nothing is known about it. From its sequence it is predicted to have protein binding function. In the microarray experiments it is up-regulated in S*yn*^{97CS} (14 fold), *Sap47*^{156} (10 fold), *V2* and *V3* double mutants (10 fold) (Table 4-6) (Fig. 28). However, the qPCR results are quite variable (Fig. 28)

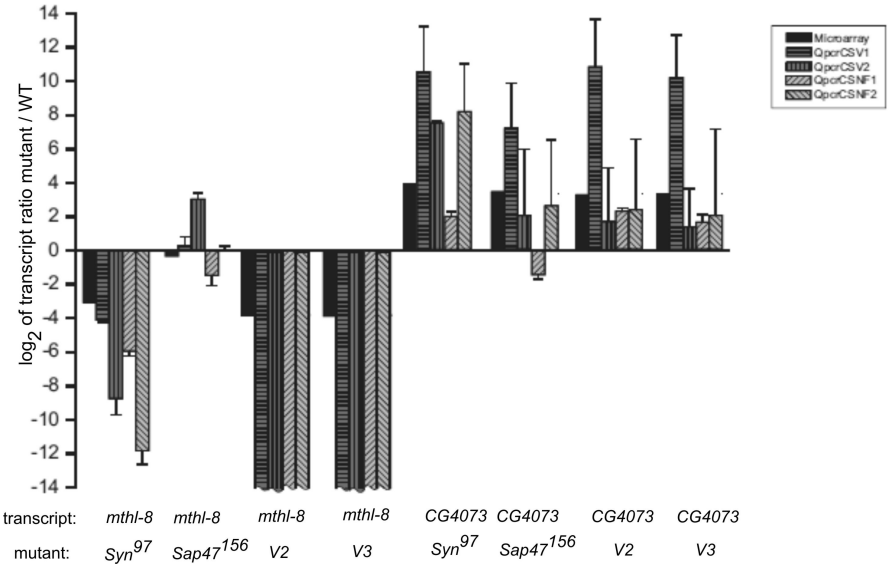


Fig. 28 *mthl8 transcript levels are strongly down-regulated in Syn^{97}, V2 and V3 mutants, whereas no change is observed in Sap47^{156}. CG4073 expression is variable but may be up-regulated in Syn^{97}, Sap47^{156}, V2 and V3 double mutants.*

In the microarray experiment ***nebbish*** gene expression was found to be down-regulated ~4 fold in the *Sap47* mutant (Table 4). This was reproduced by qPCR (Fig 29).However, in the *V2* double mutant contradictory results were obtained by the two different techniques (Fig. 29). This gene is specifically expressed in male testes (Andrews et al., 2000). It codes for kinesin-like protein 38B (Klp 38B). Klp38B is known to function as a mitotic kinesin that binds to chromatin and microtubules helping in the formation of bipolar spindle and attachment of the chromosomes to the spindle (Molina et al., 1997; Ohkura et al., 1997). It also may function as a transcription factor (Dimova et al., 2003).

Gene ***CG9279*** expression was consistently down-regulated ~4 fold only in *Sap47* mutants whereas no change was observed in other genotypes. Not much is known about this gene, but based on sequence similarity it is predicted to be involved in microtubule-based movement (Goldstein and Gunawardena, 2000).

***Transcription Factor II ß* (*TFIIß*)** gene expression was not reliably reproduced in any of the genotypes. It is known to be involved in regulation of DNA binding (Hansen et al., 1997) and transcription factor binding.

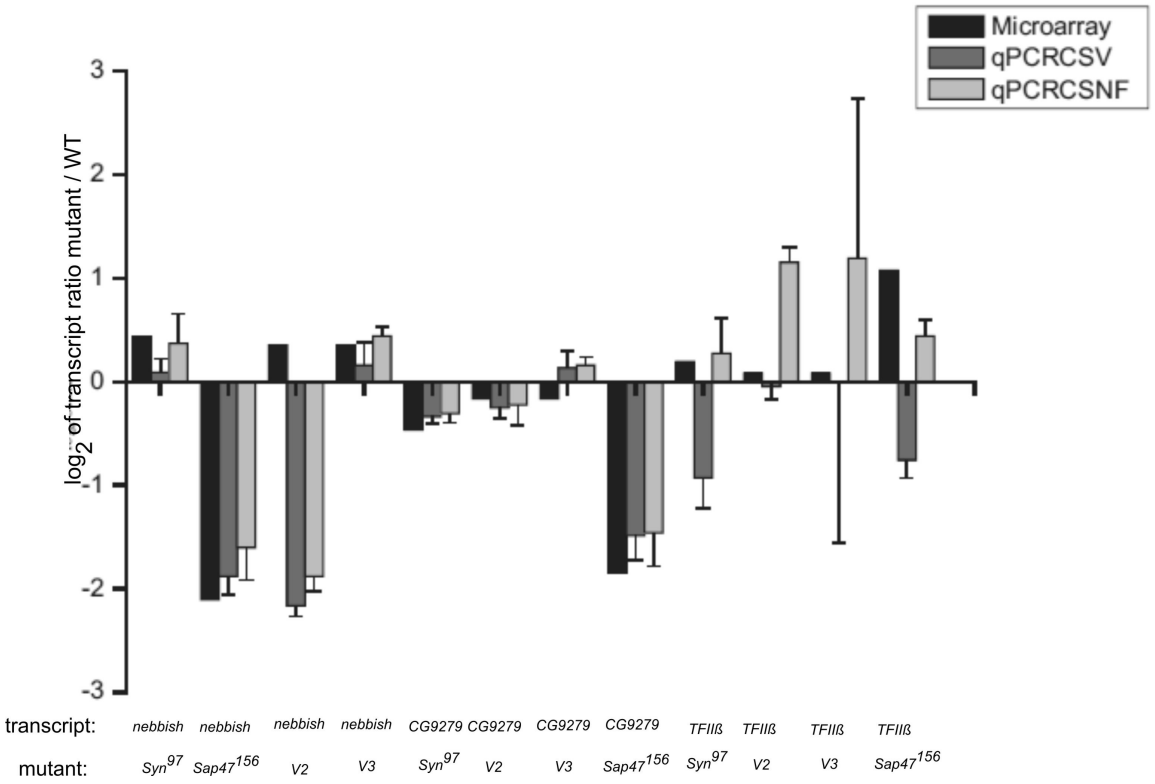


Fig. 29 *Nebbish transcript levels are significantly down-regulated in Sap47^{156} but results are inconsistent for V2. No change is observed in Syn^{97}and V3. CG9279 transcript is significantly down-regulated in Sap47^{156} but, Syn^{97}, V2 and V3 mutants do not show any change. TFIIß transcript levels are not reproducibly changed in any of the four experimental groups.*

The ***fruitless*** gene was a false positive candidate and was not reproducibly altered in any of the examined genotypes (Fig. 30). It has been shown to be involved in courtship (Wheeler et al., 1989) and aggression (Chan and Kravitz, 2007) behavior. It is also known to play a role in nervous system development (Song et al., 2002a).

takeout gene emerged as a strong candidate which was down-regulated in all four experimental genotypes (*Syn^{97}, Sap47^{156}, V2* and *V3* double mutants) (Fig. 30). It is believed to interact with *fru* (Dauwalder et al., 2002) and regulate courtship behavior. It is a gene which functions under the control of circadian clock transcription regulation (So et al., 2000) and in adult feeding behavior (Wong et al., 2009).

zeelin-1 gene expression is significantly down regulated in *Sap47*^{156} whereas, *Syn*^{97}, *V2* and *V3* do not show any change in gene expression levels (Fig. 30). *Zeelin-1* is known to be involved in assembly of muscle thick filaments (Qiu et al., 2005).

***Nicotinic Acetyl Choline Receptor* (*nAChR*)** gene expression was not consistently changed in any of the four experimental groups (*Syn*^{97}, *Sap47*^{156}, *V2* and *V3* double mutants) (Fig. 30). *nAChR* are known to mediate fast excitatory Synaptic transmission in *Drosophila* (Lee and O'Dowd, 1999), specifically also in mushroom body. The activation of nAChR is believed to induce a calcium dependent plasticity in Kenyon cells which can contribute in mediating adult behaviors (Campusano et al., 2007).

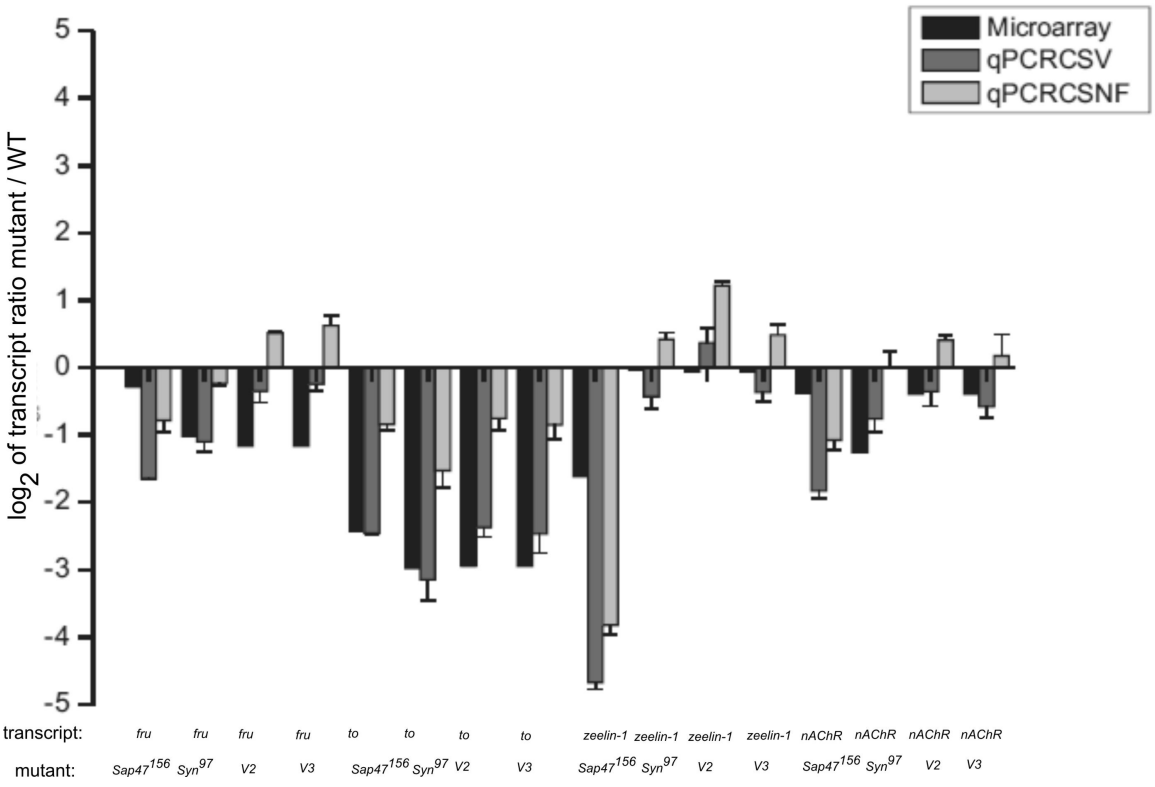


Fig. 30 *fru transcript levels are not reproducibly changed in Syn*^{97}*, Sap47*^{156}*, V2 and V3, takeout transcript levels are consistently down-regulated in Syn*^{97}*, Sap47*^{156}*, V2 and V3. zeelin transcript is significantly down-regulated in Sap47*^{156}*, but Syn*^{97}*, V2 and V3 mutants do not show any consistent change. nAChR transcript levels are not significantly changed in any of the four experimental groups.*

The 5' region of the *Synapsin* gene which is deleted in the *Syn*, *V2* and *V3* mutants was tested by qPCR as the negative control. No transcripts were detected by this primer pair in *Synapsin* mutants and double mutant *V2* and *V3* (Fig.31).

Gene ***CG14305*** was not significantly changed in all four experimental genotypes. From sequence similarity it is predicted to have protein kinase activity (serine threonine kinase), it is also predicted to have ATP and Mg^{2+} binding domains (Fig.31).

CG31097 was down-regulated in $Sap47^{156CS}$ whereas no change was observed in Syn^{97CS}, *V2* and *V3*. The gene is uncharacterized, but from its sequence it is predicted to function as a protein kinase.

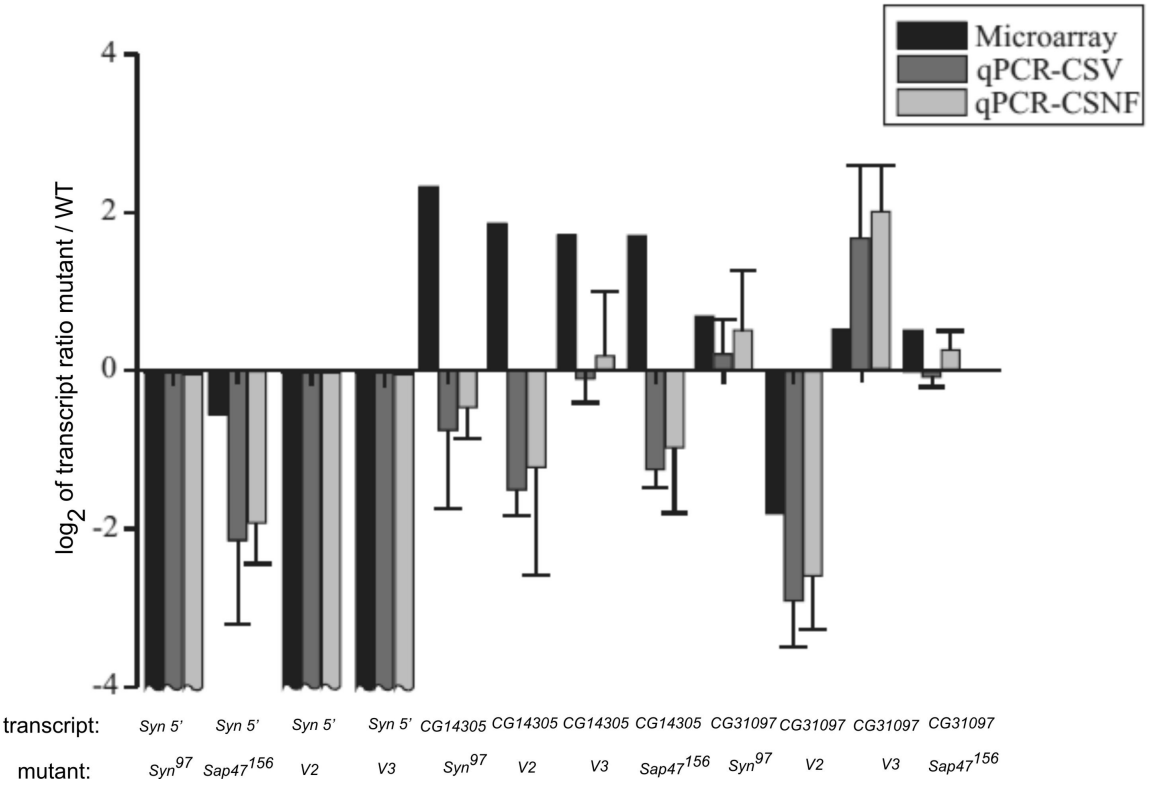


Fig. 31 *Syn 5' transcript levels are not detected in Syn^{97}, V2 and V3, thereby acting as negative control, black bar indicate microarray data for the Affymetrix probes designed with 3' end of Synapsin gene. The CG14305 transcript levels are not significantly changed in Syn^{97}, $Sap47^{156}$, V2 and V3. CG31097 transcript is significantly down-regulated in $Sap47^{156}$ but, Syn^{97}, V2 and V3 mutants do not show any change*

4. Discussion

Data of gene expression studies with mutants can be difficult to interpret at times as apart from the genotype of the animals, gene expression is also dependent on various other factors. Technically all experiments were done in a similar manner, but factors like minor variations in tissue homogenization, RNA isolation, reverse transcription, in vitro transcription, labeling and hybridization cannot be excluded. Also it was attempted to minimize these differences between stocks that were unrelated to the genotype. For example health conditions of the different genotypes could be an important determinant in our case as after the experiments were completed it was found that some strains were infected with *microsporidia*. The table below indicates the infection severity based on a small sample of 4 animals from each stock, illustrating the variability of infection. It is not possible to infer from these data the degree of infection at the time of RNA isolation. However it seems possible that the differential observed expression in the same WT stocks in January compared to November (Fig.16 and 23) may be partly due to such an infection. The infection becomes apparent only in aged flies, thereby minimizing the possibility that the flies used for extraction were severely infected as we used 2-3 day old flies for microarray experiments.

Strain	Microsporidia infection
CS^{V}	negative
CS^{NF}	2 out of 4 positive
$Sap47^{156CS}$	negative
Syn^{97CS}	strongly positive
V2 double mutant	3 out of 4 positive
V3 double mutant	negative

Our intention was to keep as many factors constant as possible for the 6 stocks. They were always raised on same batch of food, equal number of males and females were used for breeding the next generation. Ideally one would have chosen equal number of non mated males and females for RNA extraction (Guan et al., 2005) (to make sure that gender and mating dependent changes in gene expression do not have an influence on our experiments). Also, to minimize food vial to food vial variation within the same genotype

we decided to collect flies for a given genotype always from the same vial. However, the long time required to collect equal numbers of fly heads on dry ice lead to degradation of RNA samples. Therefore for the follow on experiments we collected 400 fly heads with undetermined ratio of males to females, and irrespective of mating status which might account for some of the disparities in expression between the two WT and the double mutants *V2* and *V3*.

The RNA quality was tested before each reverse transcription and seemed comparable as on normalization the data fit quite well (Fig 13, 14). For further analysis a short gene list was generated using fold change and significance (adjusted p value) as selection criteria (sorting and selection was done using Microsoft Access). This list was further analysed using DAVID software (http://david.abcc.ncifcrf.gov/) and NetAffx™ software supported by Affymetrix Inc.

The two double mutants V2 and V3 resulted from the same recombination event but have been maintained separately for 4 years. Also, the two wild types have been maintained separately for the last 10 years. In most cases the founder effect of the independently established lines for the double mutant was conserved indicating that these two independently maintained strains did not show significantly different genetic drift effects over generations, except for the gene *nebbish* (Fig. 29) which frpm qPCR data seems to be down-regulated in V2 but not in V3 double mutant flies. The two wild types used as controls in our experiments did not differ significantly from each other but higher variability was observed within the same WT between the two extractions performed in November and January. Preliminary analysis shows that the majority of altered genes belong to the citric acid cycle (TCA cycle) indicating that basal metabolism was affected in flies from January batch. A plausible explanation of this observation cannot be given.

Our experiments with qPCR indicate the reliability of the microarray technique although the agreement is not absolute. Our analysis revealed that qPCR experiment showed similar trends of down or up-regulation of candidate gene transcripts as microarray experiments. However, the magnitude of alteration indicated by microarray experiments was different from what was observed from qPCR experiments.

One of the candidate genes in the microarray experiments, *mthl-8* showed an 8-16 fold down-regulated transcript level in *Synapsin* mutants, *V2* and *V3* double mutants, but expression was below the detection range in the qPCR experiments with *V2* and *V3* as would be expected for a null mutant for the *mthl-8* gene. A recent study (Kern and Begun, 2008) reports that the *mthl-8* gene which is located at the tip of the chromosome 3L close to telomere is prone to dynamic deletion polymorphisms and shows presence/absence in various WT stocks. Thus, one possibility to interpret the *mthl-8* expression data is to assume that *V2* and *V3* stocks have suffered such a deletion of *mthl-8* gene which by genetic drift had been fixed by the time the qPCR experiments were done. For this reason the *mthl-8* gene is not considered as a candidate for further investigation of interaction with SAP or SYN.

The only gene that based on the present experiments clearly should be analysed further as a candidate for interaction with *SYN* is the latrotoxin receptor gene *Cirl*. However, even for this gene first a genetic rescue experiment should be performed, where the *Synapsin* and *Sap47* transcripts levels have been restored in the *Syn* mutant by expression of *Syn* cDNA as a transgene (refer to Ph.D thesis of T. Nuwal for probable transcript copy number alterations for each gene). If the rescue restores the amount of *Cirl* transcript to WT levels one would conclude that the alteration of transcript number in *Syn* mutant was indeed due to this mutation and not due to any other secondary effect like genetic background. In this case it would be interesting to investigate why in the double mutant *Cirl* expression is observed at wild type levels (Fig. 27). Again a “rescue” of the double mutant with *Sap* cDNA should be performed to determine if this restores the reduced *Cirl* expression of the *Syn* mutant.

For all other genes listed as candidates it seems that either changes in expression in mutants were too small or were not reliably supported by qPCR results such that further efforts to study their interactions with *Sap* or *Syn* genes cannot be recommended.

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6. Summary

PART I

Animals need to constantly evaluate their external environment in order to survive. In some cases the internal state of the animal changes to cope with it's surrounding. In our study we wanted to investigate the role of amines in modulating internal states of Drosophila. We have designed a behavioral paradigm where the flies are fixed in space but can walk on a small styrofoam ball suspended by a gentle stream of air. The walking activity of flies was used as behavioral readout. An operant training paradigm was established by coupling one of the walking directions to incidence of heat punishment. We observed that animals quickly realized the contingency of punishment with walking direction and avoided walking in the punished direction in the presence of punishment, but did not continue walking in the unpunished direction in the absence of the punishment. This would indicate that the flies do not form a memory for the punished direction or rapidly erase it under new conditions.

On having established the paradigm with heat punishment we have attempted to activate selected subsets of neuronal populations of Drosophila while they were walking on the ball. The selective activation of neurons was achieved by expressing the light-activated ion channel channelrhodopsin-2 (ChR2) using the Gal4-UAS system and coupling the unidirectional walking of the animals on the ball with the incidence of blue light required to activate the channels and depolarize the neurons. The feasibility of this approach was tested by light-activating sugar sensitive gustatory receptor neurons expressing ChR2, we found that when the light was actuated the flies preferred to turn in one direction the optically "rewarded" direction. Next we similarly activated different subsets of aminergic neurons. We observed that in our setup animals avoided to turn in the direction which was coupled to activation of dopaminergic neurons indicating that release of dopamine is disliked by the animals. This is in accordance with associative learning experiments where dopamine is believed to underlie the formation of an association between a neutral conditioned stimulus with the aversive unconditioned stimulus. However, when we activated tyraminergic/octopaminergic neurons we did not observe any directional preference. The activation of dopaminergic and tyraminergic/octopaminergic neurons led to arousal of the animals indicating that we

were indeed successful in activating those neurons. Also, the activation of serotonergic neurons did not have any effect on directional preference of the animals.

With this newly established paradigm it will be interesting to find out if in insects like in mammals a reward mediating system exists and to test subsets of aminergic or peptidergic neurons that could possibly be involved in a reward signaling system which has not been detected in our study. Also, it would be interesting to localize neuropile regions that would be involved in mediating choice behavior in our paradigm.

PART II

In collaboration with S. Kneitz (IZKF Wuerzburg) and T. Nuwal we performed genome-wide expression analysis of two pre-synaptic mutants - Synapsin (Syn^{97}) and Synapse associated protein of 47 kDa ($Sap47^{156}$). The rationale behind these experiments was to identify genes that were up- or down-regulated due to these mutations. The microarray experiments provided us with several candidate genes some of which we have verified by qPCR. From our qPCR analysis we can conclude that out of the verified genes only Cirl transcripts seem to be reproducibly down regulated in Synapsin mutants. The Cirl gene codes for a calcium independent receptor for latrotoxin. Further qPCR experiments need to be performed to verify other candidate genes. The molecular interactions between CIRL and SYN or their genes should now be investigated in detail.

7. Zusammenfassung

Teil I

Lebewesen müssen beständig ihre äußere Umgebung auswerten, um überleben zu können. Manchmal ändern sich innere Zustände der Tiere, damit sie sich der Außenwelt anpassen. In unseren Untersuchungen wollten wir die Rolle von Aminen untersuchen, die für die Modulation von inneren Zuständen bei *Drosophila* notwendig sind. Wir haben ein Verhaltensparadigma entwickelt, bei dem die Fliege räumlich fixiert ist, aber auf einem Styroporball laufen kann, der auf einem Luftpolster schwebt. Die Laufaktivität der Fliege wird durch die Ballbewegungen anzeigt. Mit diesem Versuchsaufbau wurde ein operantes Lernparadigma etabliert, bei dem eine Laufrichtung mit Bestrafung durch Hitze gekoppelt wird. Wir konnten feststellen, dass die Tiere schnell den Zusammenhang zwischen dem Auftreten der Bestrafung und der Laufrichtung realisieren und die bestrafte Seite vermeiden. Wurde die eine Laufrichtung nicht mehr bestraft, so vermieden die Fliegen sie nicht mehr. Dies zeigt dass die Fliegen kein Gedächtnis für die bestrafte Richtung ausbilden oder es bei veränderten Bedingungen rasch löschen . Nachdem sich der Versuchsaufbau mit Hitzebestrafung bewährt hatte, wurde versucht, bestimmte Sub-population von Neuronen von *Drosophila* zu aktivieren, während die Fliege auf dem Ball läuft. Die selektive Aktivierung von Neuronen wurde durch die Expression des lichtaktivierten Jonenkanals Channelrhodopsin-2 (ChR-2) mit Hilfe des Gal4-UAS-System und Beleuchtung der Fliege mit Blaulicht erreicht, das die Kanäle aktiviert. Nun erfolgte eine Kopplung einer Laufrichtung auf dem Ball mit dem Auftreten von blauem Licht. Die Durchführbarkeit derartiger Experimente wurde durch die Aktivierung von zuckersensitiven gustatorischen Rezeptorneuronen getestet. Die Ergebnisse zeigten, dass die Tiere bevorzugt die Richtung wählen, welche die Zuckerrezeptorneurone aktiviert. Anschließend aktivierten wir verschiedene Untergruppen von Neuronen des aminergen Systems. In diesem Versuchsaufbau beobachteten wir, dass die Tiere die Laufrichtung vermieden, die gekoppelt war mit der Aktivierung dopaminerger Neurone. Diese Ergebnisse stehen in Übereinstimmung mit Versuchen zum assoziativen Lernen, bei dem Dopamin wahrscheinlich notwendig ist für die Assoziation zwischen dem neutralen Konditionierungsstimulus und dem aversiven unkonditionierten Stimulus. Wenn wir jedoch die tyraminergen/oktopaminergen Neurone aktivierten, konnte keine gerichtete Präferenz nachgewiesen werden. Die Aktivierung dopaminerger und

tyraminger/oktopaminerger Neurone führte jedoch zur Aktivitätssteigerung der Tiere, wodurch die erfolgreiche der Aktivierung der Neurone belegt wurde. Die Aktivierung serotonerger Neurone führte ebenfalls zu keinem Effekt in der Präferenz der Tiere.
In zukünftigen Experimenten mit dem neu entwickelten Paradigma wäre es interessant, herauszufinden, ob in Insekten auch ein belohnungsabhängiges System existiert, vergleichbar dem von Säugern. So wäre die Identifizierung von Subpopulationen aminerger oder peptiderger Neurone des Belohnungssystems bei Insekten wichtig, da dies nicht in unseren Experimenten entdeckt wurde. Eine weitere interessante Fragestellung wäre, welche Gehirnstruktur die Richtungswahl auf dem Ball vermittelt.

Teil II

In Zussamenarbeit mit S. Kneitz (IZKF, Würzburg) und T. Nuwal wurde in der vorliegenden Arbeit die genomweite Genexpression einer *Synapsin*-Mutante (Syn^{97}) und einer Mutante für das Synapsen-assoziierte-Protein von 47kDa ($Sap47^{156}$) untersucht. Bei beiden Proteinen handelt es sich um präsynaptische Proteine von *Drosophila*. Ziel dieses Experiments war es, Gene zu identifizieren die aufgrund dieser Mutationen hoch bzw. herunterreguliert vorliegen. Durch das Microarray-Experiment wurden mehrere Kandidatengene entdeckt, wovon einige dieser Gene per qPCR-Versuchen verifiziert wurden. Aufgrund der qPCR-Analysen lässt sich schlussfolgern, dass nur das *Cirl*-Transkript in den *Synapsin*-Mutanten reproduzierbar herunterreguliert vorliegt. Das *Cirl* gene kodiert für einen Calcium independent receptor for Latrotoxin Weitere qPCR-Experimente sind notwendig, um die anderen Kandidatengene zu bestätigen. Die molekularen Interaktionen zwichen CIRL und Synapsin oder ihren Genen müssen nun im Detail untersucht werden.

Erklärung

Erklärung gemäß § 4 Absatz 3 der Promotionsordnung der Fakultät für Biologie der Bayerischen Julius-Maximilians-Universität zu Würzburg vom 15. März 1999.

Hiermit erkläre ich, die vorgelegte Dissertation selbständig angefertigt zu haben und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt zu haben. Alle aus der Literatur entnommenen Stellen sind als solche kenntlich gemacht. Des Weiteren erkläre ich, dass die vorliegende Arbeit weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat. Zuvor habe ich keine akademischen Grade erworben oder zu erwerben versucht.

Würzburg, den .....

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Publications

1. Salt Processing in Larval Drosophila: Choice, Feeding, and Learning Shift from Appetitive to Aversive in a Concentration-Dependent Way (Chemical Senses)
Niewalda, T., **Singhal, N**., Fiala, A., Saumweber, T., Wegener, S., and Gerber, B.

2. Optogenetically induced olfactory stimulation in Drosophila larvae reveals the neuronal basis of odor-aversion behavior (Frontiers of Neuroscience)
Bellmann, D., Richardt, A., Freyberger, R., **Nuwal, N**., Schwärzel, M., Fiala, A and Störtkuhl, K.F.

3. Avoidance of optogenetically induced dopamine release in operant behavior of Drosophila (submitted)
Nuwal, N., Stock, P., Hiemeyer, J., Schmid, B., Buchner, E & Fiala, A

Selected abstract and poster presentations

4. **Singhal N**, Buchner E, Fiala A*:* Optogenetic investigation of nervous system functionusing walking behavior in *Drosophila* (Abstract and poster presentation, 32nd Goettingen neurobiology conference, Goettingen, Germany).

5**. Singhal N**, Buchner E, Fiala A: Operant conditioning in walking *Drosophila* (Abstract and poster presentation, 12th European *Drosophila* Neurobiology Conference. September 6-10, 2008. Wuerzburg, Germany, Journal of Neurogenetics 2009 ,23 Suppl 1, pp V.35).

6. **Singhal N**, Hiemeyer J, Schmid B, Buchner E, Fiala A Optogenetic analysis of nervous sytem function by exploiting walking behavior in *Drosophila*- A novel paradigm, Neurobiology of *Drosophila,* September 29- October 3, 2009. CSHL, USA

Wuerzburg, **.....**
Nidhi Nuwal

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