

Dissertation

Neural circuits mediating aversive olfactory
conditioning in *Drosophila*

Zur Erlangung des akademischen Grades Doktor der Naturwissenschaften
(Dr. rer. nat.)

Fakultät für Biologie

Ludwigs-Maximilians-Universität München

Dana Shani Galili

Datum der Einreichung: 5. August 2014

Die vorliegende Arbeit wurde im Max-Planck-Institut für Neurobiologie in der Arbeitsgruppe von Dr. Hiromu Tanimoto, in der Zeit vom 1. März 2009 bis 31. Juli 2014 ausgeführt.

Erstgutachter: Prof. Dr. Mark Huebener

Zweitgutachter: Prof. Dr. Andreas Herz

Tag der mündlichen Prüfung: 14. Oktober 2014

In memory of my grandfather David Holzer, 1928-2014.

לזכרו של סבי דוד הולצר, 1928-2014.

Contents

Contents.....	4
1 List of publications and declaration of self-contributions	6
2 Table of figures	7
3 List of abbreviations.....	8
4 Zusammenfassung	9
5 Summary	11
6 Introduction	13
6.1 Neural pathways of odor and reinforcement	13
6.1.1 Olfactory pathway.....	13
6.1.2 Electric shock sensation	15
6.1.3 Temperature sensation.....	16
6.2 Learning and memory in <i>Drosophila</i>	18
6.2.1 Classical conditioning of fruit-flies	18
6.2.2 Reinforcement pathway: the role of dopamine	20
6.2.3 The role of mushroom bodies as coincidence detectors.....	21
6.2.4 Importance of timing for associative learning: Trace, delay and relief learning	23
6.3 Advantages of fly neuroscience: the genetic tool box.....	26
6.4 Aims of the thesis: exploring the CS and US representations in the nervous system	28
7 Results.....	29
7.1 First paper: Galili DS, Lüdke A, Galizia CG, Szyszka P, Tanimoto H. (2011) Olfactory trace conditioning in <i>Drosophila</i> . <i>J Neurosci</i> . 31(20):7240-8.....	29
7.2 Second paper: Galili DS, Dylla KV, Lüdke A, Friedrich AB, Yamagata N, Wong JY, Ho CH, Szyszka P, Tanimoto H. (2014) Converging circuits mediate temperature and shock aversive olfactory conditioning in <i>Drosophila</i> . <i>Curr Biol</i> . 24(15):1712-22.	29
8 Discussion.....	30
8.1 Olfactory trace conditioning in <i>Drosophila</i>	30
8.1.1 Trace and delay memories are based on the same odor perception	30
8.1.2 Odor trace may be less salient than the odor itself.....	30
8.1.3 Identity and location of the odor trace.....	31
8.1.4 Former experience improves trace conditioning.....	33
8.2 Comparing the neural circuits of aversive reinforcers: temperature and shock.....	34

8.2.1	Temperature and shock sensation are separate	35
8.2.2	Detection of increased temperature during reinforcement learning occurs via a specific sensory pathway	36
8.2.3	Dopamine integrates aversive reinforcement of both temperature and shock (Figure 9A) 37	
8.2.4	Overlapping subsets of dopaminergic neurons for temperature and shock reinforcement 38	
8.3	General conclusions and outlook.....	40
9	References	41
10	Acknowledgements.....	50
11	Curriculum Vitae	51
12	Declaration (Eidesstattliche Versicherung).....	55

1 List of publications and declaration of self-contributions

Galili DS, Lüdke A, Galizia CG, Szyszka P, Tanimoto H. (2011) Olfactory trace conditioning in *Drosophila*. *J Neurosci*. 31(20):7240-8.

D.S.G., A.L., C.G.G., P.S., and H.T. designed the research;

D.S.G. and A.L. collected the data: D.S.G. performed the experiments in figures 1, 2, 3A, 3B, 3C, 5, 7; ~80% of the data. A.L. performed the experiments in figures 3D, 3E, 4; ~20% of the data.

D.S.G., A.L., C.G.G., P.S., and H.T. analyzed the data: D.S.G. together with H.T. analyzed the data for figures 1, 2, 3A, 3B, 3C, 5, 7. ~80% of the data. A.L., C.G.G. and P.S. analyzed ~20% of the data (Figures 3D, 3E, 4).

D.S.G., A.L., P.S., and H.T. wrote the paper.

Self-contribution: I collected and analyzed ~80% of the data.

Galili DS, Dylla KV, Lüdke A, Friedrich AB, Yamagata N, Wong JY, Ho CH, Szyszka P, Tanimoto H. (2014) Converging circuits mediate temperature and shock aversive olfactory conditioning in *Drosophila*. *Curr Biol*. 24(15):1712-22.

D.S.G. and H.T. designed the research;

D.S.G., K.V.D., J.H.W., C.H.H., N.Y. and A.B.F. collected the data: K.V.D. performed the experiments described in figure 4 and Figure S3 (~15% of the data). J.H.W., C.H.H., N.Y. collected the data for Figure 6 E, F; 7C; S6C (~5% of data). A.B.F. contributed confocal images for Figure 6 D and Figure S5 (~5% of data). D.S.G. collected the data for Figures 1, 2, 3, 5, 6 A, B, C, 7, S1, S2, S4, S6 A, B (~75% of data).

Self-contribution: I collected the data for Figures 1, 2, 3, 5, 6 A, B, C, 7, S1, S2, S4, S6 A, B (~75% of data).

D.S.G. and H.T. analyzed the data, with the exception of Figure 4 and Figure S3 which were analyzed by K.V.D., P.S. and A.L. (~15% of the data).

Self-contribution: I analyzed ~85% of the data.

D.S.G., K.V.D., P.S. and H.T. wrote the paper.

2 Table of figures

Figure 1: Overview of the olfactory system of <i>Drosophila</i>	14
Figure 2: Temperature sensation in flies	18
Figure 3: Classical olfactory conditioning in <i>Drosophila</i>	19
Figure 4: Dopaminergic neurons innervate the mushroom bodies (MBs) and play a role in learning and memory	21
Figure 5: Schematic representation of the molecular mechanisms involved in aversive olfactory conditioning	23
Figure 6: Behavioral design and memory performance in delay and trace conditioning.....	25
Figure 7: Simplified schematic of the olfactory conditioning circuit in flies.....	28
Figure 8: Possible locations of the odor trace	33
Figure 9: Summary and model for sensory and reinforcement signaling.....	39

3 List of abbreviations

CS	Conditioned Stimulus
US	Unconditioned Stimulus
ORN	Olfactory Receptor Neuron
MB	Mushroom Body
KC	Kenyon Cells
TRP	Transient Receptor Potential
cAMP	cyclic Adenosine Monophosphate
AC	Anterior Cell
UAS	Upstream Activating Sequence
Rdl	Resistance to dieldrin

4 Zusammenfassung

Die Assoziation eines sensorischen Umweltreizes mit einem Verstärkungsreiz, auch Belohnungs- oder Bestrafungslernen genannt, verschafft Tieren einen Überlebensvorteil. Während dieses assoziativen Lernens wird der sensorische Reiz (konditionierter Reiz; CS) zeit- und raum- nahe zu dem verstärkenden Reiz (nicht konditionierter Reiz; US) präsentiert, sowohl auf neuronaler als auch auf Verhaltensebene. Auf diese Weise wird der CS mit einem positiven oder negativen Vorhersagewert aufgeladen, der das zukünftige Antwortverhalten des Tieres gegenüber dem CS negativ oder positiv beeinflusst.

In meiner Doktorarbeit verwendete ich die olfaktorische Konditionierung, das Bestrafungslernen, am Modellorganismus *Drosophila* um herauszufinden, wo und wie diese Reize im Nervensystem abgebildet werden um eine Assoziation zu ermöglichen.

Im ersten Teil meiner Doktorarbeit untersuchte ich die Repräsentation eines Duftreizes und dessen zu Grunde liegenden neuronalen Signalweg. Es ist möglich, eine Assoziation zwischen Duftreiz (CS) und Elektroschock (US) herzustellen, selbst wenn der zuerst präsentierte Duft schon verfliegen ist und erst dann der Elektroschock gegeben wird („Duftspur- Konditionierung“). Die durch Präsentation zeitlich nicht-überlappender Reize hervorgerufene Konditionierung habe ich mit der durch zeitlich überlappende Reize erzeugten Konditionierung („Verzögerungs-Konditionierung“) verglichen. Hierdurch fand ich heraus, dass Fruchtfliegen sogar eine Duftspur mit einem Elektroschock, der in 15 s Abstand präsentiert wurde, assoziieren können. Die Gedächtnisse, welche beim „Duftspur-“ und „Verzögerungs-Konditionieren“ gebildet werden, sind sich auffallend ähnlich: beide zeigen eine Sättigungskurve der Lernwerte, jedoch mit unterschiedlicher Geschwindigkeit, und beide Gedächtnisse besitzen ähnliche Zerfallskinetik und hochkorrelierte Generalisierungsprofile über verschiedene Düfte hinweg. Zusammengefasst weisen diese Ergebnisse auf Gemeinsamkeiten in der Duftwahrnehmung hin, welche wahrscheinlich während und nach der Duftreizpräsentation im Nervensystem aufrechterhalten wird. Zur Erforschung des physiologischen Korrelates dieser Duftspur haben wir (Zusammenarbeit mit Alja Luedke, Universität Konstanz) ein auf Calcium basiertes bildgebendes Verfahren an der lebenden Fruchtfliege verwendet. Nach Beendigung der Duftreizpräsentation zeigten die duftspezifischen Neurone (ORN) im Antennallobus ein einige Sekunden andauerndes Antwortverhalten, welches sich fundamental vom Antwortverhalten während der Duftreizpräsentation unterschied. Diese schwache Übereinstimmung zwischen dem Duftgeneralisierungsprofil während des „Duftspur-Konditionierens“

und des physiologischen Duftgeneralisierungsprofils im Antennallobus deuten darauf hin, dass die zum assoziativen Lernen benutzte Duftspur in einer den ORN nachfolgenden Gehirnstruktur codiert ist.

Der zweite Teil meiner Doktorarbeit beschäftigt sich mit der Repräsentation von bestrafenden Reizen (US) und deren zu Grunde liegenden neuronalen Signalwegen. Dafür habe ich eine Duft-Temperatur-Konditionierungseinrichtung etabliert, welche vergleichbar ist zur Duft-Elektroschock-Konditionierung. Somit konnte ich auch die den beiden Gedächtnissen zu Grunde liegenden neuronalen Signalwege besser vergleichen. Es war mir möglich einen spezifischen sensorischen Signalweg zu beschreiben, der für erhöhte Temperatur als Bestrafung benötigt wird: die sogenannten AC-Neuronen, die als Wärmesensoren dienen durch Expressieren des Rezeptors dTrpA1. Trotz unterschiedlicher sensorischer Signalwege für Duft-Temperatur- und Duft-Elektroschock-Konditionierung, konvergieren beide in einem zentralen Signalweg: den Dopamin-Neuronen, welche generell die Verstärkerinformation weiterleiten. Obwohl eine gemeinsame Population von Dopamin-Neuronen Informationen von Temperatur- und Elektroschockbestrafung übermittelt, ist die Population, die für Temperaturbestrafung zuständig ist, kleiner, und wahrscheinlich in der Population von Dopamin-Neuronen, die Elektroschockbestrafung weiterleiten, mit inbegriffen. Daraus ziehe ich folgenden Schluss: Dopamin-Neurone integrieren unterschiedliche schädliche Reize in einen generellen Bestrafungs-Signalweg.

Zusammenfassend tragen meine Ergebnisse zu unserem Verständnis von olfaktorischem Bestrafungslernen bei. Insbesondere konnte ich bisher nicht beschriebene Verhaltensfähigkeiten von Fliegen und die zu Grunde liegenden neuronalen Repräsentationen darlegen.

5 Summary

For all animals it is highly advantageous to associate an environmental sensory stimulus with a reinforcing experience. During associative learning, the neural representation of the sensory stimulus (conditioned stimulus; CS) converges in time and location with that of the reinforcer (unconditioned stimulus; US). The CS is then affiliated with a predictive value, altering the animal's response towards it in following exposures. In my PhD thesis I made use of olfactory aversive conditioning in *Drosophila* to ask where these two different stimuli are represented and how they are processed in the nervous system to allow association.

In the first part of my thesis, I investigated the presentation of the odor stimulus (CS) and its underlying neuronal pathway. CS-US association is possible even when the US is presented after the physical sensory stimulus is gone ('trace conditioning'). I compared such association of temporally non-overlapping stimuli to learning of overlapping stimuli ('delay conditioning'). I found that flies associate an odor trace with electric shock reinforcement even when they were separated with a 15 s gap. Memories after trace and delay conditioning have striking similarities: both reached the same asymptotic learning level, although at different rates, and both memories have similar decay kinetics and highly correlated generalization profiles across odors. Altogether, these results point at a common odor percept which is probably kept in the nervous system throughout and following odor presentation. In search of the physiological correlate of the odor trace, we used in vivo calcium imaging to characterize the odor-evoked activity of the olfactory receptor neurons (ORNs) in the antennal lobe (in collaboration with Alja Luedke, Konstanz University). After the offset of odor presentation, ORNs showed odor-specific response patterns that lasted for a few seconds and were fundamentally different from the response patterns during odor stimulation. Weak correlation between the behavioral odor generalization profile in trace conditioning and the physiological odor similarity profiles in the antennal lobe suggest that the odor trace used for associative learning may be encoded downstream of the ORNs.

In the second part of the thesis I investigated the presentation of different aversive stimuli (USs) and their underlying neuronal pathways. I established an odor-temperature conditioning assay, comparable to the commonly used odor-shock conditioning, and compared the neural pathways mediating both memory types. I described a specific sensory pathway for increased temperature as an aversive reinforcement: the thermal sensors AC neurons, expressing dTrpA1 receptors. Despite the separate sensory pathways for odor-temperature and odor-shock conditioning, both converge to one central

pathway: the dopamine neurons, generally signaling reinforcement in the fly brain. Although a common population of dopamine neurons mediates both reinforcement types, the population mediating temperature reinforcement is smaller, and probably included within the population of dopamine neurons mediating shock reinforcement. I conclude that dopamine neurons integrate different noxious signals into a general aversive reinforcement pathway.

Altogether, my results contribute to our understanding of aversive olfactory conditioning, demonstrating previously undescribed behavioral abilities of flies and their neuronal representations.

6 Introduction

6.1 Neural pathways of odor and reinforcement

The *Drosophila melanogaster* brain has been studied for decades, and therefore much is known about specific neuronal pathways encoding sensory stimuli. In my project, I focus on the association of olfactory information with aversive reinforcement, applying either electric shock or increased temperature. The pathway underlying olfactory sensation has been intensively studied and is well described; however pathways underlying aversive stimuli sensation, especially for electric shock, are still poorly described although electric shock is widely used for aversive conditioning experiments.

6.1.1 Olfactory pathway

In *Drosophila*, odors are detected by olfactory receptor neurons (ORNs), which are located in sensilla on the antenna and maxillary palps. In general, all the ORNs of one receptor type project onto one glomerulus of the antennal lobe (Hallem et al., 2004), where they synapse with projection neurons as well as with local interneurons (Figure 1). Projection neurons further transmit odor information to the lateral horn and the mushroom body (MB). Within the MB calyx, projection neurons synapse onto Kenyon cells (KCs), the major intrinsic neurons. The lateral horns are considered centers for innate olfactory behaviors, whereas the MBs are regarded as centers for learned olfactory behaviors (Tanaka et al., 2004), as will be discussed later (chapter 6.2.3). The output regions of MBs and lateral horns are not yet well characterized, but include premotor areas (Ito et al., 1998), and eventually control odor avoidance and attraction behaviors. The olfactory pathway is overviewed in figure 1.

Along this pathway, each relay has a stereotyped odor-evoked map of activity, and the neural representation of an odor is modified as it is transmitted from one relay to the next, through excitatory and inhibitory interactions (Perez-Orive et al., 2002, Wang et al., 2003, Bhandawat et al., 2007, Olsen et al., 2007, Silbering and Galizia, 2007, Root et al., 2008). In general, each ORN expresses one type of odorant receptor and responds to a number of odorants, so each odorant is encoded by a combinatorial pattern of activated ORNs (Galizia and Szyszka, 2008, Masse et al., 2009), although some odors activate more unique pathways (Suh et al., 2004, Jones et al., 2007, Semmelhack and Wang, 2009, Stensmyr et al., 2012). In the antennal lobes, odors are represented by specific combinations of activated glomeruli, and in the MBs by specific ensembles of KCs (Wang et al., 2003, Wang et al., 2004, Masse et al., 2009). Local neurons in the antennal lobes greatly contribute to the modification of olfactory representation as

it is transmitted from ORNs to projection neurons (Wilson and Laurent, 2005, Root et al., 2008, Tanaka et al., 2009, Olsen et al., 2010, Das et al., 2011). The population of local neurons in the antennal lobe is highly diverse and includes neurons innervating single, multi or all glomeruli (Chou et al., 2010). The majority of local neurons are inhibitory although some excitatory local neurons are also important for olfactory modification (Olsen et al., 2007, Shang et al., 2007, Huang et al., 2010). Lateral connections between the glomeruli might add to odor modification (Bhandawat et al., 2007, Masse et al., 2009). In the MBs, the olfactory information is further processed by a 10 to 1 convergence of input from projection neurons to KCs (Turner et al., 2008, Caron et al., 2013). This convergence and a high firing threshold leads to sparse firing of KCs for each given odor (Turner et al., 2008). Lateral connections between KCs, and between KCs and modulatory neurons innervating the MBs may further modify olfactory representations and behaviors. Some of these modulatory neurons are important for olfactory memory formation and consolidation, such as the GABAergic anterior paired lateral neuron (Liu and Davis, 2009), or the dorsal paired medial neuron (Keene et al., 2004, Yu et al., 2005). To conclude, representation of most odors starts as a unique combination of many active ORNs, and becomes sparse as it reaches the KCs. This sparseness separates representations of different odors from each other and serves a basis for memory formation. Representations of different odors do indeed become less correlated as they progress through the olfactory system (Turner et al., 2008).

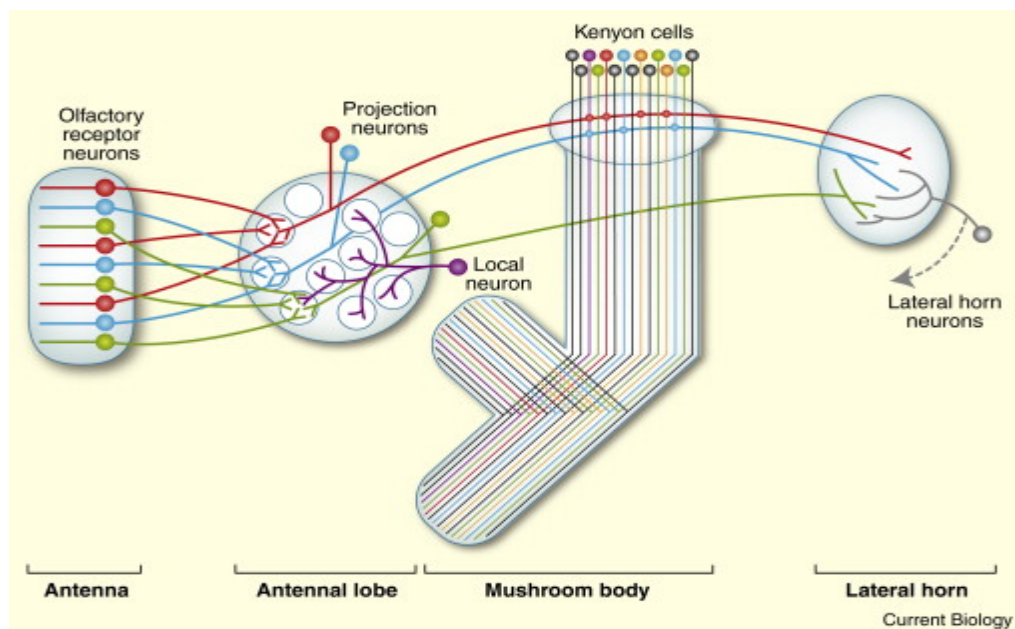


Figure 1: Overview of the olfactory system of *Drosophila*

Olfactory receptor neurons in the antennae and maxillary palps send axons to specific glomeruli in the antennal lobe. All olfactory receptor neurons expressing the same odorant receptor (same color) converge at a single glomerulus. There they form synaptic contacts with projection neurons and local neurons. Projection neurons send axons either directly to the lateral horn (green projection neuron) or via the calyx of the mushroom bodies (red and blue projection neurons), where they form synapses with Kenyon cells. Figure is taken from (Masse et al., 2009).

6.1.2 Electric shock sensation

Electric shock is a potent noxious stimulus which is easy to control, hence application of electric shock is the most common way of inducing aversion in animals and humans (Handwerker and Kobal, 1993). Nociceptors are the sensory receptors that respond to different kinds of noxious stimuli which can potentially damage tissues. In the hairy skin of mammals there are a few types of nociceptors. Some are polymodal receptors, responding to thermal, mechanical and irritant chemical stimuli. Other types of nociceptors are only sensitive to one modality and even to a specific intensity of that modality by having distinct activation thresholds. Sensory receptors are located in peripheral nerve endings and send sensory information through many types of fibers. These fibers include thickly myelinated A-beta-fibers carrying motor response and/or low threshold mechanical information, as well as thinly myelinated A-delta-fibers or C-fibers carrying information from thermo-receptors and nociceptors. Transcutaneous electrical stimulation of peripheral nerves excites almost all of these fibers, rendering the response non-specific for any modality (Baumgartner et al., 2012). Electric shock can excite the full spectrum of peripheral nerve fibers (depending on the stimulation method). Additionally, the sensory nerve endings are probably not recruited when an electrical stimulus is given, and the signal bypasses a transduction mechanism (Handwerker and Kobal, 1993). Being a potent and unnatural stimulus, electric shock might recruit general sensory pathways rather than dedicated ones.

For flies, similarly to mammals, exposure to electric shock leads to immediate avoidance, and they show stereotypical behaviors including rolling over, jumping, flying, and general increased activity (personal observations). Thus, shock is undoubtedly sensed by flies and perceived as aversive. Although electric shock has been widely used in fly research, the molecular and cellular mechanisms of its perception are poorly understood. The responses of flies to other nociceptive stimuli such as harsh touch, irritant chemicals and extreme temperature have been studied, and their molecular correlates are characterized (see next paragraph and “temperature sensation” chapter 6.1.3). But whether shock sensation utilizes any of the characterized nociceptive sensory pathways, or novel ones, is still an open question.

Nociception studies in *Drosophila* larvae led to the discovery of polymodal nociceptors in their body walls, the class IV multiple-dendritic neurons (Hwang et al., 2007). These neurons detect both noxious heat and noxious mechanical stimuli and are responsible for avoidance of larvae from increased temperature, mechanical pain, dry surfaces and bright light (Hwang et al., 2007, Xiang et al., 2010, Im and Galko, 2012, Johnson and Carder, 2012). These cells express Painless, Piezo, Pickpocket and dTrpA1, all proteins that function in perception of noxious mechano- and/or thermal stimuli (Zhong et al., 2010, Neely et al., 2011, Hwang et al., 2012, Kim et al., 2012, Zhong et al., 2012). The role of some proteins in aversive sensation is also multimodal: Painless is involved in thermal, mechanical and chemical nociceptive perception (Zhong et al., 2010), and dTrpA1 in the sensation of mild heat, noxious heat and chemical avoidance (Rosenzweig et al., 2005, Kang et al., 2010, Neely et al., 2011, Kang et al., 2012). While larvae share a single class of neurons for sensing different modalities of aversive stimuli, no such neurons have been identified in adult flies. For example, the expression pattern of *painless* in adults is distributed in the central and peripheral nervous system (Xu et al., 2006). Other transmitters and proteins are involved in mechanical nociception: histamine (Buchner et al., 1993, Melzig et al., 1996), Pickpocket (Zhong et al., 2010) and Piezo (Kim et al., 2012) but their cellular distribution is not fully characterized. It is not known whether electric shock stimulation is sensed through a specific sensory pathway, or if it leads to unspecific activation such as it is the case in mammals (Handwerker and Kobal, 1993, Baumgartner et al., 2012). Electric shock may be sensed by any of the identified pain receptors, or by novel ones.

6.1.3 Temperature sensation

Drosophila melanogaster have a narrow range of preferred temperatures and will actively avoid too hot or cold environments (Sayeed and Benzer, 1996). Thermo-sensors responding to different temperature ranges were identified and characterised in *Drosophila*: Brivido, TRP and TRPL for sensing cold temperatures (Rosenzweig et al., 2008, Gallio et al., 2011), and dTrpA1, GR28B, painless and pyrexia for sensing warm and hot temperatures (Tracey et al., 2003, Lee et al., 2005, Rosenzweig et al., 2005, Hamada et al., 2008, Ni et al., 2013). With the exception of GR28B, all other thermal receptors belong to the transient receptor potential (TRP) family. These are cation channels which possess temperature sensitivity, and open directly when the surrounding temperature reaches a certain level. Molecularly, temperature sensitivity of TRP channels is modulated by the transmembrane voltage, and changes in ambient temperature result in graded shifts of the voltage dependence of channel activation (Voets et al., 2004). Temperature sensitivity occurs whenever the activation energies associated with the opening

and closing transitions of a channel are sufficiently different. Accordingly, thermal sensitivity is possible when the energy needed for channel opening is much greater than that needed for closing, such as in the case of thermo-sensitive TRP channels (Voets et al., 2004). Each of these channels opens at a distinct temperature, allowing for graded activation of variable receptors with temperature elevation. While dTrpA1 and GR28B are receptors for mild heat (higher than 27°C), pyrexia and painless are only activated by temperatures higher than 38°C or 40°C, respectively (reviewed by Sokabe and Tominaga, 2009). Other proteins and channel-subunits are involved in thermal sensation although they are probably not direct temperature receptors, such as Straightjacket (Neely et al., 2010), histamine receptors (Hong et al., 2006), the cAMP pathway in the mushroom bodies (Hong et al., 2008, Kang et al., 2011), amnesiac (Aldrich et al., 2010) and others. The main characterized thermo-sensory membrane proteins are summarized in Figure 2A.

A few studies speculated that mild heat perception in adult flies may take place by two separate cellular pathways, one by sensory cells located in the antenna and the other by sensory cells located inside the head (Sayeed and Benzer, 1996, Zars, 2001). Indeed, two groups of receptor neurons were recently identified (Fig.2B), which sense environmental temperature elevation: anterior cell (AC) neurons are two pairs of neurons located in the central brain close to the antennal nerve, serving as internal thermal receptors, and expressing dTrpA1 channels as a molecular sensor (Hamada et al., 2008). Hot cells are the external thermal receptors. These are three neurons in the third segment of the antenna which innervate the antennal lobe (Gallio et al., 2011) and express the recently identified thermo-receptor, from a gustatory receptor family, GR28B (Ni et al., 2013). AC neurons and hot cells have shared innervation sites, glomeruli VP2 and VP3 of the antennal lobe (Gallio et al., 2011, Tanaka et al., 2012), however the majority of AC neurons' axon terminals are in the posterior protocerebrum (Hamada et al., 2008, Shih and Chiang, 2011, Tanaka et al., 2012).

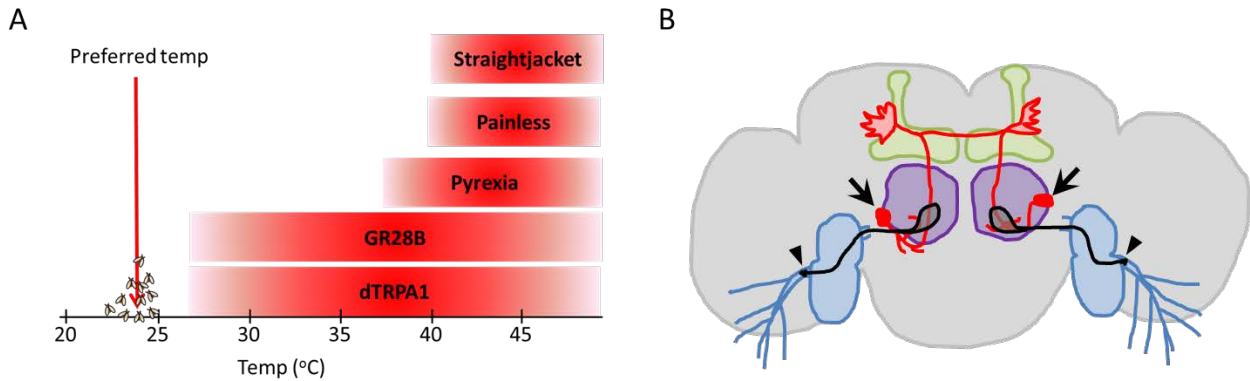


Figure 2: Temperature sensation in flies

A. Threshold activation temperature of thermo-sensory membrane proteins. Flies narrowly distribute around their preferred temperature, 24°C. dTrpA1 is activated above 27°C and contributes to avoidance of warmer temperatures. Pyrexia is activated above 38°C and prevents paralysis during high temperature stress. Painless is activated above 40°C, and is essential for avoiding hazardous temperatures. These channels belong to the TrpA subfamily and possess temperature sensitivity. GR28B is a thermal receptor belonging to the gustatory receptor family, with a similar activation threshold to dTrpA1. Straightjacket is a sub-unit of a calcium channel needed for sensation of high temperatures, with a similar threshold to Painless. **B.** Schematic of two pathways for elevated temperature reception in the fly brain. Red: the internal AC neurons which express dTrpA1 (arrows mark cell bodies). Black: the antennal hot cells which express GR28B (arrowheads mark cell bodies). VP2 and VP3 glomeruli of the antennal lobes (black innervation sites) are innervated both by hot cells and by AC neurons. AC neurons mostly terminate in the posterior protocerebrum (red innervation sites). Blue: antenna; purple: antennal lobes; Green: MBs. Adapted from Galili et al., 2014, figure 3A there, with changes.

6.2 Learning and memory in *Drosophila*

It is important for animals to rapidly detect meaningful stimuli in their surroundings, and use these to predict the value of co-occurring stimuli. Indeed, fruit-flies can learn to associate overlapping stimuli, remember and use this knowledge in following exposures. Since the pioneering work of Seymour Benzer beginning in the 1960's, learning and memory in *Drosophila melanogaster* has been a research topic in laboratories world-wide. Benzer and his colleagues were first to use behavioral genetics in flies to dissect the factors important for behaviors such as vision, locomotion, sexual function, circadian rhythm, learning and memory.

6.2.1 Classical conditioning of fruit-flies

Ivan Pavlov first described classical conditioning in the beginning of the 20th century. In his classic experiments, dogs were conditioned to respond with increased salivation to a whistle sound that was coupled with food presentation. The tone served as a conditioned stimulus (CS), and food as an

unconditioned stimulus (US), which led to a measurable behavioral response, salivation. After the CS-US coupling, the animal started responding to the CS with a conditioned response (Pavlov, 1911). Classical conditioning has been studied extensively in many model organisms since, including humans. A rather neutral CS is coupled to a meaningful US. The US can either be aversive and elicit an avoidance response or appetitive and elicit attraction. Fruit-flies can easily be trained and tested with classical conditioning (Quinn et al., 1974, Tully and Quinn, 1985). The most commonly used CSs for flies are olfactory or visual cues. Here, I will focus on aversive olfactory classical conditioning. Multiple stimuli can serve as aversive USs for *Drosophila* flies: mechanical vibration (Folkers, 1982, Eschbach et al., 2011, van Swinderen, 2011), courtship refusal (Siegel and Hall, 1979, Gailey et al., 1984), bitter substances and irritant chemicals (Gerber and Hendel, 2006, Schnaitmann et al., 2010, El-Keredy et al., 2012), the initial effect of ethanol (Kaun et al., 2011), humidity (Le Bourg, 2005), elevated temperature (Wolf and Heisenberg, 1991, Wustmann and Heisenberg, 1997, Ofstad et al., 2011), and electric shock (Quinn et al., 1974) (for more information see reviews: (Davis, 1996, Pitman et al., 2009)). The most commonly used aversive classical conditioning paradigm in flies is coupling an odor (CS) to electric shock (US), followed by a test for conditioned odor avoidance (Figure 3; Quinn et al., 1974, Tully and Quinn, 1985). This coupling leads to strong and stable odor avoidance, which is easy to quantify based on the distribution of flies between two tubes, one containing the CS and the other a control odor (or odorless solution). Depending on the training design, the associative memory can last as long as a few days (Tully et al., 1994). Much of the current knowledge about the neural circuit of olfactory conditioning in flies emerged from using electric shock olfactory conditioning (reviewed in: Davis, 2005), which I also utilized during my PhD project.

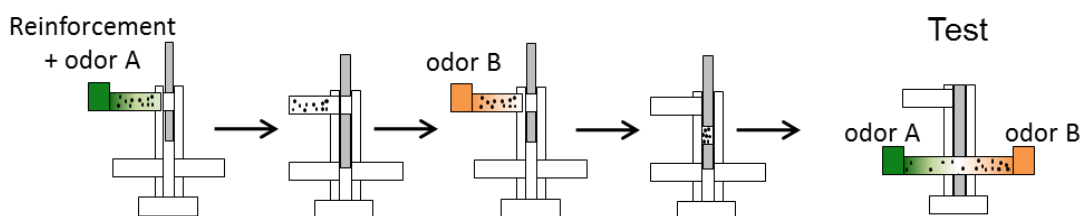


Figure 3: Classical olfactory conditioning in *Drosophila*

During training, an odor (CS) is presented together with reinforcement, here, electric shock (US). In a differential design, another odor is presented without reinforcement, as a control odor (odor B). During a subsequent test period, flies are given a choice between the two odors, and their distribution is counted to calculate conditioned odor avoidance. If the flies learned the association between the CS and US, they will avoid the CS during the test (although the US is not presented). A single odor design is also possible, where following the CS-US coupling, a choice is given between the CS odor and an odorless solution. Figure adapted from Galili et al., 2014, figure 1A there, with changes.

6.2.2 Reinforcement pathway: the role of dopamine

Although not much is known about electric shock sensation in flies (see chapter 6.1.2 “electric shock sensation”), it is widely used as a potent aversive reinforcer in olfactory conditioning. There are multiple lines of evidence that electric shock reinforcement information is eventually conveyed to dopaminergic neurons (Schwaerzel et al., 2003, Riemensperger et al., 2005, Kim et al., 2007, Claridge-Chang et al., 2009, Aso et al., 2010, Waddell, 2013). During training, these dopaminergic neurons are thought to signal reinforcement information to the axons of KCs, and modulate the strength of synaptic connections between KCs and their output partners (Figure 4). Dopamine type 1 receptors on KC axons play a role in this plasticity, resulting in alteration of the conditioned odor avoidance behavior (Kim et al., 2007). Recently, specific subsets of dopaminergic neurons were identified to play a role in olfactory conditioning with electric shock (Claridge-Chang et al., 2009, Aso et al., 2010, Aso et al., 2012).

The advantage of using flies for analyzing neural circuits lies in the ability to genetically manipulate small subsets of neurons independently. The function of dopaminergic neurons in aversive reinforcement was pinpointed to specific cellular subsets. Dopaminergic neurons in the fly brain are concentrated in a few clusters (Nassel and Elekes, 1992, Mao and Davis, 2009). Three of those clusters, the PPL1, the PPL2ab and the PAM clusters, include neurons which innervate the MBs, the site of coincidence detection (Figure 4A). By activating restricted subsets of dopaminergic neurons together with odor presentation, the source of aversive reinforcement signals was mapped to the 12 cells of the PPL1 cluster (Claridge-Chang et al., 2009). Even more specifically, a small subset of 1-2 neurons within the PPL1 cluster termed MB-MP1, was shown necessary for transmitting reinforcement information to the MBs during learning of olfactory shock conditioning (Figure 4B; Aso et al., 2010, Aso et al., 2012). These neurons terminate in the spur of γ lobe of the MBs (Mao and Davis, 2009, Aso et al., 2010), an area shown to be necessary during olfactory shock conditioning (Qin et al., 2012). Additionally, a type of dopamine neuron within the PAM cluster termed MB-M3, innervating the tip of β lobe, was shown necessary for intact olfactory conditioning (Figure 4B). Flies formed aversive odor memory without electric shock when MB-M3 was selectively stimulated together with odor presentation (Aso et al., 2010). Other dopaminergic neurons in the PPL1 cluster MB-MV1 and MB-V1 also play a role in learning during electric shock conditioning, at different time points after memory formation (Figure 4B). These dopaminergic neurons MB-MP1, MB-MV1, MB-V1 and MB-M3 terminate in spatially segregated regions of the MBs, and their activation affects different temporal stages of memory (Figure 4B; Aso et al., 2012). Thus, it is likely that electric shock creates parallel memory traces which are signaled by distinct subsets of dopamine neurons.

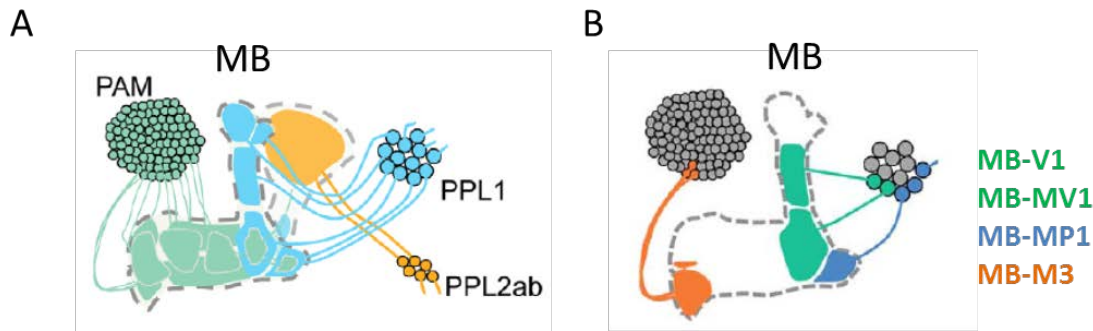


Figure 4: Dopaminergic neurons innervate the mushroom bodies (MBs) and play a role in learning and memory

A. Three clusters of dopaminergic neurons directly innervate the MBs: PAM, PPL1, PPL2ab (nomenclature according to Nassel and Elekes, 1992). **B.** Four types of dopaminergic neurons were found important for memory formation in olfactory shock conditioning (Aso et al., 2010, Aso et al., 2012). From the PPL1 cluster the neurons MB-V1, MB-MV1 (both depicted in green), MB-MP1 (depicted in blue). Additionally, from the PAM cluster the neuron MB-M3 (depicted in orange) (Figure modified from Aso et al., 2012).

However, there is only sparse evidence for the role of dopamine in signaling aversive reinforcers other than electric shock (Unoki et al., 2005, Honjo and Furukubo-Tokunaga, 2009). In some cases dopamine was not found necessary for conditioning of flies with variable aversive reinforcers (Sitaraman et al., 2008, Yarali and Gerber, 2010). In addition, serotonin is involved in reinforcement signaling in *Drosophila* and other insects (Sitaraman et al., 2008, Wright et al., 2010, Sitaraman et al., 2012). Particularly, thermal reinforcement in *Drosophila* place learning requires serotonin, but not dopamine (Sitaraman et al., 2008). Therefore, it is unknown whether the role of dopaminergic neurons in shock learning is restricted to signaling electric shock, or if they are part of a general aversive reinforcement pathway. To test this question, it is important to compare aversive conditioning with different reinforcers under otherwise similar experimental conditions.

6.2.3 The role of mushroom bodies as coincidence detectors

From studies of structural mutants, the mushroom bodies emerged as the main brain region needed for flies' olfactory learning and memory (Heisenberg et al., 1985, de Belle and Heisenberg, 1994, Connolly et al., 1996). In olfactory shock conditioning, information on electric shock reinforcement converges in the MBs with olfactory information. Synaptic plasticity mechanisms in the Kenyon cells (KCs) of the MBs were shown necessary and sufficient for olfactory memory formation (reviewed in: Gerber et al., 2004). Olfactory information is conveyed by projection neurons to the MB calyx, where they synapse onto a pattern of KCs representing the odor. KCs can be loosely divided into three types, forming the different MB lobes: $\alpha\beta$, $\alpha'\beta'$ and γ . Reinforcement information arrives to the MB lobes via modulatory

dopaminergic neurons which innervate KCs axons in different regions of the lobes. When both olfactory and reinforcement events reach the KCs simultaneously (or within a short range, as will be discussed), output from these activated KCs onto MB-output neurons is thought to be strengthened. The KCs project to a variety of target regions including premotor areas (Ito et al., 1998). Strengthened output is thought to mediate conditioned odor avoidance during the test period, when odor is encountered without reinforcement (reviewed in: Gerber et al., 2004). Indeed, blocking MB-output specifically during test period impaired memory performance, indicating that MB-output is needed for behavior execution (Dubnau et al., 2001, McGuire et al., 2001).

Behavioral screens of flies were used to discover genes involved in learning and memory. By inducing random mutations into the fly genome, many learning mutants were isolated in the past decades, among them *dunce* and *rutabaga* which are a part of the cAMP signaling pathway (Dudai et al., 1976, Duerr and Quinn, 1982, Dudai et al., 1983, Shotwell, 1983). It has been suggested that the type I adenylate cyclase, encoded in flies by the *rutabaga* gene, acts as a molecular coincidence detector between odor and reinforcement information, since *rutabaga* mutants are impaired in olfactory learning (Davis et al., 1995, Zars et al., 2000, Gerber et al., 2004). Activation of this enzyme requires both calcium influx and active G-protein; therefore it is capable of integrating information from two independent sources such as neuronal depolarization following odor activation (calcium influx), and dopamine receptor activation following reinforcement (G-protein activation), (Tomchik and Davis, 2009), as summarized in Figure 5. Restoring *rutabaga* expression specifically to the MBs of *rutabaga* mutant flies was enough to restore the learning ability (Zars et al., 2000). Activation of adenylate cyclase leads to the conversion of ATP to cAMP. cAMP, a major signal transducer of the cell, then activates protein kinase A, which in turn initiates a phosphorylation cascade leading to the induction of genes involved in learning (Davis et al., 1995, Connolly et al., 1996, Davis, 1996). Along with *rutabaga*, other genes in the cAMP signaling pathway have proven important for intact olfactory learning: *amnesiac* (encoding a product similar to adenylate cyclase activating peptides), *dunce* (cAMP phosphodiesterase), *DCO* (a subunit of protein kinase A), and *dCREB2* (cAMP-response element binding protein). All these genes are expressed preferentially in the MBs (reviewed in: Davis et al., 1995). The adenylate cyclase and cAMP signaling pathway is conserved among many species, and was shown important for learning and memory in other organisms beyond flies (Mons et al., 1999, Wang and Zhang, 2012).

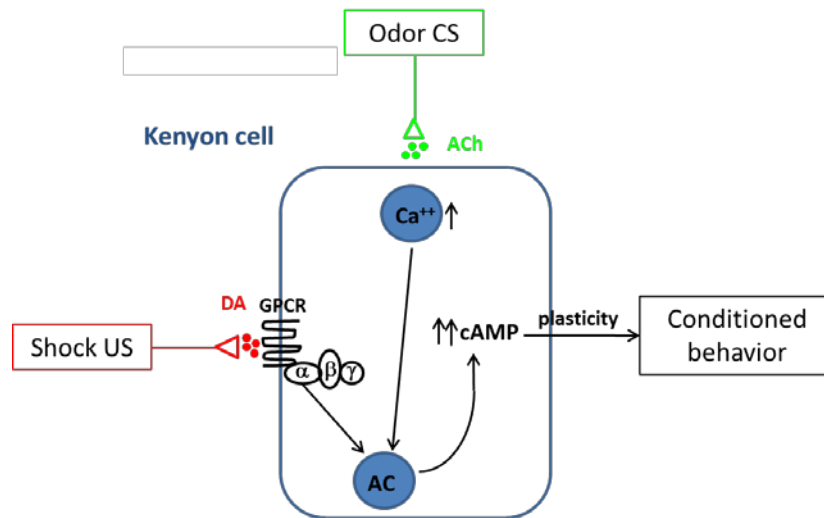


Figure 5: Schematic representation of the molecular mechanisms involved in aversive olfactory conditioning

CS information reaches the Kenyon cell via projection neurons releasing acetylcholine (ACh), leading to opening of voltage-gated calcium channels and increased intracellular calcium. Calcium binds to calmodulin and activates the *rutabaga* protein adenylate cyclase (AC). The shock US induces dopamine (DA) release, which binds to the alpha subunit of the G protein coupled receptor (GPCR), and activates AC. During coincident occurrence of CS and US, the two types of input act synergistically on AC, triggering cAMP signaling, and leading to the strengthening of the output from this Kenyon cell (Busto et al., 2010).

6.2.4 Importance of timing for associative learning: Trace, delay and relief learning

For efficient associative learning to take place, an overlap between CS and US presentation is beneficial. However, animals can also associate two temporally separated stimuli. This ability is important, since often in nature the cause and outcome of stimuli are not contiguous. In many behaviors critical for survival such as searching for a mate or food, or escaping predators, there can be a temporal gap between the sensory stimulus and the reward or punishment it predicts. In 1927, Ivan Pavlov had already noted that dogs were able to learn the CS-US association in classical conditioning, even after training with a whistle-sound CS and a food reward US, separated by several minutes (Pavlov, 1927). The conditioned salivary response was delayed in proportion to the duration of the CS-US interval, suggesting that the dogs learned the existence of the gap and anticipated the US.

To enable association of stimuli which are separated in time, the preceding CS must induce a representation in the form of a stimulus trace in the neuronal network, which persists after the stimulus has terminated. The stimulus trace can then be associated with the following reinforcing US. This form of learning where CS and US are separated by a temporal gap is termed trace conditioning, in contrast to delay conditioning, where both CS and US occur with a temporal overlap (Figure 6A; Tanimoto et al., 2004). When an aversive US is presented before the CS, animals can associate the CS with the end of the

aversive US, and show attraction towards it. This type of learning is termed relief learning (Tanimoto et al., 2004, Yarali and Gerber, 2010). Thus, the relative timing of CS and US presentation is a critical parameter of learning. Trace conditioning was studied in many organisms, using different paradigms (Rescorla, 1988, Figure 1 there). In general, aversive learning is highest when the CS and the US overlap, such as in delay conditioning, and it is lower for trace conditioning. The shape of the CS-US interval function in olfactory learning of flies (Figure 6B) is strikingly similar to those in mammals, across different conditioning paradigms with different stimuli (Rescorla, 1988, Figure 1 there), suggesting that stimuli traces are kept for a variety of modalities and are commonly used for conditioning by many species.

Are the memories formed during delay and trace conditioning governed by different neuronal pathways? In mammals, delay and trace eyeblink conditioning engage similar brain circuits, but in trace conditioning there is additionally a requirement of the hippocampus (Solomon et al., 1986, Woodruff-Pak and Disterhoft, 2008) and in humans also a state of awareness is required (Clark and Squire, 1998, Clark et al., 2002, Christian and Thompson, 2003). Therefore, trace memory was proposed to be qualitatively and anatomically distinct from delay memory, and serve as a model of declarative memory (Clark et al., 2002, Woodruff-Pak and Disterhoft, 2008). However, other views have been proposed (LaBar and Disterhoft, 1998). The demands on neural resources increase with task complexity for both trace and delay conditioning (Knuttnen et al., 2001, Carter et al., 2003). Thus the differential requirement of the hippocampus for trace conditioning might be a result of task complexity and not of the discontinuity between stimulus and reinforcement (Carrillo et al., 2000, Beylin et al., 2001, Walker and Steinmetz, 2008, Kehoe et al., 2009). In addition, a few examples of trace conditioning in humans without awareness have been observed (Bekinschtein et al., 2009, Arzi et al., 2012). Therefore, the anatomical and mechanistic distinction between the associative memory in delay and trace neuronal pathways in mammals is still an open question.

Insects show simple yet stereotypical behaviors and are an attractive model to study trace conditioning both behaviorally and physiologically. However, only a few attempts to study the mechanisms of stimulus traces and trace memories have been performed in insects (Figure 6B; Tully and Quinn, 1985, Tanimoto et al., 2004, Ito et al., 2008, Tomchik and Davis, 2009). A systematic comparison between delay and trace conditioning memories is missing. Do delay and trace memories in insects involve separated neural circuits, as was suggested in mammals? Additionally, trace conditioning is a good way to study the neural correlates of sensory traces. How does the percept of a stimulus evolve after the

stimulus termination? The engram of the olfactory trace and its temporal properties are unknown. In order to tackle these open questions it is important to first establish a robust experimental design where trace and delay conditioning can be compared.

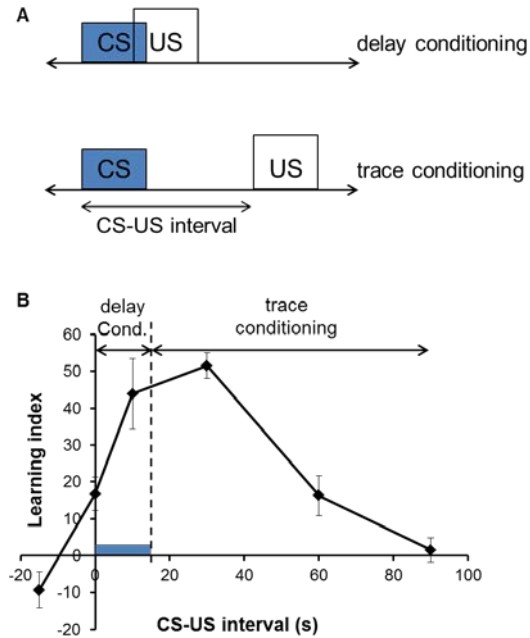


Figure 6: Behavioral design and memory performance in delay and trace conditioning

A. Behavioral design of delay (upper) and trace (lower) conditioning. While in delay conditioning CS and US presentations overlap, in trace conditioning there is a stimulus-free gap between them. The time between CS-onset and US-onset is termed the CS-US interval. **B.** Memory performance after aversive conditioning in *Drosophila* as a function of CS-US interval. Blue bar indicates the 15 s odor CS, its end is indicated by a dashed line. US is 4 electric shocks of 90 V within 16 s. Delay conditioning occurs when the CS-US interval ≤ 15 s, and trace conditioning when the CS-US interval > 15 s. Figure is modified from (Dylla et al., 2013). Data for B is taken from (Tanimoto et al., 2004).

When using odor as the CS it is critical to control for a clean offset of the stimulus presentation, to assure a real physiological trace, rather than a residual trace of the odor in the experimental set-up. This control was missing in all the former attempts to study trace conditioning in flies, which makes interpretation of these results ambiguous (Tully and Quinn, 1985, Tanimoto et al., 2004, Ito et al., 2008, Tomchik and Davis, 2009).

6.3 Advantages of fly neuroscience: the genetic tool box

Drosophila melanogaster has long been a popular model organism for biological and developmental studies. In recent decades, the fruit fly has also become an important model organism in behavioral neuroscience for several reasons. *Drosophila* are small, inexpensive, easy to cultivate, have a short generation time and perform highly stereotyped behaviors. Flies have a numerically reduced nervous system compared to mammals ($\sim 10^5$ vs. $\sim 10^{11}$ cells in a *Drosophila* and a human brain, respectively). In addition to the anatomical simplicity, studies in *Drosophila* are supported by a wealth of genetic techniques (Venken and Bellen, 2005). With only four pairs of chromosomes and knowledge of the complete sequence of the genome, genetic analysis of behaviour is facilitated. Among the most common genetic techniques used in *Drosophila* is the generation of mutant flies. Hundreds of mutants that affect behavioral functions have been identified in genetic screens. Usually, mutagenic treatments such as the feeding of specific chemicals or UV-radiation allow an unbiased generation of mutations (Ashburner, 1989). Another method to induce mutations is by randomly inserting transposable genetic elements (P-elements) into the fly genome (O'Kane and Gehring, 1987). Simple behaviors can be tested across many mutants, to identify specific genes required for these behaviors. Using a genetic screen, the gene *period* was among the first to be discovered (Konopka and Benzer, 1971). This gene plays a role in circadian rhythm of flies, and demonstrated that mutations in a single gene can alter circadian behavior, a first step towards a molecular analysis of circadian rhythms in several species. Generation and use of mutant flies tremendously advanced our understanding of the genetic basis of behaviour, yielding principles applicable to several other species. Since the fly genome is sequenced, targeted mutations are also possible by inserting P-elements into known locations in the genome, thus disrupting genes of interest. Different types of insertion and deletion mutations were generated using specifically designed P-elements (Hummel and Klambt, 2008).

One of the most important genetic techniques in *Drosophila* is the GAL4/UAS gene expression system allowing spatial and temporal manipulations of target cells [(Brand and Perrimon, 1993), reviewed in (Duffy, 2002)]. The GAL4/UAS system allows for the expression of any cloned gene in a cell population defined by an activated enhancer. This system comprises two kinds of transgenics: GAL4 driver strains and UAS effector strains. In the GAL4 strain, the yeast transcription factor GAL4 is expressed in a tissue-specific manner, under the control of a cloned or neighbouring genomic enhancer (so-called "GAL4 enhancer-trap"). The UAS (upstream activating sequence) strain carries a cloned arbitrary transgene of interest under the control of UAS promoter, which is exclusively activated in the presence of GAL4. The

two components are brought together with a simple genetic cross. In the progeny of the cross, the effector transgene will be expressed specifically in a defined set of cells that have the tissue specific promoter activated and express the GAL4 protein (Brand and Perrimon, 1993). Common effector transgenes used in *Drosophila* neuroscience research under UAS control include blockers of neuronal activity such as the potassium channel Kir2.1 (Baines et al., 2001) or the light-chain of tetanus toxin (Sweeney et al., 1995); neuronal activators such as the temperature-activated channel dTrpA1 (Hamada et al., 2008); and marker proteins such as GFP linked to mCD8 to allow plasma membrane labelling (Lee and Luo, 1999). For knocking-down specific genes in a restricted cellular population, one can exploit the RNA interference method, and express a double stranded RNA sequence complementary to the sequence of a gene of interest, under UAS control (Enerly et al., 2003). Expression of any of these transgenes in a specific cellular population by using the GAL4-UAS transcription system enables labelling, regulating, blocking or activating specific subsets of neurons, and testing the effect of these manipulations on behaviour.

Many tools were developed to complement and control the GAL4/UAS expression system, enabling specific spatial and temporal manipulations. By splitting the GAL4 construct into two parts, the DNA-binding domain and the activation domain, it is possible to use an intersection of different lines and thus reduce the number of labelled cells, in some cases to the level of single cells (Luan et al., 2006). Additionally, the yeast protein GAL80, a repressor of GAL4, binds the transactivation domain of GAL4 and prevents GAL4 activated transcription. The use of GAL80 allows for refinement of the expression pattern (Lee and Luo, 1999). A further modification of GAL80 allows for exact temporal control of the repression: the temperature sensitive version of GAL80 only represses GAL4 at certain temperatures (McGuire et al., 2003), providing an “on-off” temporal switch for GAL4 expression.

Altogether, these sophisticated genetic tools enable convenient and non-invasive neuronal manipulations in intact behaving animals, thus being optimal for the functional and structural characterization of neural circuits.

6.4 Aims of the thesis: exploring the CS and US representations in the nervous system

What happens to CS representation after CS is gone?

What is the US representation in different modalities?

My PhD project focuses on two open questions in the field of *Drosophila* olfactory learning (figure 7). The first question concerns the temporal requirement for the CS-US association. What do flies learn when a gap is inserted between the odor and the reinforcement? In order to answer this question, I established a robust paradigm for trace conditioning of flies using odor and electric shock which were temporally separated. Flies can learn the association even when a gap of 15 seconds is separating the end of odor presentation from the beginning of electric shock (Galili et al., 2011). I study the commonalities and differences between trace conditioning and delay conditioning (when odor and shock overlap), and look for the identity and location of the olfactory trace. The second part of my project concerns the neuronal representation of reinforcement. Almost all of the current knowledge on aversive reinforcement pathway was derived using electric shock. However, we do not know the sensory correlates of shock sensation. Electric shock is eventually signaled through dopamine neurons, but the generality of this representation was never tested. I compared electric shock reinforcement with a more ecologically relevant reinforcement: increased temperature. I established olfactory temperature conditioning, using the same experimental set-up and design as olfactory shock conditioning, and used genetic manipulations to compare the sensory and central neurons needed for forming these memories.

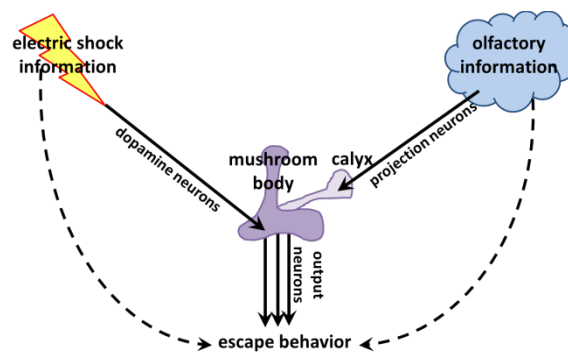


Figure 7: Simplified schematic of the olfactory conditioning circuit in flies

Olfactory information reaches the mushroom body calyx via projection neurons, while electric shock information arrives at the mushroom body lobes via dopaminergic neurons. When both events happen simultaneously, mechanisms of synaptic plasticity are strengthening the synapses between the mushroom body and its output neurons, modifying the behavioral output to favor escape behavior. Naïve escape behavior can also be triggered by unconditioned odors or electric shocks. My PhD project focuses on two aspects of this learning circuit: 1) the CS pathway and its temporal requirements for association with US (by studying trace conditioning); 2) the US pathway and its neural representation (by comparing temperature and shock conditioning).

7 Results

- 7.1 First paper: Galili DS, Lüdke A, Galizia CG, Szyszka P, Tanimoto H. (2011) Olfactory trace conditioning in *Drosophila*. *J Neurosci*. 31(20):7240-8.

<http://www.jneurosci.org/content/31/20/7240.long>

DOI: 10.1523/JNEUROSCI.6667-10.2011.

- 7.2 Second paper: Galili DS, Dylla KV, Lüdke A, Friedrich AB, Yamagata N, Wong JY, Ho CH, Szyszka P, Tanimoto H. (2014) Converging circuits mediate temperature and shock aversive olfactory conditioning in *Drosophila*. *Curr Biol*. 24(15):1712-22.

<http://www.sciencedirect.com/science/article/pii/S0960982214007799>

DOI: 10.1016/j.cub.2014.06.062.

8 Discussion

8.1 Olfactory trace conditioning in *Drosophila*

I showed that flies can associate an odor CS with a shock US even when a gap is separating them, in olfactory trace conditioning. Olfactory stimuli are often hard to switch-off as odor molecules may linger in the set-up in the form of residual odor, preventing a clean stimulus offset. I controlled for precise odor presentation, by testing whether flies are able to form an association between reinforcement and the residual odor, and then chose an odor which did not lead to learning of the residual. Thus the odor trace I studied originated from the nervous system of the fly itself and not the apparatus (Galili et al., 2011, Fig. 2).

8.1.1 Trace and delay memories are based on the same odor perception

By comparing the behavioral parameters of trace and delay memories, I discovered that they share striking commonalities. My data suggest that both memory forms engage similar odor percepts, although odor saliency may decline between odor presentation and its trace. This means that the odor percept probably does not undergo qualitative changes after offset. This conclusion is based on three findings: (1) Trace and delay conditioning have similar generalization profiles (Galili et al., 2011, Fig. 5). When one odor is presented during training and a new odor is presented during test, flies generalize their conditioned odor avoidance response depending on the degree of perceived similarity between the new odor and the trained one. With four different odors, the generalization index after training with trace or with delay conditioning was identical (correlation coefficient of $r^2=0.99$), suggesting the odor itself and its trace share a common (or extremely similar) percept. (2) Trace and delay memories reached the same asymptote in memory acquisition after sufficient trials (Galili et al., 2011 Fig. 6A). See discussion in the next chapter 9.1.2. (3) Trace and delay memories have similar decay patterns. When starting from similar levels of initial memory, both memories decay within the same duration and their decay curves are not significantly different (Galili et al., 2011, Fig. 6B). Together, these results suggest that a similar perception of odor quality may be engaged in both trace- and delay memories.

8.1.2 Odor trace may be less salient than the odor itself

Compared with delay conditioning, trace conditioning was less effective, and initial trials led to higher memory performance with delay than with trace conditioning. Although reaching similar levels of learning after sufficient training, acquisition rate of trace memory is slower (Galili et al., 2011, Fig. 6A).

This difference might be explained by a reduced CS salience in trace conditioning. One of the classical models of learning acquisition (Rescorla and Wagner, 1972) suggests that learning improvement during acquisition depends on three factors: the asymptotic level of memory acquisition, the strength of the reinforcement and the saliency of the stimulus. In the trace conditioning paradigm I established, the asymptotic levels are identical between trace and delay acquisition, and the same shock stimulus was used as reinforcement; hence the difference in the learning rates can be attributed to different saliencies of the sensory stimuli. According to Rescorla and Wagner (1972), CS saliency specifically changes the rate, but not the asymptote of learning acquisition. During trace conditioning, the salience of the odor stimulus probably decays after CS presentation until the US is applied. So the odor trace is probably fading with time [this fits also to the lower memory scores after longer intervals, as seen in the CS-US interval function, Figure 6B, and (Galili et al., 2011; Figure 3B there)]. In insect olfactory learning, changing odor saliency was indeed reported to effect memory formation (Pelz et al., 1997, Masek and Heisenberg, 2008, Yarali et al., 2009). In these studies, higher odor intensity supported stronger association, implying that the saliency of the odor can determine its effectiveness for memory formation. Together, my data suggest that the odor trace retains stimulus specificity but might be less salient than that odor during its presentation.

8.1.3 Identity and location of the odor trace

Successful trace conditioning in *Drosophila* was possible after a single trial (Galili et al., 2011), implying that a lingering physiological trace of the stimulus must be stored somewhere in the nervous system. Together with my collaborators from Konstanz University we showed that there are post-odor representations in ORNs which are different than the ORN responses during odor presentation (Galili et al., 2011, Fig. 4). However, these post-odor activations do not reflect the odor traces used for trace conditioning, as the behavioral generalization profile of different odors does not correlate with the physiological similarity profile of different odors. A similar approach was implemented by imaging projection neurons during and after odor presentation, by our collaborator Alja Luedke from Konstanz University (personal communication). Projection neurons also exhibit post-odor responses which differ from their odor responses, but also the projection neurons' post-odor responses do not correlate to behavioral generalization profiles. So, the physiological odor similarity profile in ORNs or projection neurons after odor presentation does not predict the perceived odor similarity, which is used for generalization learning. These findings indicate that the odor trace does not consist of persistent neuronal activity in ORNs or projection neurons. However, the odor trace may be encoded in activity

other than persistent calcium signals. For example, long-lasting biochemical modifications or “tags” (Perisse and Waddell, 2011, Dylla et al., 2013) can signal an odor trace. ‘Eligibility traces’ are transient synaptic changes which can theoretically last for several seconds, for example the activation of an enzyme with slow kinetics, important for synaptic plasticity. In the model suggested by Izhikevich (2007), after stimulus presentation, the slow kinetics of synaptic plasticity are sensitive to changes in the extracellular dopamine concentration signaling reinforcement, during a critical period of a few seconds. The synaptic ‘eligibility trace’ creates a time window in which a global diffusive reinforcement signal in the form of extracellular dopamine can selectively influence the right synapses at the right time (Izhikevich, 2007). Alternatively, subtle network activity, such as changes in the correlation of glomerular spontaneous activity following odor presentation (Galan et al., 2006) might also be an underlying mechanism for maintaining a sensory trace for a short while. In a study by Galan et al. (2006), a single odor presentation changed the relative timing of spontaneous firing across glomeruli, recapitulating the odor-induced activation, and this modification lasted for a few minutes. Both these scenarios suggest a trace representation which is reminiscent of the stimulus itself, a prediction which fits my results of trace and delay memories sharing a common odor percept (see chapter 9.1.1). Either a synaptic ‘eligibility trace’ or a modification of the network activity could represent a sensory trace in any of the neuronal relays along the olfactory pathway, without being reflected in our calcium imaging. Alternatively, the trace can be maintained as an increased firing in other neurons along the olfactory system, besides ORNs or projection neurons. Calcium imaging may be used to test for activity related to trace maintenance in these regions. Reasonable candidates are the local neurons in the antennal lobe, or the Kenyon cells of the MBs (Figure 8). Modulatory neurons of the olfactory system like the anterior paired lateral neuron (Liu and Davis, 2009) or the dorsal paired medial neuron (Waddell et al., 2000) may also play a role in keeping the olfactory trace.

Shuai et al. (2011) suggested a role for *Drosophila* Kenyon cells in trace conditioning, based on studies with Rac, a small G protein belonging to the Rho family of GTPases, which plays a role in actin cytoskeleton remodeling. In trace conditioning, inhibition of Rac specifically in the mushroom bodies enhanced trace- but not delay-memory formation. Furthermore, rescue experiments in dopamine receptor mutants showed that restoring type 1 dopamine receptor expression to mushroom bodies recovered trace conditioning, which was impaired in mutants (Shuai et al., 2011). Thus it will be interesting to study the functional correlations of trace conditioning in the MBs’ Kenyon cells. Inhibition of the MBs by the anterior paired lateral neuron might also play a role in odor trace maintenance. The anterior paired lateral neuron is a single GABAergic neuron in each hemisphere, which probably supplies

the main GABAergic innervation to the MB lobes through the GABA-A receptors, resistance to dieldrin (Rdl; Liu and Davis, 2009)). Rdl levels in the MBs were negatively correlated to olfactory memory performance (Liu et al., 2007). In preliminary experiments I knocked-down the GABA-A receptors Rdl in the MBs and trained flies for delay or trace conditioning. Decreased levels of Rdl receptors in the MBs improved trace conditioning, compared to delay conditioning (data not shown). These data hint that inhibition of the MBs may contribute to the fading of the olfactory trace, hence trace duration may be prolonged when inhibition of the MBs is blocked. Future experiments are needed to dissect the contribution of the MBs and MB-inhibition to olfactory traces and trace conditioning (Figure 8).

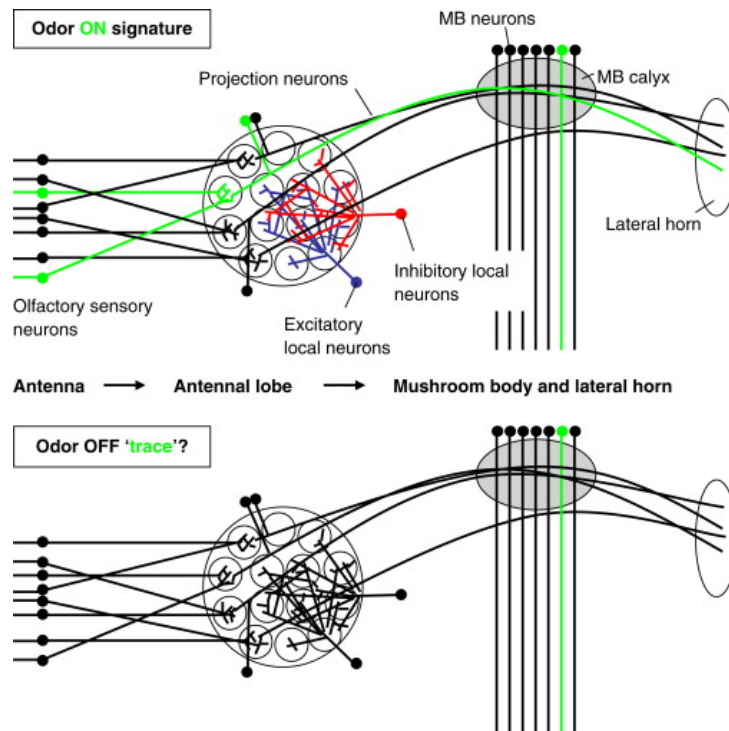


Figure 8: Possible locations of the odor trace

During odor presentation, olfactory sensory neurons (ORNs) send olfactory information to projection neurons in the antennal lobe, which further transmit the information to the mushroom bodies (MBs; upper panel; see olfactory pathway chapter 6.1.1). After the odor presentation is over, the odor trace is maintained in the nervous system. Our collaborator from Konstanz University, Alja Luedke, showed that the odor trace is not kept in calcium signals of ORNs or projection neurons (Galili et al., 2011; and personal communication). Possibly, the odor trace is located in the firing patterns or in biochemical alterations (eligibility traces) of the Kenyon cells of the MBs (Perisse and Waddell, 2011, Shuai et al., 2011, Dylla et al., 2013). Figure taken from Perisse and Waddell (2011).

8.1.4 Former experience improves trace conditioning

One of the most interesting and apparently conserved features of trace conditioning is that performance can be improved with prior experience. This was shown in mammals (Clark and Squire, 1998, Woodruff-

Pak and Disterhoft, 2008), and here is shown for the first time in insects (Galili et al., 2011, Szyszka et al., 2011). Successful trace conditioning with a short time interval between CS and US extends the interval that can be bridged in subsequent trials, enabling learning with gaps that were too long for naïve flies. Thus it appears that flies benefit from being familiar with the gap between the stimuli. There are two possible mechanisms to explain how the experience gained from trace conditioning training can help bridge a longer gap in subsequent trials. The first mechanism is altering the CS representation pathway. Behavioral experience could enhance the CS saliency, so that greater importance will be assigned to the fading CS trace by the fly during later trials. Enhanced saliency would result in a stronger association (Rescorla and Wagner, 1972). At the level of neuronal activity, associative learning may change the dynamics of odor-evoked activity and strengthen or extend the odor trace until the US arrival. Indeed, calcium imaging in odor-activated dopamine neurons revealed that olfactory conditioning prolonged the response to the trained but not to the control odor (Riemensperger et al., 2005). An alternative possible mechanism is a temporal shift of the onset of activity in reinforcement neurons, after the initial training. In this case the activation of US-representing neurons would start already during CS presentation. This is the case in monkeys, which can be trained to anticipate the arrival of a future US in trace conditioning, and show earlier activation of reinforcement neurons following training ('US anticipation', reviewed by Schultz, 2006). Insects can also learn to anticipate a US after associative conditioning (Gil et al., 2007, Gil et al., 2008). It was shown that honeybees' experience with increasing magnitudes of reinforcement during associative learning changed their subsequent behavior towards the CS; the animals spent longer time inspecting CS that predicted a bigger reward, indicating they learned to expect the US (Gil et al., 2007). Since flies can learn trace conditioning with a single training trial (Galili et al., 2011), US anticipation can be excluded as an explanation for 1-trial trace learning. Nonetheless, after several trials, US anticipation may develop. Whether experience with trace conditioning alters the CS pathway by prolonging the trace, or the US pathway by earlier activation of US-representation is still an open question. These proposed mechanisms may also act together to improve consecutive trials.

8.2 Comparing the neural circuits of aversive reinforcers: temperature and shock

Drosophila flies naturally avoid hot environments, thus increased temperature can be used as aversive reinforcement during associative learning. I established olfactory temperature conditioning, using similar conditions to those used for the well-studied olfactory shock conditioning. By using genetic manipulations of sensory proteins and sensory neurons, as well as aminergic neurons, I dissected the

commonalities and differences between the neural representations of these aversive reinforcements: temperature and shock. I found that the sensory correlates of temperature and shock perception are different: elevated temperature is sensed through Anterior Cell (AC) neurons expressing the thermo-sensitive cation channel dTrpA1, while shock sensation does not require these neurons. Although reception engages different pathways, eventually both shock and temperature reinforcement converge onto dopaminergic neurons, generally signaling aversive reinforcement. Within the small population of dopaminergic neurons necessary for both temperature and shock conditioning, a smaller subgroup of neurons seems to represent temperature than those representing shock.

8.2.1 Temperature and shock sensation are separate

I revealed the sensory neurons specific for temperature punishment: dTrpA1-expressing internal thermal receptors, AC neurons (Galili et al., 2014; Figure 3). Blocking neuronal transmission in AC neurons or knocking down dTrpA1 levels specifically in these cells impaired temperature, but not shock conditioning. In addition, using *in-vivo* calcium imaging of AC neurons (done in collaboration with Kristina V. Dylla, Konstanz University) we showed that these neurons are activated by increased temperature, but not by electric shock (Galili et al., 2014; Figure 4). I validated the temperature-specific requirement of AC neurons using shock conditioning with reduced level of electric shock. This calibrated shock conditioning induced similar memory performance as the temperature conditioning (Galili et al., 2014; Figure 1C), but did not require AC neurons or dTrpA1 (Galili et al., 2014; Figures S2A, S2E). Altogether, my results imply that the perception of increased temperature and shock during aversive conditioning is separated at the receptor level. In addition, as the separation of requirement is also true when using mild electric shock, I conclude that AC neurons' temperature-sensation is stimulus-specific and not merely intensity dependent. In the context of associative learning, AC neurons mediate the reinforcing property of mild temperature increase. None of the characterized temperature or pain receptors that I tested were necessary during shock conditioning, suggesting that shock perception may not be sensed by a specific sensory pathway. Shock sensation might utilize other, uncharacterized receptors, or activate a combination of sensory pathways in parallel. In mammals, for example, shock activates multiple peripheral nerve fibers (Handwerker and Kobal, 1993, Baumgartner et al., 2012). It is possible that stimuli which are met by animals in their natural environments would have specific sensory pathways, whereas artificial stimuli would utilize the existing sensors without stimulus-specificity. Future experiments should focus on the sensory correlates of electric shock sensation.

8.2.2 Detection of increased temperature during reinforcement learning occurs via a specific sensory pathway

While temperature sensors are distributed in different types of cells in the fly brain and peripheral tissues (Tracey et al., 2003, Lee et al., 2005, Rosenzweig et al., 2005, Hamada et al., 2008, Neely et al., 2010, Gallio et al., 2011), the sensory neurons and molecules mediating thermal punishment are selective. Out of the five receptor proteins I tested, only dTrpA1 was required for temperature conditioning (although this result is specific for the conditions I used). In addition, I showed that the internal receptors AC neurons are required for temperature conditioning, while the antennal thermal receptors and dTrpA1-expressing neurons in the labral sense organ seem to be dispensable for temperature punishment. Both AC neurons and the antennal thermal receptors serve in sensing and avoiding ambient temperature elevation in a similar range of above 27°C (Rosenzweig et al., 2005, Ni et al., 2013). However, these two receptors play a different behavioral role (Hamada et al., 2008, Gallio et al., 2011, Ni et al., 2013): Blocking AC neurons impaired long-term thermal preference behavior (Hamada et al., 2008), and blocking the antennal thermal receptor hot-cells impaired immediate thermal avoidance ((Ni et al., 2013) and confirmed by personal observations, data not shown). Hence, there are parallel pathways for sensing temperature information required for reflexive avoidance of hot environments, and they have separate behavioral outputs. In addition to the separation of reflexive thermal behaviors, only the internal temperature receptors AC neurons are required for learning temperature reinforcement during conditioning. It is possible that the antennal thermal receptors play a role in comparing temperatures between the two antennae and sense the direction of a temperature gradient. Thus AC neurons and hot cells seem to be employed in segregated pathways for sensing the same single stimulus, leading to specialized behavioral outputs as the sensory information is further processed (see Figure 9B, red pathways). An example for a similar circuit configuration may be seen in sugar sensation in *Drosophila*. Flies have multiple sugar taste organs; sugar taste receptors are distributed on legs, labellum, and pharynx, and these pathways innervate separate regions in the subesophageal ganglion in the brain (Isono and Morita, 2010). It has been suggested that the map in subesophageal ganglion may reflect distinct output signaling circuits according to different peripheral inputs. For example, leg taste neurons may trigger searching or avoidance behavior while labellar or pharyngeal taste neurons control initiation or rejection of feeding behavior (Isono and Morita, 2010). In general, behaviorally relevant natural stimuli may activate parallel sensory pathways, each functioning for a different behavioral purpose. A simplified model is suggested in Figure 9B (red pathways).

Although I showed the separation of sensory pathways to specific circuits regarding reinforcement signaling, other functions may be shared between these sensory pathways. For example, glomeruli VP2 and VP3 of the antennal lobe are suggested to be commonly innervated by both AC neurons and hot cells (Gallio et al., 2011, Tanaka et al., 2012). It was also shown that a few Kenyon cells get thermal information from glomeruli VP2 and VP3 through projection neurons (Caron et al., 2013, Figure 3 there). It is an open question which behavioral functions this circuit does serve, but the role of the MB in thermal behaviors may depend on these inputs (Hong et al., 2008). To conclude, mild temperature increase activates separate sensory pathways, and can lead to different behavioral outputs, some are segregated between these inputs and some may be shared.

8.2.3 Dopamine integrates aversive reinforcement of both temperature and shock (Figure 9A)

I showed that dopaminergic neurons are required for both shock and temperature reinforcement signaling. Dopamine neurons are not needed for innate perception, but for assigning the appropriate aversive value of the reinforcement to the olfactory stimulus (Galili et al., 2014; Figure 5). Evidence from my anatomical characterization suggest that AC neurons contact dopamine neurons, possibly transmitting temperature reinforcement information during conditioning (Galili et al., 2014; Figure 6). My results join a line of research on the role of dopamine in reinforcement signaling, both in vertebrates and invertebrates. Many studies in recent years support a role for the dopamine system in both negative and positive reinforcement signaling. In insects, aversive learning of electric shock is conveyed by dopaminergic neurons (Schwaerzel et al., 2003, Riemensperger et al., 2005, Kim et al., 2007, Vergoz et al., 2007, Claridge-Chang et al., 2009, Aso et al., 2010, Waddell, 2013). Aversive conditioning using quinine taste in fly larvae (Honjo and Furukubo-Tokunaga, 2009) or saline solution in water-deprived crickets (Unoki et al., 2005) also depend on dopaminergic neurons. Although reward signaling in flies was initially attributed to octopamine (Schwaerzel et al., 2003, Schroll et al., 2006), recent papers show that reward learning depends additionally on dopaminergic neurons (Kim et al., 2007, Burke et al., 2012, Liu et al., 2012a, Perry and Barron, 2013) that are probably innervated by octopaminergic neurons signaling the appetitive value of sweet taste.

My results add a significant contribution to this emerging percept of dopamine as a general signal of value. Not only is dopamine required for aversive reinforcement signaling in flies using the very potent, artificial electric shock punishment, but also ecologically relevant temperature reinforcement is conveyed by dopaminergic neurons (Figure 9A,B).

The convergence of different inputs to dopaminergic neurons may be conserved among different animal species beyond insects. Similar to the organization in the fly, midbrain dopaminergic neurons in mammals have been shown to respond to different punishing events in rodents and primates (Brischoux et al., 2009, Bromberg-Martin et al., 2010, Lammel et al., 2011, Wang and Tsien, 2011, Zweifel et al., 2011). Although dopamine in mammals was affiliated with reward signaling, it is emerging that dopamine plays a role in signaling aversive conditioned stimuli as well (Matsumoto and Hikosaka, 2009, Schultz, 2010, Lammel et al., 2011, Zweifel et al., 2011). Thus, dopamine may be a signal encoding general motivational values for decision making regardless of stimuli and valence.

Temperature and shock are sensed by different sensory pathways, converging to the same dopamine system. A similar circuit configuration was described for sugar reinforcement in flies. Sugar includes two separate reinforcing qualities- sweet taste and nutritional value. These qualities are sensed by separate pathways and converge onto the dopaminergic system, which signals reinforcement (Burke et al., 2012). The sweet taste is probably signaled by octopamine and conveyed to dopaminergic PAM neurons, while nutritional value is sensed independently of octopamine, and eventually signaled by dopamine too (Burke et al., 2012). Similarly, in the case of temperature and shock, sensory pathways differ and reinforcement signaling converges to the dopaminergic system. This convergence may be a typical property of reinforcement systems. While the stimulus identity is initially separated, a general system attaches a value to an environmentally relevant reinforcer. A simplified model is suggested in Figure 9B.

8.2.4 Overlapping subsets of dopaminergic neurons for temperature and shock reinforcement

Next, I dissected the contribution of specific subsets of dopaminergic neurons to temperature and shock conditioning. Dopaminergic neurons in the fly brain are concentrated in a few clusters (Nassel and Elekes, 1992, Mao and Davis, 2009), and specific cellular subsets were shown to participate in different behaviors: ethanol-induced locomotion, sleep and arousal, courtship suppression learning, proboscis extension, olfactory learning and aggression were all attributed to specific clusters, sometimes even single dopaminergic neurons (Kong et al., 2010, Keleman et al., 2012, Liu et al., 2012a, Liu et al., 2012b, Marella et al., 2012, Ueno et al., 2012, Alekseyenko et al., 2013). Here I show that temperature and shock reinforcement signaling require an intact small population of ~20 dopaminergic neurons labeled by *TH-D'-GAL4* line (Galili et al., 2014; Figure 7). One population of neurons may commonly signal temperature and shock reinforcement. Moreover, I found the neurons labeled by *TH-F3-GAL4* necessary for shock but not temperature conditioning, suggesting a smaller set of dopamine neurons is mediating increased temperature than those mediating shock reinforcement (Figure 9A). Alternatively, different

subsets of neurons within the *TH-D'-GAL4* may separately be responsible for shock- and for temperature olfactory memories. In order to find out whether there are specific dopaminergic neurons which signal temperature reinforcement, I screened a library of driver lines labeling variable sub-populations, small clusters or single dopaminergic neurons (data not shown). However, I did not find drivers which specifically impaired temperature but not shock memories. Thus, the signal for temperature reinforcement may be included within the population of dopaminergic neurons signaling shock reinforcement. It is possible, that the artificial electric shock stimulus recruits more than one specific pathway (Claridge-Chang et al., 2009, Aso et al., 2010, Aso et al., 2012), while the ecologically relevant thermal stimulus recruits a more specific subset of dopaminergic neurons within those, to signal reinforcement.

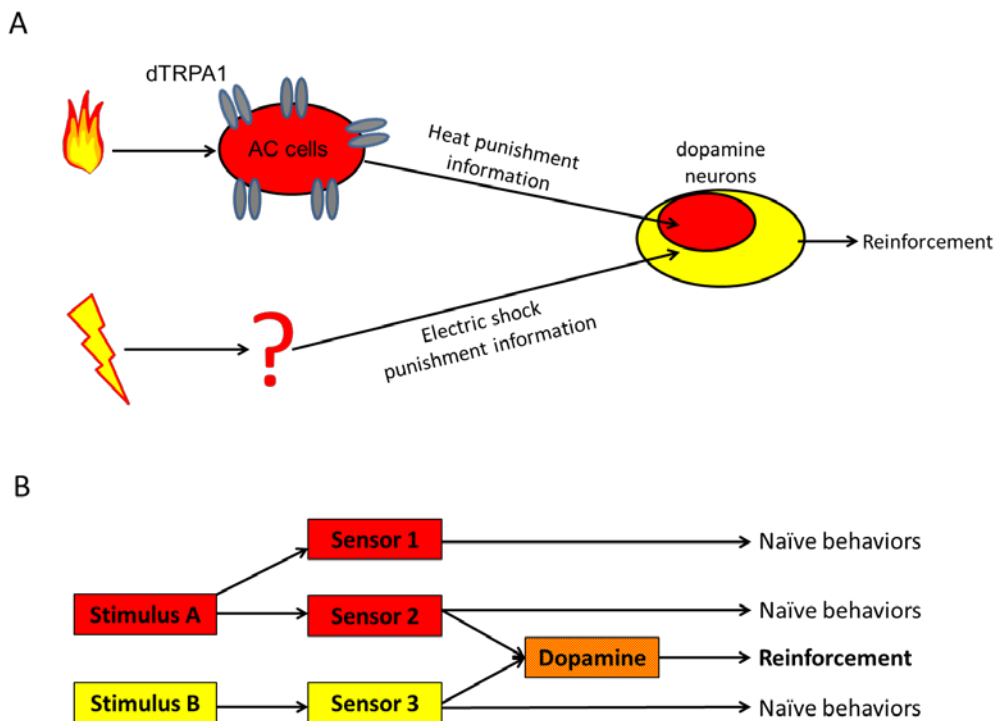


Figure 9: Summary and model for sensory and reinforcement signaling

A. While electric shock receptors are unknown, elevated temperature is sensed through AC neurons expressing dTrpA1 channels. The information of both temperature and electric shock is conveyed to dopaminergic neurons, generally signaling reinforcement. The population of dopaminergic neurons signaling increased temperature is probably contained within those signaling shock. **B.** A general model for sensory and reinforcement signaling. A single stimulus can be separately sensed by different receptors, leading to different behavioral outputs. In other circuits, different stimuli are separately sensed but converge later onto dopaminergic neurons, signaling reinforcement. For example, temperature can be represented as stimulus A, sensed separately by AC neurons and hot-cells. Shock can be represented as Stimulus B, sensed by an unknown sensor (see text for more examples). I showed here that both temperature and shock converge onto dopamine neurons for reinforcement signaling.

8.3 General conclusions and outlook

Brains have the unique ability to change and adapt according to environmental experience. Animals must learn to attach values to meaningful sensory stimuli they are exposed to, in order to approach stimuli which predict positive outcome, and avoid those which predict negative outcome. Hence the study of associative learning is an important and fundamental field in neuroscience. Associative learning involves detection and encoding of two stimuli: the CS and the US. My results provide important insights into the neuronal pathways transmitting both these stimuli, until they converge and modulate behavioral output. In my PhD project I studied two phenomena in the field of olfactory conditioning in *Drosophila* concerning either the CS pathway or the US pathway: olfactory trace conditioning, and aversive temperature and shock reinforcement signaling. Flies served as an excellent model system to study these behavioral, genetic and physiological questions in neuroscience. Establishing a clean paradigm for trace conditioning and my behavioral characterization of trace memories will help to continue the search for the identity of odor trace in future studies (Galili et al., 2011, Perisse and Waddell, 2011, Shuai et al., 2011, Szyszka et al., 2011, Dylla et al., 2013). Since I could show that dopamine representation of aversive reinforcement is shared between different modalities (electric shock and increased temperature), the neurons responsible for other aversive reinforcements may be localized in future studies. Additionally, my results demonstrate a specific pathway for temperature sensation and reinforcement, which may encourage others to study the sensory correlates of other stimuli, to reach a broader picture of aversive reinforcement signaling.

9 References

- Aldrich BT, Kasuya J, Faron M, Ishimoto H, Kitamoto T (2010) The amnesiac gene is involved in the regulation of thermal nociception in *Drosophila melanogaster*. *J Neurogenet* 24:33-41.
- Alekseyenko OV, Chan YB, Li R, Kravitz EA (2013) Single dopaminergic neurons that modulate aggression in *Drosophila*. *Proc Natl Acad Sci U S A* 110:6151-6156.
- Arzi A, Shedlesky L, Ben-Shaul M, Nasser K, Oksenberg A, Hairston IS, Sobel N (2012) Humans can learn new information during sleep. *Nat Neurosci* 15:1460-1465.
- Ashburner M (1989) *Drosophila: A Laboratory Handbook*. New York: Cold Spring Harbor Laboratory.
- Aso Y, Herb A, Ogueta M, Siwanowicz I, Templier T, Friedrich AB, Ito K, Scholz H, Tanimoto H (2012) Three dopamine pathways induce aversive odor memories with different stability. *PLoS genetics* 8:e1002768.
- Aso Y, Siwanowicz I, Bracker L, Ito K, Kitamoto T, Tanimoto H (2010) Specific dopaminergic neurons for the formation of labile aversive memory. *Curr Biol* 20:1445-1451.
- Baines RA, Uhler JP, Thompson A, Sweeney ST, Bate M (2001) Altered electrical properties in *Drosophila* neurons developing without synaptic transmission. *J Neurosci* 21:1523-1531.
- Baumgartner U, Greffrath W, Treede RD (2012) Contact heat and cold, mechanical, electrical and chemical stimuli to elicit small fiber-evoked potentials: merits and limitations for basic science and clinical use. *Neurophysiologie clinique = Clinical neurophysiology* 42:267-280.
- Bekinschtein TA, Shalom DE, Forcato C, Herrera M, Coleman MR, Manes FF, Sigman M (2009) Classical conditioning in the vegetative and minimally conscious state. *Nat Neurosci* 12:1343-1349.
- Beylin AV, Gandhi CC, Wood GE, Talk AC, Matzel LD, Shors TJ (2001) The role of the hippocampus in trace conditioning: temporal discontinuity or task difficulty? *Neurobiol Learn Mem* 76:447-461.
- Bhandawat V, Olsen SR, Gouwens NW, Schlieff ML, Wilson RI (2007) Sensory processing in the *Drosophila* antennal lobe increases reliability and separability of ensemble odor representations. *Nat Neurosci* 10:1474-1482.
- Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118:401-415.
- Brischoux F, Chakraborty S, Brierley DI, Ungless MA (2009) Phasic excitation of dopamine neurons in ventral VTA by noxious stimuli. *Proc Natl Acad Sci U S A* 106:4894-4899.
- Bromberg-Martin ES, Matsumoto M, Hikosaka O (2010) Dopamine in motivational control: rewarding, aversive, and alerting. *Neuron* 68:815-834.
- Buchner E, Buchner S, Burg MG, Hofbauer A, Pak WL, Pollack I (1993) Histamine is a major mechanosensory neurotransmitter candidate in *Drosophila melanogaster*. *Cell and tissue research* 273:119-125.
- Burke CJ, Huetteroth W, Oswald D, Perisse E, Krashes MJ, Das G, Gohl D, Silies M, Certel S, Waddell S (2012) Layered reward signalling through octopamine and dopamine in *Drosophila*. *Nature* 492:433-437.
- Busto GU, Cervantes-Sandoval I, Davis RL (2010) Olfactory learning in *Drosophila*. *Physiology (Bethesda)* 25:338-346.
- Caron SJ, Ruta V, Abbott LF, Axel R (2013) Random convergence of olfactory inputs in the *Drosophila* mushroom body. *Nature* 497:113-117.
- Carrillo MC, Gabrieli JDE, Disterhoft JF (2000) Selective effects of division of attention on discrimination conditioning. *Psychobiology* 28:293-302.
- Carter RM, Hofstotter C, Tsuchiya N, Koch C (2003) Working memory and fear conditioning. *Proc Natl Acad Sci U S A* 100:1399-1404.

- Chou YH, Spletter ML, Yaksi E, Leong JC, Wilson RI, Luo L (2010) Diversity and wiring variability of olfactory local interneurons in the *Drosophila* antennal lobe. *Nat Neurosci* 13:439-449.
- Christian KM, Thompson RF (2003) Neural substrates of eyeblink conditioning: acquisition and retention. *Learn Mem* 10:427-455.
- Claridge-Chang A, Roorda RD, Vrontou E, Sjulson L, Li H, Hirsh J, Miesenbock G (2009) Writing memories with light-addressable reinforcement circuitry. *Cell* 139:405-415.
- Clark RE, Manns JR, Squire LR (2002) Classical conditioning, awareness, and brain systems. *Trends Cogn Sci* 6:524-531.
- Clark RE, Squire LR (1998) Classical conditioning and brain systems: the role of awareness. *Science* 280:77-81.
- Connolly JB, Roberts IJ, Armstrong JD, Kaiser K, Forte M, Tully T, O'Kane CJ (1996) Associative learning disrupted by impaired Gs signaling in *Drosophila* mushroom bodies. *Science* 274:2104-2107.
- Das S, Sadanandappa MK, Dervan A, Larkin A, Lee JA, Sudhakaran IP, Priya R, Heidari R, Holohan EE, Pimentel A, Gandhi A, Ito K, Sanyal S, Wang JW, Rodrigues V, Ramaswami M (2011) Plasticity of local GABAergic interneurons drives olfactory habituation. *Proc Natl Acad Sci U S A* 108:E646-654.
- Davis RL (1996) Physiology and biochemistry of *Drosophila* learning mutants. *Physiological reviews* 76:299-317.
- Davis RL (2005) Olfactory memory formation in *Drosophila*: from molecular to systems neuroscience. *Annu Rev Neurosci* 28:275-302.
- Davis RL, Cherry J, Dauwalder B, Han PL, Skoulakis E (1995) The cyclic AMP system and *Drosophila* learning. *Molecular and cellular biochemistry* 149-150:271-278.
- de Belle JS, Heisenberg M (1994) Associative odor learning in *Drosophila* abolished by chemical ablation of mushroom bodies. *Science* 263:692-695.
- Dubnau J, Grady L, Kitamoto T, Tully T (2001) Disruption of neurotransmission in *Drosophila* mushroom body blocks retrieval but not acquisition of memory. *Nature* 411:476-480.
- Dudai Y, Jan YN, Byers D, Quinn WG, Benzer S (1976) *dunce*, a mutant of *Drosophila* deficient in learning. *Proc Natl Acad Sci U S A* 73:1684-1688.
- Dudai Y, Uzzan A, Zvi S (1983) Abnormal activity of adenylate cyclase in the *Drosophila* memory mutant *rutabaga*. *Neuroscience letters* 42:207-212.
- Duerr JS, Quinn WG (1982) Three *Drosophila* mutations that block associative learning also affect habituation and sensitization. *Proc Natl Acad Sci U S A* 79:3646-3650.
- Duffy JB (2002) GAL4 system in *Drosophila*: a fly geneticist's Swiss army knife. *Genesis* 34:1-15.
- Dylla KV, Galili DS, Szyszka P, Ludke A (2013) Trace conditioning in insects-keep the trace! *Frontiers in physiology* 4:67.
- El-Keredy A, Schleyer M, König C, Ekim A, Gerber B (2012) Behavioural analyses of quinine processing in choice, feeding and learning of larval *Drosophila*. *PLoS One* 7:e40525.
- Enerly E, Larsson J, Lambertsson A (2003) Silencing the *Drosophila* ribosomal protein L14 gene using targeted RNA interference causes distinct somatic anomalies. *Gene* 320:41-48.
- Eschbach C, Cano C, Haberkern H, Schraut K, Guan C, Triphan T, Gerber B (2011) Associative learning between odorants and mechanosensory punishment in larval *Drosophila*. *J Exp Biol* 214:3897-3905.
- Folkers E (1982) Visual learning and memory of *Drosophila melanogaster* wild type CS and the mutants *dunce1*, *amnesiac*, *turnip* and *rutabaga*. *Journal of Insect Physiology* 28:535-539.
- Gailey DA, Jackson FR, Siegel RW (1984) Conditioning Mutations in *DROSOPHILA MELANOGASTER* Affect an Experience-Dependent Behavioral Modification in Courting Males. *Genetics* 106:613-623.
- Galan RF, Weidert M, Menzel R, Herz AV, Galizia CG (2006) Sensory memory for odors is encoded in spontaneous correlated activity between olfactory glomeruli. *Neural computation* 18:10-25.

- Galili DS, Dylla KV, Lüdke A, Friedrich AB, Yamagata N, Wong JH, Ho CH, Szyszka P, Tanimoto H (2014) Converging circuits mediate temperature and shock aversive olfactory conditioning in *Drosophila*. *Curr Biol* 24.
- Galili DS, Ludke A, Galizia CG, Szyszka P, Tanimoto H (2011) Olfactory trace conditioning in *Drosophila*. *J Neurosci* 31:7240-7248.
- Galizia CG, Szyszka P (2008) Olfactory coding in the insect brain: molecular receptive ranges, spatial and temporal coding. *Entomologia Experimentalis et Applicata* 128:81-92.
- Gallio M, Ofstad TA, Macpherson LJ, Wang JW, Zuker CS (2011) The coding of temperature in the *Drosophila* brain. *Cell* 144:614-624.
- Gerber B, Hendel T (2006) Outcome expectations drive learned behaviour in larval *Drosophila*. *Proc Biol Sci* 273:2965-2968.
- Gerber B, Tanimoto H, Heisenberg M (2004) An engram found? Evaluating the evidence from fruit flies. *Curr Opin Neurobiol* 14:737-744.
- Gil M, De Marco RJ, Menzel R (2007) Learning reward expectations in honeybees. *Learn Mem* 14:491-496.
- Gil M, Menzel R, De Marco RJ (2008) Does an insect's unconditioned response to sucrose reveal expectations of reward? *PLoS One* 3:e2810.
- Hallem EA, Ho MG, Carlson JR (2004) The molecular basis of odor coding in the *Drosophila* antenna. *Cell* 117:965-979.
- Hamada FN, Rosenzweig M, Kang K, Pulver SR, Ghezzi A, Jegla TJ, Garrity PA (2008) An internal thermal sensor controlling temperature preference in *Drosophila*. *Nature* 454:217-220.
- Handwerker HO, Kobal G (1993) Psychophysiology of experimentally induced pain. *Physiological reviews* 73:639-671.
- Heisenberg M, Borst A, Wagner S, Byers D (1985) *Drosophila* mushroom body mutants are deficient in olfactory learning. *J Neurogenet* 2:1-30.
- Hong ST, Bang S, Hyun S, Kang J, Jeong K, Paik D, Chung J, Kim J (2008) cAMP signalling in mushroom bodies modulates temperature preference behaviour in *Drosophila*. *Nature* 454:771-775.
- Hong ST, Bang S, Paik D, Kang J, Hwang S, Jeon K, Chun B, Hyun S, Lee Y, Kim J (2006) Histamine and its receptors modulate temperature-preference behaviors in *Drosophila*. *J Neurosci* 26:7245-7256.
- Honjo K, Furukubo-Tokunaga K (2009) Distinctive neuronal networks and biochemical pathways for appetitive and aversive memory in *Drosophila* larvae. *J Neurosci* 29:852-862.
- Huang J, Zhang W, Qiao W, Hu A, Wang Z (2010) Functional connectivity and selective odor responses of excitatory local interneurons in *Drosophila* antennal lobe. *Neuron* 67:1021-1033.
- Hummel T, Klambt C (2008) P-element mutagenesis. *Methods Mol Biol* 420:97-117.
- Hwang RY, Stearns NA, Tracey WD (2012) The ankyrin repeat domain of the TRPA protein *painless* is important for thermal nociception but not mechanical nociception. *PLoS One* 7:e30090.
- Hwang RY, Zhong L, Xu Y, Johnson T, Zhang F, Deisseroth K, Tracey WD (2007) Nociceptive neurons protect *Drosophila* larvae from parasitoid wasps. *Curr Biol* 17:2105-2116.
- Im SH, Galko MJ (2012) Pokes, sunburn, and hot sauce: *Drosophila* as an emerging model for the biology of nociception. *Developmental dynamics : an official publication of the American Association of Anatomists* 241:16-26.
- Isono K, Morita H (2010) Molecular and cellular designs of insect taste receptor system. *Frontiers in cellular neuroscience* 4:20.
- Ito I, Ong RCY, Raman B, Stopfer M (2008) Sparse odor representation and olfactory learning. *Nature neuroscience* 11:1177-1184.
- Ito K, Suzuki K, Estes P, Ramaswami M, Yamamoto D, Strausfeld NJ (1998) The organization of extrinsic neurons and their implications in the functional roles of the mushroom bodies in *Drosophila melanogaster* Meigen. *Learn Mem* 5:52-77.

- Izhikevich EM (2007) Solving the distal reward problem through linkage of STDP and dopamine signaling. *Cereb Cortex* 17:2443-2452.
- Johnson WA, Carder JW (2012) *Drosophila* nociceptors mediate larval aversion to dry surface environments utilizing both the painless TRP channel and the DEG/ENaC subunit, PPK1. *PLoS One* 7:e32878.
- Jones WD, Cayirlioglu P, Kadow IG, Vosshall LB (2007) Two chemosensory receptors together mediate carbon dioxide detection in *Drosophila*. *Nature* 445:86-90.
- Kang J, Kim J, Choi KW (2011) Novel cytochrome P450, *cyp6a17*, is required for temperature preference behavior in *Drosophila*. *PLoS One* 6:e29800.
- Kang K, Panzano VC, Chang EC, Ni L, Dainis AM, Jenkins AM, Regna K, Muskavitch MA, Garrity PA (2012) Modulation of TRPA1 thermal sensitivity enables sensory discrimination in *Drosophila*. *Nature* 481:76-80.
- Kang K, Pulver SR, Panzano VC, Chang EC, Griffith LC, Theobald DL, Garrity PA (2010) Analysis of *Drosophila* TRPA1 reveals an ancient origin for human chemical nociception. *Nature* 464:597-600.
- Kaun KR, Azanchi R, Maung Z, Hirsh J, Heberlein U (2011) A *Drosophila* model for alcohol reward. *Nat Neurosci* 14:612-619.
- Keene AC, Stratmann M, Keller A, Perrat PN, Vosshall LB, Waddell S (2004) Diverse odor-conditioned memories require uniquely timed dorsal paired medial neuron output. *Neuron* 44:521-533.
- Kehoe EJ, Ludvig EA, Sutton RS (2009) Magnitude and timing of conditioned responses in delay and trace classical conditioning of the nictitating membrane response of the rabbit (*Oryctolagus cuniculus*). *Behav Neurosci* 123:1095-1101.
- Keleman K, Vrontou E, Kruttner S, Yu JY, Kurtovic-Kozaric A, Dickson BJ (2012) Dopamine neurons modulate pheromone responses in *Drosophila* courtship learning. *Nature* 489:145-149.
- Kim SE, Coste B, Chadha A, Cook B, Patapoutian A (2012) The role of *Drosophila* Piezo in mechanical nociception. *Nature* 483:209-212.
- Kim YC, Lee HG, Han KA (2007) D1 dopamine receptor *dDA1* is required in the mushroom body neurons for aversive and appetitive learning in *Drosophila*. *J Neurosci* 27:7640-7647.
- Knuttinen MG, Power JM, Preston AR, Disterhoft JF (2001) Awareness in classical differential eyeblink conditioning in young and aging humans. *Behavioral neuroscience* 115:747-757.
- Kong EC, Woo K, Li H, Lebestky T, Mayer N, Sniffen MR, Heberlein U, Bainton RJ, Hirsh J, Wolf FW (2010) A pair of dopamine neurons target the D1-like dopamine receptor *DopR* in the central complex to promote ethanol-stimulated locomotion in *Drosophila*. *PLoS One* 5:e9954.
- Konopka RJ, Benzer S (1971) Clock mutants of *Drosophila melanogaster*. *Proc Natl Acad Sci U S A* 68:2112-2116.
- LaBar KS, Disterhoft JF (1998) Conditioning, awareness, and the hippocampus. *Hippocampus* 8:620-626.
- Lammel S, Ion DI, Roeper J, Malenka RC (2011) Projection-specific modulation of dopamine neuron synapses by aversive and rewarding stimuli. *Neuron* 70:855-862.
- Le Bourg E (2005) Humidity as an aversive stimulus in learning in *Drosophila melanogaster*. *Learning & behavior* 33:265-276.
- Lee T, Luo L (1999) Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* 22:451-461.
- Lee Y, Lee J, Bang S, Hyun S, Kang J, Hong ST, Bae E, Kaang BK, Kim J (2005) Pyrexia is a new thermal transient receptor potential channel endowing tolerance to high temperatures in *Drosophila melanogaster*. *Nature genetics* 37:305-310.
- Liu C, Placais PY, Yamagata N, Pfeiffer BD, Aso Y, Friedrich AB, Siwanowicz I, Rubin GM, Preat T, Tanimoto H (2012a) A subset of dopamine neurons signals reward for odour memory in *Drosophila*. *Nature* 488:512-516.

- Liu Q, Liu S, Kodama L, Driscoll MR, Wu MN (2012b) Two dopaminergic neurons signal to the dorsal fan-shaped body to promote wakefulness in *Drosophila*. *Curr Biol* 22:2114-2123.
- Liu X, Davis RL (2009) The GABAergic anterior paired lateral neuron suppresses and is suppressed by olfactory learning. *Nat Neurosci* 12:53-59.
- Liu X, Krause WC, Davis RL (2007) GABAA receptor RDL inhibits *Drosophila* olfactory associative learning. *Neuron* 56:1090-1102.
- Luan H, Peabody NC, Vinson CR, White BH (2006) Refined spatial manipulation of neuronal function by combinatorial restriction of transgene expression. *Neuron* 52:425-436.
- Mao Z, Davis RL (2009) Eight different types of dopaminergic neurons innervate the *Drosophila* mushroom body neuropil: anatomical and physiological heterogeneity. *Frontiers in neural circuits* 3:5.
- Marella S, Mann K, Scott K (2012) Dopaminergic modulation of sucrose acceptance behavior in *Drosophila*. *Neuron* 73:941-950.
- Masek P, Heisenberg M (2008) Distinct memories of odor intensity and quality in *Drosophila*. *Proc Natl Acad Sci U S A* 105:15985-15990.
- Masse NY, Turner GC, Jefferis GS (2009) Olfactory information processing in *Drosophila*. *Curr Biol* 19:R700-713.
- Matsumoto M, Hikosaka O (2009) Two types of dopamine neuron distinctly convey positive and negative motivational signals. *Nature* 459:837-841.
- McGuire SE, Le PT, Davis RL (2001) The role of *Drosophila* mushroom body signaling in olfactory memory. *Science* 293:1330-1333.
- McGuire SE, Le PT, Osborn AJ, Matsumoto K, Davis RL (2003) Spatiotemporal rescue of memory dysfunction in *Drosophila*. *Science* 302:1765-1768.
- Melzig J, Buchner S, Wiebel F, Wolf R, Burg M, Pak WL, Buchner E (1996) Genetic depletion of histamine from the nervous system of *Drosophila* eliminates specific visual and mechanosensory behavior. *J Comp Physiol A* 179:763-773.
- Mons N, Guillou JL, Jaffard R (1999) The role of Ca²⁺/calmodulin-stimulable adenylyl cyclases as molecular coincidence detectors in memory formation. *Cellular and molecular life sciences : CMLS* 55:525-533.
- Nassel DR, Elekes K (1992) Aminergic neurons in the brain of blowflies and *Drosophila*: dopamine- and tyrosine hydroxylase-immunoreactive neurons and their relationship with putative histaminergic neurons. *Cell and tissue research* 267:147-167.
- Neely GG, Hess A, Costigan M, Keene AC, Goulas S, Langeslag M, Griffin RS, Belfer I, Dai F, Smith SB, Diatchenko L, Gupta V, Xia CP, Amann S, Kreitz S, Heindl-Erdmann C, Wolz S, Ly CV, Arora S, Sarangi R, Dan D, Novatchkova M, Rosenzweig M, Gibson DG, Truong D, Schramek D, Zoranovic T, Cronin SJ, Angjeli B, Brune K, Dietzl G, Maixner W, Meixner A, Thomas W, Pospisilik JA, Alenius M, Kress M, Subramaniam S, Garrity PA, Bellen HJ, Woolf CJ, Penninger JM (2010) A genome-wide *Drosophila* screen for heat nociception identifies alpha2delta3 as an evolutionarily conserved pain gene. *Cell* 143:628-638.
- Neely GG, Keene AC, Duchek P, Chang EC, Wang QP, Aksoy YA, Rosenzweig M, Costigan M, Woolf CJ, Garrity PA, Penninger JM (2011) TrpA1 regulates thermal nociception in *Drosophila*. *PLoS One* 6:e24343.
- Ni L, Bronk P, Chang EC, Lowell AM, Flam JO, Panzano VC, Theobald DL, Griffith LC, Garrity PA (2013) A gustatory receptor paralogue controls rapid warmth avoidance in *Drosophila*. *Nature* 500:580-584.
- O'Kane CJ, Gehring WJ (1987) Detection in situ of genomic regulatory elements in *Drosophila*. *Proc Natl Acad Sci U S A* 84:9123-9127.

- Ofstad TA, Zuker CS, Reiser MB (2011) Visual place learning in *Drosophila melanogaster*. *Nature* 474:204-207.
- Olsen SR, Bhandawat V, Wilson RI (2007) Excitatory interactions between olfactory processing channels in the *Drosophila* antennal lobe. *Neuron* 54:89-103.
- Olsen SR, Bhandawat V, Wilson RI (2010) Divisive normalization in olfactory population codes. *Neuron* 66:287-299.
- Pavlov I (1911) Lectures on conditioned reflexes. In: Transactions of the Society of Russian Physicians.
- Pavlov IP (1927) Conditioned reflexes: an investigation of the physiological activity of the cereb cortex. London: Oxford University Press.
- Pelz C, Gerber B, Menzel R (1997) Odorant intensity as a determinant for olfactory conditioning in honeybees: roles in discrimination, overshadowing and memory consolidation. *J Exp Biol* 200:837-847.
- Perez-Orive J, Mazor O, Turner GC, Cassenaer S, Wilson RI, Laurent G (2002) Oscillations and sparsening of odor representations in the mushroom body. *Science* 297:359-365.
- Perisse E, Waddell S (2011) Associative memory: without a trace. *Curr Biol* 21:R579-581.
- Perry CJ, Barron AB (2013) Neural mechanisms of reward in insects. *Annual review of entomology* 58:543-562.
- Pitman JL, DasGupta S, Krashes MJ, Leung B, Perrat PN, Waddell S (2009) There are many ways to train a fly. *Fly* 3:3-9.
- Qin H, Cressy M, Li W, Coravos JS, Izzi SA, Dubnau J (2012) Gamma neurons mediate dopaminergic input during aversive olfactory memory formation in *Drosophila*. *Curr Biol* 22:608-614.
- Quinn WG, Harris WA, Benzer S (1974) Conditioned behavior in *Drosophila melanogaster*. *Proc Natl Acad Sci U S A* 71:708-712.
- Rescorla RA (1988) Behavioral studies of Pavlovian conditioning. *Annual review of neuroscience* 11:329-352.
- Rescorla RA, Wagner AR (1972) A theory of Pavlovian conditioning: variations in the effectiveness of reinforcement and nonreinforcement. In: Classical conditioning II: current research and theory (Black, A. H. and Prokasy, W. F., eds), pp 64-99 New-York: Appleton-Century-Crofts.
- Riemensperger T, Voller T, Stock P, Buchner E, Fiala A (2005) Punishment prediction by dopaminergic neurons in *Drosophila*. *Curr Biol* 15:1953-1960.
- Root CM, Masuyama K, Green DS, Enell LE, Nassel DR, Lee CH, Wang JW (2008) A presynaptic gain control mechanism fine-tunes olfactory behavior. *Neuron* 59:311-321.
- Rosenzweig M, Brennan KM, Tayler TD, Phelps PO, Patapoutian A, Garrity PA (2005) The *Drosophila* ortholog of vertebrate TRPA1 regulates thermotaxis. *Genes & development* 19:419-424.
- Rosenzweig M, Kang K, Garrity PA (2008) Distinct TRP channels are required for warm and cool avoidance in *Drosophila melanogaster*. *Proc Natl Acad Sci U S A* 105:14668-14673.
- Sayeed O, Benzer S (1996) Behavioral genetics of thermosensation and hygrosensation in *Drosophila*. *Proc Natl Acad Sci U S A* 93:6079-6084.
- Schnaitmann C, Vogt K, Triphan T, Tanimoto H (2010) Appetitive and aversive visual learning in freely moving *Drosophila*. *Front Behav Neurosci* 4:10.
- Schroll C, Riemensperger T, Bucher D, Ehmer J, Voller T, Erbguth K, Gerber B, Hendel T, Nagel G, Buchner E, Fiala A (2006) Light-induced activation of distinct modulatory neurons triggers appetitive or aversive learning in *Drosophila* larvae. *Curr Biol* 16:1741-1747.
- Schultz W (2006) Behavioral theories and the neurophysiology of reward. *Annu Rev Psychol* 57:87-115.
- Schultz W (2010) Multiple functions of dopamine neurons. *F1000 biology reports* 2.
- Schwaerzel M, Monastirioti M, Scholz H, Friggi-Grelin F, Birman S, Heisenberg M (2003) Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila*. *J Neurosci* 23:10495-10502.

- Semmelhack JL, Wang JW (2009) Select *Drosophila* glomeruli mediate innate olfactory attraction and aversion. *Nature* 459:218-223.
- Shang Y, Claridge-Chang A, Sjulson L, Pypaert M, Miesenbock G (2007) Excitatory local circuits and their implications for olfactory processing in the fly antennal lobe. *Cell* 128:601-612.
- Shih HW, Chiang AS (2011) Anatomical characterization of thermosensory AC neurons in the adult *Drosophila* brain. *J Neurogenet* 25:1-6.
- Shotwell SL (1983) Cyclic adenosine 3':5'-monophosphate phosphodiesterase and its role in learning in *Drosophila*. *J Neurosci* 3:739-747.
- Shuai Y, Hu Y, Qin H, Campbell RA, Zhong Y (2011) Distinct molecular underpinnings of *Drosophila* olfactory trace conditioning. *Proc Natl Acad Sci U S A* 108:20201-20206.
- Siegel RW, Hall JC (1979) Conditioned responses in courtship behavior of normal and mutant *Drosophila*. *Proc Natl Acad Sci U S A* 76:3430-3434.
- Silbering AF, Galizia CG (2007) Processing of odor mixtures in the *Drosophila* antennal lobe reveals both global inhibition and glomerulus-specific interactions. *J Neurosci* 27:11966-11977.
- Sitaraman D, LaFerriere H, Birman S, Zars T (2012) Serotonin is critical for rewarded olfactory short-term memory in *Drosophila*. *J Neurogenet* 26:238-244.
- Sitaraman D, Zars M, Laferriere H, Chen YC, Sable-Smith A, Kitamoto T, Rottinghaus GE, Zars T (2008) Serotonin is necessary for place memory in *Drosophila*. *Proc Natl Acad Sci U S A* 105:5579-5584.
- Sokabe T, Tominaga M (2009) A temperature-sensitive TRP ion channel, *Painless*, functions as a noxious heat sensor in fruit flies. *Communicative & integrative biology* 2:170-173.
- Solomon PR, Vander Schaaf ER, Thompson RF, Weisz DJ (1986) Hippocampus and trace conditioning of the rabbit's classically conditioned nictitating membrane response. *Behav Neurosci* 100:729-744.
- Stensmyr MC, Dweck HK, Farhan A, Ibba I, Strutz A, Mukunda L, Linz J, Grabe V, Steck K, Lavista-Llanos S, Wicher D, Sachse S, Knaden M, Becher PG, Seki Y, Hansson BS (2012) A conserved dedicated olfactory circuit for detecting harmful microbes in *Drosophila*. *Cell* 151:1345-1357.
- Suh GS, Wong AM, Hergarden AC, Wang JW, Simon AF, Benzer S, Axel R, Anderson DJ (2004) A single population of olfactory sensory neurons mediates an innate avoidance behaviour in *Drosophila*. *Nature* 431:854-859.
- Sweeney ST, Broadie K, Keane J, Niemann H, O'Kane CJ (1995) Targeted expression of tetanus toxin light chain in *Drosophila* specifically eliminates synaptic transmission and causes behavioral defects. *Neuron* 14:341-351.
- Szyszkka P, Demmler C, Oemisch M, Sommer L, Biergans S, Birnbach B, Silbering AF, Galizia CG (2011) Mind the gap: olfactory trace conditioning in honeybees. *J Neurosci* 31:7229-7239.
- Tanaka NK, Awasaki T, Shimada T, Ito K (2004) Integration of chemosensory pathways in the *Drosophila* second-order olfactory centers. *Curr Biol* 14:449-457.
- Tanaka NK, Endo K, Ito K (2012) Organization of antennal lobe-associated neurons in adult *Drosophila melanogaster* brain. *The Journal of comparative neurology* 520:4067-4130.
- Tanaka NK, Ito K, Stopfer M (2009) Odor-evoked neural oscillations in *Drosophila* are mediated by widely branching interneurons. *J Neurosci* 29:8595-8603.
- Tanimoto H, Heisenberg M, Gerber B (2004) Experimental psychology: event timing turns punishment to reward. *Nature* 430:983.
- Tomchik SM, Davis RL (2009) Dynamics of learning-related cAMP signaling and stimulus integration in the *Drosophila* olfactory pathway. *Neuron* 64:510-521.
- Tracey WD, Jr., Wilson RI, Laurent G, Benzer S (2003) *painless*, a *Drosophila* gene essential for nociception. *Cell* 113:261-273.
- Tully T, Preat T, Boynton SC, Del Vecchio M (1994) Genetic dissection of consolidated memory in *Drosophila*. *Cell* 79:35-47.

- Tully T, Quinn WG (1985) Classical conditioning and retention in normal and mutant *Drosophila melanogaster*. *J Comp Physiol A* 157:263-277.
- Turner GC, Bazhenov M, Laurent G (2008) Olfactory representations by *Drosophila* mushroom body neurons. *J Neurophysiol* 99:734-746.
- Ueno T, Tomita J, Tanimoto H, Endo K, Ito K, Kume S, Kume K (2012) Identification of a dopamine pathway that regulates sleep and arousal in *Drosophila*. *Nat Neurosci* 15:1516-1523.
- Unoki S, Matsumoto Y, Mizunami M (2005) Participation of octopaminergic reward system and dopaminergic punishment system in insect olfactory learning revealed by pharmacological study. *The European journal of neuroscience* 22:1409-1416.
- van Swinderen B (2011) Conditioning to colors: a population assay for visual learning in *Drosophila*. *Cold Spring Harbor protocols* 2011:1334-1336.
- Venken KJ, Bellen HJ (2005) Emerging technologies for gene manipulation in *Drosophila melanogaster*. *Nature reviews Genetics* 6:167-178.
- Vergoz V, Roussel E, Sandoz JC, Giurfa M (2007) Aversive learning in honeybees revealed by the olfactory conditioning of the sting extension reflex. *PLoS One* 2:e288.
- Voets T, Droogmans G, Wissenbach U, Janssens A, Flockerzi V, Nilius B (2004) The principle of temperature-dependent gating in cold- and heat-sensitive TRP channels. *Nature* 430:748-754.
- Waddell S (2013) Reinforcement signalling in *Drosophila*; dopamine does it all after all. *Curr Opin Neurobiol* 23:324-329.
- Waddell S, Armstrong JD, Kitamoto T, Kaiser K, Quinn WG (2000) The amnesiac gene product is expressed in two neurons in the *Drosophila* brain that are critical for memory. *Cell* 103:805-813.
- Walker AG, Steinmetz JE (2008) Hippocampal lesions in rats differentially affect long- and short-trace eyeblink conditioning. *Physiology & behavior* 93:570-578.
- Wang DV, Tsien JZ (2011) Convergent processing of both positive and negative motivational signals by the VTA dopamine neuronal populations. *PLoS One* 6:e17047.
- Wang H, Zhang M (2012) The role of Ca(2+)-stimulated adenylyl cyclases in bidirectional synaptic plasticity and brain function. *Reviews in the neurosciences* 23:67-78.
- Wang JW, Wong AM, Flores J, Vosshall LB, Axel R (2003) Two-photon calcium imaging reveals an odor-evoked map of activity in the fly brain. *Cell* 112:271-282.
- Wang Y, Guo HF, Pologruto TA, Hannan F, Hakker I, Svoboda K, Zhong Y (2004) Stereotyped odor-evoked activity in the mushroom body of *Drosophila* revealed by green fluorescent protein-based Ca²⁺ imaging. *J Neurosci* 24:6507-6514.
- Wilson RI, Laurent G (2005) Role of GABAergic inhibition in shaping odor-evoked spatiotemporal patterns in the *Drosophila* antennal lobe. *J Neurosci* 25:9069-9079.
- Wolf R, Heisenberg M (1991) Basic organization of operant behavior as revealed in *Drosophila* flight orientation. *J Comp Physiol A* 169:699-705.
- Woodruff-Pak DS, Disterhoft JF (2008) Where is the trace in trace conditioning? *Trends Neurosci* 31:105-112.
- Wright GA, Mustard JA, Simcock NK, Ross-Taylor AA, McNicholas LD, Popescu A, Marion-Poll F (2010) Parallel reinforcement pathways for conditioned food aversions in the honeybee. *Curr Biol* 20:2234-2240.
- Wustmann G, Heisenberg M (1997) Behavioral manipulation of retrieval in a spatial memory task for *Drosophila melanogaster*. *Learn Mem* 4:328-336.
- Xiang Y, Yuan Q, Vogt N, Looger LL, Jan LY, Jan YN (2010) Light-avoidance-mediating photoreceptors tile the *Drosophila* larval body wall. *Nature* 468:921-926.
- Xu SY, Cang CL, Liu XF, Peng YQ, Ye YZ, Zhao ZQ, Guo AK (2006) Thermal nociception in adult *Drosophila*: behavioral characterization and the role of the painless gene. *Genes, brain, and behavior* 5:602-613.

- Yarali A, Ehser S, Hapil FZ, Huang J, Gerber B (2009) Odour intensity learning in fruit flies. *Proc Biol Sci* 276:3413-3420.
- Yarali A, Gerber B (2010) A Neurogenetic Dissociation between Punishment-, Reward-, and Relief-Learning in *Drosophila*. *Front Behav Neurosci* 4:189.
- Yu D, Keene AC, Srivatsan A, Waddell S, Davis RL (2005) *Drosophila* DPM neurons form a delayed and branch-specific memory trace after olfactory classical conditioning. *Cell* 123:945-957.
- Zars T (2001) Two thermosensors in *Drosophila* have different behavioral functions. *J Comp Physiol A* 187:235-242.
- Zars T, Fischer M, Schulz R, Heisenberg M (2000) Localization of a short-term memory in *Drosophila*. *Science* 288:672-675.
- Zhong L, Bellemer A, Yan H, Ken H, Jessica R, Hwang RY, Pitt GS, Tracey WD (2012) Thermosensory and nonthermosensory isoforms of *Drosophila melanogaster* TRPA1 reveal heat-sensor domains of a thermoTRP Channel. *Cell reports* 1:43-55.
- Zhong L, Hwang RY, Tracey WD (2010) Pickpocket is a DEG/ENaC protein required for mechanical nociception in *Drosophila* larvae. *Curr Biol* 20:429-434.
- Zweifel LS, Fadok JP, Argilli E, Garelick MG, Jones GL, Dickerson TM, Allen JM, Mizumori SJ, Bonci A, Palmiter RD (2011) Activation of dopamine neurons is critical for aversive conditioning and prevention of generalized anxiety. *Nat Neurosci* 14:620-626.

10 Acknowledgements

I want to thank my advisor Hiromu Tanimoto; for teaching me how to think in a scientific manner, how to provoke the important questions, how to build hypotheses and interpret results, and most importantly for sharing with me his passion for science.

I thank my doktorvater Mark Huebener and my PhD thesis committee for putting the time and effort to read my thesis, comment on it, and take part in my PhD defense.

I wish to thank my colleagues in the Tanimoto lab: Katrin, Yoshi, Vlad, Toshi, Christopher, Chang, Mirjam, Ayse, Nobu, Stephan, Anja, Igor, Sibel, for helpful discussions and comments, and their endless willingness to assist and support. Our weekly group meetings and their presence throughout my project was inspiring and irreplaceable.

I wish to thank my collaborators Kristina V. Dylla, Alja Luedke, Paul Szyszka and C. Giovanni Galicia from Konstanz University for enthusiastic discussions and fruitful exchange of ideas. It was a great pleasure to collaborate with them.

I wish to thank my partner Roni Jortner for his support, both emotionally and scientifically. His belief in me taught me to believe in myself. My daughter Einav Jortner, for showing me there is always a reason to smile. My parents, for teaching me to love the nature and encouraging me to explore. And finally my grandfather for motivating me to keep pushing my limits.

11 Curriculum Vitae

EDUCATION

- 2009-2014 PhD, Max Planck Institute for Neurobiology, Martinsried, Germany.
- 2006-2008 M.Sc, Weizmann institute, Rehovot, Israel. Life science. Summa Cum Laude.
- 2003-2006 B.Sc, Hebrew University of Jerusalem, Israel. Combined biology and psychology, "Amirim" honorary program of the faculty of mathematics and sciences. Summa Cum Laude.

SCHOLARSHIPS AND AWARDS

- 2009-2012 Minerva Stiftung. Fellowship for PhD students.
- 2008 Weizmann Institute, Rehovot, Israel. Dean's prize for M.Sc. students
- 2006-2008 Weizmann Institute, Rehovot, Israel. Fellowship for M.Sc. students.
- 2004-2006 Hebrew University, Jerusalem, "Amirim" honorary program for B.Sc students in the faculty of mathematics and sciences. Merit-based fellowship.
- 2005 Hebrew University, Jerusalem, Dean's list for honorary B.Sc. students
- 2004 Hebrew University, Jerusalem, Dean's prize for honorary B.Sc. students
- 2003 Hebrew University, Jerusalem, Merit-based applicant award

RESEARCH EXPERIENCE

- 2009-2014 Max Planck Institute for Neurobiology, Martinsried, Germany, in the lab of Dr. Hiromu Tanimoto. Research focus is learning and memory in *Drosophila Melanogaster*, using genetic manipulations and behavior techniques, conditioning assays and fly brain anatomy.
- 2007-2008 Weizmann Institute of Science, Rehovot, Israel, in the lab of Prof. Alon Chen. Research on the neurobiology of stress and anxiety-like behaviors. Main research tools included behavioral tests, molecular biological techniques (e.g. cloning, real-time PCR, in-situ hybridization), mice surgery and brain microinjections, histological techniques, immunoassays and cell-culture methods.

2004-2006 Hadassah Medical School, the Hebrew University, Jerusalem, Israel, in the lab of Prof. David Lichtstein. Undergraduate research on molecular biology of the sodium-potassium ionic pump and its involvement in human affective disorders.

EMPLOYMENT

2004-2006 Nature Park & Galleries, Hebrew University nature museum, instructor in university fauna collections.

2002-2003 Ortal Manpower Services Inc., placement coordinator

2000-2002 Military service, IDF. Served as a psychometric examiner of pre-military candidates. Honorary discharge, sergeant.

PEER-REVIEWED PUBLICATIONS

Galili DS, Dylla KV, Lüdke A, Friedrich AB, Yamagata N, Wong JY, Ho CH, Szyszka P, Tanimoto H. (2014) Converging circuits mediate temperature and shock aversive olfactory conditioning in *Drosophila*. *Curr Biol*. 24(15):1712-22.

Dylla KV, Galili DS, Szyszka P, Lüdke A. (2013) Trace conditioning in insects – Keep the trace! Review, *Front Physiol. - Invertebrate Physiology*, 4:67; Research Topic: Plasticity in the sensory systems of invertebrates. doi: 10.3389/fphys.2013.00067.

Galili DS, Lüdke A, Galizia CG, Szyszka P, Tanimoto H. (2011) Olfactory trace conditioning in *Drosophila*. *J Neurosci*. 31(20):7240-8.

Deutsch J, Jang HG, Mansur N, Ilovich O, Shpolansky U, Galili D, Feldman T, Rosen H, Lichtstein D. (2006) 4-(3'alpha15'beta-dihydroxy-5'beta-estran-17'beta-yl)furan-2-methyl alcohol: an anti-digoxin agent with a novel mechanism of action. *J Med Chem*. 49(2):600-6.

Goldstein I, Levy T, Galili D, Ovadia H, Yirmiya R, Rosen H, Lichtstein D. (2006) Involvement of Na⁺, K⁺-ATPase and Endogenous Digitalis-Like Compounds in Depressive Disorders. *Biol Psychiatry*. 60(5):491-9.

Feldmann T, Glukmann V, Friedman E, Shpolansky U, Galili D, Lichtstein D, Rosen H. (2007) Role of endosomal Na⁺, K⁺-ATPase and cardiac steroids in the regulation of endocytosis. *Am J Physiol Cell Physiol*. 293(3):C885-96.

CONFERENCE ABSTRACTS

Galili D, Tanimoto H: Comparing aversive olfactory memories with different reinforcers. Annual meeting of the Israeli Society for Neuroscience, Eilat 18-21 Dec 2012.

Galili D, Tanimoto H: Aversive olfactory learning with different reinforcers. Learning and memory in insects: new insights from honeybee and *Drosophila*. Konstanz 25-26 September 2012.

Galili D, Tanimoto H: Aversive olfactory memories with different reinforcers. Invertebrate Neurobiology. Cold spring harbor Asia meeting, Suzho, China, June 18-22 2012.

Galili D, Tanimoto H: Aversive olfactory memories with different reinforcers. Neurobiology of *Drosophila*. Cold spring harbor Laboratory meeting, New-York, October 3–7, 2011.

Galili D, Tanimoto H: from bad to worse: neural pathways of different punishments in *Drosophila* associative learning. Structure and Function of Neural Circuits, EMBL Heidelberg, September 2010.

Galili D, Tanimoto H: trace conditioning in fruit flies. Insect olfaction workshop, Annual meeting of the Computational Neuroscience Society. Berlin, May 2010.

Galili D, Tanimoto H: trace conditioning in fruit flies. Berlin-Wuerzburg meeting. Munich, December 2009.

Galili D, Chen A: Mice lacking the Urocortin-1 gene show anxiety-like behaviors: on the role of Urocortin-1 neurons in Edinger-Westphal. Annual Charles E. Smith lecture of the National Institute for Psychobiology (NIPI), Israel, June 2008.

Galili D, Chen A: Urocortin-1 deficient mice exhibit a robust anxiogenic phenotype: the role of Edinger-Westphal Urocortin-1 neurons. The 37th annual meeting of the Israel Endocrine Society, April 2008.

Galili D, Chen A: Urocortin-1 deficient mice exhibit a robust anxiogenic phenotype: the role of Edinger-Westphal Urocortin-1 neurons. Annual Conference of the Israeli Society for Biological Psychiatry (ISBP), March 2008.

INVITED TALKS

“Memory traces of olfactory learning in the fruit fly”; Institute of Biophysics, Chinese Academy of Sciences, Beijing, China. July 12, 2012.

“Memory traces of olfactory learning in the fruit fly”; Leibniz Institute for Neurobiology Magdeburg, Germany, April 25, 2013.

“Neural circuits of learning and memory in fruit-flies”; Opening retreat of AMGEN scholars program, Burghotel Aschau, Germany. July 07, 2013.

12 Declaration (Eidesstattliche Versicherung)

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

München, den
.....
(Unterschrift)

Erklärung

Hiermit erkläre ich, *

- dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist.
- dass ich mich anderweitig einer Doktorprüfung ohne Erfolg **nicht** unterzogen habe.
- dass ich mich mit Erfolg der Doktorprüfung im Hauptfach
..... und in den Nebenfächern
.....
bei der Fakultät für der
(Hochschule/Universität)
unterzogen habe.
- dass ich ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen.

München, den
.....
(Unterschrift)

*) Nichtzutreffendes streichen