Dissertation

A Single Pair of Neurons Defines a Neuropeptide-Dependent Aversive Memory Channel in *Drosophila melanogaster*

Jan Niklas Hörtzsch

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Referees:

Prof. Dr. Christoph M. Schuster

Prof. Dr. Hilmar Bading

Science is a way of life. Science is a perspective.
Science is the process that takes us from confusion to understanding in a manner that's precise,
predictive and reliable - a transformation, for those lucky enough to experience it,
that is empowering and emotional.
(Brian Greene)

The most exciting phrase to hear in science,
the one that heralds new discoveries, is not 'Eureka!' but 'That's funny...'
(Isaac Asimov)

I react on outer influences, therefore I am.
(*Drosophila melanogaster*)

Ut sementem feceris, ita metes.
(Cicero)

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Materials and Methods

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ABBREVIATIONS

ACT	Antennocerebral tract
AGT	Antennoglumerular tract
AL	Antennal lobe
amn	*amnesiac* gene
AMN	Amnesiac neuropeptide
AMON	Amontillado (homologue of the prohormone convertase 2)
AMPAR	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AN	Antennal nerve
APL	*Anterior paired lateral* neurons
ARM	*Anesthesia-resistant memory*
ASM	*Anesthesia-sensitive memory*
ATF	Activating transcription factor
AUC	Area under the curve
BSA	Bovine serum albumin
Ca	Calyx
CA	Corpora allata
Ca^{2+}	Calcium
CaM	Calmodulin
CaMBP	Calcium/Calmodulin binding peptide
CaMK	Ca^{2+}/calmodulin-dependent kinase
cAMP	3',5'-cyclic adenosine monophosphate
CaRE	Cognate Ca^{2+} response element
CBP	CREB binding protein
CC	Corpora cardiac
CNV	Copy number variation
CR	Conditioned response
CRE	cAMP response element
CREB	cyclic AMP-responsive element-binding protein
CS	Conditioned stimulus
DA	Dopaminergic neurons

DAPI	4',6-diamidino-2-phenylindole
DCO	PKA catalytic subunit
DILP	*Drosophila melanogaster* insulin-like peptide
dInR	*Drosophila* Insulin receptor
DLG	Discs large protein
DLP	*Dorsal lateral peptidergic* neurons
DNA	Deoxyribonucleic acid
dnc	cAMP specific phosphodiesterase
dnc	*dunce* gene
dncPDE	cAMP phosphodiesterase encoded by the *dunce* gene
DPM	*Dorsal paired medial* neurons
dsRNA	Double-stranded RNA
EGF-R	Epidermal growth factor-receptor
EIF2	Eukaryotic initiation factor 2
eLNs	excitatory local interneurons
ER	Endoplasmic reticulum
FasII	*fascicilinII* gene product
FFPE	Formalin-fixed, paraffin-embedded
GCaMP	Fusion of GFP, calmodulin and M13
GFP	Green fluorescent protein
Gs	Stimulatory G protein
HDAC	Histone deactylase
HS	Heat shock
iACT	Inner antennocerebral tract
IHC	Immunohistochemistry
iLNs	Inhibitory local interneurons
IN	Interneurons
InsP_3R	Inositol (1,4,5)-trisphosphate receptor
IPCs	Insulin-producing cells
iSTM	Immediate short-term memory
KC	Kenyon cells
KD	Knock down

KID	Kinase inducible domain
KIX	KID interaction domain
LH	Lateral horn (dorsolateral protocerebrum)
LT-ARM	*Long-term ARM*
LTM	*Long-term memory*
LTP	Long-term potentiation
M13	Peptide sequence from MLCK
MAPK	Mitogen-activated protein kinase
MB	Mushroom body
MBN	Mushroom body neurons
MCH	4-methyl-cyclohexanol
MLCK	Myosin light chain kinase
MT-ARM	*Middle-term ARM*
MTM	*Middle-term memory*
NF1	Neurofibromin
NGS	Normal goat serum
NMDAR	N-methyl-D-aspartate receptor
NSC	Neurosecretory cell
OCT	3-octanol
ORN	Olfactory receptor neurons
OSN	Olfactory sensory neurons
P	Peduncle
PACAP	Pituitary adenylyl cyclase activating peptide
PAT	Process analytical technology
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	*Pars intercerebralis*
PI	Performance index
PKA	Protein kinase A
PKA-R	PKA regulatory subunit
PKR	Protein kinase R
PL	*Pars lateralis*

PN	Projection neurons
POL II	RNA polymerase II
PTG	Prothoracic gland
PTSD	Posttraumatic stress disorders
RHA	RNA helicase A
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RNase-L	Ribonuclease-L
ROI	Region of interest
ROS	Reactive oxygen species
rsh	*radish* gene
rsh	*radish* gene product
RT PCR	Real time PCR
RT	Room temperature
rut	*rutabaga* gene
RUT	Type I adenylyl cyclase
RyR	Ryanodine receptor
SEM	Standard error calculator
Ser133	Serine 133
siRNA	Small interfering RNA
SMP	Superior medial protocerebrum
sNPF	Short neuropeptide F
ST-ARM	*Short-term ARM*
STM	*Short-term memory*
TARGET	Temporal and regional gene expression targeting
TBP	TATA binding protein
TF	Transcription factor
TS	Temperature sensitive
UAS	Upstream activating sequence
UR	Unconditioned response
US	Unconditioned stimulus

VDRC	Vienna *Drosophila* Resource Center
VGCC	Voltage-gated calcium channel
VOL	*volado* gene product
VSCC	Voltage-sensitive Ca^{2+} channels

1. SUMMARY

Parallel information processing in distinct channels is a common functional principle of nervous systems to facilitate rapid and precise extraction of specific features. A hallmark of such parallel processing is that the originally acquired information is initially segregated into individual processing channels that are tuned to extract distinct features of the input before re-converging them to guide appropriate responses. Parallel processing also applies to aversive olfactory memories in *Drosophila* where the metabolically costly and more enduring memory channel is sensitive to cold anesthesia (*ASM*) whereas the parallel anesthesia resistant memory channel (*ARM*) is only transient. The molecular basis and functional significance of this segregation of aversive olfactory memories in parallel channels is currently unclear. Here, we show that an aversive unconditioned stimulus (US) used in classical olfactory conditioning experiments is responsible for synaptic activity-driven neuronal nuclear calcium transients in distinct areas of the fly brain. These areas include the fly's association center, the mushroom bodies (MBs), as well as the fly's master regulator of its neuropeptidergic system, the *pars intercerebralis* (*PI*). Blockade of nuclear calcium signaling allowed us to functionally and morphologically separate the role of cAMP, a classical signaling pathway in learning and memory, and nuclear calcium signaling in the establishment of consolidated long-term memories (*LTM*) (Weislogel et al., 2013). In addition, we show that the US activates the fly's widespread neuropeptidergic system and, in particular, the *PI* which results in multiple local signaling events or even systemic responses. Furthermore, we show that the acquisition and formation of all *ASM* phases requires additional release of mature neuropeptides from a single pair of *dorsal paired medial* (*DPM*) neurons. *DPM* neurons form a recurrent network with mushroom body neurons that has been shown to be involved in the formation of serotonin-dependent *ARM,* consolidation of memory and linking these consolidation processes to sleep. Our results reveal that *DPMs* define a qualitatively distinct parallel memory channel that strictly depends on mature neuropeptides and that is, within the first hours after training, behaviorally additive to the neuropeptide-independent *ARM* channel. Afterwards, in its subsequent consolidated phase, the *ASM* channel becomes exclusive towards the *ARM* channel. Thus, we propose that *DPM* neurons are capable of gating the simultaneous formation of two parallel memory channels by means of using two distinct signaling systems. Finally, given that neuropeptide signaling appears to be more widely involved in the

processing of the US, it could represent a general mean of defining parallel processing channels.

2. ZUSAMMENFASSUNG

Die parallele Verarbeitung, bzw Aufspaltung von Informationen in verschiedene Kanäle ist ein allgemeines Funktionsprinzip von Nervensystemen um schnelle und präzise Reaktionen auf bestimmte Stimuli zu erleichtern. Ein Kennzeichen dieser parallelen Verarbeitung ist, dass die ursprünglich gewonnenen Informationen zunächst in einzelne, reizspezifische Verarbeitungskanäle aufgetrennt werden, bevor sie erneut konvergieren um die entsprechende Reaktion auf den Reiz zu ermöglichen. Dieses Prinzip wird auch von *Drosophila* bei der klassischen olfaktorischen Konditionierung angewendet, bei der zwei verschiedene Kanäle simultan etabliert werden. Der eine ist metabolisch aufwendig und langlebig, während seiner Etablierung jedoch Kälteschock sensitiv (*ASM*), der andere ist für den Organismus einfacher zu etablieren und Kälteschock resistent, allerdings auch kurzlebiger (*ARM*). Die molekularen Grundlagen sowie die funktionelle Bedeutung dieser Aufteilung in zwei parallele Kanäle ist jedoch zur Zeit noch unklar. Hier zeigen wir dass die Präsentation des aversiven, unkonditionierten Stimulus (US) verantwortlich ist für zeitlich begrenzte Kalziumeinströme in den Zellkern bestimmter Neurone in bestimmten Hirnarealen der Fliege, vornehmlich in die zentralen Assoziationszenter - die Pilzkörper -, sowie die Hauptregulierungsregion des neuropeptidergen Systems - den *pars intercerebralis* (*PI*). Blockierung der Kernkalziumsignale ermöglichte uns die funktionelle sowie morphologische Separierung der Notwendigkeit von cAMP -einem klassischen Signalweg in Gedächtnisbildung- und Kernkalziumsignalen, in der Etablierung von konsolidiertem Langzeitgedächtnis (*LTM*) (Weislogel et al., 2013). Daneben wirkt der US auch aktivierend auf das neuropeptiderge System und besonders auf dessen Organisator den *PI*, was sich in verschiedenen lokalen Signalereignissen und systemischen Reaktionen zeigt. Des Weiteren zeigen wir, dass sowohl der Erwerb als auch die Bildung aller *ASM* Phasen von der zusätzlichen Freisetzung von reifen Neuropeptiden abhängig ist, welche von einem einzigen dorsal-medial liegendem Paar von Neuronen (*DPM*) ausgeht. Die *DPMs* bilden ein rekursives Netzwerk mit Neuronen des Pilzkörpers welches bei der Bildung von Serotonin-abhängigen *ARM*-Phasen, sowie der Konsolidierung von Gedächtnis und der Abfolge von Schlafmustern eine Rolle spielt. Die *DPMs* definieren somit einen streng neuropeptidabhängigen Gedächtniskanal der Anfangs additiv und später exklusiv mit dem parallelen *ARM* Kanal interagiert. Unsere Hypothese lautet daher dass die *DPM* Neurone die gleichzeitige Bildung von zwei parallelen Gedächtniskanälen mittels zweier unterschiedlicher Signalsysteme steuern. Da

Neuropeptidsignale anscheinend einen viel größeren Einfluß auf die Prozessierung des US haben, könnten sie sich darüber hinaus als ein allgemeines Mittel zur Definition von parallelen Verarbeitungskanälen heraustellen.

3. INTRODUCTION

3.1 *Drosophila* as a model organism

The fruit fly *Drosophila melanogaster* is one of the most extensively used and best understood model organisms of all time. It has been the primal organism for genetic studies due to its giant polytene, salivary gland chromosomes, which show a barcode like banding pattern and allow easy identifications of chromosomal rearrangements and deletions even with standard optical microscopes. Since then *Drosophila* has been the subject of countless biological studies in the context of development, neurobiology, behavior and genetics since the early years of the 20th century and onwards. This extensive research resulted in the publication of its complete genomic sequence in the year 2000 (Adams et al., 2000; Myers et al., 2000), revealing that the genome of *Drosophila* consists of 142.573.017 base pairs encoding for 13.918 protein coding genes, 3.384 non coding genes and 257 pseudogenes which are located on four chromosomes and result in 34.749 gene transcripts (For further and permanent updated information check also http://flybase.org/ - the central information hub for *Drosophila*).

Drosophila is easy to handle and inexpensive to maintain since it basically requires only a simple diet of carbohydrates (cornmeal and corn syrup) and proteins (yeast extract). It has a relatively simple and short reproduction cycle, normally about 8-14 days (depending on the environmental temperature) which enables scientists to breed and observe several generations in a matter of months. Moreover the reproduction rate is quite high, as females, at room temperature, lay around 30-50 eggs per day throughout their lifetime, resulting in about 750-1.500 eggs, providing a sufficient amount of offspring for e.g., screens for behavioral analysis.

Although the size of the fly genome is around 5% of the human genome (3.2 billion base-pairs on 23 chromosomes) the amount of coding genes is by far not as small, since *Drosophila* have approximately 15.500 genes compared to around 22.000 genes in humans. Thus the density of genes per chromosome is much higher in the fly genome. Nonetheless most important is the fact that humans and flies show a close relationship between their genes, since they have retained around 60% of homologue genes from a common ancestor (Bier, 2005). From these

homologue genes a match of approximately 75% of already identified genes, which are mutated, amplified or deleted and play diverse roles in human diseases, is present. For all of these genes, functional counterparts have to be shown to be present in the fly too (Lloyd and Taylor, 2010; Pandey and Nichols, 2011; Reiter et al., 2001) and *Drosophila* mutants have been widely used to model neurological diseases in humans such as Alzheimer's, Parkinson's and Huntington's disease (Feany and Bender, 2000; Finelli et al., 2004; Iijima et al., 2004; Lee et al., 2004; Shulman et al., 2003), as well as in obesity (Liu et al., 2012b; Skorupa et al., 2008) and alcoholism (Devineni and Heberlein, 2009; Kong et al., 2010; Rodan and Rothenfluh, 2010). The genomic relationship between the two species is so close that often the sequences of newly discovered human genes can be matched with equivalent genes in the fly. Hence medical studies benefit immensely from examining the function of these genes in *Drosophila* and therefore bypassing potential ethical issues of biomedical research on human subjects or mamalian models. In addition, also on the molecular level many features and pathways are similar, making *Drosophila* a prime candidate for clinical studies concerning cancer, hypoxic responses, developmental defects, ageing and neurological and infectious diseases which will hopefully result in the development of new, potent, therapeutical drugs (Pandey and Nichols, 2011). Besides the close relationship of the genomes, it is relatively simple to induce mutations through disruptions or general alterations in fly genes, making *Drosophila* a simple means for creating transgenic animals. This has resulted in a huge amount of stable mutant strains, as well as hundreds of Gal4 driver lines for the use in the Gal4/UAS system (Brand and Perrimon, 1993). These drivers are created by the enhancer trap method, using the pGAWB construct (Duffy, 2002) to express the transcription factor Gal4 in numerous different patterns (see also 4.7.1). Naturally the amount of different possible proteins that can be expressed through Gal4 has also increased resulting in fluorescent reporter-, gene transcript knock down-, nuclear signaling influencing-, or apoptosis inducing-, effector strains (see 4.9 and 4.10). In combination with other genetic tools (e.g., $Gal80^{ts}$) these constructs can now be controlled not only in their spatial, but also in their temporal expression, ensuring the avoidance of developmental defects through prolonged expression of the construct already during the larval states (4.7.2). Yet the power of the *Drosophila* genetics being further enhanced to enable even more precise expression of Gal4 through the combination of two additive Gal4 drivers (split Gal4 system) in which Gal4 is only active in the overlapping parts of the two driver lines used (Luan et al., 2006), or the insertion or removal of single nucleotides to whole genes

(genome editing) using the CRISPR/Cas system (Fineran and Charpentier, 2012) to edit, regulate and target the genome (Sander and Joung, 2014). Taken together all these advantages clearly point out the importance and usefulness of *Drosophila* as a model organism in biological and medical studies.

3.2 Learning and memory

3.2.1 Memory formation - a dynamic process

Memory refers to the processes that take place to store, retain and later retrieve information that concerns past experiences and impressions. Therefore it follows the initial learning and acquisition processes which take place during the initial confrontation with the stimulus. For us, as humans, it empowers us with the capability to learn and adapt from previous incidents, experiences and tasks and ensures our survival by permitting the retrieval of learned facts, impressions, habits or skills. Whereas short-term memory (*STM*) reveals limited capacity and transient nature, long-term memory (*LTM*) refers to a robust and lasting storage of information. Although the majority of these accumulated data is most of the time outside of our awareness, once stored information regularly can be recalled into working memory when necessary. The process by which memories are stabilized and integrated into *LTM* after learning is called consolidation. This process is dependent on de-novo protein synthesis and marks a crucial phase that enables us to maintain specific memories and protect them from any interfering treatments, as new information becomes fixed at a cellular level (McGaugh, 2000; McGaugh and Petrinovich, 1966). Whereas standard consolidation theories describe this process as an irreversible passage (McGaugh, 1966; Müller and Pilzecker, 1900) actual studies revealed that retrieval of a once consolidated memory sets this information in a labile state, enabling its re-processing and therefore facilitate different possible outcomes (Nader et al., 2000a; Sara, 2000a, b). Thus memory retrieval is a dynamic process during which reactivation of an already stabilized *LTM* can destabilize the initial memory trace resulting in either weakening (extinction) or strengthening (reconsolidation) the already consolidated memory (see also 3.3).

3.2.2 Memory formation in *Drosophila*

In animals and especially solitary living insects such as *Drosophila* learning and memory differs immensely from that in higher animals, or humans, because basically they follow genetically fixed and stable routines. This results in a predetermined life-cycle of unvarying events, such as that females lay their eggs on a suitable food source for the larvae. The offspring hatches, starts feeding and developing through a sequence of different stages, resulting in pupation and subsequent hatching. Adult flies recognize appropriate mates by a set of fixed signs, perform static courtship behavior and pass their genes onto the next generation before they die. This cycle repeats itself unchanged from generation to generation and is, in general, outstandingly successful. This set of stable responses is triggered by a stable set of stimuli from the environment, but nonetheless they are adaptive (Britannica; McLaren and Mackintosh, 2000). As long as the outer influences remain stable there is no need for an animal to change its behavior, but, if alterations in the stimuli/circumstances occur adaptions in the behavior often must follow to ensure the survival of the organism. Therefore we confronted naïve *Drosophilae* exclusively with a set of non-natural stimuli (synthetic odors and electrical foot shocks), hence ensuring the novelty of the environment for the flies during the conditioning. Subsequently observed performance in the odor choice situation (see 4.6) can thence be considered as adaptions to changed environmental influences and thus be considered as an indicator for the capacity of either learning or memory, depending on the time interval between conditioning and testing, respectively.

We distinguish between different phases of memory in *Drosophila*. The initial learning, or acquisition phase which is tested in *immediate Short Term Memory* (*iSTM*) tasks as well as regular *STM*, middle-term memory (*MTM*), anesthesia-resistant memory (*ARM*) and *LTM* (Dubnau and Tully, 1998; Isabel et al., 2004b; Tully et al., 1994a; Tully and Quinn, 1985), (for details about the different induction protocols see 4.5.2). The establishment of these different memory phases and especially of consolidated *LTM* is a dynamic process which depends on different effector molecules and signaling cascades, such as nuclear calcium and/or 3',5'-cyclic adenosine monophosphate (cAMP) signaling (see 3.4) and/or neuropeptide signaling (see 3.5) (Alberini, 2011; Comas et al., 2004; Feany and Quinn, 1995a; Limback-Stokin et al., 2004; Miyashita et al., 2012; Perazzona et al., 2004), to trigger the transition from labile *STM* traces into resilient *LTM* (McGaugh, 2000). This procedure includes biochemical processes in the

neurons such as protein synthesis, which is considered as a distinctive hallmark of *LTM* formation in many species, (Davis and Squire, 1984) although translation of new proteins may be the second step after new transcripts have been produced. Blocking transcription rather than translation results in an impairment in *LTM* formation in a wide range of species (Igaz et al., 2002; Neale et al., 1973; Pedreira et al., 1996). Thus is it now a commonly accepted view that the activation or repression of transcriptional activation in defined time windows is required for proper consolidation (Bailey et al., 1996; Stork and Welzl, 1999). First, a subset of genes named immediate-early genes which encode for transcription factors are activated or unrepressed during and/or very shortly after learning (Abraham et al., 1991; Tischmeyer and Grimm, 1999). Second, several hours later the newly expressed early gene proteins start to modulate the expression of a wider set of target genes leading to stable changes in synaptic transmission through protein synthesis (Bailey et al., 1996) and therefore the functional modification of synapses (Lefer et al., 2013).

3.3 Classical avoidance conditioning of *Drosophila* in the context of anxiety disorders

3.3.1 Anxiety disorders - a basic overview

Anxiety disorders and especially Posttraumatic stress disorders (PTSD) emerge as a response of a human experiencing terrifying and usually life-threatening events (Wessa and Flor, 2007), such as rape (Foa and Rothbaum, 2001), childhood abuse (Bremner et al., 1995), accidents (McFarlane et al., 1997), catastrophes (Salcioglu et al., 2007) or combat (Yehuda et al., 1995). These adaptions result in severe anxiety complaints, sleep deprivation and drastic mood changes such as depression (Davidson et al., 1998; Spoormaker and van den Bout, 2005) causing serious restrictions in the daily life of patients. Today in the clinical practice patients suffering from phobias, traumas, PTSD and, or addictions are treated by therapies in which they are exposed to the trauma, or addiction related cues but in the absence of the associated aversive or rewarding stimuli (Singewald et al., 2015). These kind of therapies are called “exposure-based therapies” and are thought to bring the once consolidated memory back into a labile state in which the original memory can be modified (renewal), strengthened (reconsolidation), suppressed (extinction) or even erased (blocked reconsolidation) (McGaugh, 2000; Monfils et al., 2009; Nader, 2003; Nader et al., 2000b; Reichelt and Lee, 2013). The problem is that extinction creates a conflict in the behavioral output between the

original aversive and the newly acquired memory, since the original CS+ remains the same in both memory phases. Therefore, it is not easy to suppress already consolidated traumatic responses and thus extinction is a process that demands time to slowly enable the subject to uncouple the triggered response from the inducing stimulus, resulting in a diminishment of the intensity of the conditioning over time (Pedreira et al., 2004). It is known that extinction is an active process which depends on protein synthesis (Pedreira and Maldonado, 2003) and can therefore not be seen as forgetting, but rather as a new form of memory. Recent research revealed that the permanence of consolidated forms of memory is depending on its reconsolidation. Brought back into its labile state, the once acquired memory must be re-approved to persist. This phenomenon is, like extinction, protein synthesis dependent and has been observed in many different species, including invertebrates such as nematodes (Rose and Rankin, 2006), honeybees (Stollhoff et al., 2008) and crabs (Nmda-type et al.) as well as vertebrates, including mice (Kida et al., 2009), rats (Nader et al., 2000a), rabbits (Coureaud et al., 2009) and humans (Hupbach et al., 2007; Schwabe et al., 2014). If reactivated memories must be reconsolidated in order to continue, a blockage of reconsolidation would probably result in a disruption of the original memory trace and subsequently result in its complete obliteration. This would, in return, offer a novel treatment for PTSD patients (Pitman, 2011; Soeter and Kindt, 2011; Stern et al., 2012).

The problems of treating PTSD and anxiety disorders occur in the everyday use of these therapies in the clinical practice. Firstly, extinction is associated with spontaneous or induced relapses into the original pathological state (reinstatement, reacquisition) since the original memory is not erased and the original association remains, at least in parts, intact (Myers and Davis, 2007; Vervliet et al., 2013) and secondly, blocked reconsolidation requires drugs that often themselves cause severe problems for the patients (Monfils et al., 2009) (see also 3.2.3). A deeper understanding of the underlying neurobiological principles of these memory forming and/or affecting phenomena may lead to more potent and efficient types of clinical treatments for the affected patients. For example patients suffering from PTSD regularly show fear responses to trauma reminders outside of contexts in which these cues would reasonable predict danger (Fig. 5.17). This leads to a generalization of the traumatic experience in every type of context, turning fear from a helpful survival instinct into a permanent stressor, affecting heavily the well-being of the concerned (see also 5.7). Finding a method to overcome

this generalization phenomena might be helpful in designing novel therapeutic strategies which could lead to a decrease of PTSD symptoms.

3.3.2 Classical conditioning in *Drosophila*

The findings of Pavlov in the early years of the last century about the possibility to implement conditioned responses due to repeated presentation of a conditioned stimulus paired with an unconditioned stimulus, have led to a wide field of behavioral research, making use of this approach. Usually, the conditioned stimulus (CS) is a neutral stimulus, the unconditioned stimulus (US) is biologically potent and the unconditioned response (UR) to the US is an innate reflex response. After successful conditioning the conditioned response (CR) is triggered already through the sole presentation of the CS (Pavlov, 1927; Pawłow, 1927). In our laboratory we have established an associative learning paradigm for *Drosophila melanogaster* that is based on previously developed classical conditioning procedures (Quinn et al., 1974; Tully and Quinn, 1985) which were established over forty years ago. This olfactory conditioning paradigm gives us the possibility to establish different kinds of memory phases in the fly with robustness and reproducibility and enables us in combination with the power of the *Drosophila* genetics to have a deeper insight into the underlying mechanisms of memory formation. To establish memories a group of approximately fifty flies per trial are confronted with two different slightly aversive odors in which one of the odors acts as the conditioned stimulus (CS+) and is paired during its presentation with aversive electrical foot shocks, which represent US. The second odor (CS-) is presented in the same context but without the US. Through different training protocols varying in number and spacing of the conditioning trials *Drosophila* develops various phases of memories: *STM*, *MTM*, *ARM* and *LTM* (Heisenberg, 2003). The success of the memory acquisition and maintenance can be scored in an odor-T-maze in which the CS+ and CS- are presented simultaneously and the flies have a determined amount of time to choose between the odors.

3.3.3 *Drosophila* as a model for anxiety disorders

Despite the possibilities to implement different forms of short, unconsolidated and long lasting, consolidated memory phases and the examination of the underlying mechanisms crucial for their correct formation (which is the main focus in this thesis), preliminary data from our laboratory (Khouaja et al., in preparation) show that *Drosophila* is also a prime

candidate to examine the underlying mechanisms of trauma formation and anxiety disorders. We were able to implement extinction after previous conditioning and our analysis of *Drosophila LTM* has shown that extinguished memory is not erased since the original acquisition can be recalled by reacquisition (recall via one *STM* conditioning trial), reinstatement (recall via presentation of the US alone), renewal (sole presentation of the CS+ acting as a reminder in a new context) or spontaneous recovery (no obvious inducer). Moreover our results show that a mild recall of once consolidated aversive olfactory memories evoke an initial transient extinction of the conditioned behavior, followed by a robust recovery within the following day. We further revealed that this recovery depends on intact nuclear calcium signaling in distinct cells, since extinction of previously formed *LTM* did not occur when the nuclear Ca^{2+}/calmodulin signaling blocker CaMBP4 (see 3.3.3 and 4.9) was simultaneously expressed. These findings are consistent with data from mammalian studies in which extinction training failed to extinguish previously formed *LTM* after application of the protein synthesis blocker cycloheximide (Pedreira and Maldonado, 2003; Pedreira et al., 2004), clearly underlining the necessity of translational processes in the formation of extinction memory in both invertebrates and vertebrates. Surprisingly knock down of the prohormone convertase 2 - Amontillado (see 3.4 and 4.10) during extinction training showed no effect and left the originally formed aversive memory unaltered, indicating a lack of involvement of neuropeptidergic signaling in generating extinction. Furthermore, we could show that extinction memory in flies displays comparable phenomena to human psychopathology, namely reinstatement, which means spontaneous relapse into the original conditioned state (Vervliet et al., 2013). These findings demonstrate that the recovery of formerly established conditioned behavior is based on an active, protein synthesis requiring reconsolidation process. Importantly, preliminary evidence suggests that blocked reconsolidation can completely erase the original aversive memory (Pitman, 2011; Soeter and Kindt, 2011; Stern et al., 2012). Flies which expressed CaMBP4 to block nuclear calcium signaling during the reconsolidation process, could not reacquire their original conditioned behavior. Thus, it seems plausible that consolidated aversive olfactory memories in *Drosophila* are subject to extinction and reconsolidation processes and that blocked reconsolidation likely erases the original aversive memory. These surprisingly extensive similarities between insect and mammalian memory phenomena suggest, that, the underlying functional principles are evolutionary conserved and have most likely already existed in a common ancestor of both

lineages. Revealing the basic mechanisms underlying reinstatement and the understanding of their functional processes and subsequently their suppression, enabling life-long establishment of extinction memory, would lead to a breakthrough in treating traumatized patients suffering from PTSD and other anxiety disorders. Therefore, extinction related research is of high clinical significance, since the first promising treatment to suppress reinstatement with the β-adrenergic receptor antagonist propranolol (Kindt et al., 2009) turned out to affect only declarative memory in humans (Bos et al., 2012) and was useless in an animal model for PTSD (Cohen et al., 2011). This displays a potentially harmful side effect of the drug when it is dispensed in exposure-based treatments of anxiety disorders (Vervliet et al., 2013). Taken together these findings underscore again the importance of *Drosophila* in serving as a model organism, to monitor even complex behavioral adaptions and their underlying physiological and molecular mechanisms (see also 6.4.3).

3.4 Memory formation and the underlying molecular mechanisms

3.4.1 Nuclear calcium signaling

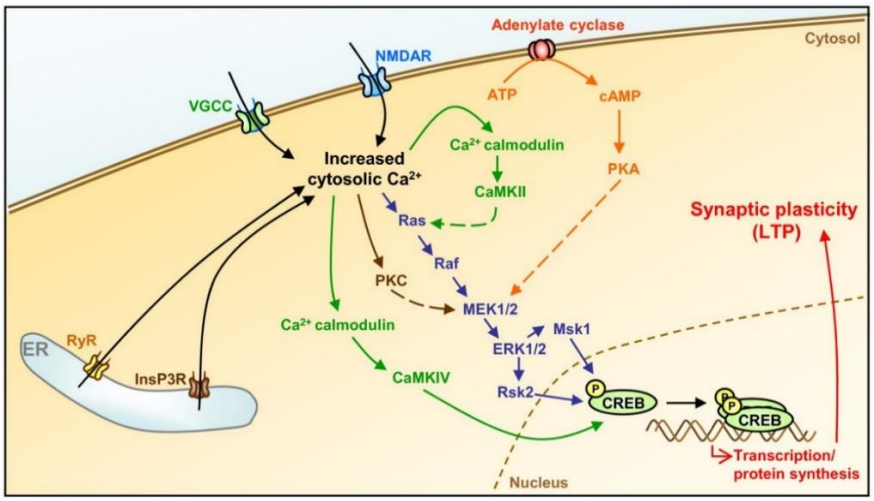


Fig. 3.1. Calcium signaling in synaptic plasticity. Synaptic activity results in the elevation of cytosolic calcium levels by promoting extracellular calcium influx (through opening of specific cell surface calcium channels, e.g. voltage-gated calcium channels (VGCCs) or N-methyl-D-aspartate receptors (NMDAR) or endoplasmic reticulum (ER) calcium efflux - via activation of ryanodine receptors (RyRs) or Inositol (1,4,5)-trisphosphate receptors (InsP_3Rs). Increased cytosolic calcium concentrations initiate the activation of several kinase-dependent signaling cascades leading to cyclic AMP-responsive element-binding protein (CREB) activation and phosphorylation at Serine 133 (Ser133), a process critical for protein synthesis-dependent synaptic plasticity and long term potentiation (LTP) (Figure and legend from Marambaud et al., 2009 and modified for PhD thesis).

Calcium signaling plays a central role as a mediator of fast local signals in a variety of cellular processes, such as cell differentiation, activation of transcription factors (TF), memory formation, synaptic plasticity (Fig. 3.1), the development of diseases(Fig. 3.2) and cell death (Bading, 2013; Bootman et al., 2001; Cohen and Greenberg, 2008; Marambaud et al., 2009).

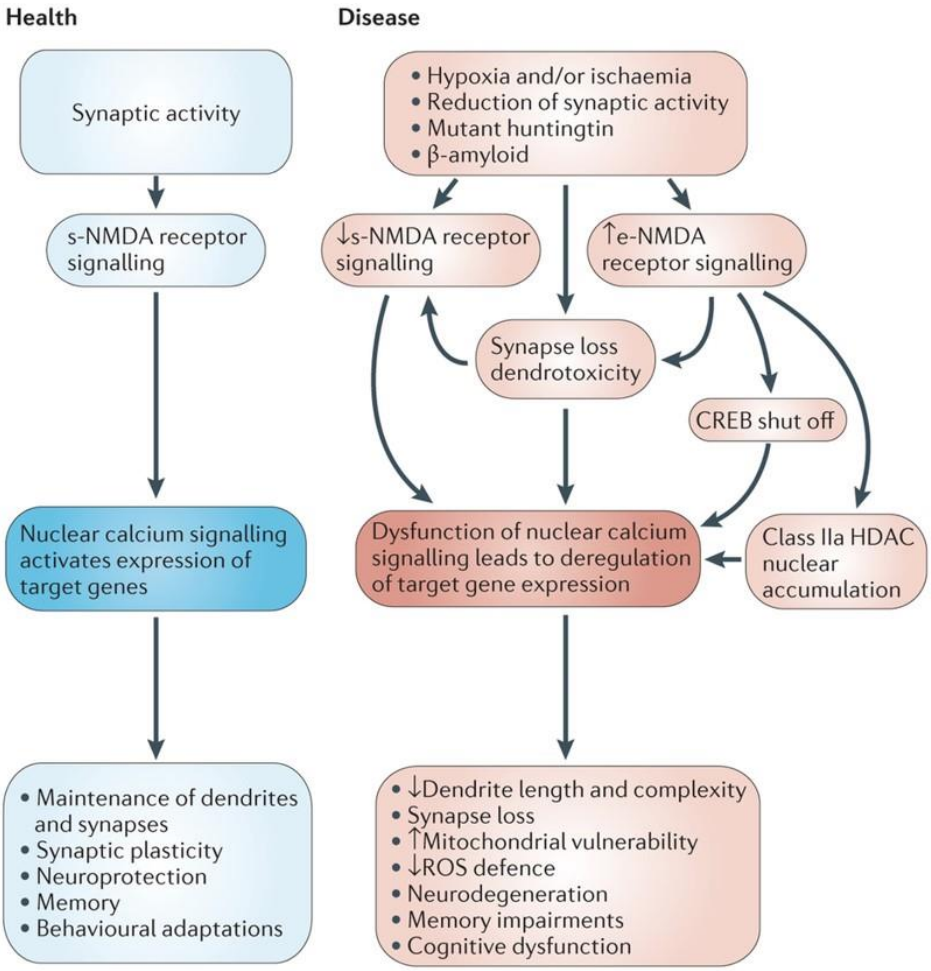


Fig. 3.2. 'Nuclear calciopathy' as a common factor in the aetiology of neurodegenerative and cognitive disorders. Nuclear calcium signaling induced by synaptic activity stimulating synaptic NMDA (s-NMDA) receptors and regulating specific target gene expression is important for neuronal health and essential for the maintenance and functional integrity of synapses and dendrites (left panel). Toxic molecules, genetic defects or harmful conditions (such as β-amyloid, mutant huntingtin, deprivation of synaptic activity or hypoxia and/or ischaemia) and possibly also ageing can lead to perturbations in the balance between s-NMDA receptor and extrasynaptic NMDA (e-NMDA) receptor signaling (right panel). An increase in the number or activity of e-NMDA receptors and/or a decrease in s-NMDA receptor function owing to synapse loss or dendrotoxicity can lead to dysfunctioning of nuclear calcium signaling, which includes the shut-off of cyclic AMP-responsive element-binding protein (CREB) function and nuclear accumulation of class IIa histone deactylases (HDACs). The resulting deficits in the expression of nuclear calcium target genes may increase mitochondrial vulnerability, decrease the neurons' antioxidant defence systems and perpetuate the disintegration of dendrites and the loss of synapses, leading to neurodegeneration and cognitive decline. ROS, reactive oxygen species (Figure and legend from Bading, 2013 and modified for PhD thesis).

Calcium is an intracellular second messenger which links synaptic activity in neurons to gene expression in the nucleus and whose cytosolic concentration increases due to an influx from the extracellular space (via VGCCs or NMDARs) or when it is released from endoplasmatic or sarcoplasmatic stores (Fig. 3.1).

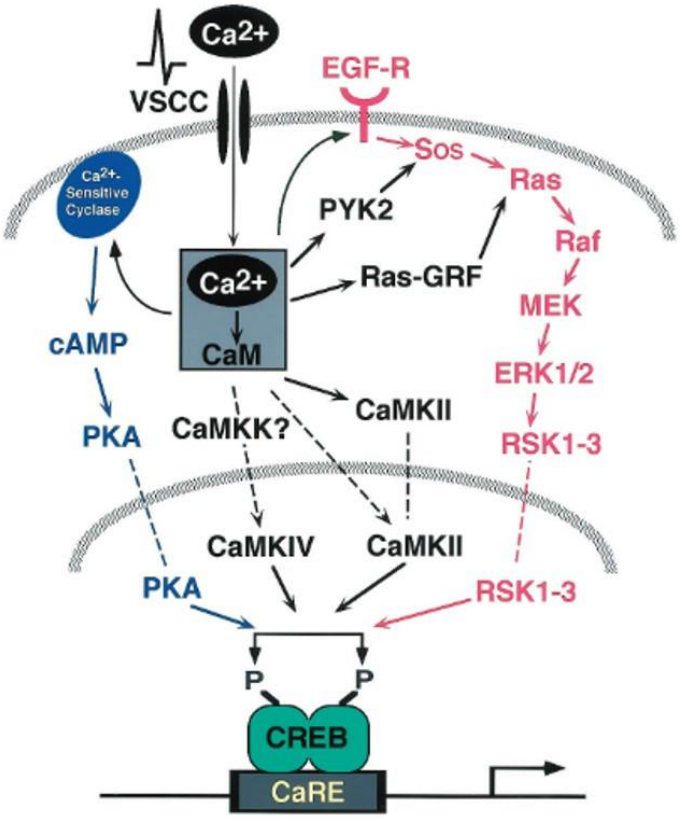


Fig. 3.3. Multiple signaling pathways contribute to cAMP response element-binding protein (CREB) Ser133 phosphorylation in response to Ca^{2+} influx. In neuronal cells, electrical activity leads to membrane depolarization, opening voltage-sensitive Ca^{2+} channels (VSCCs) in the plasma membrane and resulting in influx of extracellular Ca^{2+}. Inside the cell, calcium activates many kinases, some of which directly phosphorylate CREB at Ser133. Upon entering the cell, Ca^{2+} binds to a protein, calmodulin (CaM). The Ca^{2+}/CaM complex (shaded box) can activate the PKA pathway (blue) by directly stimulating calcium-sensitive adenylyly cyclases, leading to generation of cAMP and the activation of PKA. PKA can then translocate to the nucleus where it phosphorylates CREB at Ser133. Ca^{2+}/CaM also activates members of the Ca^{2+}/calmodulin-dependent kinase (CaMK) family (black), all of which can phosphorylate CREB at Ser133. Ca^{2+}/CaM directly activates CaMKI (not shown), CaMKII, and CaMKIV. Ca^{2+}/CaM can also activate CaMKK, which can then directly activate both CaMKIV and CaMKI (not shown). Nuclear translocation of Ca^{2+}/CaM may account for the activation of CaMKIV and CaMKII. CaMKIV is localized predominantly to the nucleus while isoforms of CaMKII are found both in the nucleus and in the cytoplasm. In addition, certain CaMKII isoforms may translocate from the cytoplasm to the nucleus. Ca^{2+}/CaM also activates the Ras/MAPK pathway (red). Ca^{2+} activation of Ras may occur through multiple mechanisms. Ca^{2+} influx can lead to the ligand-independent activation of the epidermal growth factor-receptor (EGF-R), which then leads to activation of guanine-nucleotide exchange factors, such as Sos and Ras activation. Activation of Ras stimulates the Raf, MEK, and ERK1/2 kinase cascade. The MAP kinases ERK1/2 directly activate members of the pp90 RSK family of protein kinases (RSK1-3). Activated RSKs then translocate to the nucleus where they phosphorylate CREB at Ser133. Ca^{2+}/CaM can also activate Ras by activating Ras-GRF, a Ca^{2+} -activated guanine-nucleotide exchange factor. The calcium-activated tyrosine kinase PYK2 can also activate Sos and lead to stimulation of the Ras pathway. Dashed lines indicate translocation from the cytoplasm to the nucleus (Figure and legend from Shaywitz and Greenberg, 1999 and modified for PhD thesis).

Ca^{2+} mobilizing signals can be triggered by depolarization, extracellular agonists, intracellular messengers, depletion of intracellular stores and other factors (Berridge et al., 2003).

The release of Ca^{2+} from intracellular stores, such as the nucleoplasmic reticulum is mediated by several different types of Ca^{2+} channels, of which inositol (1,4,5)-trisphosphate receptors (InsP_3Rs) and ryanodine receptors (RyRs) are the best characterized (Bootman et al., 2009; Gerasimenko and Gerasimenko, 2004).

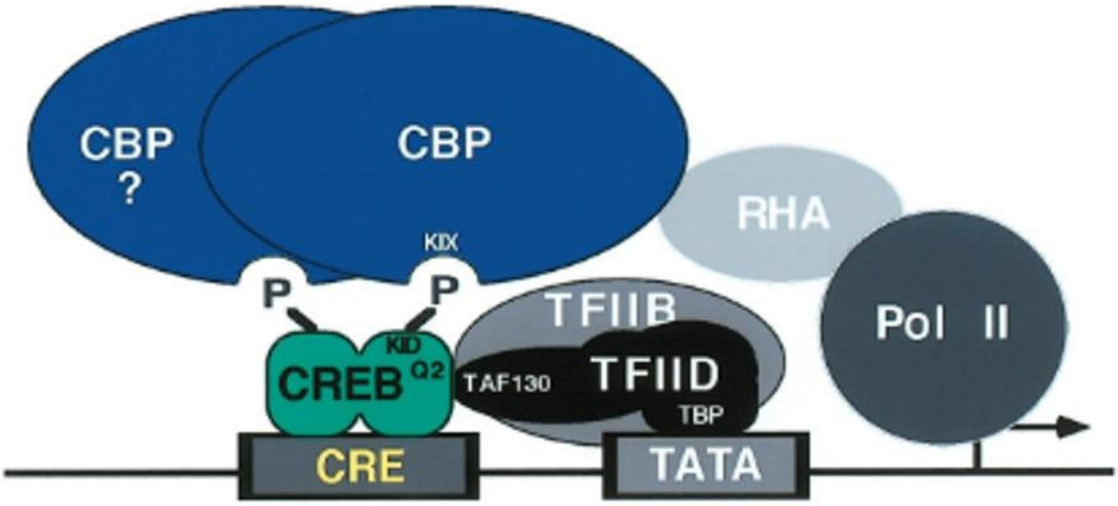


Fig. 3.4. Multiple domains of CREB contribute to transcriptional activation. Different domains of CREB bind distinct coactivators and basal transcription factors to activate transcription. Shown is a CREB dimer bound to its cognate Ca^{2+} response element (CaRE)/CRE element on the promoter of a CREB target gene. Downstream of the CaRE/CRE is the TATA box, which binds the multiprotein TFIID basal transcription factor (via the TBP protein). Another factor within TFIID, TAF130, binds to the Q2 domain of CREB. The Q2 domain of CREB has also been shown to interact with TFIIB, which is a part of the basal transcription machinery as well. A distinct domain of CREB, the kinase inducible domain (KID), contributes to signal-induced transcriptional activation. When phosphorylated at Ser133, the KID of CREB can bind to the KID interaction (KIX) domain of the CBP. It is presently unclear whether CBP associates with Ser133–phosphorylated CREB as a dimer. CBP associates indirectly with RNA polymerase II (Pol II) via the RNA helicase A (RHA) protein. Therefore, recruitment of CBP to Ser133–phosphorylated CREB results in recruitment and stabilization of Pol II on the promoter of CREB target genes, whereas the Q2 domain interacts with other elements of the basal transcription machinery that are required for transcription, such as TFIID and TFIIB (Figure and legend from Shaywitz and Greenberg, 1999 and modified for Phd thesis).

At the synase calcium influx acts locally, by activating signaling cascades which then regulate posttranslational modifications, essential for synaptic plasticity, such as the insertion of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) in the postsynaptic membrane (Ehrlich and Malinow, 2004; Soderling, 2000).

An important pathway in nuclear calcium signaling and subsequently gene pool regulation, which may be also involved in *LTM* formation and long-term potentiation (LTP), is triggered by the transcription factor cAMP response element binding protein (CREB) (Bading et al., 1993; Bading et al., 1997; Hardingham and Bading, 1999; Zhang et al., 2009). The activation of CREB by phosphorylation at serine 133 (Ser133) (Parker et al., 1996) can be driven by the Ca^{2+}/CaM-pathway (black trace in Fig. 3.3), cAMP signaling (blue trace in Fig. 3.3), calcineurin or growth and/or stress related signals which use mitogen-activated protein kinase (MAPKs) pathways (Lamprecht, 1999).

However, the additional activation of CREBs coactivator CREB binding protein (CBP) through nuclear calcium/calmodulin-dependent protein kinases (CaMK) - especially CaMKIV - which are dependent on calcium transients induced by voltage changes through L-type Ca^{2+} channels and calcium permeable NMDA type glutamate receptors (see also 6.1), is crucial (Deisseroth et al., 2003; Greer and Greenberg, 2008; Wu et al., 2007; Xia et al., 2005; Zieg et al., 2008). CREB and its coactivator CBP subsequently bind to cAMP response element (CRE) sequences on the DNA (Fig. 3.4.) to increase or decrease transcription of downstream genes (Alonso and García-Sancho, 2011; Bengtson and Bading, 2012; Hardingham et al., 2001; Shaywitz and Greenberg, 1999).

Besides, initiating the activation of several signaling cascades, Ca^{2+} can also enter the nucleus directly by crossing the nuclear pore complex to activate gene transcription (Wiegert and Bading, 2011).

Taken together, nuclear calcium acts as one of the key molecules in regulating the general physiology of cells, through the regulation of their gene pool. These adaptive mechanisms, crucial for synaptic plasticity and therefore lasting adaptions to environmental changes, are indispensable for memory forming and consolidation processes. The importance of nuclear calcium signaling may not be restricted to the nervous system and, indeed, not even restricted to the animal kingdom. Calcium signaling is important for the immune response (Lewis, 2001; Oh-hora and Rao, 2008), and in plants, calcium signaling in the nucleus of root cells is at the center of symbiosis signaling (Oldroyd and Downie, 2006). Thus, the concept that persistent adaptations take place when calcium enters the cell nucleus to activate transcription may be common to many biological systems independent of cell type or phylogenetic borders.

3.4.2 cAMP/PKA signaling

Besides activation through the nuclear calcium/calmodulin complex and its signaling cascade, CREB gets also activated via the cAMP/protein kinase A (PKA) mediated pathway (see blue trace in Fig. 3.3).

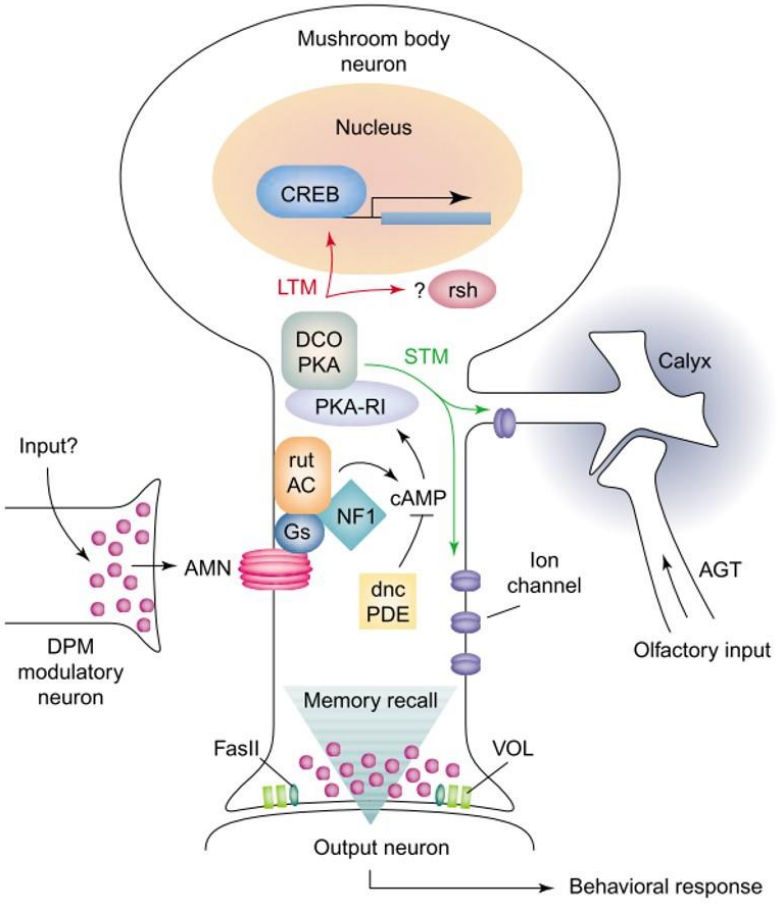


Fig. 3.5. How the cAMP cascade might mediate learning and memory in *Drosophila*. A mushroom body (MB) neuron receives olfactory input, via interneurons in the antennoglumerular tract (AGT) that synapse in the MB calyx. MBs also receive electric-shock input through unknown neurons. Presynaptic termini of the MB neuron, residing in the MB lobes, are innervated by modulatory neurons like the *dorsal paired medial* (*DPM*) neurons that might release Amnesiac (AMN) neuropeptide(s). Activation of the RUT adenylyl cyclase leads to elevation of cAMP levels in the relevant MB neurons. Longer-term stimulation of the cascade by AMN might lengthen the association and help consolidate the memory. Depending on the conditions of training and the duration of cAMP elevation, the experience results in short-lived modification of synaptic connectivity (short-term memory; *STM*) or in longer lasting functional and structural changes (long-term memory; *LTM*) in that neuron. Persistent or repeated activation of cAMP-dependent protein kinase (PKA) appears to bring about enduring synaptic changes via CREB-dependent gene activation. Recall of olfactory memory requires synaptic transmission from MB neurons. DCO, PKA catalytic subunit; PKA-R1, PKA regulatory subunit; dncPDE, cAMP phosphodiesterase endcoded by the *dunce* gene; Gs, stimulatory G protein; RUT, type I adenylyl cyclase; NF1; neurofibromin, rsh, *radish* gene product; rut, *rutabaga* gene product; VOL *volado* gene product; FasII, *fascicilin*II gene product. (Figure and legend from Waddell and Quinn, 2001b and modified for PhD thesis)

This signaling pathway was considered for a long time as the primary activation mechanism for learning associated transcription processes in *Drosophila* (Yin et al., 1994). Hormones and

neurotransmitters can raise intracellular cAMP levels by binding to receptors that activate heterotrimeric G-proteins. These G-proteins then directly activate adenylyl cyclase, which catalyzes the production of cAMP (Shaywitz and Greenberg, 1999). When intracellular cAMP levels are elevated they activate PKA in the cytoplasm (through binding onto the regulatory PKA subunits PKA_R, and consecutively triggering, their separation from the catalytic PKA subunits PKA_C), resulting in a translocation of PKA_C into the nucleus where it phosphorylates and subsequently activates the transcription factor cAMP response element binding protein-2 (dCREB-2) at Ser133 (Shaywitz and Greenberg, 1999; Yin et al., 1994).

dCREB-2 (also called ATF-4) is, together with mouse mATF-4 and the *Aplysia* ApCREB-2, a member of a subfamily of the ATF/CREB proteins (Vallejo et al., 1993). Although it was initially described as a repressor of CRE-dependent transcription (Karpinski et al., 1992) it contains a constitutive activation domain of transcription (Liang and Hai, 1997) and can directly interact with the transcriptional coactivator, CBP (Gachon et al., 2001).

CREB2 has been reported to modulate the formation of olfactory *LTM* in *Drosophila*, since the overexpression of a repressor isoform of CREB (dCREB2-b) resulted in acute blockade of *LTM,* whereas the overexpression of an activator isoform (dCREB2-a) was reported to enhance *LTM.* Therefore, it was proposed that the balance of functional dCREB2-a and dCREB2-b acts as a ratio-metric switch for memories to remain labile or to become enduring (Perazzona et al., 2004) (for a general model about how cAMP might mediate olfactory related *LTM* contents see Fig. 3.5). Besides, the importance of functional cAMP signaling in distinct cells, for correct memory formation and its segregation, was shown several times from independent studies (Blum et al., 2009; Isabel et al., 2004a; Waddell and Quinn, 2001a). Moreover, the finding of the general connection between cAMP signaling and *LTM* formation was one of the first results, in this field of research in the early eighties. This was done using the now considered "classical" cAMP/PKA signaling cascade impaired learning mutants *dunce*, which encodes for a cAMP specific phosphodiesterase and therefore shows elevated levels of cAMP (Byers et al., 1981; Davis and Kiger, 1981; Dudai et al., 1976; Tempel et al., 1983) and *rutabaga*, which encodes for a calmodulin dependent adenylate cyclase resulting in decreased cAMP levels; (Dudai et al., 1976; Livingstone et al., 1984; Tempel et al., 1983). Hence, both mutants directly interfere in the metabolism of the second messenger molecule cAMP and show disrupted *STM/LTM* formation. Double mutant flies carrying the $dunce^-$ as well as the rut^- mutation

showed near wild-type levels of cAMP, suggesting that mutations at the rutabaga locus compensate the elevated cAMP levels (Livingstone et al., 1984). Nonetheless these double mutant flies are still unable to learn, implying that the process of memory formation requires distinct spatial and temporal regulation of the cAMP level, rather than its absolute level of concentration.

3.5 Neuropeptides and the prohormone convertase Amontillado

In insects the neuroendocrine system is primarily based on neuropeptide transmitters (Nassel and Winther, 2010) that are synthesized in the cell body as inactive precursor peptides (prepropeptides) (Andrews et al., 1987) before they undergo proteolytic cleavage and further processing steps (e.g. amidation) in the endoplasmic reticulum (ER), turning them to propeptides (Chun et al., 1994; Eipper et al., 1992; Hook et al., 2008).

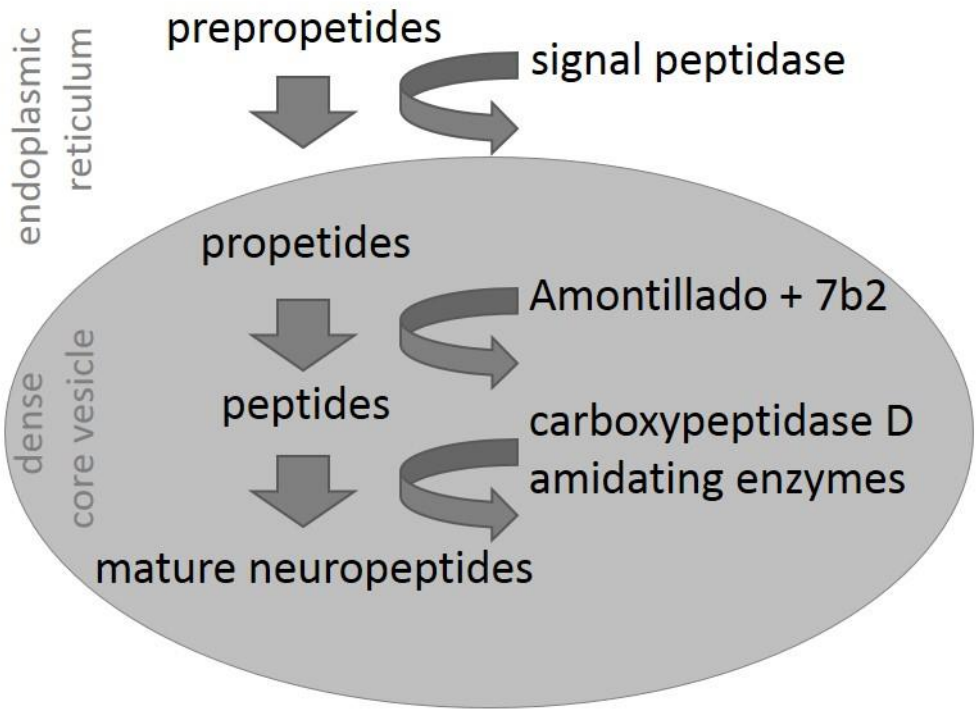


Fig. 3.6. Neuropeptide processing by the prohormone convertase Amontillado. Schematic drawing of the localization of the prohormone convertase Amontillado and its essential helper peptide 7b2 in dense core vesicles of neuropeptide releasing cells. After being synthesized in the ER as prepropeptides and packed in dense core vesicles the propeptides are cut and therefore activated by Amontillado and 7b2 before carboxypeptidase D and different amidating enzymes transfer them to their final, mature state before release.

Propeptides are later matured, through several common processing steps, (Wegener et al., 2011) to their finally active form before they are either released into the hemolymph to act in

global or regional modulatory ways as hormones, or at synapses to regulate their target cells as locally acting co-transmitters to fast neurotransmitters.

“Neuropeptide signaling is functionally very diverse and one and the same neuropeptide may act as a circulating neurohormone, as a locally released neuromodulator or even as a cotransmitter of classical fast-acting neurotransmitters..” (Nässel, 2009)

Neuropeptides show a wide array in regulating functional processes of neuronal circuits and physiological processes, including electrolyte balance (McKinley et al., 1999; Saria and Beubler, 1985), growth (Woll and Rozengurt, 1989), sleep (Foltenyi et al., 2007), presynaptic facilitation (Root et al., 2011), the modulation of locomotion through rhythmic pattern generators (Marder and Bucher, 2001) and circadian rhythmicity (Cavanaugh et al., 2014) along others, in both vertebrates and invertebrates (Strand, 1999) (see also 6.2).

Drosophila has 42 neuropeptide precursor related genes encoding approximately 75 neuropeptides (Nassel and Winther, 2010) and a common maturation step for all of these neuropeptides is mediated by the homologue of the prohormone convertase 2 - Amontillado (Amon) and its essential helper peptide 7b2 (Rayburn et al., 2009; Rhea et al., 2010; Siekhaus and Fuller, 1999; Wegener et al., 2011) which are both located inside the dense core vesicles of neuropeptide releasing cells (Fig. 3.6). Therefore, interfering with Amon, by for example performing a knock down (KD) of the *amontillado* gene in different cell specific patterns, should elicit if these cells are neurosecretory active. Furthermore, KDs performed prior to conditioning experiments should answer the question if the neuropeptidergic release of these cells is incorporated in learning and memory related context (see also 5.4 and 6.2).

3.6 Parallel processing channels

3.6.1 General overview

A general principle in neuronal information processing is to segregate incoming sensory information into parallel processing channels that are tuned to extract distinct features of the input before re-converging them to guide appropriate responses (Rauschecker and Scott, 2009; Young, 1998). Prominent examples for this principle of parallel processing can be found throughout evolution and across different sensory modalities including the visual (Nassi and

Callaway, 2009a; Paulk et al., 2008), auditory (Recanzone and Cohen, 2010; Schul et al., 1999), olfactory (Haberly, 2001; Rossler and Brill, 2013), somatosensory (Dijkerman and de Haan, 2007) and gustatory systems (Roper, 2009). More recently, parallel processing has been suggested to operate also at circuits reflecting internal states such as the control of the basal ganglia output (Kravitz et al., 2012; Lobo and Nestler, 2011) or basal ganglia associated learning processes (Belin et al., 2009; Devan et al., 2011). Furthermore, the diverse and potent neuromodulatory functions of the neuroendocrine system with its hormonal, regional or local levels of action have nourished ideas that neuropeptides, such as oxytocin, might also engage in parallel processing during the control of complex behaviors (Dolen, 2015). However, how the segregation into parallel information channels is controlled is unknown.

3.6.2 Parallel memory channels in *Drosophila*

Aversive olfactory memory phases in *Drosophila* have been described to form two parallel memory channels (Bouzaiane et al., 2015; Isabel et al., 2004b; Placais et al., 2012; Tully et al., 1994b). One of them is characterized by its resistance to cold anesthesia (*ARM*) and independence of *de novo* protein synthesis of its consolidated phase (*long-term ARM, LT-ARM*), whereas the other channel is cold anesthesia sensitive (*ASM*) and requires *de novo* protein synthesis for its consolidated phase (*LTM*). The non-consolidated short- and middle-term phases of both memory channels (*STM* and *ST-ARM, MTM* and *MT-ARM*) appear to coexist, whereas the consolidated long-term phases (*LTM* and *ARM*) seem to exclude each other (Bouzaiane et al., 2015; Isabel et al., 2004b; Placais et al., 2012) (see also 6.2 and Fig. 6.2). So far, these parallel memory channels have only been clearly distinguished in *Drosophila* but they might also exist in other invertebrates as well as in mammals (Hermitte et al., 1999; Okamoto et al., 2011). Although the importance of cAMP signaling in segregating *ARM* and *ASM* phases in the context of single trial conditioning experiments (Scheunemann et al., 2012) and the importance of the neuropeptide Amnesiac in *STM/MTM* formation has already been shown (DeZazzo et al., 1999), the molecular and anatomical bases of the segregation of memories into distinct parallel channels, as well as their functional significance, remain not fully understood.

In this study we have reexamined the aversive olfactory memory phases of *Drosophila* and found by *in vivo* calcium imaging and targeted disruptions of the maturation of neuropeptides

that the fly's neuroendocrine system is more strongly involved in the processing of the aversive US and in the formation of aversive memories than so far assumed. In particular, we found that mature neuropeptides are required in a single pair of neurons, the *DPM* neurons, for the acquisition and formation of all *ASM* phases (*STM, MTM* and *LTM*). Our results suggest that neuropeptide signaling segregates aversive memories into neuropeptide-dependent *ASM* and neuropeptide-independent *ARM* channels.

3.7 Structure of the *Drosophila* brain and its compartments

3.7.1 Basic overview

With exception of the central complex the structure of the *Drosophila* brain is mirror symmetrically build.

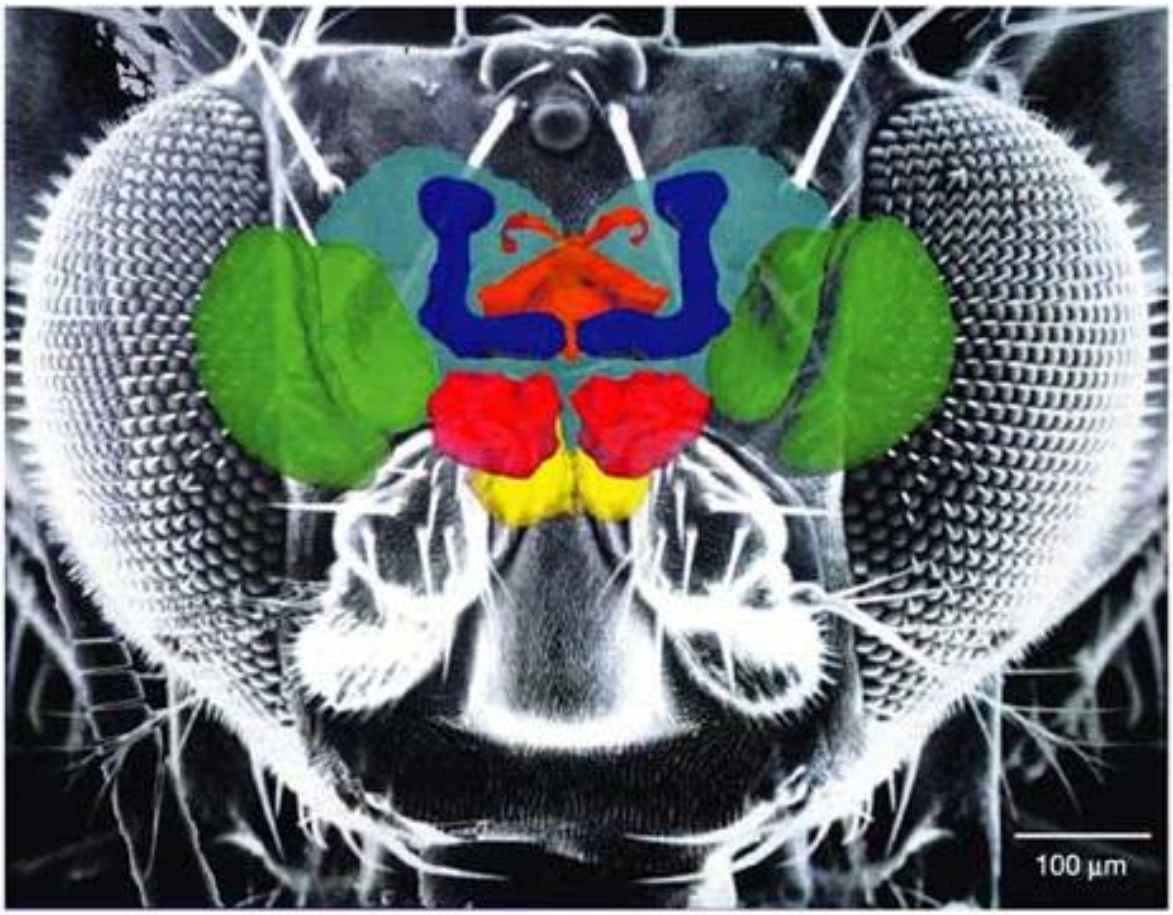


Fig. 3.7. *Drosophila* brain shown in the head capsule. The most prominent fibre assemblies are colour coded. Green, optic lobes; yellow, suboesophageal ganglion; red, antennal lobes; blue, mushroom bodies; orange, central complex. The various neuropil regions surrounding the mushroom bodies and central complex are shown in grey in the background (Figure and legend from Heisenberg, 2003).

The central complex lies sagittal in the midplane of the head capsule, flanked by a pair of mushroom bodies (MB) and both are embedded, but also separated, by glial sheaths from the many discrete, but so far barely studied, neuropil regions surrounding them. This general neuropil, the mushroom bodies and the central complex might be the three principal components in a basic functional model of the (supraoesophageal) insect brain (Heisenberg, 2003). Lateral to the neuropil the highly ordered optical lobes (Heisenberg and Wolf, 1984) and ventrally the antennal lobes, which project to the calyces of the MB, are located (Anton and Homberg, 1999; Davis, 2011). Ventral to the oesophagus lies the suboesohageal ganglion (Fig. 3.7).

3.7.2 Central complex - compartments and functions

The central complex is located ventrally between the two protocerebral hemispheres in the brain and consists of four neuropilar subunits, namely (in order from anterior to posterior): the ellipsoid body, the fan-shaped body, the underneath located, paired noduli and the protocerebral bridge (Fig. 3.8). These four structures are all interconnected by a set of columnar interneurons that form many regular patterns of projection (Hanesch et al., 1989; Heisenberg, 1994; Renn et al., 1999). The central complex receives input from most parts of the brain through large field neurons (Strauss, 2002) and is associated with functions related to higher locomotor control. Flies with mutations affecting the structure of the central complex walk more slowly than wild type flies, react less quickly to changing stimuli during flight and show altered orientation behavior toward landmarks. They are either less active or quickly loose activity, or fail to start walking or flying under circumstances in which wild type flies would readily do so (Ilius et al., 2007; Strauss et al., 1992; Strauss and Heisenberg, 1993).

Besides, the central complex also plays a role in visual pattern memory (Liu et al., 2006), multimodal information processing (Müller et al., 1997), courtship behavior (Popov et al., 2003), olfactory *LTM* (Wu et al., 2007), spatial orientation (Heinze and Homberg, 2007) and spatial orientation memory (Neuser et al., 2008). It mediates communication between the two hemispheres and is believed to be a control center for many behavioral outputs (Heisenberg and Wolf, 1992) and therefore it is considered as the flies homologue to the vertebrate hippocampus.

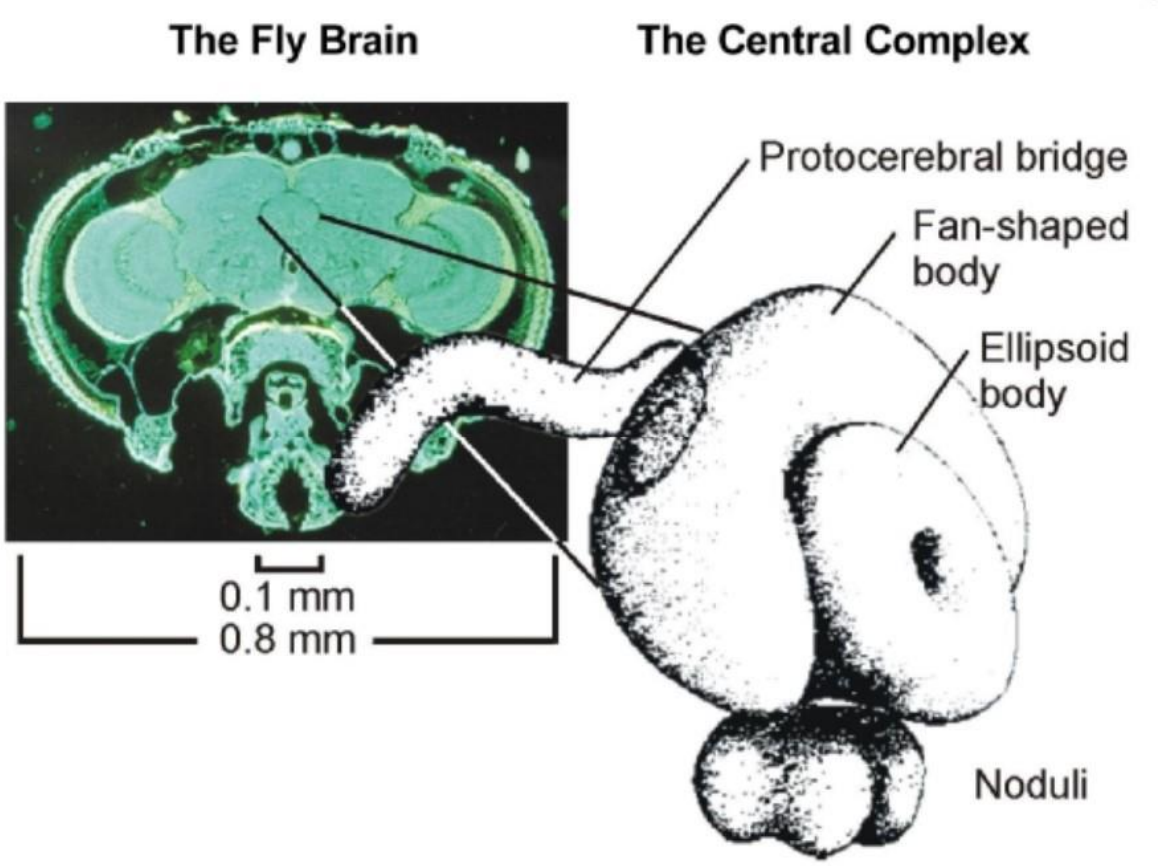


Fig. 3.8. Location and organization of the central complex. Frontal sections through the head and brain of a *Drosophila* fly. Autofluorescence highlights all of the neuropils in green and the cell bodies in yellow. The central complex is located in the middle, between the protocerebral brain hemispheres. It comprises four interconnected neuropilar regions: the fan-shaped body, the ellipsoid body, the protocerebral bridge and the paired noduli (Figure and legend from Strauss, 2002)

3.7.3 Mushroom bodies compartments and functions

The mushroom bodies (MB) are two mirror-symmetrical stalks (peduncles) with large cup-shaped protruberances (calyces) at their dorsocaudal ends. They extending from dorsocaudal to rostroventral through the midbrain and dividing frontally into a medial and a vertical lobed neuropil, namely α, α', β, β' and γ lobes (Fig. 3.9) (Strausfeld et al., 1998). Most of this structure is contributed by the Kenyon cells (about 2500 in *Drosophila*), with their small cell bodies densely packed above and beside the calyces in the dorsocaudal cell body rind. They send out thousands of their long thin axons in parallel, forming the peduncle and lobes (Fig. 3.7, Fig. 3.12). MBs occur in a wide array across invertebrate phyla (Brown and Wolff, 2012; Heuer and Loesel, 2009; Kenyon, 1896; Strausfeld et al., 2009; Wolff et al., 2012) in which they share a neuroanatomical ground pattern, as well as proteins required for memory formation. For example chemosensory afferents which supply thousands of intrinsic neurons, parallel

processes which establish orthogonal networks with feedback loops as well as modulatory inputs, and efferents (Wolff and Strausfeld, 2015). In insects and spiders, but also in annelids they represent sensory-associative brain centers implicated in olfactory discrimination, as well as in olfactory learning and memory acquisition, consolidation and retrieval (Heisenberg, 2003; McGuire et al., 2001; Strausfeld et al., 2009; van Swinderen, 2009; Wang et al., 2008). In support of this idea, the mushroom bodies are, relatively, largest in social insects, which excel in chemical communication (Heisenberg, 2003).

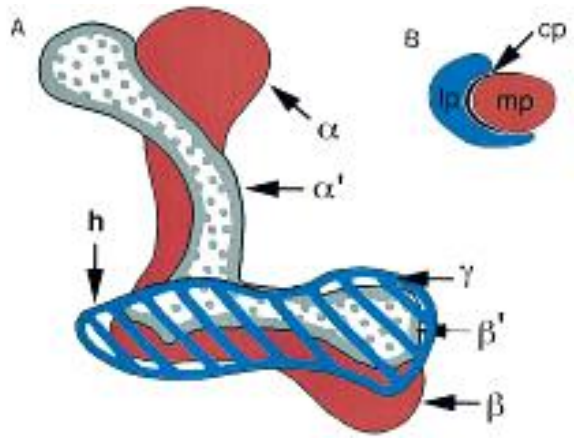


Fig. 3.9. Cartoon of the mushroom body lobes depicted from an anterior viewpoint. Although the mushroom bodies are bilateral, this diagram depicts only the left lobe structure. Dorsal is up; medial is to the right. The peduncle would extend behind the plane of paper toward the Kenyon cells. The most anterior lobe, γ, is shown striped in blue, and is continuous with the heel (h). Just behind the γ lobe are the α' and β' collateral lobes, stippled in gray. The β lobe, ventral to the β' lobe, and its collateral α, are in brown (A). Cartoon of a cross section through the peduncle at the level of the fan-shaped body. The lateral peduncle is in blue, the central peduncle in black, and the medial peduncle in brown, corresponding to the coloration of the lobes to which they project (B) (Figure and legend from Crittenden et al., 1998).

Electrophysiological experiments have shown that mushroom body neurons are also responsive to visual, tactile, and gustatory stimuli (Erber, 1978; Erber et al., 1980; Gronenberg, 1986) The prominent antennoglomerular tract and anterior superior optic tract convey olfactory and visual information to the mushroom body calyces, whereas additional afferents relay mechanosensory information (Mobbs, 1982; Rybak and Menzel, 1993; Strausfeld, 1976). This convergence suggests that the mushroom bodies may be sites of sensory integration, an essential component to associative learning (Crittenden et al., 1998). Therefore, they are the invertebrate homologue of the mammalian pallium with which they share a common origin (Tomer et al., 2010). The pallium represents the most highly developed part of the forebrain as it harbors huge densities of interneurons arranged in cortical layers around a central neuropil (cortex). Although it is less elaborated in other vertebrates it is generally considered

to function as a sensory-associative center integrating primarily olfactory information (Nieuwenhuys, 2002) and serves as the central structure for learning and memory (Kandel et al., 2000).

Moreover MB build up a tight, recurrent feedback loop with two *dorsal paired medial* (*DPM*) neurons and their directly coupled *anterior paired lateral* (*APL*) neurons (see 6.2 and Fig. 6.3 and Fig. 6.4) (Liu and Davis, 2009; Wu et al., 2011b; Wu et al., 2013). This network is supposed to play a key role in the segregation of neuropeptide dependent *ASM* and neuropeptide independent *ARM* phases (see 6.2) and linking memory consolidation processes to sleep (Crocker et al., 2010; Haynes et al., 2015; Joiner et al., 2006; Liu et al., 2008). For additional information about *DPM* neurons see chapters: 5.4.5, 5.4.6 and 6.2 and figures: 5.4e, 5.8b, 6.3 and 6.4.

3.7.4 The *pars intercerebralis* and the neuroendocrine system

The insect neuroendocrine system consists of several populations of neurosecretory cells (NSCs) with peripheral axons terminating in contact with specialized neurohemal glands where the neurohormones are released (Raabe, 1982, 1989; Schooneveld, 1998; Veelaert et al., 1998; Siegmund and Korge, 2001). The majority of NSCs are found in the dorso-medial protocerebrum, the so-called *pars intercerebralis* (*PI*) and *pars lateralis* (*PL*). The *PI,* a part of the *superior medial protocerebrum* (*SMP*), is a small cluster of cells that constitutes the master structure of this wide spread neuroendocrine system (Nassel et al., 2008; Nassel and Homberg, 2006) and thus is often referred to as the functional equivalent of the mammalian hypothalamus (de Velasco et al., 2007; Veelaert et al., 1998). The *PI* and the *PL* project their axons towards a set of small glands, the corpora cardiaca (CC), and corpora allata (CA). In *Drosophila*, the CC and CA, along with a third neuroendocrine gland, the prothoracic gland (PTG), are fused into a single complex, the ring gland, which surrounds the anterior tip of the aorta (Fig. 3.10). The *PI-PL*/ring gland complex of insects has been repeatedly compared to the hypothalamus–pituitary axis in vertebrates (e.g.,Veelaert et al., 1998), based on clear anatomically and functionally similarities between the two (i.e., their shared role in energy metabolism, growth, water retention, and reproduction; reviewed in (de Velasco et al., 2007; Nässel, 2002).

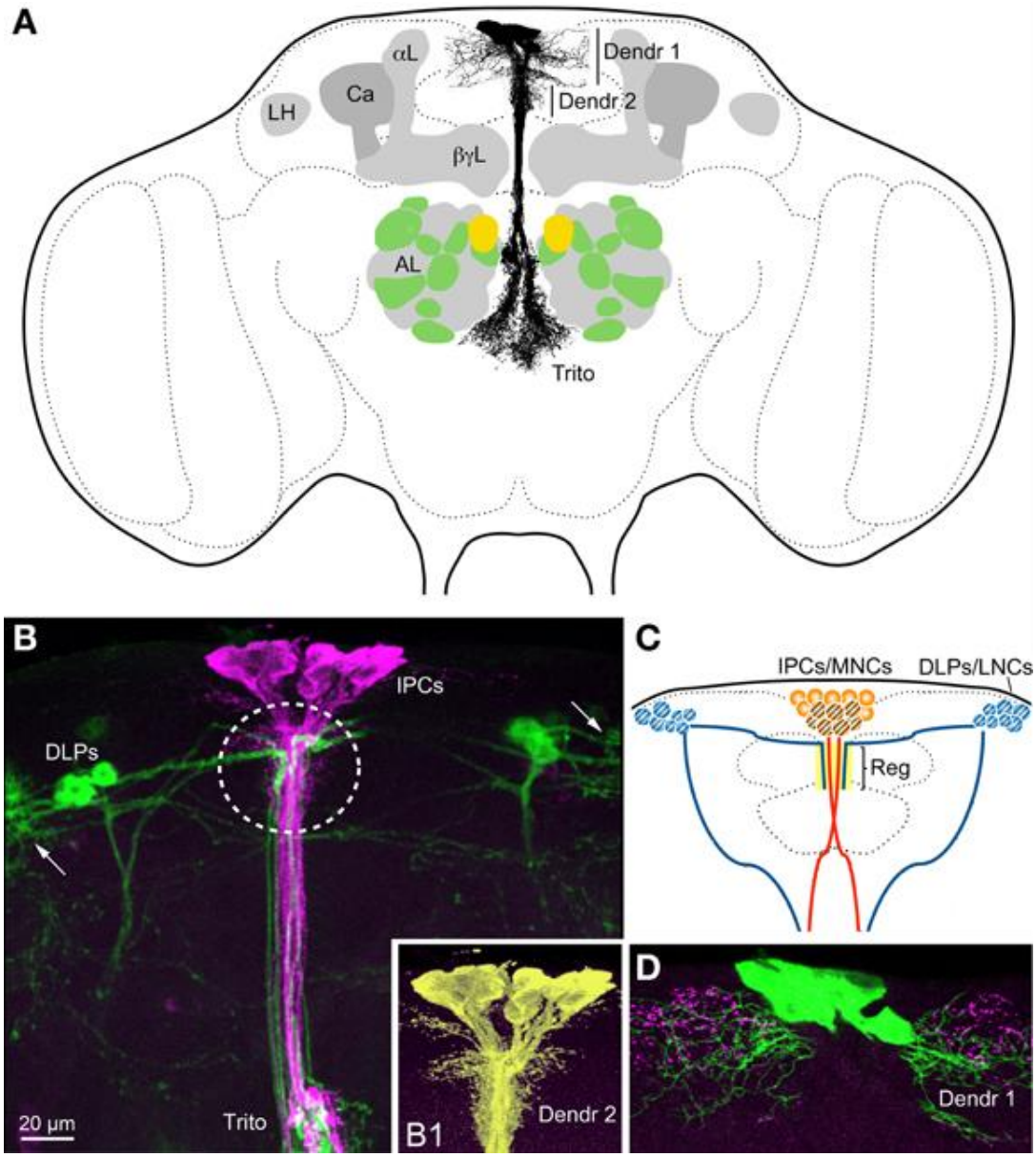


Figure 3.10. Insulin-producing cells (IPCs) and other neurons in the *Drosophila* brain. (A) The IPCs are seen with their cell bodies dorsally, two sets of presumed dendrites (Dendr 1 and 2) in the *pars intercerebralis* and processes branching in the (tritocerebrum Trito). It is not known whether these branches are dendrites or axon terminations, or both. The axons that exit to the corpora cardiaca and aorta are not displayed (they exit above the tritocerebrum, in a direction toward the reader). The antennal lobes (AL) are depicted with the anterior 10 (green and yellow) of the about 14 glomeruli that contain olfactory sensory neurons (OSNs) expressing short neuropeptide F (sNPF). The yellow glomeruli are DM1 that receive OSNs expressing odorant receptor Or42b and sNPF, known to be essential for food search. These sNPF-expressing OSNs also express the insulin receptor (dInR) and the sNPF receptor. DILPs are known to modulate odor sensitivity of these OSNs (Root et al., 2011). The mushroom bodies with calyx (Ca), α-, β- and γ-lobes (α L, β γ L) and the lateral horn (LH) are also depicted. The mushroom bodies also seem to be targeted by DILPs, at least in larvae (Zhao and Campos, 2012). **(B)** The IPCs (magenta, anti-DILP2) and corazonin-expressing *dorsal lateral peptidergic* (*DLP*) neurons (GFP, green) converge medially in the *pars intercerebralis* (encircled) and in the tritocerebrum (Trito). The *DLP*s are known to regulate IPC activity (Kapan et al., 2012). Arrows indicate the likely dendrites of the *DLP*s. **(B1)** Detail of IPCs (enhanced color) visualizing the short dendrites (Dendr 2) that seem to receive inputs from *DLP*s. **(C)** Schematic depiction of IPCs, *DLP*s and their point of convergence in the *pars intercerebralis* (Reg). The IPCs are located in the median neurosecretory cells cluster and the *DLP*s among the lateral neurosecretory cells. **(D)** The IPCs (green) may receive inputs from serotonin-producing neuron branches (magenta) both at the long dendrites (Dendr 1) and the short (not shown here). Panel **(B)** is altered from (Kapan et al., 2012) and 2D from (Luo et al., 2012) (Figure and legend from Nässel et al., 2013 and modified for PhD thesis).

Furtermore, the identification and characterization of *Drosophila melanogaster* insulin-like peptides (DILPs), together with the examination of intracellular signaling mechanisms in neurosecretory cells, in which these DILPs are produced in the brain, have revealed a functional conservation in nutrient sensing and the underlying signaling mechanisms between mammals and fruit flies (Haselton and Fridell, 2010). Besides, DILPs and growth factors do not only regulate development, growth, reproduction, metabolism, stress resistance and lifespan, but also certain behaviors and cognitive functions (Nässel et al., 2013). DILPs are expressed in a variety of tissues including the larval ventral nerve cord, larval salivary glands, larval midgut, ovaries, and the larval and adult brain (Brogiolo et al., 2001; Haselton and Fridell, 2010; Ikeya et al., 2002; Rulifson et al., 2002)

Studies investigating the function of DILPs have found that they all are co-expressed in 5-7 pairs of bilaterally symmetrical, clustered median neurosecretory cells in the *pars intercerebralis* (*PI*) region of the protocerebrum in both larvae and adults (Fig. 3.10) (Broughton et al., 2005; Ikeya et al., 2002; Rulifson et al., 2002). Axonal processes originating from these DILP-producing median neurosecretory cells in the *PI* terminate in neurohemal areas of the aorta and CC tissue-containing ring gland, thus providing a route for DILPS to be released directly into the circulatory system (Haselton and Fridell, 2010; Ikeya et al., 2002; Rulifson et al., 2002).

3.8 The olfactory system in *Drosophila*

Drosophila primarily detect odors through about 60 olfactory receptor proteins, one of which is expressed in each of the approximately 1400 olfactory receptor neurons (ORNs), which are located in the sensory bristles on the antennae and the maxillary palps on each side of the head (Clyne et al., 1999; Lessing and Carlson, 1999) It has been shown that ORNs expressing the same olfactory receptor project to the same, odor specific synapse cluster called glomerulus. All together there are about 40 glomeruli located in the antennal lobe (Fig. 3.11) and they serve as morphological distinguishable areas, harboring the presynaptic terminals of the ORNs (Fishilevich and Vosshall, 2005; Gao et al., 2000; Keene and Waddell, 2007; Vosshall et al., 2000). In the antennal lobe, the cholinergic ORNs form excitatory synapses with at least three classes of neurons: excitatory cholinergic projection neurons (PNs), inhibitory GABAergic local interneurons (iLNs) and excitatory cholinergic local interneurons (eLNs) (Jefferis et al., 2007; Shang et al., 2007; Stocker et al., 1997).

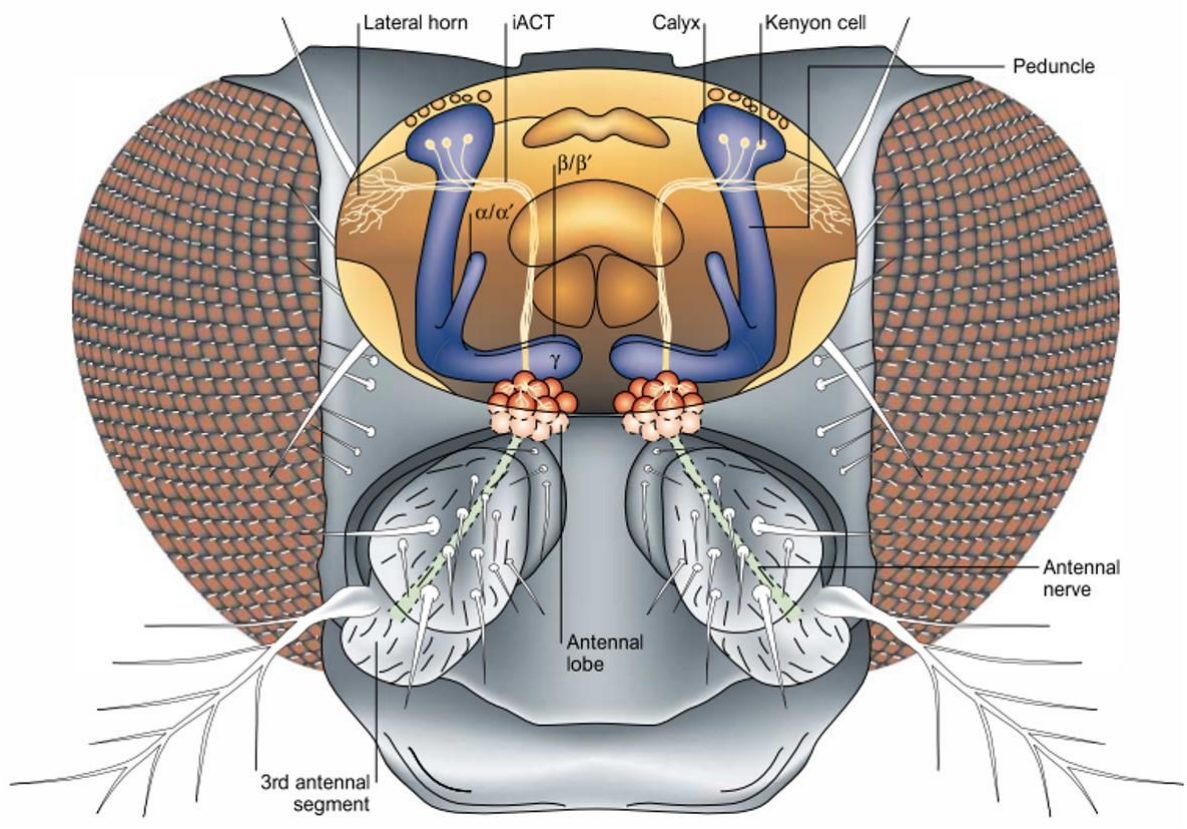


Fig. 3.11. Olfactory pathway. Odor information is carried from the third antennal segments and maxillary palps (not shown) to the antennal lobe, where receptor fibres are sorted according to their chemospecificities in about 40 glomeruli. These represent the primary odor qualities, which are reported to two major target areas in the brain, the dorsolateral protocerebrum (lateral horn) and the calyx of the mushroom body. The inner antennocerebral tract (iACT) connects individual glomeruli to both areas. α/α′, β/β′ and γ mark the three mushroom body subsystems described by (Crittenden et al., 1998) (Figure and legend from Heisenberg, 2003 and modified for PhD thesis).

Since flies have approximately 180 PNs each glomerulus is sampled on average by 3-5 PNs (Stocker et al., 1997). The PNs extend dendrites into a single antennal lobe glomerulus and transmit the olfactory information to the calyx of MBs, which are considered as the primary association centers of olfactory and aversive or appetitive stimuli (see 3.6.3) (Davis, 1993; de Belle and Heisenberg, 1994; Heisenberg, 2003; Heisenberg et al., 1985; Krashes et al., 2007; Pascual and Préat, 2001; Zars et al., 2000). Besides, PN show also connections to other higher centers of learning and integration, such as the lateral horn (Jefferis et al., 2001; Marin et al., 2002; Wong et al., 2002).

The PNs are organized into at least two different neural tracts - the inner and the medial antennocerebral tract (ACT). The inner ACT project onto Kenyon cells (KC) in the MB calyx

(Keene and Waddell, 2007) as well as towards the lateral horn, whereas the medial ACT is only connected to the lateral horn (Fig. 3.12) (Stocker et al., 1997).

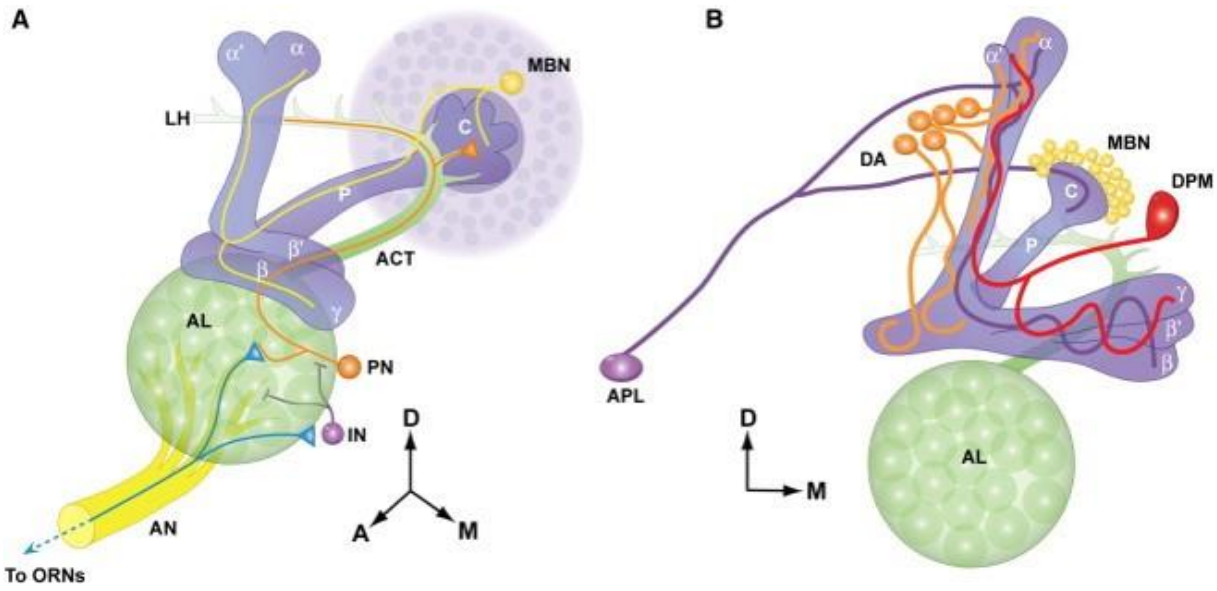


Fig. 3.12. Anatomical Organization of the Olfactory Nervous System in *Drosophila* (**A**) Olfactory nervous system viewed from the left-front and slightly dorsal position of the fly. Olfactory information is transmitted from olfactory receptor neurons (ORNs) located on the antennae (not shown) via the antennal nerve (AN) to the antennal lobe (AL), where the axons of ORNs synapse on two types of secondary olfactory neurons, the projection neurons (PN) and the AL interneurons (IN). The INs are known to be either excitatory or inhibitory. PNs send their axons via a nerve known as the antennal cerebral tract (ACT) to the mushroom body neurons (MBN) and to the lateral horn (LH). The PNs synapse with MBNs in a neuropil region known as the calyx (C). Three classes of MBNs have been described according to their axonal collaterals (α/β, α'/β', and γ). The axons extended by MBNs follow the pedunculus (P) to reach the MB lobes (α, α', β, β', and γ). For simplicity, only one ORN axon (green), one PN (orange), one IN (purple), and one α/β MB neuron (yellow) have been superimposed on a schematic of one hemisphere of the fly brain. Axis arrows: A = anterior, D = dorsal, M = medial. Adapted from (Busto et al., 2010). (**B**) Frontal perspective of neurons that are extrinsic to the MBs in one hemisphere showing the dorsal paired medial (*DPM*) neuron, *anterior paired lateral* (*APL*) neuron, and dopaminergic (DA) neurons. The *DPM* neuron (red) extends a single neurite which bifurcates to innervate the vertical lobes (α and α') and the horizontal (β, β', and γ) lobes of the MBs. Only five of the DA neurons (DA, orange) in the *PPL1* cluster are illustrated. These neurons innervate distinct zones of the MB vertical lobes. The *APL* neuron (magenta) broadly innervates the calyx and the MB lobes. Axis arrows: D = dorsal, M = medial. (Figure and legend from Davis, 2011 and modified for PhD thesis).

As the structure and function of the insects' olfactory nervous system is remarkably homologous to that of vertebrates we can assume that the principles have been conserved across animal phyla (Busto et al., 2010), making Drosophila a prime candidate to serve as model organism in olfactory reception and olfactory memory related studies.

4. MATERIALS AND METHODS

4.1 Material: media, buffer and antibodies

4.1.1 Fruit fly: preparation and *in vivo* imaging of adult flies

Este's Ringer solution adjusted to pH 7.3:

Hepes 5mM
NaCl 130mM
KCl 5mM
$MgCl_2$ 2mM
$CaCl_2$ 2mM
Sucrose 36mM

4.1.2 Fruit fly: immunohistochemistry (IHC)

1x Phosphate buffered saline (PBS) pH7.4:

8g/l NaCl
0.2g/l KCl
1.44g/l Na2HPO4
0.24g/l KH_2PO_4

Fixative solution used for whole mount fly brains:

Paraformaldehyde 4%
Sucrose 4%
1xPBS to 100ml

Washing and antibody incubation buffer for whole mount fly brains:

Bovine serum albumin (BSA) 1%
Triton X-100 0.5%
Na-azide 0.05%
1xPBS to 100ml

Dilution and blocking buffer: Immunohistochemistry

BSA 2%
Triton X-100 0.1%
NGS (normal goat serum) 5% (added before blocking step)
1xPBS to 100ml

Primary antibodies: Immunohistochemistry of whole mount brains

Mouse monoclonal anti Myc SC-40 (IHC 1:100) Santa Cruz Biotechnology
Mouse monoclonal anti α-tubulin (IHC 1:100) Santa Cruz Biotechnology
Mouse anti Bruchpilot nc-82 (IHC 1:50) Erich Buchner, Würzburg
Rabbit "polyclonal" anti GFP (IHC 1:200) Molecular Probes
Rabbit "polyclonal" anti GFP conjugated to Alexa 488 (IHC 1:200) Invitrogen
Rabbit "polyclonal" anti DLG (IHC 1:1000) Invitrogen

Secondary antibodies: Immunohistochemistry of whole mount brains

Goat anti mouse Cy3 (IHC 1:200) Dianova
Goat anti mouse Alexa488 (IHC 1:200) Molecular Probes
Goat anti mouse Alexa568 (IHC 1:500) ThermoFisher Schientific
Goat anti rabbit Alexa488 (IHC 1:200) Molecular Probes
Goat anti rabbit conjugated to Cy3 (IHC 1:500) Dianova

Antibodies are diluted in process analytical technology (PAT)

4.2. Fly genetics and culture

4.2.1 Fly stocks

Name	Expression profile	Genotype	Source	Product number or publication
WT2202U	none	wild type *Drosophila*	Y.Zhong	(Wang et al., 2008)
amnesiac^{x8}	null mutant of the *amn* gene	Null mutant of the Amnesiac gene	C.Wegener, Würzburg	(Moore et al., 1998)
OK107	Gal4 expression in α/β, α'/ β' and γ-lobes of MBs	w*; P{GawB}ey^{OK107}	Bloomington	#106098
C739	Gal4 expression in α/β-lobes of MBs	y^{1} w^{67c23}; P{GawB}Hr39^{c739}	Bloomington	#7362
201Y	Gal4 expression in γ-lobes of MBs	w^{1118}; P{GawB}Tab2^{201Y}	Bloomington	#4440
hs-P26	Heat shock promoter drives Gal4 expression ubiquitously and randomly	P{hs-GAL4.P26}	Y. Zhong	(Wang et al., 2008; Xia et al., 2005)

amon-91D	Expresses Gal4 in the pattern of the amon gene	w*; P{amon-Gal4.R}91D	Bloomington	#30554
386Y	Expresses Gal4 in peptidergic neurons. Reflects expression of amon gene	w[*]; P{GawB}386Y	Bloomington	#25410
c316	Expresses Gal4 in *DPM* neurons	w*; P{GawB}c316	Bloomington	#30830
VT-064246	Gal4 expression restricted to *DPM* neurons	P{VT064246-Gal4}attP2	VDRC	#204311
UAS-mCD8::GFP	MARCM set, GFP labels the cell surface (mouse CD8 is a transmembrane protein), highly concentrated in neuronal processes	y[1] w[*]; P{UAS-mCD8::GFP.L}Ptp 4E^{LL4}; Pin^{Yt}/CyO	Bloomington	#5136
UAS-GCaMP3	Expresses a fluorescent calcium reporter protein under control of 10 UAS sequences.	w^{1118}; P{UAS-GCaMP3.T}attP40	Bloomington	#32116
UAS-GCaMP3.NLS	Expresses a fluorescent calcium reporter protein under control of 10 UAS sequences and harbors a nuclear localization sequence	w*; +; UAS-GCaMP3.NLS	J.M. Weislogel	(Weislogel, 2008; Weislogel et al., 2013)
UAS-CaMBP4	Expresses nuclear calcium signaling inhibitor under UAS-control	w*; +; UAS-CaMBP4 myc	J.M. Weislogel	(Weislogel, 2008; Weislogel et al., 2013)
UAS-amon-RNAi28b	Expresses a dsRNA under UAS control for RNAi of amon.	w*; P{UAS-amon-RNAi}28b	Bloomington	#29009
UAS-amon.R-40L	Expresses wild type amon under UAS control	w*; P{UAS-amon.R}40L	Bloomington	#29008
UAS-dFmrf-RNAi	Expresses dsRNA for RNAi of Fmrf under UAS control, TRiP	y^{1} v^{1}; P{TRiP.JF01909} attP2	Bloomington	#25870
UAS-Nplp3-RNAi	Expresses dsRNA for RNAi of Nplp3 under UAS control, TRiP	y^{1} v^{1}; P{TRiP.JF03188} attP2	Bloomington	#28760

UAS-CCHa2-RNAi	Expresses dsRNA for RNAi of CCHa2 under UAS control, TRiP	y^1 sc^* v^1; P{TRiP.HMC 04565}attP40	Bloomington	#57183
UAS-Acp70A-RNAi	Expresses dsRNA for RNAi of Acp70A under UAS control, TRiP	y^1 v^1; P{TRiP.JF02022} attP2	Bloomington	#25998
UAS-Acp70A	Expresses Acp70 under UAS control, also in females (induces ovulation in virgin females)	$P\{SP^{g.Yp1.hs}\}G1$	Bloomington	#4365

Tab. 1. Fly stocks: Genetically modified fly lines are commercially available at the Vienna *Drosophila* Resource Centre (VDRC) and the Bloomington *Drosophila* Stock Center at Indiana University, where new transgenic lines are created and verified stocks are maintained.

All crossings (except for the single neuropeptide experiments) were set up with virgin females from the reporter lines (GCaMP3, GCaMP3.NLS, mCD8::GFP, CaMBP4, amon.R-40L and amon-RNAi28b) crossed to males carrying the Gal4-driver constructs. For the single neuropeptide experiments virgin females of the hs-Gal4-P26 strain were crossed to males carrying the RNA neuropeptide knock down (dFMRF, Nplp3, CCHa2, Acp70A) or overexpression constructs (Acp70A) respectively. All flies were cultured on standard fly food at 75% relative humidity and at the restrictive temperature of 18°C to prevent developmental defects. Transgene expression in F1 offspring from these crossings were used for behavioral analysis and were induced by heat shock or by 6 days de-repression temperature paradigm 2-3 days after hatching (for details see 4.8). The non-induced siblings served as controls and were of the same age when used for behavioral analysis but had remained the whole time at 18°C to prevent expression of the transgene constructs. For *in vivo* imaging in adult flies, flies carrying UAS transgenes containing GCaMP3 or GCaMP3.NLS were crossed with mushroom body and PI expressing driver lines (Gal4-OK107 and amon-Gal4-91D). Newly hatched flies (males and females) were collected and cultured for an additional 5-6 days on standard fly food at room temperature (RT) before being used on the next day for imaging.

4.2.2 Fly culture

Flies were cultured on standard fly food in incubators (KMF 720, Binder GmbH, Tuttlingen, Germany) at a constant temperature of 18°C and 75% relative humidity. 2-3 days after hatching flies were placed in fresh food vials containing a small strip of blotting paper (Rotilabo®-Blotting paper, CL66.1, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) to absorb

excessive humidity and were then either treated by one of the induction protocols (see 4.8) or were brought back to 18°C to serve as controls. Standard fly food was made according to a protocol from Bloomington Drosophila Stock Center (homepage http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/bloomfood.htm) with some slight modifications.

Standard fly food:

9g/l agar
18g/l yeast
10g/l soy flour
90g/l yellow corn meal
44g/l sugar beet syrup
80g/l malt extract
62,3ml/l propionic acid
6,23ml/l phosphoric acid

4.3 *In vivo* calcium imaging

Flies were briefly immobilized with CO_2 and dissected on ice. The flys' wings were either removed or together with their eyes and thorax were glued with dental cement (either ProtempTM II or Transbond Supreme Low Viscosity UV Light Cure Adhesive, 3M Unitek ESPE Dental Products, Seefeld, Germany) to a thin plastic coverslip covered with thin polyethylene foil, thereby leaving their feet and antennae free of glue. A hole was cut in the foil and head cuticle under a droplet of Este's Ringers solution and the trachea and overlying fatty tissue were removed to reveal the underlying brain.

The preparation was then mounted onto a wide-field upright microscope (BX51WI, Olympus, Hamburg, Germany) equipped with a 20x immersion objective (XLUMPLFL20xW, N.A. 0.95, Olympus) and an EMCCD camera (Andor iXon DV885, BFi OPTiLAS, Groebenzell, Germany) connected through a software interface (Cell^R, Olympus) to a Xenon fluorescent excitation source and filter wheel (MT-20, Olympus). GCaMP3.NLS was imaged with 470/40 nm excitation and 525/50 emission filters (AHF Analysentechnik, Tuebingen, Germany). During recordings, a continuous stream of air was presented to the fly through Teflon tubing connected to an empty glass vial. Airflow could be switched by solenoid valves (Lee Company, Westbrook, USA) to vials containing 3-Octanol, 4-methylcyclohexanol or mineral oil. Electrical shocks (10 to 70µA for 1.5s repeated every 5s for one minute) generated by an isolated pulse stimulator (AM Systems Model 2100, Science Products GmbH, Hofheim, Germany) in constant

current mode were delivered to the fly's feet through a copper grid brought into contact with the feet of the fly with a micromanipulator (Narishige NMN-25, Science Products GmbH, Hofheim, Germany).

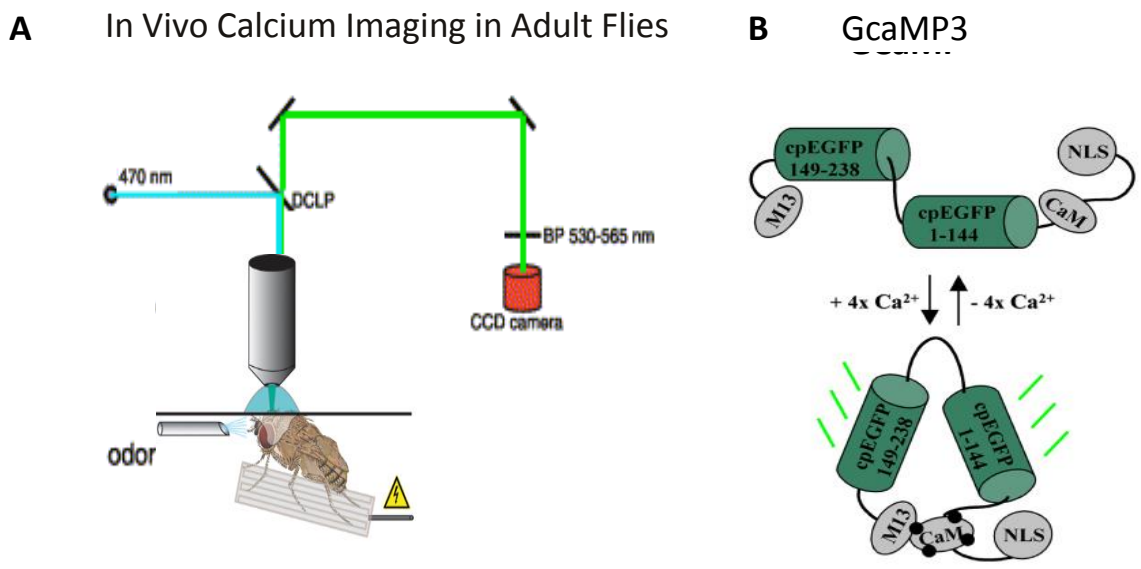


Fig. 4.1. Schematic representation of calcium live imaging in *Drosophila*. (**A**) Schematic drawing of the live-imaging set-up in which cells were excited through an opening in the skull with wavelength of 470nm and emission light was detected at wavelengths of 530-565nm with a CCD camera during the presentation of odor and electric foot shocks. (**B**) Schematic drawing of the reporter construct GCaMP3 (Tian et al., 2009) which undergoes a conformational change when binding to nuclear calcium after excitation by light of the wavelength of 470nm. (Figure created by Dr.J.M.Weislogel and Dr.C.P.Bengtson (**A**) and from Nakai et al., 2001 (**B**) modified for PhD thesis).

All experiments were performed at a constant exposure (15-30ms with 2x2 binning) and imaging rate (2Hz) and stimulations were commenced after baseline intensities had stabilized. All images were corrected for background fluorescence using a measurement from the same image in a region devoid of detectable recombinant fluorescent protein. Quantitative data using GCaMP3 is presented for each region of interest (ROI) as: $\Delta F/F_0 = (F-F_0)/F_0$ where F represents the background subtracted emission fluorescence intensity of GCaMP3 and F_0 represents the baseline F measured prior to each stimulation series. Area under the curve (AUC) was calculated as the mathematical integral of the $\Delta F/F_0$ trace during stimulation.

4.4 Whole mount immunostaining of adult brains

Fly brains were prepared similar as described (Krashes et al., 2007). Briefly, fly brains were dissected in ice-cold Schneider's Drosophila Medium (Gibco Invitrogen, Gaithersburg, MD,

USA) and incubated in fixative solution overnight at 4°C. Brains were washed in Drosophila washing buffer (PAT) at room temperature (3 x 20min). After blocking with 5% normal goat serum (NGS) overnight, primary antibodies (diluted in antibody incubation buffer) were added and incubated for 48h at 4°C. Next day, brains were washed again in PAT (3 x 10min on a rocker) before secondary antibodies (diluted in antibody incubation buffer) were added and again incubated for 48h at 4°C. Next day, brains were washed again (3 x 10min on a rocker) in PAT. Finally, brains were counterstained and mounted in VECTASHIELD® (Vector Laboratories, Burlingame, USA) containing DAPI (1.5µg/ml) and equilibrate overnight. Brains were imaged using either a Leica SP2 confocal microscope with HCX PL APO CS 40x 1.25 oil UV objective (Leica Microsystems GmbH, Wetzlar, Germany) or a Zeiss LSM 5 Exciter with a Zeiss 40x EC Plan-NEOFLUAR objective (Zeiss Application Center, Heidelberg, Germany). Time series as well as confocal z-stacks were processed using ImageJ (Image Processing and Analysis in Java, W.Rasband, National Institute of Health, Maryland, USA) and Adobe Photoshop software (Adobe Systems Software Limited, Dublin, Ireland).

4.5 Behaviour assays

4.5.1 Conditioning paradigm

Aversive olfactory associated learning was performed with a Pavlovian conditioning procedure (Pavlov, 1927; Tully and Quinn, 1985) (see Fig. 4.2) in a climate chamber (Unit. No. 59226090300010, Weiss Umwelttechnik GmbH, Reiskirchen-Lindenstruth, Germany) at 25°C and 75% relative humidity under dim red light (Parathom CL-A 80064, Osram GmbH, Munich, Germany). Therefore groups of approximately 60 flies were placed into the training chambers lined with an electrifying grid (Fig. 4.2g) and exposed to a constant humidified air stream of 750 ml/min generated by a vacuum pump (Type: N810 3FT.18, KNF Neuberger GmbH, Freiburg, Germany). A constant flow of air was ensured by a system of airflow meters with valves (Meterate Tube, Nr.314-146/090 by GPE Scientific Ltd, Bedfordshire, England) (Fig. 4.3c). Tubing used for connections was either BEKHA-LIT (8x2mm Art.Nr.84000420, APD Petzetakis Schlauchtechnik GmbH, Schwalmtal, Germany) between pump and elevators or Masterflex Precision Pump Tubing (#06424-25, Cole-Parmer Instrument Company, LLC., Vernon Hills, USA) between the rest of the components. Adapters and distributors for tubing came from Carl Roth (E773.1, E808.1 and E809.1) and Cole-Parmer (#31501-55).

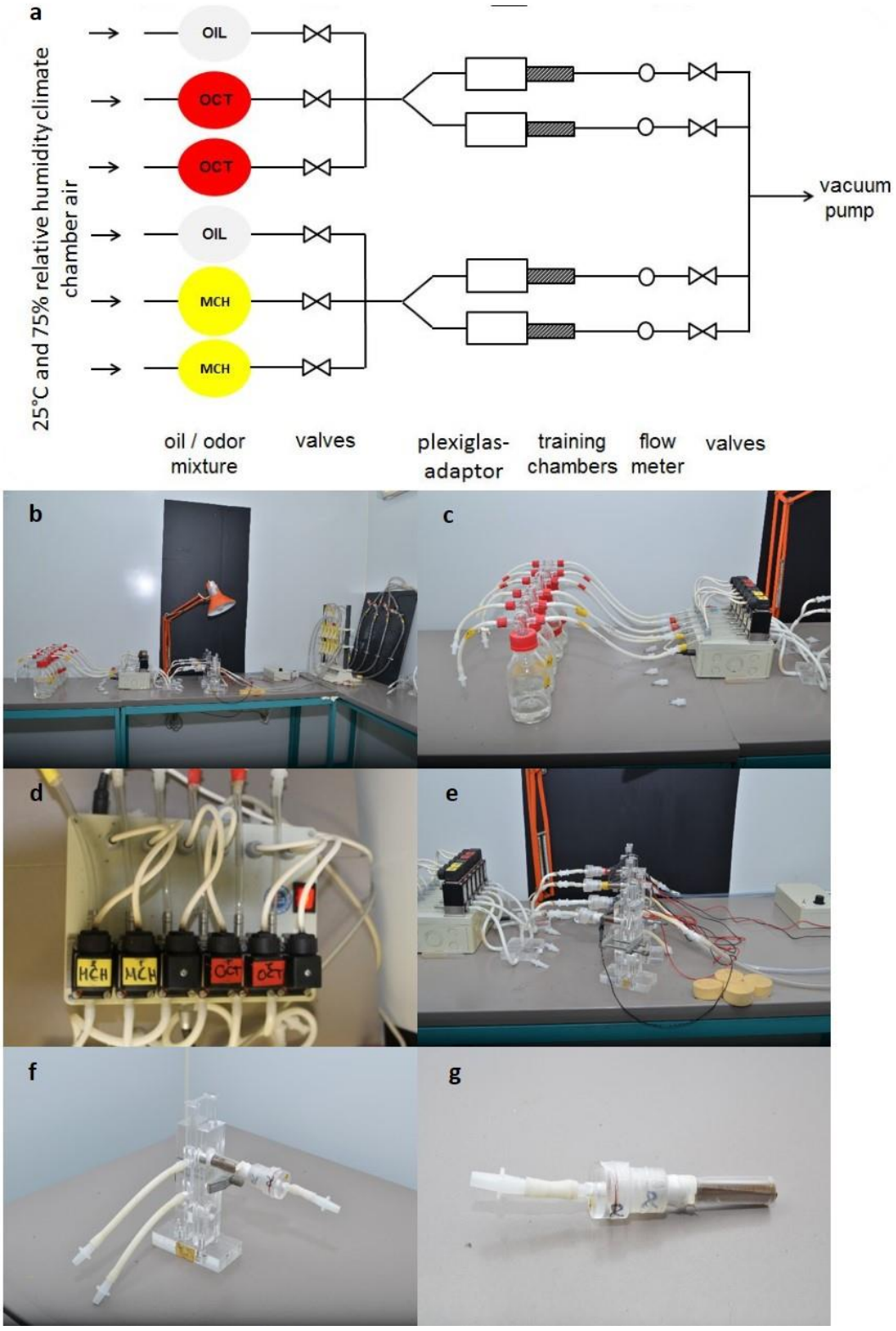


Fig. 4.2. Olfactory aversive conditioning paradigm. (**a**) Schematic drawing (kindly provided by B.Sc.R.Hoffmann) of the conditioning set-up. The computer-controlled set-up enables an automated conditioning protocol. The air flow is displayed by a flow meter and is adjusted to 750ml/min (**b**). Detailed pictures of individual components such as gas-washing bottles (**c**), solenoid valves (**d**) and a full conditioning situation with four chambers running simultaneously (**e**) as well as close-ups of an Plexiglas® elevator (**f**) and a training chamber with electrifying grid (**g**).

The flies were exposed to this novel environment for 90 seconds before they were sequentially exposed in random order for 60sec to two odors, MCH (4-methyl-cyclohexanol - $CH_3C_6H_{10}OH$; Cat.:66360, Sigma Aldrich Chemie GmbH, Munich, Germany) or OCT (3-octanol - $C_8H_{18}O$; Sigma Aldrich Cat.:74870), one of which acted as the conditioned stimulus (CS+) paired with 60V electrical shocks (US), and the other odor, presented without shock, served as the control stimulus (CS-, OCT or MCH). A 45sec purging interval without odor always separated CS+ and CS- presentation.

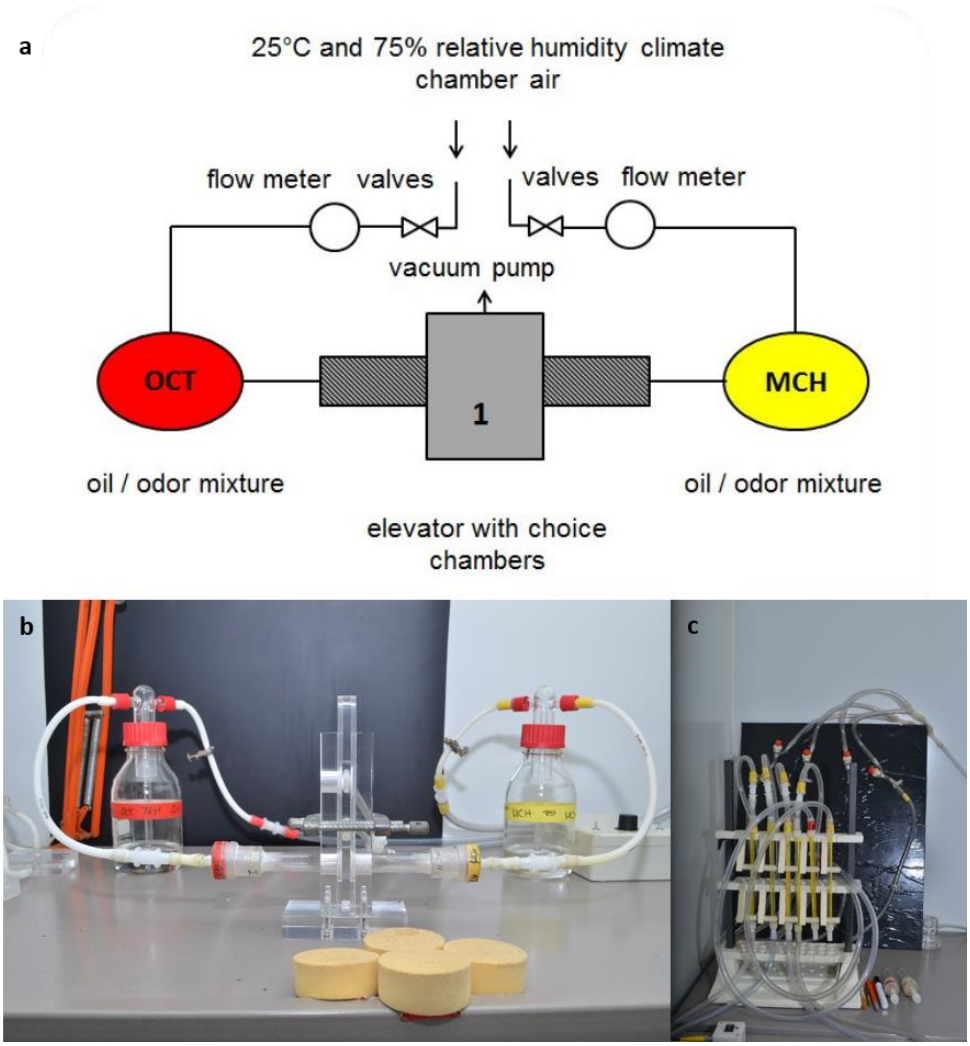


Fig. 4.3. Overview of the T-maze odor-choice situation. (**a**) Schematic drawing (kindly provided by B.Sc.R.Hoffmann) of the odor presentation apparatus serving as a T-maze to assess odor preference. Flies are transferred to the center of the T-maze and are simultaneously exposed to the CS- and CS+ from opposite arms of the T-maze. An equal distribution of flow-through was hand-regulated by little clamps on the gas-washing bottles used during the choice situation. Flies were trapped inside their respective arms after a 120s period for decision making, killed and counted. (**b**-**c**) Pictures of the odor-choice set-up (**b**) and the air flow meters with valves (**c**) in detail.

The learning experiments are representing therefore a counterbalanced design in which the results are averaged, with one group of flies being trained to associate shock with the first odor and a second group to associate shock with the second odor and the odors (MCH or OCT) were randomly assigned to first or second in the sequence. The correct sequence and timing of the induction protocol was secured by a computer controlled switching device with solenoid valves (Nr.122101, Bürkert GmbH & Co KG, Ingelfingen, Germany) (Fig. 4.2d) which was custom made by the Abteilung Elektronik of the Universität Heidelberg (Zentralbereich Universität Heidelberg, INF 367, Heidelberg, Germany).

Odors were diluted in 100ml heavy mineral oil (Sigma Aldrich Cat.:330760) to an end concentration of 1:1000 (MCH) and 1.5:1000 (OCT) to eliminate naïve odor bias and to achieve an equal preference for the odors in T-maze behavioral tests of unconditioned flies. Oil/odor mixtures were prepared in gas-washing bottles with frits (Duran 500ml bottle, retrace code: 10011389, Schott AG, Mainz, Germany) (Fig. 4.2c). The 60V electrical shocks were delivered by a generator (Natus Neurology Incorporated - Grass Products, S48 Stimulator, Warwick, USA) in twelve pulses (1.5 sec each) with a 3.5sec rest interval in between. The conditioning and testing chambers as well as the elevators (Fig. 4.2f-g) were custom made by the Abteilung Feinmechanik of the Universität Heidelberg (Zentralbereich Universität Heidelberg). Air leakage from the adapter flanges in the elevators was prevented using Teflon O-ring seals (T017: .676x.070 and T020: .864x.070, MS Wil GmbH, Zurich, Switzerland). To test the conditioned avoidance responses the flies were transported to a T-maze (Fig. 4.3a-b) choice situation (consisting of the same elevators and chambers used during conditioning with the only difference that the chambers in the choice situation lacked the electrifying grid and the air flow rate had to be regulated by small clamps on the gas-washing bottles) in which the CS+ and CS- were presented simultaneously. After 2 minutes time to allow flies voluntarily to enter one of the T-maze arms, flies were trapped in either one of the arms, killed in a freezer at -80°C for 15min (HERAfreezeTM HFU240BV, Thermoscientific Germany BV & Co. KG, Braunschweig, Germany) and then counted to calculate a performance index (PI) (see 4.6). As flies react very sensitive to any distractive environmental changes such as noise, vibrations and light or temperature and humidity shifts the environment was kept as stable as possible and the flies were disturbed as less as possible, during the conditioning and testing phases, to obtain constant and maximal learning scores. Odors had to be refreshed every two weeks in order to guarantee their constant intensity. To avoid mixing up the odors a color code was

used for all bottles and tubes: red for Octanol and yellow for MCH (see Fig. 4.2 and 4.3 respectively).

4.5.2 Conditioning protocols

For a single conditioning trial (n=1) F1 flies from the same crossing were divided into two groups of approximately 60 flies each and were then moved into vials without food but containing a piece of Rotilabo®-Blotting paper to absorb excessive humidity on the feet of the flies to avoid excessive shock potentially caused by wet feet on an electric grid. After a drying period of about 1h in which the flies could acclimatize to the temperature and the humidity conditions of the climate chamber, they were conditioned separately, but simultaneously (except for *iSTM*) whereby one group experienced MCH as CS+ (forward) while the other group experienced OCT as CS+ (reverse) before both groups were tested for odor preference in the T-maze from which results were pooled together to get one performance index (for further details see 4.5.1 and 4.6).

Flies underwent one single conditioning trial (duration 5min) before both groups (forward and reverse) were tested for memory retention of conditioned avoidance within 6min (*STM*) or 3h (*MTM*) of completing conditioning. Long lasting forms of memory are: *ARM* which requires a repetition of the conditioning in a 10x massed (no breaks between the single trials; duration of conditioning 50min) manner or *LTM* which was implemented by 10x spaced (15min break between the single trials; duration of conditioning 3h 5min) conditioning. *ARMs* and *LTMs* were tested for memory retention of conditioned avoidance 24h after completing conditioning training (Tully et al., 1994a). For *MTM*, *ARM* and *LTM* the flies were immediately removed from the conditioning paradigm and transferred back to their food vials after the conditioning phase. The vials were stored at 18°C for 2h (*MTM*) or 23h (*ARM* and *LTM* respectively), before the vials were brought back into the climate chamber to allow the flies to acclimate again for 1h before they were tested. For *iSTM*s forward and reverse scores were obtained independently to achieve testing of the flies within 40s of the conditioning trial to avoid potential contamination with *MTM*.

training cycle (5min):

- 90s air flow
- 1 min odor one (CS+) + shock (US) / 12 pulses 60V
- 45s rest (purging interval)
- 1 min odor 2 (CS-)
- 45s rest

4.6. Performance index calculation and data analysis

The following formula was used to calculate the performance index:

$$PI = (((Oct\text{-}MCH^*)/(Oct+MCH^*)) + ((MCH\text{-}Oct^*)/(MCH+Oct^*))) \times 100/2$$

"forward" side "reverse" side

* indicates the odor acting as CS+

The performance index was calculated as the number of flies avoiding the shocked odor minus that avoiding the non-shocked odor divided by the total number of flies. This was done independently for both odors (forward and reverse) before the two scores were summed up and finally got multiplied by 100 and divided by 2. The PI ranges from -100 to +100 and accordingly a PI of 0 indicates an equal distribution of flies in each arm of the T-maze, while a PI of 100 indicates an avoidance of the conditioned stimulus by all of the tested flies (Xia et al., 2005). The PI therefore reflects learning and memory success.

All data are presented as means ± standard error calculator (SEM) and were analyzed in a Student's t-test. Asterisks indicate the statistical significance (*, $p \leq 0,05$, **, $p \leq 0,01$, ***, $p \leq 0,001$).

4.7 Transgene expression systems

4.7.1 UAS/Gal4 System

The UAS/Gal4 system is a system for targeted gene expression that allows the selective activation of any cloned gene in a wide variety of tissue- and cell-specific patterns. The gene encoding the yeast (*Saccharomyces cerevisiae*) transcriptional activator Gal4 is inserted

randomly into the *Drosophila* genome to generate "enhancer-trap" lines that express Gal4 under the control of nearby genomic enhancers. By screening these randomly created Gal4 lines it is possible to get drivers with a very restricted expression patterns for example in only a subset of neurons (Busto et al., 2010; Duffy, 2002). There is now a large collection of lines that express Gal4 in a huge variety of cell-type and tissue-specific patterns (Brand and Perrimon, 1993; Johnson et al., 1990). Gal4 encodes for a protein of 881 amino acids and is induced by galactose (Laughon and Gesteland, 1984; Oshima, 1982). Importantly, expression of Gal4 alone appears to have no overt deleterious phenotypic effects. It is then possible to introduce a gene containing GAL4 binding sites (UAS element) within its promoter, to activate it in those cells where GAL4 is expressed (Brand and Perrimon, 1993) (Fig. 4.4).

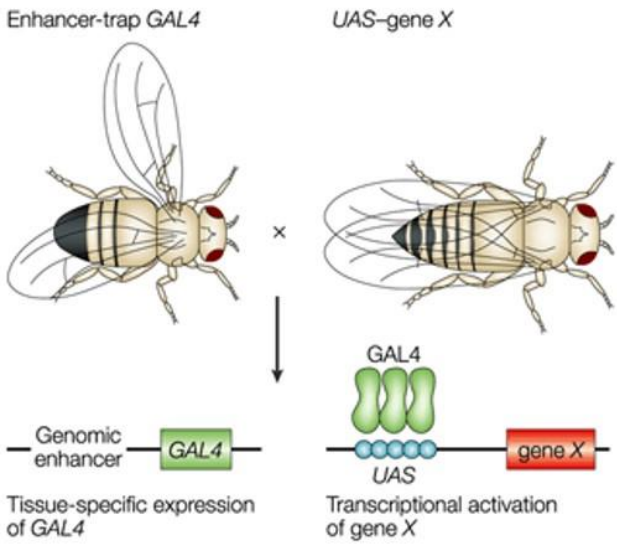


Fig. 4.4. The UAS/Gal4 system. The yeast transcriptional activator Gal4 can be used to regulate gene expression in *Drosophila* by inserting the upstream activating sequence (UAS) to which it binds next to a gene of interest (geneX).The GAL4 gene has been inserted at random positions in the *Drosophila* genome to generate 'enhancer-trap' lines that express GAL4 under the control of nearby genomic enhancers, and there is now a large collection of lines that express GAL4 in a huge variety of cell-type and tissue-specific patterns. Therefore, the expression of gene X can be driven in any of these patterns by crossing the appropriate GAL4 enhancer- trap line to flies that carry the UAS–gene X transgene. This system has been adapted to carry out genetic screens for genes that give phenotypes when misexpressed in a particular tissue (modular misexpression screens) (Figure and legend from St Johnston, 2002 and modified for PhD thesis).

In 1993 Brand and Perrimon published a bipartite approach for directing gene expression *in vivo.* In this system, expression of the gene of interest, the responder, is controlled by the presence of the UAS element. Because transcription of the responder requires the presence of Gal4, the absence of Gal4 in the responder lines maintains them in a transcriptionally silent

state (Brand and Perrimon, 1993). To activate their transcription, responder lines are mated to flies expressing Gal4, termed the driver, in a particular topographical pattern. The resulting progeny then express the responder in a transcriptional pattern that reflects the Gal4 driver expression pattern (Duffy, 2002).

4.7.2 TARGET System

To prevent constitutive expression of Gal4 and its transgenic target construct during the developmental phase of the flies, potentially resulting in developmental defects, and to provide temporal control over the expression of the reporter constructs, we chose to use the well-established temporal and regional gene expression targeting (TARGET) System (McGuire et al., 2003).

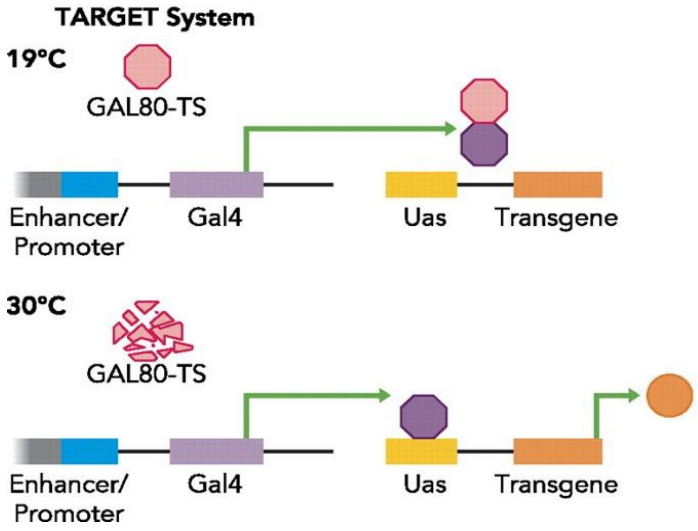


Fig. 4.5. The TARGET system. In the conventional GAL4/UAS system a P element carrying the GAL4 coding region drives the expression of GAL4 protein in a specific tissue on the basis of proximity of the P element to a tissue-specific enhancer. GAL4 protein then binds to its cognate UAS binding site and activates transcription of the downstream effector gene. In the TARGET system, a temperature-sensitive GAL80 protein ($GAL80^{ts}$), expressed ubiquitously from the tubulin 1α promoter, represses the transcriptional activity of GAL4 at 18°C and thus prevents the expression of the UAS-transgene, but becomes inactive at 30°C, allowing GAL4 to drive the expression of the UAS-transgene in its expression-specific pattern (Figures and legend from Busto et al., 2010).

In this system the activity of the Gal4 drivers is restricted by the expression of the temperature sensitive Gal4 repressor $Gal80^{ts}$ which is ubiquitously expressed under the control of the tubulin 1α promoter. $Gal80^{ts}$ binds to Gal4 and disables its transcriptional activity at a restrictive temperature of 18°C. At a permissive temperature of 31-33°C $Gal80^{ts}$ starts to undergo conformational changes that disrupt its binding to Gal4, resulting in a de-repression

of Gal4 transcription and consequently in Gal4-dependent transgene expression (McGuire et al., 2003) (Fig. 4.5).

This process of Gal4-de-repression is according to our own experiences a rather slow process requiring an incubation period of 5-6 days at the permissive temperature to achieve good transgene expression (Weislogel et al., 2013).

4.8 Induction protocols

4.8.1 De-repression paradigm

To achieve spatiotemporal control of the expression of our UAS transgenes (CaMBP4, GCaMP3, GCaMP3.NLS, mCD8::GFP, amon.R-40L and amon-RNAi28b) we used the TARGET System (see 4.7.2, Fig. 4.5). Therefore our triple transgenic flies (F1 offspring harboring: Gal4-x; Gal80^{ts}; UAS-x) were raised under restrictive conditions to avoid transgene expression during development and hence secure avoidance of possible developmental defects. 2-3 days after eclosion, flies were shifted to permissive conditions (for 6 days at a 12h:12h light-dark regime) to achieve expression of the reporter constructs (Fig. 4.6a-b). During this time frame the flies were transferred into new food vials every three days to maintain a stable food quality.

4.8.2 Heat shock paradigm

For heat shock induction, flies were collected within 1 to 2 days after eclosion, placed in fresh food vials containing a strip of Whatman filter paper to absorb extra humidity, and kept at 18°C. Twelve to 18 hours before training, the vials were, after an acclimation phase of 1h, submerged in a 37°C water bath (Type VF, Grant Instruments Ltd., Cambridge, England) until the bottom of the foam stopper (inside the vials) was below the surface of the water, thereby ensuring that the flies could not escape the heat shock. After the vials remained submerged for 30 min, they were transferred to the climate chamber (25°C and 75% relative humidity). Training began immediately after the incubation period (for details see Weislogel et al., 2013) (Fig. 4.6c-d).

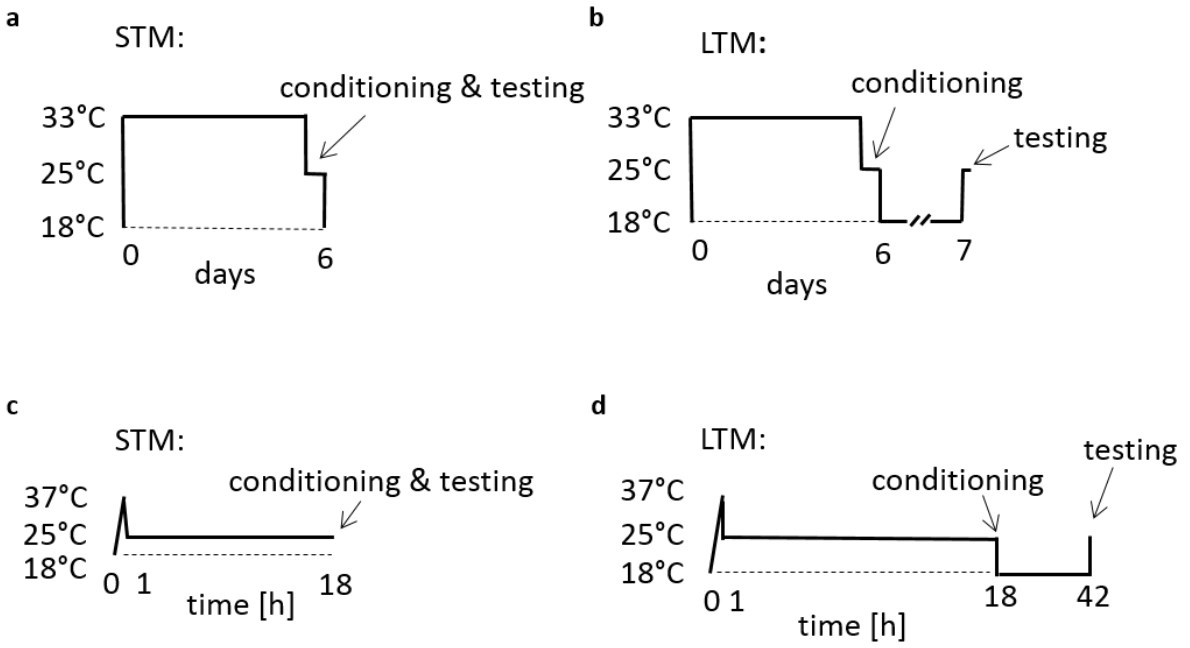


Fig. 4.6. Schematic representation of the different induction protocols. (**a**-**b**) Flies expressing their reporter construct through the TARGET system were induced for 6d at 33°C before training and testing. (**c**-**d**) Flies harboring the heat shock inducible hs-Gal4-P26 driver line received a 30min heat shock at 37°C and were then stored at 25°C for 18h before conditioning. Note that for *MTM* the training protocol is the same as for *STM* but flies were switched to 18°C for 3h before testing and *ARM* has the same protocol as for *LTM* but the training cycles are not spaced. All flies were trained and tested at 25°C.

The use of the heat shock expression system (hs-Gal4-P26) offers a tight temporal control of transgene expression in a ubiquitously distributed random subset of cells throughout the whole fly brain (Weislogel, 2008; Weislogel et al., 2013; Xia et al., 2005).

4.9 CaMBP4 – the calcium/calmodulin binding polypeptide

To screen for a requirement of nuclear calcium signaling in olfactory learning, Dr. Jan Weislogel created a fly line containing the myc-tagged nuclear calcium/calmodulin (CaM) signaling blocker CaMBP4 (Wang et al., 1995; Zhang et al., 2007; Zhang et al., 2009). CaMBP4 is a nuclear protein that contains four identical copies of the M13 peptide (Fig. 4.7a), the calmodulin binding sequence of skeletal muscle myosin light chain kinase (MLCK) (Nakai et al., 2001; Wang et al., 1995), which binds to and thus inhibits in a competitive fashion the calcium-CaM complex and therefore selectively blocks the activation of calcium-CaM dependent kinases known to mediate nuclear calcium regulated gene expression (Fig. 4.7b-c). Nuclear calcium is known to act as a key regulator of CREB dependent gene transcription which is crucial for indispensable cellular adaptions affecting neuronal survival (Ahlgren et al., 2014; Lau et al., 2015), morphology (Mauceri et al., 2015) and synaptic plasticity (Bading, 2000;

Hardingham et al., 2001; Soderling, 2000). Imaging studies showed that CaMBP4 is exclusively located in the neuronal nucleus (Wang et al., 1995; Weislogel, 2008; Weislogel et al., 2013). It has been shown that CaMBP4 binds CaM in a Ca^{2+}-dependent manner and inhibits competitively several CaM-dependent enzymes (Blumenthal and Krebs, 1986; Blumenthal et al., 1985; Wang et al., 1996). In mice, it has previously been shown that CaMBP4 transgenic mice have impaired long-term memory formation (Limback-Stokin et al., 2004). This transgenic construct was cloned downstream to an activator sequence, UAS (see 4.7.1, Fig. 4.4), before generation of a CaMBP4-UAS transgenic fly line for use in our olfactory learning assays. The genetic mutated control of CaMBP4 is the non-functional equivalent mM13.NLS S2 that has an altered order of amino acids (For details see Weislogel et al., 2013).

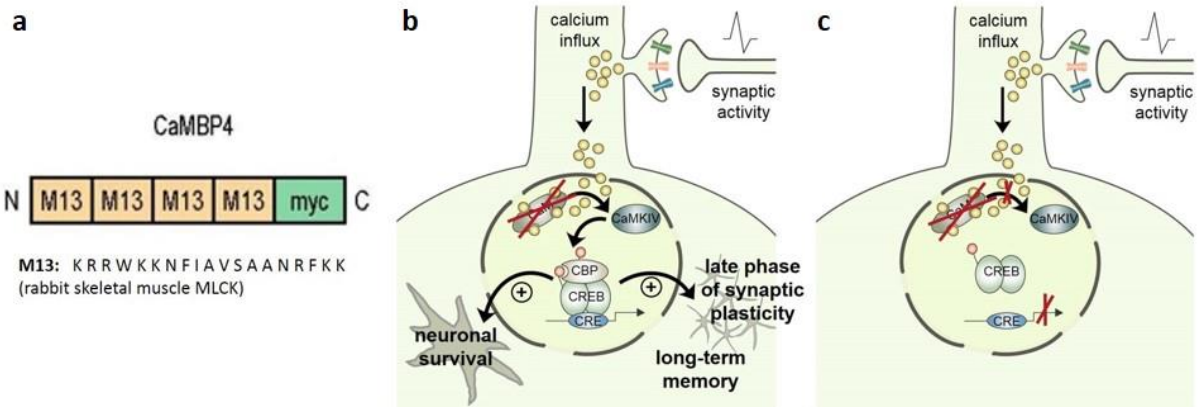


Fig. 4.7. CaMBP4 – the nuclear calcium signaling inhibitor. Schematic drawings of the Ca^{2+}/CaM signaling inhibitor CaMBP4 harboring four identical copies of the M13 peptide together with a myc-tag for immunostaining. Displayed is also the amino acid sequence of M13 (Wang et al., 1995) (**a**). Overview of the interference between blocked nuclear calcium signaling and transcriptional and translational processes in the nucleus of neurons (**b**-**c**).

4.10 Gene Silencing 'Knock down' by RNA Interference

The term RNA interference (RNAi) refers to the phenomenon of post-translational silencing of gene expression that occurs in response to the introduction of double-stranded RNA (dsRNA) into a cell (Fire et al., 1998). This phenomenon results in highly specific suppression of gene expression. Introduction of long dsRNA into nearly any eukaryotic cell triggers a strong nonspecific shutdown of transcription and translation, in part due to activation of dsRNA-dependent protein kinase-R (PKR) (Waechter et al., 1997). Activated PKR phosphorylates the translation eukaryotic initiation factor 2 (EIF2), which in association with activation of

ribonuclease-L (RNase-L) and induction of interferon production, stops protein synthesis and promotes apoptosis. Overall, this is believed to represent an antiviral defense mechanism (Williams, 1999). Though its mechanisms are not fully elucidated, RNAi represents the result of a multistep process (Fig. 4.8). Upon entering the cell, long dsRNAs are first processed by the RNAse III enzyme Dicer (Knight and Bass, 2001).

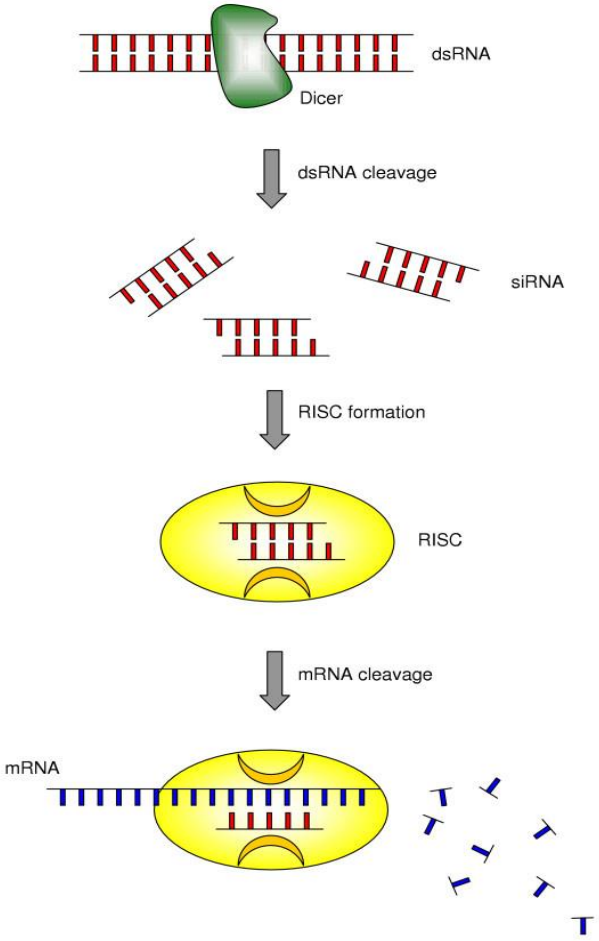


Fig. 4.8. Mechanism of RNA interference (RNAi). The appearance of double stranded RNA (dsRNA) within a cell (e.g. as a consequence of viral infection) triggers a complex response, which includes a cascade of molecular events known as RNAi. During RNAi, the cellular enzyme Dicer binds to the dsRNA and cleaves it into short pieces of ~ 20 nucleotide pairs in length (siRNA). These RNA pairs bind to the cellular enzyme complex (RISC) that uses one strand of the siRNA to bind to single stranded RNA molecules (i.e. mRNA) of complementary sequence. The nuclease activity of RISC then degrades the mRNA, thus silencing expression of the target viral gene. RNAi therefore can be used to knock down target genes of interest with high specificity (Figures and legend from Mocellin and Provenzano, 2004).

This functional dimer contains helicase, dsRNA binding domains. The Dicer enzyme produces 21–23 nucleotide dsRNA fragments with two nucleotide 3' end overhangs named small interfering RNAs (siRNAs). RNAi is mediated by the RNA-induced silencing complex (RISC)

which, guided by siRNA, recognizes mRNAs containing a sequence homologous to the siRNA and cleaves the mRNA at a site located approximately in the middle of the homologous region (Bernstein et al., 2001). Thus, gene expression is specifically inactivated at a post-transcriptional level. This natural cellular antiviral response can therefore be used to specifically inhibit the function of any chosen target gene. A growing library of validated siRNAs directed toward frequently targeted genes exists. RNAi therefore makes it possible to analyze the function of a gene by the selective elimination of its transcript (gene knockdown) (Mocellin and Provenzano, 2004).

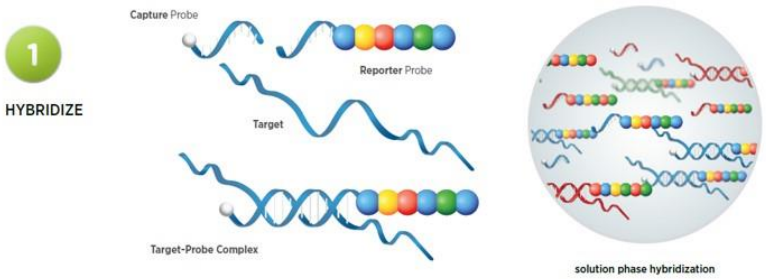
4.11 Transcriptome analysis

Transcriptome analysis was performed at the nCounter Core Facility at the UniversitätsKlinikum Heidelberg. This current *state of the art* expression profiling technology is a fully automated system of digital gene expression analysis (nCounter system, NanoString Technologies, Inc., Seattle, USA). It is an instrument designed for multiplexed measurement of gene expression using fluorescently labeled reporter probes, so called 'codesets'. The codeset probes are ca 100 bases in length. Therefore, the system is very resistant to lower RNA quality and is perfectly suited for critical samples such as formalin-fixed, paraffin-embedded (FFPE) samples. Applying a unique coding technology enables direct counting of individual RNA molecules across all levels of biological expression, with sensitivity and specificity comparable to Real Time PCR (RT PCR). The main advantage is that no enzymatic reactions are involved, in particular no reverse transcription is necessary. In addition, it is suitable for analysis of as little as 600 ng of genomic DNA (karyotyping and copy number variation (CNV) analysis).

For further details see: http://www.nanostring.com/applications/technology

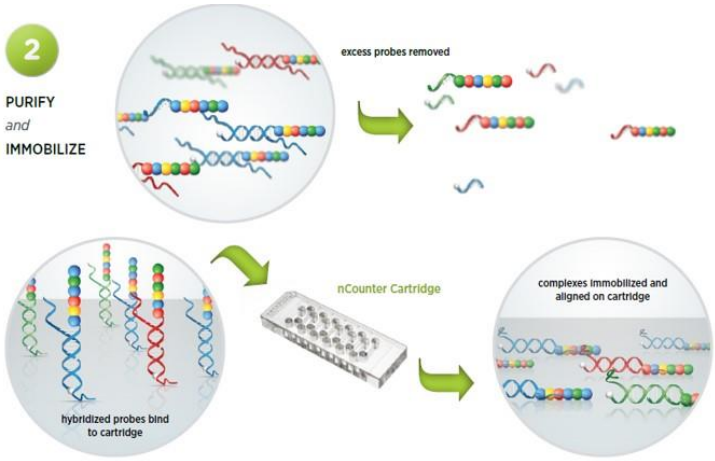
Hybridization

NanoString's Technology employs two ~50 base probes per mRNA that hybridize in solution. The Reporter Probe carries the signal; the Capture Probe allows the complex to be immobilized for data collection.



Purify and Immobilize

After hybridization, the excess probes are removed and the probe/target complexes aligned and immobilized in the nCounter Cartridge.



Count

Sample Cartridges are placed in the Digital Analyzer for data collection. Color codes on the surface of the cartridge are counted and tabulated for each target molecule.

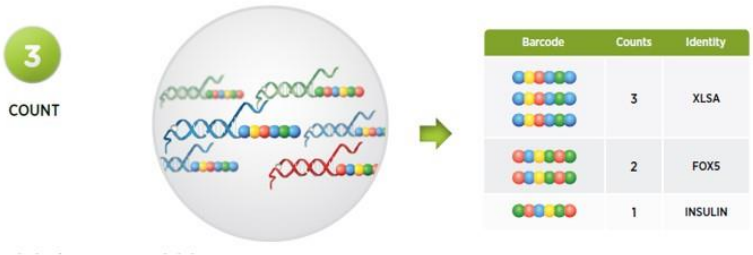


Fig. 4.9. The nCounter System for transcriptome analysis. The system utilizes a novel digital color-coded barcode technology that is based on direct multiplexed measurement of gene expression and offers high levels of precision and sensitivity (<1 copy per cell). The technology uses molecular "barcodes" and single molecule imaging to detect and count hundreds of unique transcripts in a single reaction. Each color-coded barcode is attached to a single target-specific probe corresponding to a gene of interest. Mixed together with controls, they form a multiplexed CodeSet (Figures and legend from www.nanostring.com).

5. RESULTS

5.1. Small subsets of mushroom body and *pars intercerebralis* neurons carry a nuclear calcium-dependent *LTM*-trace

In a recent collaboration with Dr. Jan Marek Weislogel we have shown that nuclear calcium signaling is essential for the formation of long term aversive olfactory memory (*LTM*) in flies whereas all other non-consolidated forms of memory do not require nuclear calcium signaling (Weislogel et al., 2013). These results were initially based on the transient but ubiquitous expression of the nuclear calcium signaling blocker CaMBP4 and a scrambled control of a related construct (S2mM13.NLS) with a heat shock activated Gal4 driver (for details see Weislogel et al., 2013). I joined this project when we attempted to map the brain regions and cell populations that could mediate nuclear calcium signals during the formation of *LTM*. Since the mushroom bodies are the association centers of all olfactory and shock related aversive cues we reasoned that the MB is a likely brain region that may employ nuclear calcium signaling. We therefore focused our mapping of nuclear calcium requirements on Gal4 driver lines with preferential expression in MBs.

In order to target MB neurons or subpopulations of them we used three different Gal4 lines: OK107-Gal4 expressing in α/β, α'/ β' and γ-lobes, c739-Gal4 expressing in α/β-lobes and 201Y-Gal4 expressing in the γ-lobes (Aso et al., 2009) (Fig. 5.1A). Note, that the expression patterns of c739 and 201Y do not overlap, inside the MB, with each other and hence target non-overlapping subsets of the MB-wide expressing OK107 line. Nonetheless, all drivers show, although they are often considered as MB specific, also expression in other brain regions (see Fig. 6.1 Aso et al., 2009). However, all three Gal4 drivers express constitutively in their respective cells, which is problematic if one expresses a transgene that might potentially interfere with developmental processes. We therefore chose to temporally restrict the activity of the Gal4 drivers by expressing the temperature sensitive Gal4 repressor $Gal80^{ts}$ under the control of the tubulin 1α promoter resulting in ubiquitous expression (McGuire et al., 2003). $Gal80^{ts}$ binds to Gal4 at restrictive temperature (18°C) and disables its transcriptional activity. At permissive temperature (31-33°C) $Gal80^{ts}$ starts to undergo conformational changes that disable its binding to Gal4 resulting in a de-repression of Gal4 and consequently in Gal4-dependent transgene expression (McGuire et al., 2003).

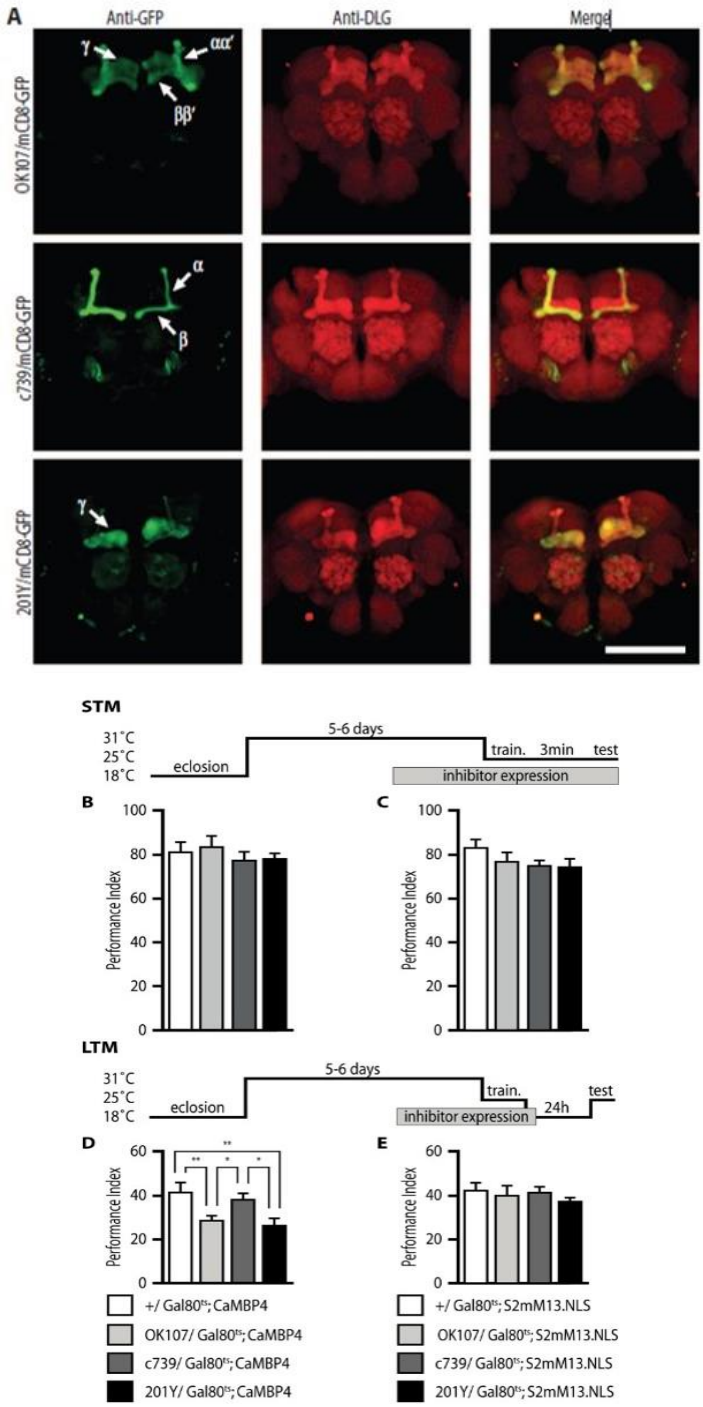


Fig. 5.1. Flies expressing a nuclear calcium signaling blocker in small subsets of neurons show impaired *LTM*. **(A)** Confocal images of the mushroom bodies in whole-mount preparations of adult fly brains expressing membrane-anchored GFP (mCD8-GFP) under the control of the indicated Gal4 driver lines, stained with an antibody against GFP (green) to visualize the α/β, α'/ β' and γ neurons and with an antibody against DLG (red) to show brain structures. Scale bar, 200 µm. **(B-E)** Flies were kept for 5 to 6 days at the permissive temperature of 31°C before conditioning and testing for *STM* **(B-C)** or *LTM* **(D-E)**. All Data are presented as mean ± SEM where * represents $p < 0.05$, ** $p < 0.01$ using the Student's t-Test (n = 8 to 13 experiments per group). (Pictures by Dr.C.P.Bengtson, behavioral data created in collaboration with Dr.J.M.Weislogel see also Weislogel et al., 2013).

This process of Gal4-de-repression is according to our own experiences a rather slow process requiring an incubation period at permissive temperature of 5-6 days to achieve good transgene expression (data not shown; Weislogel, personal communication). We therefore incorporated such a temperature treatment in all experiments that required Gal80^{ts}/Gal4-de-repression (see Materials and Methods 4.8.1.).

Expression of CaMBP4 with the Gal4 driver line OK107 revealed a significant decrease in *LTM* performance scores compared to its controls (flies that carry only the effector construct without the Gal4 driver - light grey and white bars in Fig. 5.1D respectively). In contrast *STM*, *MTM* and *ARM* were not affected (Fig. 5.1B, data for *MTM* and *ARM* not shown (see Weislogel et al., 2013). Expression of the scrambled nuclear calcium inhibitor construct S2mM13.NLS showed no impairment in any kind of tested memory when crossed to OK107-Gal4 (light grey bar in Fig. 5.1E). These results for the first time confirmed that MB neurons require nuclear calcium signaling specifically for the formation of *LTM*. Interestingly, we also found that expression of CaMBP4 or its control construct S2mM13.NLS exclusively in α and β lobes of the mushroom bodies (c739-Gal4) had no effect on either shorter or longer lasting forms of memory (dark grey bars in Fig. 5.1B-E). However, the third line tested in this assay, namely 201Y-Gal4, showed strong deficits in *LTM* formation when CaMBP4 but not S2mM13.NLS was expressed, while *STM* performance was not impaired by either construct (black bars in Fig. 5.1B-E). The impairments observed were significant and comparable to those found in OK107. Note that the expression patterns of OK107 and 201Y overlap exclusively in the γ-lobes suggesting that in these lobes a nuclear calcium dependent *LTM* trace is stored. However, if this is truly the case, since all lines also show expression outside of the MBs still remains unknown.

5.2. Expression of nuclear calcium signaling blocker causes no permanent damage

Since the expression of CaMBP4 alters gene transcription (Bading et al., 1997; Hardingham et al., 1997) and therefore also translational processes the possibility exists that these changes could lead to permanent alterations in the physiology of the affected cells. This could also explain the observed memory defects of suppressed nuclear calcium signaling. To assess whether this is or isn't the case we designed an experiment that allowed us to perform the conditioning experiments after the cessation of transiently expressed CaMBP4 (Fig. 5.2B). If CaMBP4 causes permanent damage one would expect to see memory defects even after

cessation of CaMBP4 expression, whereas if CaMBP4 affects cells reversibly no defect should be observable following cessation.

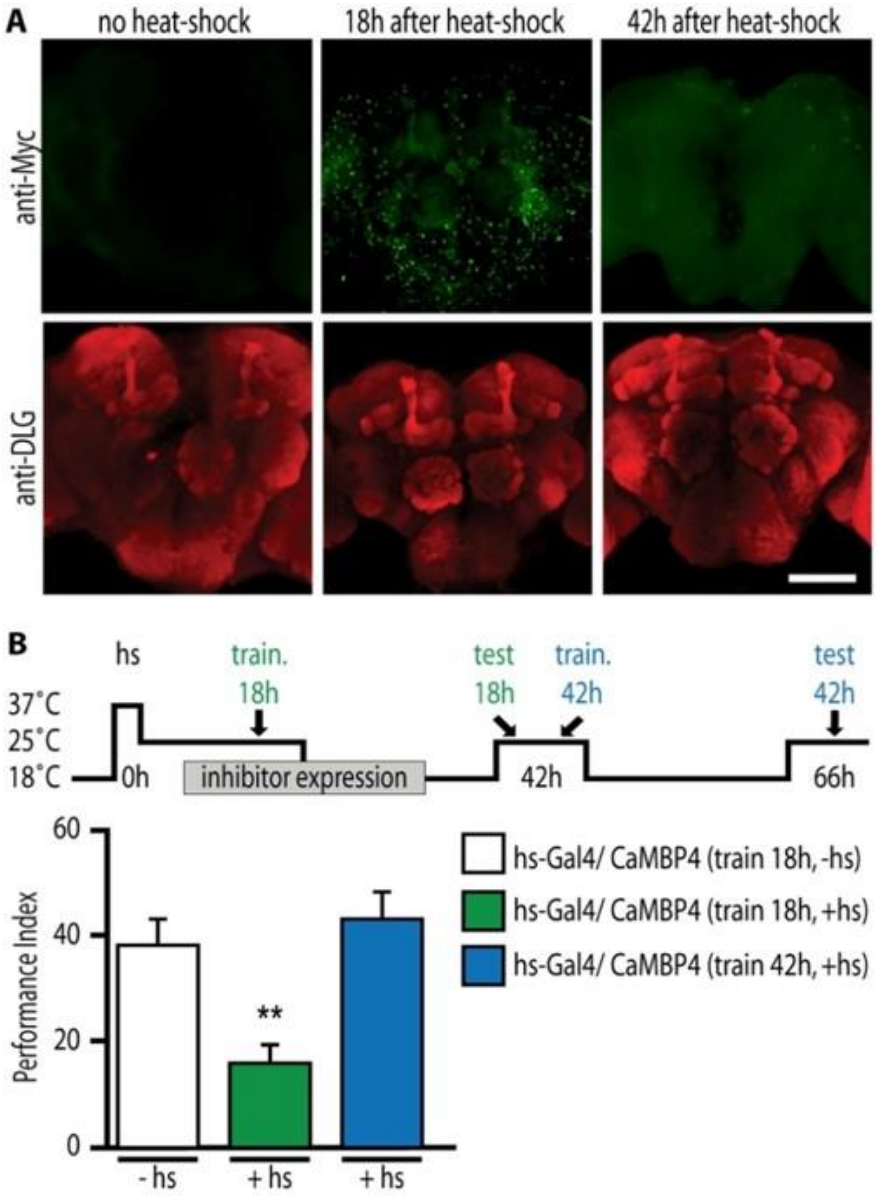


Fig. 5.2. The block in *LTM* mediated by inhibition of nuclear calcium signaling is reversible. (**A**) Confocal images show the nuclear calcium signaling blocker CaMBP4 in whole-mount brain preparations from flies without heat shock or 18 or 42 hours after heat shock. CaMBP4 was detected with an antibody against the myc epitope (green). Fly brains were counterstained with an antibody against discs large protein (DLG) to show its major neuropil structures (red). Scale bar: 200µm (**B**) Flies were trained for *LTM* 18 hours (when CaMBP4 was actively produced and abundant) and 42 hours (when CaMBP4 was no longer produced) after heat shock induction of CaMBP4 expression. *LTM* was tested in all groups 24 hours after training. Flies conditioned during CaMBP4 expression are *LTM* impaired (green bar and symbols) whereas flies conditioned after the cessation of CaMBP4 expression showed *LTM* performance indices comparable to non-induced flies of the same genotype (blue and white bar, respectively). (Data represent means ± SEM ** $P < 0.01$ using the Student's t-Test, n = 8 experiments per group) (Figure and legend from Weislogel et al., 2013 and modified for PhD thesis).

Flies carrying the heat shock inducible, ubiquitously expressing Gal4 line P26 (HS-P26-Gal4) (Wu et al., 2007; Xia et al., 2005) were conditioned and tested first without Gal4-induction (Fig. 5.2A, left panel). This was done to show that carrying the constructs does not result in background CaMBP4 expression, altered performance scores or changes in the ability of the flies to detect the odors or the foot shocks (white bar in Fig. 5.2B).

Second, CaMBP4 expression was induced in flies from the same crossing by heat shock (see Materials and Methods 4.8.2). 18h after the HS, when CaMBP4 is well detectable in fly brains (Fig. 5.2A, middle panel) the flies were conditioned and then tested after another 24h (green bar in Fig. 5.2B). As expected from our previous experiments these flies showed significantly decreased *LTM* performance indices. Third, when similarly treated flies were conditioned not 18h after HS, but 42h later, when CaMBP4 expression has ended (right panel in Fig. 5.2A) the memory defect was gone. These flies showed no impairment in *LTM* anymore and were not significantly different from the uninduced flies in their performance (blue bar in Fig. 5.2B). These results demonstrate that CaMBP4 does not cause permanent damage to the cells. We therefore conclude that the impairments in memory formation as well as the expression of CaMBP4 *per se* are temporally restricted and reversible.

[The content of the following chapters (5.3.-5.4.6) as well as the content of Fig. 5.3-Fig. 5.9 are the original figures and words from which the manuscript for Hörtzsch et al., 2016 (in revision) was created.]

5.3. In vivo calcium imaging in *Drosophila melanogaster*

We have recently shown by *in vivo* calcium imaging in the context of aversive olfactory conditioning of adult *Drosophila melanogaster* that subsets of their MB neurons respond to odor stimuli with immediate and rapidly decaying cytoplasmic calcium transients whereas foot shock stimulation resulted in somewhat delayed and longer lasting calcium transients (Weislogel et al., 2013). The kinetic differences between these fluorescence changes are consistent with the differences in how both stimuli are processed and transferred to MBs: odor information is directly channeled from the olfactory bulbs to the Kenyon cells of MBs via projection neurons (Vosshall and Stocker, 2007) whereas information of the aversive foot shocks is thought to reach the MBs indirectly (Galili et al., 2014). During the course of these imaging experiments done by Dr. C.P.Bengtson and by M.Sc. C.Schäfer using the OK107-Gal4

line to drive expression of either cytoplasmic GCaMP3 (Nakai et al., 2001) or nuclear-targeted GCaMP3-NLS (Weislogel et al., 2013), the focus of our analysis was on the responses of MB neurons to the applied conditioning stimuli (odors or foot shocks). GCaMP3 serves as a calcium indicator through increasing its fluorescence after a conformational change induced by calcium binding (Tian et al., 2009) (for further Details see Materials and Methods: 4.3).

5.3.1 Neurons of the *pars intercerebralis* are strongly activated by the unconditioned stimulus during olfactory conditioning

During the course of the in vivo live imaging experiments, we noticed robust shock-induced cytoplasmic fluorescence changes in a brain region dorso-medial to the MBs: the *pars intercerebralis* (*PI*; Figs. 5.3a-d). The *PI,* a part of the *superior medial protocerebrum* (*SMP*), is a small cluster of neurosecretory cells that constitutes the master structure of the fly's wide spread neuroendocrine system (Nassel et al., 2008; Nassel and Homberg, 2006) and that is often referred to as the functional equivalent of the mammalian hypothalamus (de Velasco et al., 2007; Veelaert et al., 1998). Note that OK107-Gal4 (Fig. 5.4a) although typically referred to as a MB-specific Gal4 driver, expresses strongly also in *PI* cells (Aso et al., 2009). A comparative analysis of the cytoplasmic response profiles of *PI* cells and Kenyon cells of MBs to stimuli that are used in aversive olfactory conditioning experiments revealed that a subset of *PI* cells is strongly activated by each aversive foot stimulus (unconditioned stimulus, US; blue trace in lower panel of Fig, 5.3b, right blue bar in Fig. 5.3c) whereas Kenyon cells showed comparably weak activation by the US (red trace in lower panel of Fig. 5.3b, right red bar in Fig. 5.3c). Conversely, odor stimulation (the conditioned stimulus, CS, in olfactory conditioning experiments), which evokes strong calcium transients in antennal lobes (Data not shown Dr. C.P.Bengtson personal communication), produced large calcium transients in Kenyon cells (red trace in middle panel in Fig. 5.3b; left bars in Fig. 5.3c) but negligible responses in *PI* cells (compare control traces in upper panel of Fig. 5.3b to odor-evoked traces in middle panel). These differences in the stimulus-response characteristics of *PI* and Kenyon cells were particularly obvious in our paired recordings from both structures of the same brain (Fig. 5.3d) suggesting that the observed amplitude differences in the response profiles of both cell types are part of the physiological representations of US and CS in individual fly brains.

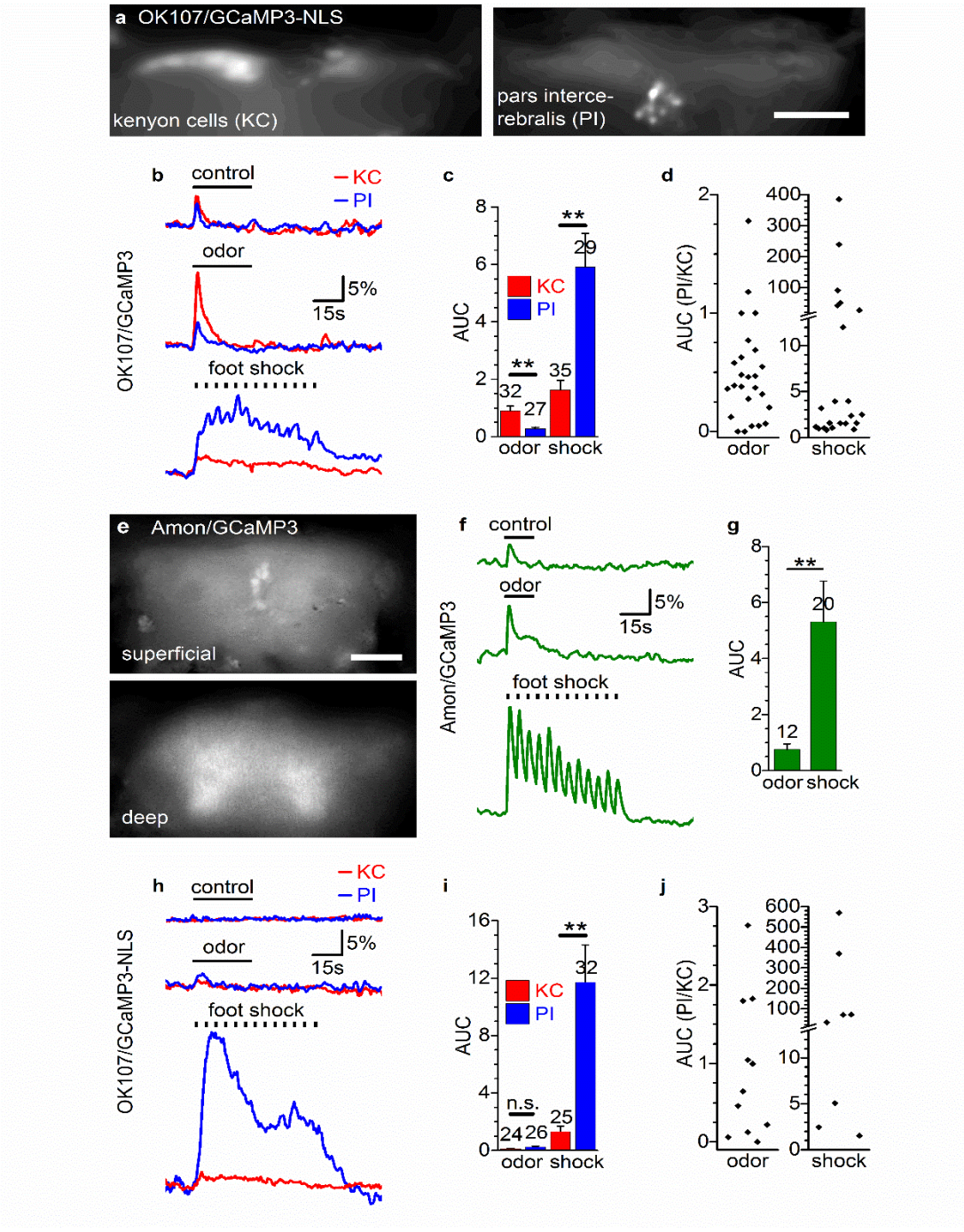


Fig. 5.3. Calcium responses of Kenyon Cells and the *pars intercerebralis* to odor and foot shock. (**a**) Images show the expression of GCaMP3 in the nuclei of an OK107;GCaMP3.NLS fly at the focal plane of the Kenyon cells (KC, left panel) and the *pars intercerebralis* (*PI*, right panel). Kenyon Cell nuclei are visible on both sides of the brain while *pars intercerebralis* expression is seen in the center. Scale bar 50µm. (**b-j**) Calcium responses to 15 or 30s of odor (3-Octanol or 4-methyl-cyclohexanol) or 60s of foot shock (10-70µA, 1.5s repeated 13 times) recorded in OK107/GCaMP3 (**b-d**) Amon;GCaMP3 (**f-g**) and OK107/GCaMP3.NLS (**h-j**) flies. Traces in b, f and h show the average Delta (F)/F0 of all flies for the most responsive region of interest. Histograms in c, g and i show the n value, mean and standard error of the area under the curve during stimulation (integral, AUC). Scatter plots in d and j show the individual AUC results from all flies where data measurements from both *PI* and KCs were recorded. *PI* responses are normalized to the KC responses. (**e**) Images show the expression of GCaMP3 in an Amon;GCaMP3 fly at a shallow and a deep focal plane. Scale bar 50µm (Data was provided by Dr. C.P.Bengtson and M.Sc.C.Schäfer for Hörtzsch et al., 2016 in revision).

It emerges that *PI* cells are strongly and apparently more directly activated by the US than Kenyon cells that are known to receive aversive and appetitive US information via dopaminergic signaling pathways (Aso et al., 2014a; Aso et al., 2012; Aso et al., 2010; Liu et al., 2012a; Mao and Davis, 2009; Riemensperger et al., 2005; Waddell, 2013; Yamagata et al., 2015). In contrast, information about the CS is more directly relayed from the primary olfactory processing center, the antennal lobes to the MB and the lateral horn (Jefferis et al., 2007; Stocker et al., 1990; Wong et al., 2002) explaining the significantly larger odor responses in Kenyon cells relative to *PI* cells (Fig. 5.3c). These results indicate that the US-triggered activation of subsets of *PI* neurons might play a role in processing the aversive stimuli.

The *PI* and the downstream neuropeptidergic signaling systems have been implicated in the regulation of several behaviors and processes (Nassel and Winther, 2010) including sleep (Crocker and Sehgal, 2010; Crocker et al., 2010; Foltenyi et al., 2007), sexual behavior (Belgacem and Martin, 2002; Belgacem and Martin, 2006; Gatti et al., 2000), metabolism (Broughton et al., 2005), circadian behavior (Cavanaugh et al., 2014), aggression (Davis et al., 2014), development and ecdysis (Ewer, 2005). Of note is the apparent involvement of neuroendocrine signaling in olfactory information processing at the level of local interneurons in the antennal lobes where neuropeptide signaling seems to adjust the dynamic ranges and sensitivities towards certain odors (Carlsson et al., 2010; Ignell et al., 2009; Winther and Ignell, 2010). A potential involvement of neuropeptidergic signaling in processing the aversive US, however, has so far not been observed.

5.3.2 Activation of subsets of neuropeptidergic cells in fly brains by US and CS stimuli

The neuropeptidergic system of insects is a widely dispersed system of neurons and neurosecretory cells that are in part grouped in specialized structures like the *PI* and the downstream glands of the *corpora cardiaca* and *corpora allata*, but individual neuropeptidergic neurons are found widespread throughout the fly's brain (Johard et al., 2008; Nassel and Winther, 2010). Often, neuropeptidergic signaling is organized in a hierarchical manner such that a specific spatio-temporal sequence of different peptide release events is triggered to coordinate all individual steps of complex behaviors such as the ecdysis behavior (Kim et al., 2006; Žitňan et al., 2007) or the feeding behavior (Nassel and Winther,

2010). We therefore analyzed whether, apart from the *PI,* also other cells of the fly's neuropeptidergic system are sensitive to US and/or odor stimulation.

Similar to the principal response profiles of the dorsal *PI* cells we found at various deeper planes of focus (Fig. 5.3e) robust cytoplasmic calcium transients that were time locked to US-stimuli (lower panel in Fig. 5.3f-g). Some of these not further specified neuropeptidergic cells showed also weak odor responses (Fig. 5.3f-g). These observations suggest that the stimuli that are used as CS and US in aversive olfactory conditioning experiments result in a widespread activation of neuropeptidergic cells. This widespread activation is particularly prominent and temporally accurate upon US stimulation indicating that this activity pattern and perhaps an associated neuropeptidergic signaling is involved in processing the aversive US and to a lesser extent in processing the CS. Whether the coincidence of CS and US alters these response profiles is currently unclear and needs to be analyzed in detail in future studies.

5.3.3 The aversive unconditioned stimulus triggers robust nuclear calcium signaling in neurons of the *pars intercerebralis*

Nuclear calcium signaling has been well established as a mediator of lasting cellular adaptations by triggering appropriate genomic programs and cellular responses (Bading, 2013; Hardingham and Bading, 2010) affecting neuronal survival (Ahlgren et al., 2014; Lau et al., 2015), morphology (Mauceri et al., 2015) and synaptic organization (Hayer and Bading, 2015) and it is involved in learning and memory (Limback-Stokin et al., 2004; Weislogel et al., 2013), persistent pain (Simonetti et al., 2013) and ageing (Oliveira et al., 2012). In *Drosophila* we have shown that the formation of long-term aversive olfactory memory requires nuclear calcium signaling in subsets of MB and *PI* neurons which are both targeted by the Gal4-OK107 and 201Y fly lines (Weislogel et al., 2013). To assess whether CS and US stimuli are capable of triggering nuclear calcium-dependent processes also in neuropeptidergic neurons we targeted the calcium sensor GCaMP3 (Tian et al., 2009) to nuclei of these cells (right panel in Fig. 5.3a).

We found that odors alone failed to evoke detectable nuclear calcium transient in neurons of the *PI* or in MBs (middle panel in Fig. 5.3h-i). In contrast, foot shocks evoked robust nuclear calcium signals in subsets of *PI* neurons (blue trace in lower panel of Fig. 5.3h, right bar in Fig. 5.3i) that were particularly prominent in our paired recordings from both structures of the

same brain (Fig. 5.3j). These observations indicate that, apart from a direct activation (the cytoplasmic signal) of subsets of *PI* and neuropeptidergic neurons, the US also triggers robust nuclear calcium signaling in these cells. This could indicate that neuropeptidergic cells undergo nuclear calcium-dependent lasting adaptations when they are confronted with repeated US exposures perhaps to ensure appropriate neuropeptidergic signaling.

This may be particularly relevant in aversive olfactory conditioning paradigms in which aversive stimuli are presented repeatedly (see DISCUSSION). Whether neuroendocrine activation is restricted to aversive stimuli or whether it is also involved in the processing of appetitive cues remains to be clarified.

5.4 Functional interference with the neuroendocrine system

Since our live imaging experiments revealed that the neurosecretory system of flies is activated by the same aversive stimuli that are applied as US in aversive olfactory conditioning experiments we decided to have a closer look on the neuroendocrine system and its potential role in memory formation. For such experiments we wished to interfere with the functional properties of the neuroendocrine system during e.g. conditioning experiments, requiring tools to target our interfering molecules specifically to neuroendocrine cells.

5.4.1 Amontillado-related Gal4-driver lines show differential expression patterns

In insects neuroendocrine signaling is primarily based on neuropeptides (Nassel and Winther, 2010) that are synthesized as precursor peptides (Andrews et al., 1987) from which mature neuropeptides are generated by several common processing steps (Wegener et al., 2011). A common maturation step for all neuropeptides in *Drosophila* is mediated by the homologue of the prohormone convertase 2 - Amontillado (Amon) (Rayburn et al., 2009; Rhea et al., 2010; Siekhaus and Fuller, 1999) that is required for the maturation of all fly neuropeptides (Wegener et al., 2011) and is therefore thought to be expressed in all neuropeptidergic neurons. Consistent with this we found that two independent Amon-specific Gal4-driver lines, namely amon-Gal4-91D (synthetic construct with 460bp of the putative amon promoter) (Rhea et al., 2010) and 386Y-Gal4 (enhancer trap insert into the promoter of the amon gene) (Taghert et al., 2001) expressed in *PI*-cells as well as in overlapping but not identical subsets of potential neurosecretory cells throughout the fly's brain (Fig. 5.4b-c).

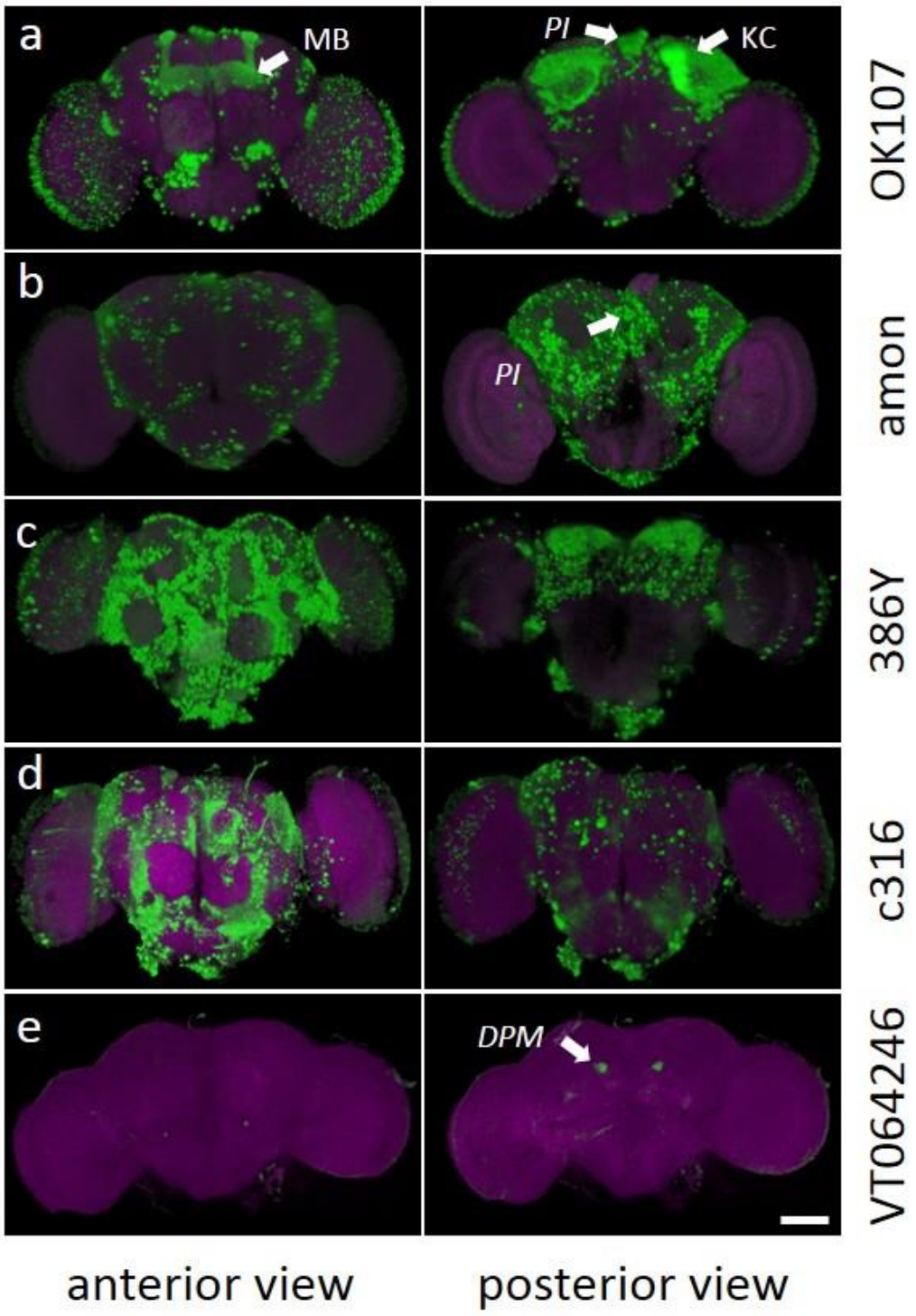


Fig. 5.4. Different expression patterns of Amontillado-related Gal4-driver lines. Confocal 3D-projections of Drosophila brains (anterior and posterior views) expressing GCaMP3-NLS in (**a**) OK107-Gal4 (**b**) amon-Gal4-91D (**c**) 386Y-Gal4 or (**d**) c316-Gal4 and (**e**) VT-064246-Gal4 cells. Immunolabeling of the cells with anti-GFP (green channel) and anti-Bruchpilot antibodies (magenta channel) show different transgenic expression patterns in cells belonging to the *PI* as well as in KCs of the MBs, *DPM*s and elsewhere. Scale bar: 50µm (Pictures by M.Sc.R.Geiger for Hörtzsch et al., 2016 in revision).

The expression profile of the OK107-Gal4 line (Fig. 5.4a) was consistent with our previous observations from the live imaging experiments (see 5.3) using GCaMP3.NLS (Fig. 5.3a) and the immunolabeling against membrane anchored GFP (mCD8::GFP) (upper panel in Fig. 5.1a) performed during the "Mushroom-Body-Mapping-Project" (see 5.1).

5.4.2 Suppressed nuclear calcium signaling in neuropeptidergic cells impairs all aversive memory phases except *ARM*

In order to address the potential relationship between nuclear calcium signaling in neuropeptidergic neurons and aversive olfactory memories we targeted CaMBP4 to subsets of neuropeptidergic cells and analyzed their behavioral consequences.

First, we used the widely expressing neuropeptidergic Amon-Gal4-91D driver (Fig. 5.4b) in our previously established Gal80^{ts} de-repression paradigm (see Materials and Methods 4.8.1) for conditional transgene expression exclusively in adult neuropeptidergic neurons. The results of the aversive olfactory conditioning procedures and the associated memory retention tests revealed that in these flies the formation of *LTM* was selectively impaired whereas all other memory phases (*STM, MTM* and *ARM*) remained unaffected (Fig. 5.5a). This is an intriguingly similar profile to that obtained previously (Fig. 5.1D) with the two independent Gal4-driver lines (OK107 and 201Y) expressing CaMBP4 in subsets of MB neurons but, notably, also in *PI* cells (Aso et al., 2009), indicating that the formation of *LTM* depends not only on intact nuclear calcium signaling in MB γ-lobes but also on neurosecretory cells outside of the MBs (see also Fig. 6.1).

In contrast, another widely expressing neuropeptidergic driver (Fig. 5.4c), 386Y-Gal4 (Taghert et al., 2001), left *ARM* and *LTM* unaffected upon CaMBP4 expression whereas *STM* and *MTM* were both strongly impaired (Fig. 5.5b). To ensure the flies' ability for proper reception of the presented odors and shocks and hence verifying that the observed deficits in *STM* and *MTM* formation in 386Y-Gal4 are not due to potential CaMBP4-induced deficits in sensory processing or failures in CS-US association caused by an impairment in the perception of the animals, sensory acuity controls were performed with this line together with a wild type strain serving as a control (Fig. 5.5c-h). These flies (both, the genetically modified as well as the wild type flies) first had to undergo the temperature treatment described earlier and were then tested with one of the odors against air to elicit if the flies showed correct odor reception.

Since both odors are slightly aversive the flies avoid them if possible, but when they are presented simultaneously the flies show no endogenous preference for one of the odors and distribute equally in the testing chambers. The second part of the acuity experiments were done in an approach in which air was applied from both sides of the T-maze, while in one of the testing chambers the shocks were presented to test correct US perception.

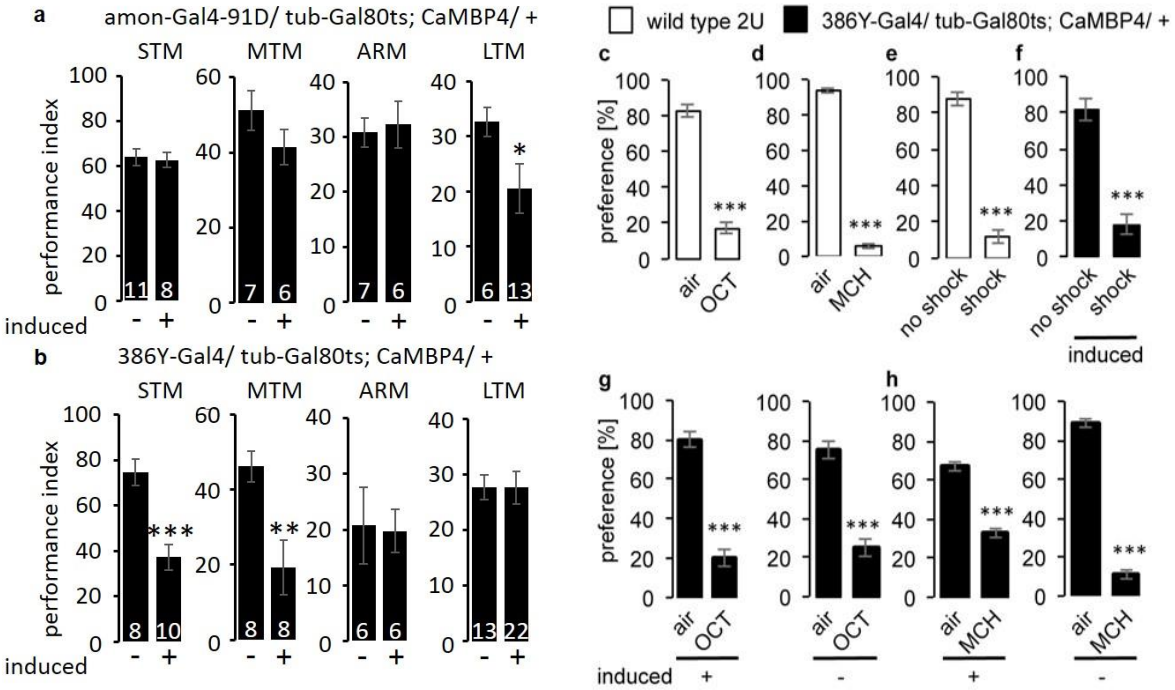


Fig. 5.5. Nuclear calcium signaling is required in different Amon cells for the formation of either *STM*, *MTM* or *LTM* formation. (**a-b**) Expression of the nuclear calcium inhibitor construct CaMBP4 in amon-Gal4 cells impairs only *LTM* formation while *STM*, *MTM* and *ARM* are not affected. Expression of CaMBP4 in 386Y-Gal4 cells affects only the formation of *STM* and *MTM* while *ARM* and *LTM* remain unaffected. (**c-h**) Sensory acuity controls for the different stimuli MCH & OCT (CS) and the electric foot shock (US) in which untrained flies had the choice between one of the odors and air or air without shock and air with shock. Note that wild type flies (white bars) avoided both CS's and the US with very high significance. Similarly strong avoidance was observed in induced (+) or untreated (-) 386Y-Gal4/ tub-Gal80ts; CaMBP4/ + flies (black bars), except that the MCH-avoidance was slightly weaker but still highly significant in CaMBP4 expressing flies (right panel in h). Data represent means ± SEM of the indicated number of conditioning experiments in a-b and n=6 in c-h. *p* values from Student's t-Tests between the pairs of data in each panel are indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0,001$) (Hörtzsch et al., 2016 in revision).

All of these tests finally were also performed with uninduced 386Y-Gal4 but not with uninduced wild type flies. All flies, independent of genotype and treatment (induced wild type and induced or uninduced 386Y-Gal4) avoided both odors as well as the shocks similarly (Fig. 5.5c-h). These results led to the conclusion that the reception of all stimuli were not affected

by the expression of CaMBP4 in a 386Y-Gal4 genetic background because the formation of *ARM* and *LTM* in these flies, as well as the formation of *STM*, *MTM* and *ARM* in amon-Gal4-91D flies was successful. These findings suggest that the observed defects were not due to incorrect perception and association of the stimuli but rather in the establishment and maintenance of the different memory phases.

Taken together these results extended our previous observations (see 5.1) in that the requirement for nuclear calcium signaling in learning and memory is not strictly confined to the formation of *LTM*. Depending on the driver used blocking nuclear calcium signaling can either interfere with the formation of aversive *LTM* or *STM/MTM.* Remarkably, CaMBP4 expression always left *ARM* unaffected*.* Furthermore, since *STM* is scored within a few minutes following single trial conditioning, a time course that is likely too fast for nuclear calcium-dependent downstream processes to already have behavioral effects, our observations suggest that US-triggered nuclear calcium signaling may not be directly involved in the formation of memory phases. It rather seems that nuclear calcium signaling may act indirectly by for example supporting processes in neuropeptidergic cells that are more directly involved in aversive memory phases except *ARM.*

Furthermore the observed defects in *STM* and *MTM* which we couldn't see in any of our former experiments with CaMBP4 (Fig. 5.1B-C) are most likely due to the different expression patterns of the driver lines. Because OK107-Gal4 and amon-Gal4-91D show expression in the *PI* but did not impair *STM* or *MTM* formation, the observed *STM* and *MTM* deficits seen in the 386Y-Gal4 fly line could have been be caused by the expression of CaMBP4 in other Amon positive cells which were not a part of the *PI* cell group labelled by the OK107-Gal4 or amon-Gal4-91D lines. Nonetheless *STM* defects in 386Y-Gal4/CaMBP4 flies seem unlikely to be caused by impaired nuclear calcium signaling since only a few minutes elapses between conditioning and testing which is not sufficient time for functional transcriptional and subsequent translational processes. This suggests that the observed *STM* impairments in 386Y-Gal4 line expressing CaMBP4 are rather indirect, non-specific effects of CaMBP4 expression.

5.4.3 The expression of Amontillado and 7b2 require nuclear calcium signaling

Following the discovery that suppressed nuclear calcium signaling in Amon positive cells has a negative effect on different aversive learning phases the question was raised if also the expression levels of molecules deriving from the neuropeptidergic system are nuclear calcium signaling dependent. This was addressed by a focused transcriptome analysis (see Materials and Methods 4.11) performed by B. sc. M. Müller at the nCounter core facility at the UniversitätsKlinikum Heidelberg (for general work flow see Fig. 5.6a) in which we attempted to quantify the mRNA expression levels of genes involved in neuroendocrine signaling (see Appendix-Table 1) in the presence or absence of the nuclear calcium signaling blocker CaMBP4. We therefore targeted CaMBP4 to all neuroendocrine cells using the widely expressing Amon-Gal4 driver (Fig. 5.4b) in a temporal and spatially controlled manner as performed before (see Materials and Methods 4.8.1 for induction protocol and Fig. 5.5a and 5.7b for behavioral analysis of Amon-Gal4). This prolonged high-temperature treatment provoking an elevated metabolism may affect the mRNA expression of many if not all genes. Therefore instead of using uninduced controls of the same genotype ("genotype controls"), we used as control group wild type flies that previously experienced the same high-temperature treatment as the CaMBP4-genotype (i.e. "treatment controls"). Furthermore, to challenge the neuroendocrine system we subjected all flies to be transcriptionally analyzed after 10 spaced repeats of the aversive conditioning trial followed by a 6h rest period before extracting the total RNA of 10 fly heads to make one RNA sample. Four such independent RNA samples (two from males and two from females) were used in our focused transcriptome analysis.

Our nCounter probes targeted 9 reference genes and 59 test genes (see Appendix Table1) including the housekeeping genes Gapdh1 and Rpl32 in the first group and in the test group the fly homologues of the nuclear calcium regulated genes *cfos* (*kajak*) and *atf3* (Bading et al., 1993; Curran and Morgan, 1995; Hudson and Goldstein, 2008; Matthews et al., 1994; Zhang et al., 2011; Zhang et al., 2009) as well as all fly genes encoding neuropeptides and genes involved in neuropeptide processing, such as Amontillado and its helper protein 7b2 (Rayburn et al., 2009; Rhea et al., 2010; Siekhaus and Fuller, 1999; Wegener et al., 2011). Our quantitative analysis of mRNA levels in the presence or absence of CaMBP4 expression revealed that all reference genes and 32 of the 59 test genes (i.e. 54.2 %) showed no significant

changes in their respective mRNA expression levels (Appendix Table 1), while 23 out of the 59 test genes (i.e. 39.0 %) showed significantly reduced mRNA expression in the presence of CaMBP4 and the remaining 4 of the 59 test genes (i.e. 6.8 %) showed significantly enhanced mRNA expression (Appendix Table 1).

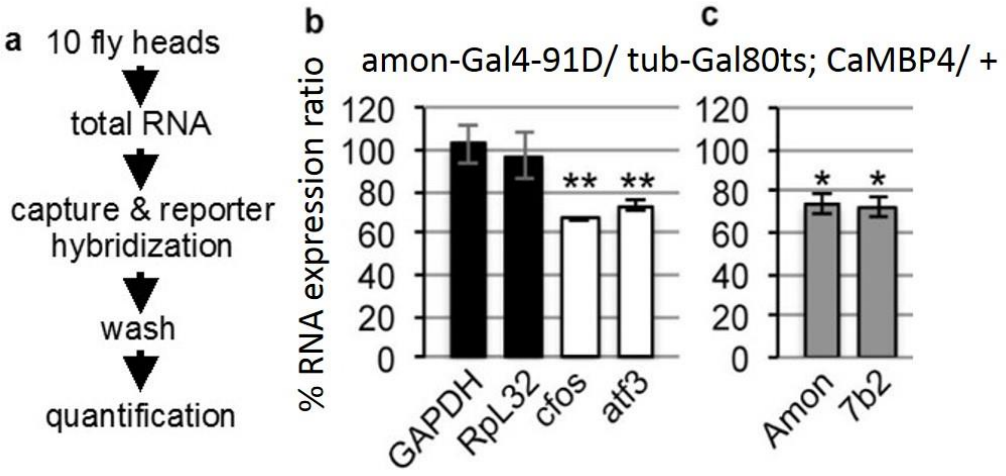


Fig. 5.6. Following aversive conditioning the mRNA-expression of Amon and its helper protein 7b2 requires nuclear calcium signaling. (**a**) Work flow of our focused transcriptome analysis (nCounter) of neuroendocrine genes in flies that have 6h before completed 10 x spaced aversive olfactory conditioning (**b-c**) Quantification of mRNA expression ratios (± SEM) from induced and trained amon-Gal4-91D/ tub-Gal80ts; CaMBP4/ + vs. induced and trained wild type fly brains (4 independent RNA samples from 10 fly heads each). Note that the housekeeping genes GAPDH and RpL32 show unaltered mRNA-expression levels in the presence or absence of CaMBP4 (black bars), while the nuclear calcium regulated genes *cfos* and *atf3* show significantly reduced mRNA expression in the presence of CaMBP4 (white bars). The mRNA-expression of Amon and its essential helper molecule 7b2 (Hwang et al., 2000) show a similar dependence on nuclear calcium signaling to that of *cfos* and *atf3*.

Figure 5.6b-c summarizes the results that are relevant for this study: consistent with data from mammals (Adler and Fink, 1993; Chen et al., 1996; Morgan and Curran, 1986; Qi et al., 1997; Tsujino et al., 2000; Xie et al., 2005) we found that the mRNA-expression of the *Drosophila* homologues of the immediate early genes *cfos (Kajak)* and *atf3* strongly depends on nuclear calcium signaling (white bars in Fig. 5.6b) whereas the expression levels of classical housekeeping genes, such as Gapdh1 and Rpl32 (Bading et al., 1993; Bettencourt et al., 2008; Nagendran and Hardy, 2011), remained unaltered (black bars in Fig. 5.6b). Importantly, the prohormon convertase PC2 Amontillado, that is required during the maturation of all neuropeptides (Wegener et al., 2011), and its essential helper protein 7b2 that is required for its maturation, activation and secretion (Hwang et al., 2000; MBIKAY et al., 2001) showed

significantly reduced mRNA expression levels in the presence of the nuclear calcium signaling blocker CaMBP4.

These results suggest that the expression of Amontillado in neurosecretory cells is regulated by nuclear calcium signaling. In addition, they provide insight into the potential cellular meaning of our observation that the aversive stimuli applied during aversive olfactory conditioning trigger nuclear calcium signaling and thereby Amontillado synthesis in neuroendocrine cells: enhanced Amon expression may be required to provide the neuroendocrine cells with sufficient amounts of freshly matured neuropeptides to perhaps replenish stores of neuropeptides (see DISCUSSION).

5.4.4 Amontillado is required for all aversive memory phases except *ARM*

It has recently been shown that knockdowns (KDs) or knockouts of the pro-hormone convertase Amontillado suppressed the maturation of all neuro-peptides (Rhea et al., 2010; Wegener et al., 2011) resulting in major defects in neuropeptide-dependent developmental processes and behaviors, such as molting and hatching (Rayburn et al., 2003; Siekhaus and Fuller, 1999), mating (Terhzaz et al., 2007) or aggression (Davis et al., 2014). We therefore chose the functionally verified Amon-RNAi-28b transgene (Davis et al., 2014; Rhea et al., 2010) for our Amon-KD experiments to analyze whether or not acutely suppressed neuropeptide maturation and thus suppressed neuropeptidergic signaling may affect the formation of aversive memory phases. Heat-shock mediated ubiquitous Amon-KD in adult flies, compared to its uninduced controls, resulted in a specific impairment of *LTM* whereas all other memory phases remained unaffected (Fig. 5.7a). This result was congruent with the prior observed results of HS-P26-Gal4 expressing CaMBP4, and first led to the presupposition that functional Amon activity is crucial, at least, in *LTM* formation. While these primary experiments were done we also crossed the $Gal80^{ts}$ construct into the Amon-RNAi reporter line to enable us spatio-temporal control of Amon-KDs exclusively in adult neuropeptidergic cells and therefore allow us to acutely interfere with neuropeptide maturation in a background free of potential developmental defects. Flies of these crossings which had not undergone the temperature treatment served as controls compared to their induced siblings of the same genotype.

When we targeted the Amon-KD with this new reporter line to neuropeptidergic cells using either the amon-Gal4-91D or the 386Y driver lines we found strong impairments in all memory

phases except *ARM* with both genotypes (Fig. 5.7b-c). The observation that *ARM* remained unaffected in all tested genotypes demonstrates that all Amon-KD flies are able to discriminate the two odors (CSs) and that they can correctly associate CS and US. Thus, aversive olfactory memories can be formed in flies with suppressed Amontillado functions, however, these memories are apparently independent of neuropeptide signaling. Conversely, all other memory phases, *STM, MTM* and *LTM*, seem to strictly depend on intact Amontillado functions indicating that these memory phases require mature neuropeptide signaling.

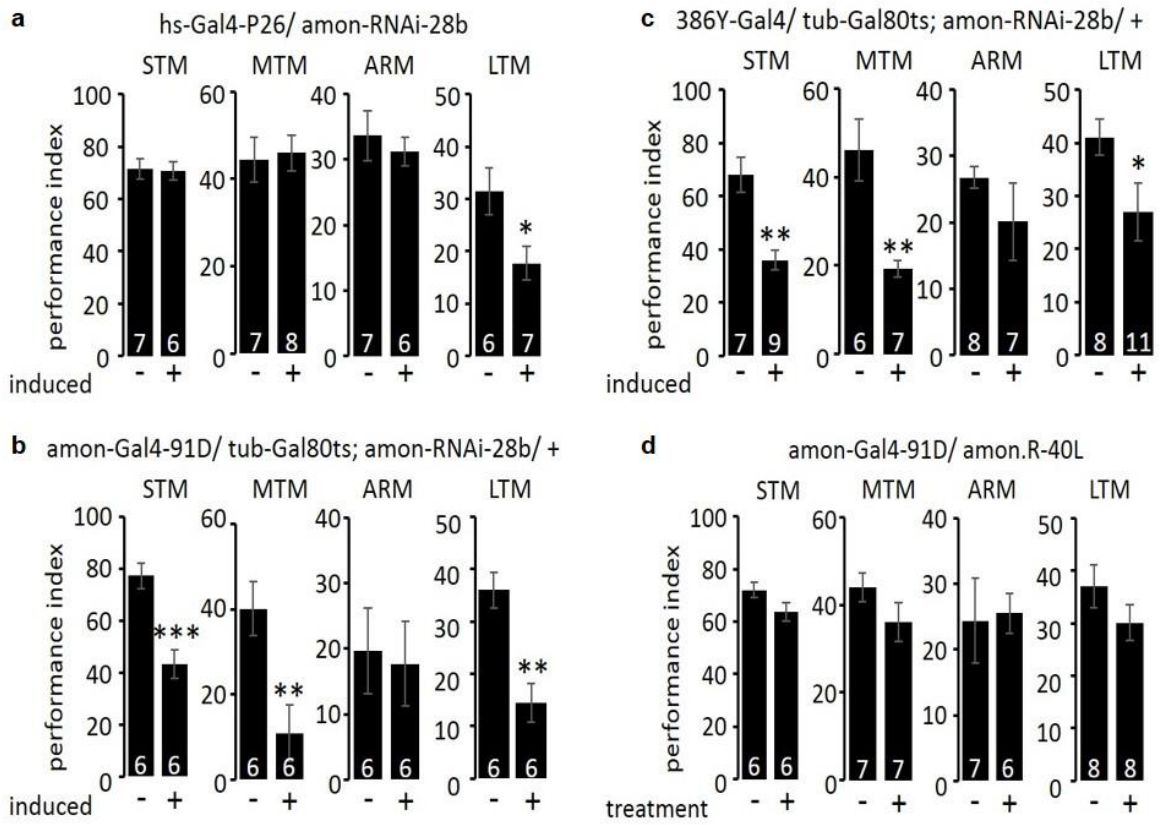


Fig. 5.7. Impaired Prohormone convertase activity can influence all types of memory except *ARM*. (**a**) Expression of prohormone convertase 2 Amontillado RNAi through heat shock Gal4 line P26 shows impaired memory only in *LTM* formation while all other memory phases remain intact. Expression of Amon-RNAi by heat induced de-repression in amon-Gal4 cells (**b**) or 386Y-Gal4 cells (**c**) impairs the formation of *STM*, *MTM* and *LTM*, while leaving *ARM* unaltered. Overexpression of Amontillado wild type RNA with amon-Gal4 shows no impairment in any of the memory phases and serves as control (**d**)**.** Data represent means ± SEM of the indicated number of conditioning experiments. *p* values from Student's t-Tests between the pairs of data in each panel are indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0,001$) (Hörtzsch et al., 2016 in revision).

Why didn't heat-shock driven ubiquitous Amon-KD result in *STM* and *MTM* impairments (Fig. 5.7a) as observed in our de-repression experiments with cell specific drivers (Fig. 5.7b-c)? The

difference in the outcomes of these two classes of experiments likely lies in the timing of the Amon-KD and its consequences on the amount of releasable mature neuropeptides. Neuropeptides are thought to be packaged into dense core vesicles in which they undergo final maturation steps turning them from propeptides to mature neuropeptides (see INTRODUCTION) while they are transported from the cell body to the periphery (Rao et al., 2001). Reaching their destination they are captured and stored at release sites before they are secreted at later time points (Bulgari et al., 2014; Shakiryanova et al., 2005; Wong et al., 2012).

It therefore seems likely that short lasting Amon-KDs, such as those triggered by heat shock induction 14h before conditioning, may leave enough mature neuropeptides in stores to be released during the first conditioning trial. This may explain the normal *STM* and *MTM* scores as well as the impaired *LTM* that may fail because of insufficient peptide replenishment and release during the 3h long spaced conditioning procedure. A longer lasting Amon-KD, such as that likely generated during the 6 days de-repression treatment, may result in depleted neuropeptide stores at the time of conditioning and will therefore impair all neuropeptide-dependent memory phases. Consistent with such a role of Amontillado in the replenishment of neuropeptide stores we found no memory effects when we overexpressed a functionally verified wild type Amontillado transgene (Rhea et al., 2010). For this we used again the amon-Gal4-91D driver to achieve permanent expression of Amontillado in all neuropetidergic cells (Fig. 5.7d). Half of the flies had to undergo the previously described temperature treatment for induction, resulting in elevated transgene expression before training and therefore also serving as treatment controls, while the other half of the flies remained at 18°C. Both groups were given 1h for acclimatization in the climate chamber prior to conditioning, as previously described (see Materials and Methods 4.5.2). None of the flies carrying the Amon overexpression construct showed any impairment in any of the memory phases tested, no matter if they were previously induced or not (Fig. 5.7d), showing clearly that an elevated level of Amon itself has no harmful effects on the flies' learning abilities.

Taken together it turned out that Amon function, and therefore correct neuropeptide maturation and secretion, has an influence on all aversive learning phases except *ARM*. This was the only memory phase showing no impairment after induction of the Amon-RNAi-28b transgene in HS-P26-Gal4, amon-Gal4-91D and 386Y-Gal4 flies, while *STM*, *MTM* and/or *LTM*

exhibited significant detriments in their performance indices. These results were observable in amon-Gal4-91D (Fig. 5.7b), as well as in 386Y-Gal4 (Fig. 5.7c) flies, revealing that the effects observed with CaMBP4 are even much stronger and more widespread when Amon function was knocked down. These findings indicate that accurate Amon function in Amon positive cells is more important for the correct generation of learning contents then proper nuclear calcium signaling in these cells. In summary these data strongly implicate a high impact of neuropeptide signaling in distinct cells in the establishment and perhaps maintenance and modification of all so far analyzed aversive memory phases except *ARM*, which appears to be resistant against most treatments.

5.4.5. Amon-KD in *DPM* neurons is sufficient to impair all aversive memory phases except *ARM*

A single aversive olfactory memory phase has previously been associated with neuropeptide signaling: *MTM* is thought to depend on the putative pituitary-adenylyl-cyclase-activating-peptide (PACAP)-like neuropeptide, Amnesiac (Amn) (DeZazzo et al., 1999; Feany and Quinn, 1995a; Quinn et al., 1979) that is released from two *dorsal paired medial* (*DPM*) neurons (Waddell et al., 2000). *DPM* neurons have been shown to receive input from MB neurons and they heavily innervate in two branches the vertical and horizontal lobes of the MBs (Keene et al., 2006; Krashes et al., 2007; Yu et al., 2005) forming a recurrent network with the fly's association center (Haynes et al., 2015; Krashes et al., 2007). Furthermore, *DPM* neurons are not only peptidergic but also serotonergic (Lee et al., 2011) and GABAergic (Haynes et al., 2015).

The GABAergic inhibitory interaction with Kenyon cell axons has been proposed to control sleep like states in flies and hence may link sleep with memory formation (Haynes et al., 2015) perhaps together with the directly coupled GABAergic and octopaminergic *anterior paired lateral* (*APL*) neurons (Liu and Davis, 2009; Wu et al., 2011b; Wu et al., 2013). The recurrent *DPM/MB*-network and its roles in sleep control (Haynes et al., 2015) and memory (Lee et al., 2011; Waddell et al., 2000; Yu et al., 2005) place this highly defined network into a prime position to serve as a general memory-relevant signaling channel (Keene et al., 2006). However, how much of this memory-relevant signaling of *DPM* cells is mediated by neuropeptides is, apart from *MTM*, currently unknown.

Neuropeptides are often co-released together with classical neurotransmitters at synapses however the postsynaptic effects of neuropeptides are slower and longer-lasting than those of classical transmitters (Nässel, 2009). Thus it is feasible that the AMN neuropeptide generates longer-lasting physiological effects within postsynaptic neurons that may lead to more permanent memory storage.

We therefore interfered with the neuropeptidergic output of *DPM* neurons by targeting Amon-KD to *DPM* neurons with two independent Gal4-drivers: c316 (Waddell et al., 2000) and VT064246 (Lee et al., 2011). The VT064246-driver is particularly interesting in this context as it has a superior selectivity for *DPM* neurons (Fig. 5.4e) whereas c316, despite being described as a "*DPM*-specific-driver" (Keene et al., 2004), targets several other neurons in addition, as documented by our confocal analysis (Fig. 5.4d).

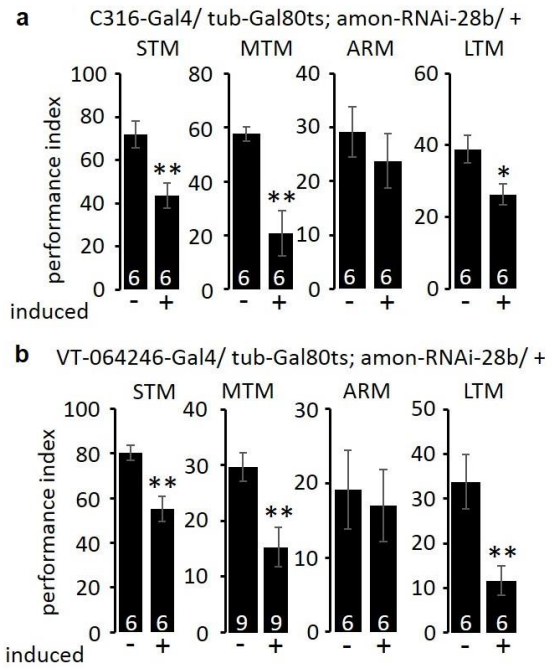


Fig. 5.8. Amontillado knock down in two peptidergic *DPM* neurons impairs all aversive memory phases except *ARM*. (**a**) Expression of Amon-RNAi by heat induced de-repression in subsets of *PI* and *DPM* neurons (c316-Gal4) or in *DPM* neurons alone (VT-064246-Gal4) (**b**) suppresses the formation of *STM*, *MTM* and *LTM* while leaving *ARM* unaltered. Data represent means ± SEM of the indicated number of conditioning experiments. *p* values from Student's t-Tests between the pairs of data in each panel are indicated by * ($p < 0.05$), ** ($p < 0.01$) (Hörtzsch et al., 2016 in revision).

Since *DPM* neurons release the AMN neuropeptide, we expected that knocking down neuropeptide maturation in *DPM* neurons would mimic the memory phenotype of the original

amn^{1} mutant (Quinn et al., 1979) which shows strongly impaired *MTM*, but no impairments in *STM* formation (Feany and Quinn, 1995b; Quinn et al., 1979). Therefore c316-Gal4 and VT-064246-Gal4 were supposed to be used as positive controls for *MTM* and negative controls for *STM* and *LTM* respectively. Intriguingly, we found that Amon-KD resulted in an almost identical pattern of memory impairments with both C316 and VT-064246 drivers, affecting all aversive memory phases except *ARM* (Fig. 5.8a-b). This is a similar picture as that seen with the other substantially more broadly expressing Gal4-driver lines amon-Gal4-91D and 386Y (Fig. 5.7b-c). Remarkably, the most selective *DPM* driver VT064246 showed either the same or even stronger memory deficits upon Amon-KD as any of the other lines, indicating that *DPM* neurons are likely the common mediators of the observed effects in all analyzed lines of the fly's neuropeptidergic system. These results strongly suggest that mature neuropeptide signaling from *DPM* neurons is necessary to allow the formation of all aversive olfactory memory phases except *ARM*.

5.4.6. Neuropeptide-dependent memories require mature neuropeptides in *DPM* neurons already during their acquisition

The finding that *DPM* neurons seem to be essential mediators of all neuropeptide-dependent aversive memory phases including *STM* (which is regularly tested within the first 6min after conditioning, depending on the procedures and set-up of the learning paradigm) appears to contradict previous reports showing that *DPM* neurons form a delayed memory trace after conditioning (Yu et al., 2005). Furthermore, synaptic transmission from *DPM* neurons has been shown to be dispensable for memory acquisition and *STM* and is apparently only required after conditioning to form *MTM* (Waddell et al., 2000). However, two distinct null mutants of the putative neuropeptide gene *amnesiac* (*amn*^{28A} and *amn*^{x8}) have been shown to not only impair *MTM* but also to affect learning and *STM* (DeZazzo et al., 1999; Moore et al., 1998; Waddell et al., 2000) even though their sensory abilities were normal (DeZazzo et al., 1999).

Although developmental influences of these constitutive mutants could not be excluded (DeZazzo et al., 1999) these data might support the idea that neuropeptide signaling is mechanistically distinct from synaptic signaling (Haynes et al., 2015) and that the putative neuropeptide Amnesiac or neuropeptide-signaling in general is required already during the acquisition of neuropeptide-dependent memories. Alternatively, it is also possible that

neuropeptides are indeed irrelevant during acquisition and that our routine *STM* scores in fact reflected impaired *MTM* behavior.

Considering these deliberations we asked ourselves whether or not a neuropeptide independent component of memory exists in a brief time window immediately after training, before neuropeptide signaling starts. Such a memory would link the odor cues of the conditioning treatment, encoded in action potentials in the neurons, to a "second messenger" like chemical signaling cascade. To differentiate between these possibilities we assessed the efficiency of memory acquisition in Amon-KD flies by scoring their odor choice behavior immediately following a single conditioning cycle (see Materials and Methods 4.5.2).

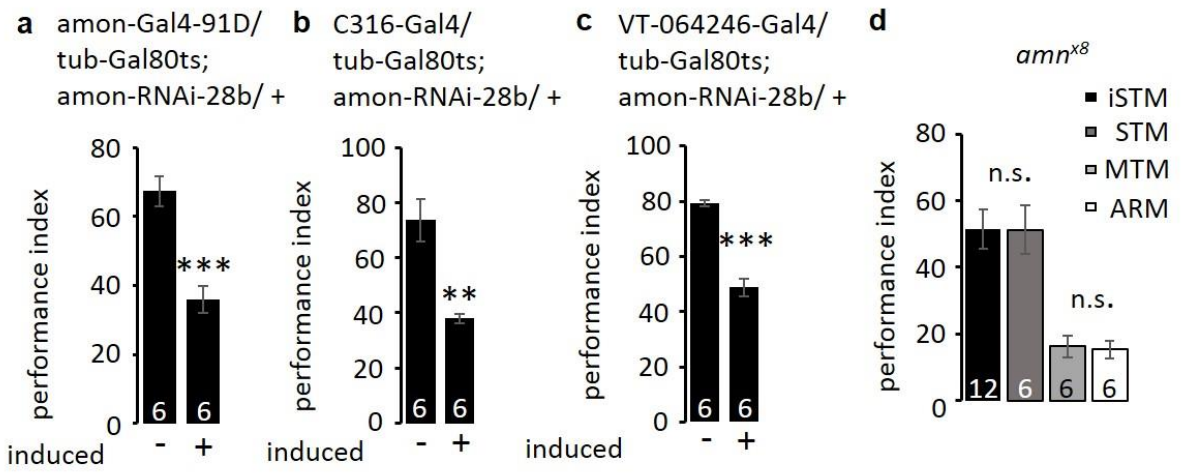


Fig. 5.9. Correct Amon function is crucial already during acquisition. *Immediate STM* (odor choice 30-40s after conditioning trial) shows a similar suppression of aversive olfactory conditioning upon Amon knock down in amon-Gal4 cells (**a**) or in *DPM* neurons (c316-Gal4 and VT-064246-Gal4) (**b** and **c**, respectively). The Amnesiac null mutant (*amn*^{x8}) shows no significantly different defects in all analyzed memory phases (**d**). Data represent means ± SEM from the indicated number of experiments. p values from Student's t-Tests between the pairs of data in each panel (**a**-**c**) and between *iSTM* vs.*STM* and *MTM* vs.*ARM* (**d**) are indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) (Hörtzsch et al., 2016 in revision).

Amon-KDs with either of the three neuropeptidergic drivers, namely amon-Gal4-91D, c316-Gal4 and VT-064246-Gal4, resulted in significantly impaired *iSTM* scores (Fig. 5.9a-c). In addition, the Amnesiac null mutant *amn*^{x8} showed in our hands very similar defects in all analyzed memory phases (Fig. 5.9d) as those obtained from Amon-KDs in *DPM* neurons (Fig. 5.9a-c, Fig. 5.7b and Fig. 5.8).

These findings strongly suggest that neuropeptide signaling in distinct Amon positive cells or more precisely in *DPM* neurons is required already during the acquisition of neuropeptide-dependent memories and that learning triggers two independent pathways of memory formation; a peptide independent and a dependent one.

Neuropeptidergic failures result in neuropeptide-independent memories that form in parallel to neuropeptide-dependent memory phases and that apparently differentiate into at least a rather efficient short-term component and a lasting longer-term (*ARM*) component (See DISCUSSION, Fig. 6.2), while in the presence of neuropeptides *STM*, *MTM* and subsequently *LTM* can form for permanent storage. However, while the responsible neuropeptide for *MTM* formation is known (a*mnesiac*) the ones which form the initial *STM* and the ones responsible for robust storage of memory in stable *LTM* still remain unclear.

5.5. *LTM* conditioning results in sex specific changes of different gene expression ratios

Despite the result from the first part of the nCounter experiment, that the expression levels of Amon and 7b2 are nuclear calcium signaling dependent (see 5.4.3 and Fig. 5.6), we had a closer look on the neuropeptide expression profile in general and thereby which genes involved in neuroendocrine signaling showed the most intense changes in their expression ratios. For this, we compared the expressed mRNA levels of naïve, untrained to trained wild type flies and also to induced and trained amon-Gal4-91D/ tub-Gal80^{ts}; CaMBP4/ + expressing flies to reveal which genes were up or downregulated 6h after the 10x spaced conditioning treatment (see Appendix Table1). This simultaneous approach of using wild type and genetically modified flies enabled us not only to observe the effects on the expression levels of different genes caused by the repeated presentation of the US, but also revealed the effects of suppressed nuclear calcium signaling in an Amon positive background on the regulatory processes in the neuroendocrine system. Finally also differences in the sex specific expression ratios were detected in this experiment, by using ten heads of each sex for separate samples (see 5.4.3). The probes we used for the nCounter targeted 9 reference genes (such as Gapdh1 and Rpl32) and 59 test genes, including all fly genes which encode for neuropeptides and/or play a role in neuropeptide processing (see Appendix Table1) (Rayburn et al., 2009; Rhea et al., 2010; Siekhaus and Fuller, 1999; Wegener et al., 2011).

The analysis of the nCounter assay revealed four candidate gene products namely dFmrf, Nplp3, Ccha2 and Acp70A which were of particular interest because they all showed intense changes in their expression ratios either through the conditioning procedure itself or through CaMBP4 expression preceding the conditioning (Fig. 5.10). Expression ratios are determined as the change of the expression level of the examined gene compared to the expression ratio of normalization genes (the most stable genes from a set of tested genes) after background correction.

5.5.1 Basic description of dFmrf expression ratios

In male flies the expression of dFmrf (Fig. 5.10a) was upregulated from 150 to 200 by the conditioning procedure and this effect was blocked completely through previous CaMBP4 expression. A similar increase in the expression ratio was observable in females (Fig. 5.10b) with the difference that the baseline expression of dFmrf is just one third compared to male flies (50 vs. 150), although the peak induced by conditioning is on the same high level for both sexes (200). The suppression of dFmrf expression through abundant CaMBP4 furthermore is not entire as it is in males and remains on a level around 80.

5.5.2 Basic description of Nplp3 expression ratios

The expression of Nplp3 was strongly reduced (from 110000 to 80000) by conditioning in males (Fig. 5.10c) and this effect was even stronger (down to a level of around 50000) following previous CaMBP4 expression. The same reduction phenomena was observable in females (Fig. 5.10d) with the difference that the baseline expression in unconditioned females is around 80000 (and therefore is on the same level as in males after conditioning) and it declined to 55000 through the conditioning procedure and remained on this extenuated level even when CaMBP4 was previously expressed.

5.5.3 Basic description of Ccha2 expression ratios

The expression level of Ccha2 in males (Fig. 5.10e) was upregulated from a value around 2200 to 2400 through the conditioning procedure and reduced to below 2100 when CaMBP4 was previously expressed. Interestingly in females (Fig. 5.10f) the conditioning treatment itself had no effects on the level of Ccha2 expression and the overall expression level remained at a

value half of the baseline expression of males (around 1000) but was increased about 200% (up to 3000) when CaMBP4 was abundant.

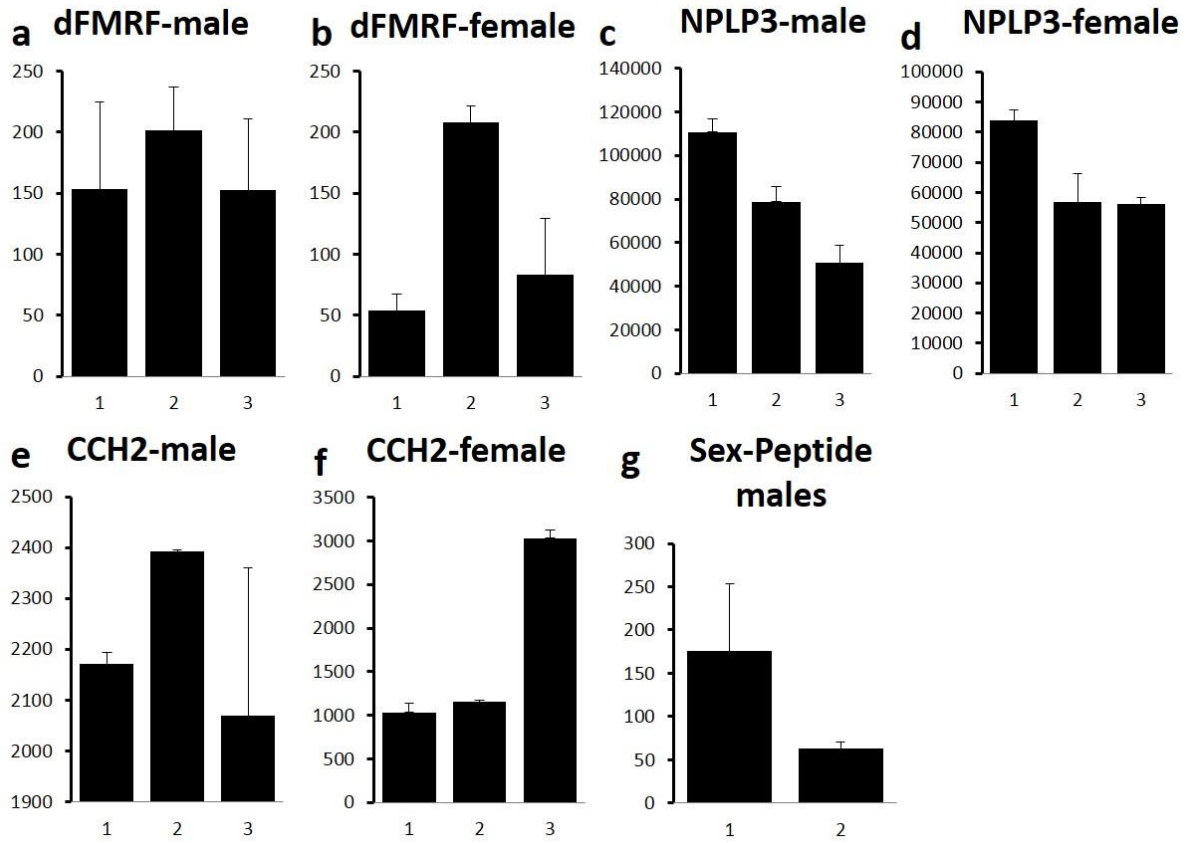


Fig. 5.10. Gene expression ratios are altered by *LTM* conditioning and are sex specific. Quantification of mRNA expression levels (2 samples per sex consisting of 10 heads each) of different genes in naïve (1) and *LTM* trained wild type flies (2) as well as in *LTM* trained amon-Gal4 91D/ tub-Gal80ts; CaMBP4/ + flies (6 days de-repression at 33°C) (3). Note that sex-peptide (Acp70-A) is only expressed in male flies.

5.5.4 Basic description of Acp70A expression ratios

The conditioning treatment resulted in a down-regulation of the expression level of Acp70A in males (Fig. 5.10g) to about two third of its original value (from around 180 to approximately 60). Furthermore Acp70A was down-regulated to a level below detection when CaMBP4 was abundant and is therefore not included in Figure 5.10g. Note that Acp70A is a male accessory gland peptide (Acp) and is therefore only expressed in male flies (Cirera and Aguadé, 1997; Kubli, 2003). Females, as expected, showed no detectable expression level of Acp70A.

5.6. Memory formation is not depending on a single neuropeptide but is rather encoded in combinatory interaction

After this preliminary analysis of the expression ratios of the four candidate genes we next crossed functionally verified RNAi lines from the Vienna Drosophila Resource Center (VDRC) with our heat shock inducible, ubiquitously expressing Gal4 line hs-P26 to perform Knock Downs (KD) of these genes, and tested these fly lines in our aversive olfactory conditioning experiments. This was done to get a closer insight on the influence of our target neuropeptides on the flies' learning abilities. For this the crosses were raised at 18°C to avoid leaky expression and 2-3 days after hatching half of the flies were induced by heat shock (as previously described) while their uninduced siblings served as control. Divergent from all previous performed learning experiments the flies were counted dioecious after freezing to discover sex specific differences in their learning performance. In addition sensory acuity controls for each crossing were performed to elicit the flies' abilities for proper odor reception, while correct shock reception was detected by preliminary, accurate performed *STM* trials. Further of note is the fact that beside the four RNAi-lines against dFmrf, Nplp3, Ccha2 and Acp70A also an overexpression line of the male accessory gland peptide Acp70A (Acp70A-G10) was used. This line served as a "gain of function" approach in males and in addition to visualize potential effects on learning and memory abilities when expressed in females. Since it is known that Acp70A is passed from males to females as a part of the seminal fluid and induces physiological and behavioral changes in the females after mating (Cirera and Aguadé, 1997; Liu and Kubli, 2003; Wigby and Chapman, 2005) such as reproductive behavior (Chen et al., 1988; Ueyama and Fuyama, 2003) and induction of ovulation (Chen, 1984) it was of particular interest to examine if Acp70A would also influence memory or conditioned odor avoidance behavior in female flies.

5.6.1. HS-P26-Gal4/ dFmrf-RNAi behavioral analysis

The dFmrf-RNAi (Ni et al., 2009; Perkins et al., 2009) KDs showed no significant difference in any of the tested memory phases, for either males (Fig. 5.11a) or females (Fig. 5.12b). Both sexes showed regular *STM* and *MTM* performance regardless of whether the transgene was induced. *LTM* capacity was generally slightly reduced in males in comparison to wild type flies

and improved mildly through dFRMF induction. In females *LTM* was not affected at all while *ARM* was mildly reduced in both sexes and improved slightly in induced females (Fig. 5.11b).

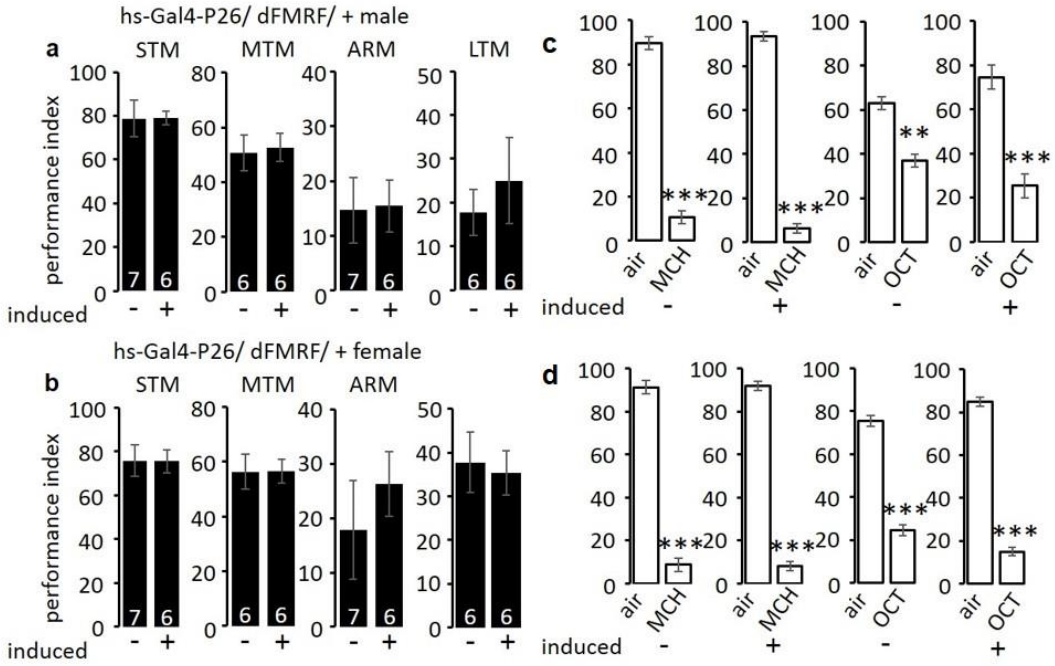


Fig. 5.11. Expression of dFmrf-RNAi has no significant impact on aversive learning. (**a-b**) Expression of dFmrf-RNAi with hs-P26-Gal4 shows in both sexes no significant impairment in any tested aversive learning phases when compared to uninduced controls. Sensory acuity controls for male (**c**) and female flies (**d**) for the different odors MCH & OCT (CS) in which untrained flies had the choice between one of the odors and air. Note that all flies – induced (+) or untreated (-) avoided both odors with very high significance. Except that the OCT-avoidance in uninduced flies was slightly weaker but still highly significant. Data represent means ± SEM of the indicated number of conditioning experiments in a-b and n=6 in c-d. *p* values from Student's t-Tests between the pairs of data in each panel are indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p <$ 0,001).

Odor perception tests revealed that males (Fig. 5.11c) avoided OCT less than females (Fig. 5.11d) and generally both sexes showed increased avoidance of OCT after induction and a higher tendency to avoid MCH than OCT. Still all tested flies avoided both odors with high significance no matter if they were induced or not. Perception of the electrical shocks is evidenced by its effect on *STM, MTM, ARM and LTM* performance.

5.6.2. HS-P26-Gal4/ Nplp3-RNAi behavioral analysis

Male flies expressing Nplp3-RNAi (Ni et al., 2009; Perkins et al., 2009) showed no significant impairment after induction in any of the tested memory phases (Fig. 5.12a), although *MTM*

and *ARM* where slightly reduced, while *STM* and *LTM* on the contrary were slightly increased after temperature treatment.

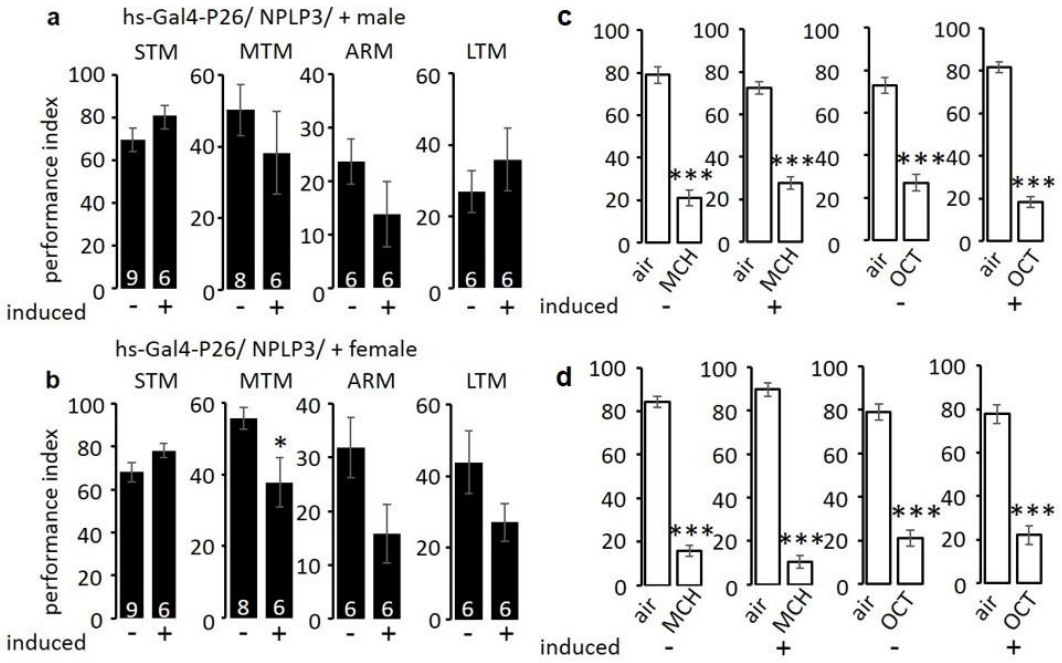


Fig. 5.12. Expression of Nplp3-RNAi shows a significant impact in *MTM* formation in females. (**a**) Expression of Nplp3-RNAi with hs-P26-Gal4 shows no significant impairment in any tested memory phase in males but reduced *MTM* in females (**b**). Females show no further significant impairment in any other tested aversive learning phase although *ARM* showed a non-significant trend toward impairment (p = 0.067). Sensory acuity controls for male (**c**) and female flies (**d**) for the the different odors MCH & OCT (CS) in which untrained flies had the choice between one of the odors and air. Note that all flies, induced (+) or untreated (-), avoided both odors with very high significance. Data represent means ± SEM of the indicated number of conditioning experiments in a-b and n=6 in c-d. *p* values from Student's t-Tests between the pairs of data in each panel are indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0,001$).

The same increased *STM* phenomena was also detectable in females, while all other memory phases decreased after induction (Fig. 5.12b) actually becoming significant in *MTM*, but not in *ARM* and *LTM* performance. Still, even after induction of Nplp3-RNAi females showed clearly detectable learning performances. Naïve, induced males (Fig. 5.12c) as well as females (Fig. 5.12d) avoided both odors with high significance in sensory acuity tests and were able to detect the electric shock correctly, as evidenced by high *STM* performance scores. The significant reduction in the *MTM* performance as well as the reduced (but not significant, p = 0.067) *ARM* scores from around 30 (uninduced) to 16 (induced) indicate that a knock down of the Nplp3-gene mimics the phenotype of the *amnesiac*^{x8} mutant in females. The Nplp3-RNAi

mutant showed comparable performance indices in these two memory phases to those of *amnesiac*^{x8} (see Fig. 5.9d). In contrast, the *STM* scores of Nplp3-RNAi mutants were much higher than those of *amnesiac*^{x8}, especially after induction of Nplp3-RNAi.

5.6.3. HS-P26-Gal4/ Ccha2-RNAi behavioral analysis

Both sexes showed regular *STM* and *MTM* performance no matter if they were induced or not although induction seemed to slightly improve the learning ability in these memory phases. Females (Fig. 5.14b) furthermore showed slightly decreased *ARM* and *LTM* performance indices in their uninduced state which improved clearly, but not significantly, after induction (p = 0.11 for *ARM* and p = 0.33 for *LTM*).

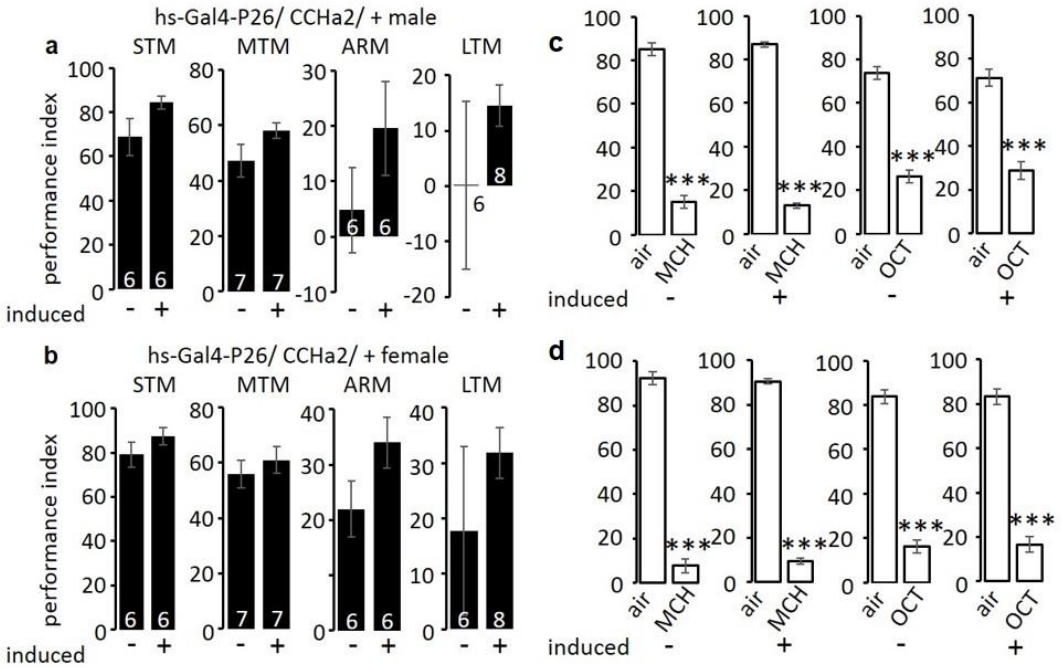


Fig. 5.13. Expression of Ccha2-RNAi has no significant impact on aversive learning. (**a**) Expression of Ccha2-RNAi with hs-P26-Gal4 shows reduced *ARM* and total blocked *LTM* performance in uninduced males, which both can be restored through induction. (**b**) Females show no impairment in any tested aversive learning phases when compared to uninduced controls. Sensory acuity controls for male (**c**) and female flies (**d**) for the different odors MCH & OCT (CS) in which untrained flies had the choice between one of the odors and air. Note that all flies – induced (+) or untreated (-) avoided both odors with very high significance. Except that the OCT-avoidance in male flies was slightly weaker than in females but still highly significant. Data represent means ± SEM of the indicated number of conditioning experiments in a-b and n=6 in c-d. *p* values from Student's t-Tests between the pairs of data in each panel are indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0,001$).

Interestingly the phenomena of improved memory performance after previous heat shock treatment to knock down Ccha2 (Keleman and Micheler; Ni et al., 2009) activity was consistent throughout all examined memory phases in this crossing. The most demonstrative example for this phenomenon is discernible in the *LTM* performance of male flies who showed no detectable performance scores at all in their uninduced state (Fig. 5.13a right panel). The odor perception tests revealed that naïve, untrained flies of both sexes no matter if they had undergone the induction treatment avoided both odors with high significance (males: Fig. 5.13c and females: Fig. 5.13d), although the OCT avoidance in males was slightly weaker than in females. Proper perception of the electrical shocks is evidenced by high *STM* performance scores in both sexes regardless of whether they were induced or not. The observation that males showed no detectable *LTM* in their uninduced state is difficult to explain as it was expected that the flies should show regular and wild type comparable *LTM* performance in their uninduced condition.

Although we could already detect decreased learning ability in the knock downs of dFmrf as well as Nplp3 in which male flies showed decreased *LTM* performance which improved after induction while their female counterparts performed similar to their wild type uninduced counterparts and showed decreased (Nplp3) learning abilities following induction (Fig. 5.12b), the effects observable in the Ccha2 crossing are much more severe and striking. Strong defects caused by leaky expression during the development of the flies are implausible since all other crossings which carried the hs-P26-Gal4 line as driver never showed comparable abnormalities under uninduced conditions. Taken together it is not really clear if the observed effects are caused by the expression or non-expression of the RNAi construct against Ccha2 or by the transgenic status of the flies *per se*.

5.6.4. HS-P26-Gal4/ Acp70A-RNAi behavioral analysis

Acp70A-RNAi (Ni et al., 2009; Perkins et al., 2009) expressing males showed regular *STM* performance no matter if they were induced or not and furthermore regular *MTM* performance if they were not induced. *MTM* decreased after induction, but not significantly ($p = 0.057$). *ARM* showed a general decrease which did not change after induction, while *LTM* was nearly gone in undinduced males and improved after induction Fig. 5.15a). This is in contrast to the results from tested females which showed decreased *ARM* and *LTM*

performance when induced but nearly regular performance scores when they were conditioned without temperature treatment. In addition *STM* and *MTM* in females was not affected at all no matter if they were induced or not (Fig. 5.15b). In the odor perception tasks all tested flies avoided both odors with high significance (males Fig. 5.15c and females Fig. 5.15d). Of note is that the avoidance behavior especially for OCT was even reinforced in both sexes by previous induction. The weakest avoidance conduction was observed in uninduced males when OCT was presented. Compared to all other tested avoidance behaviors it was the weakest but it was still significant. High *STM* performance in both sexes no matter if induced or not is a clear indication of shock perception and performance ability *per se*.

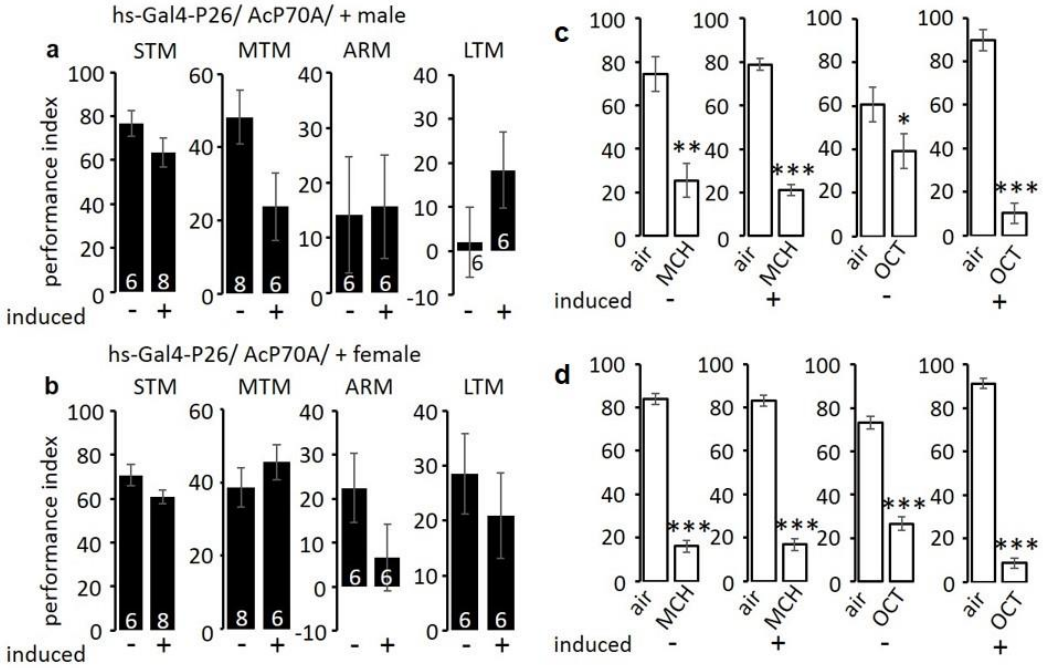


Fig. 5.14. Expression of Acp70A-RNAi has no significant impact on aversive learning. (**a**-**b**) Expression of Acp70A-RNAi with hs-P26-Gal4 shows in both sexes no significant impairment in any tested aversive learning phases when compared to uninduced controls. Sensory acuity controls for male (**c**) and female flies (**d**) for the different odors MCH & OCT (CS) in which untrained flies had the choice between one of the odors and air. Note that all flies – induced (+) or untreated (-) avoided both odors with very high significance. Except that the OCT-avoidance in uninduced flies was slightly weaker but still highly significant. Data represent means ± SEM of the indicated number of conditioning experiments in a-b and n=6 in c-d. *p* values from Student's t-Tests between the pairs of data in each panel are indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0,001$).

The increased *LTM* performance in males, after Acp70A was knocked down, is again not explainable. As mentioned before with Ccha2 (see 5.6.3) induction was instead expected to result in either decreased or unaffected learning scores. Why a diminution of a naturally

abundant neuropeptide results in an increase in avoidance behavior or memory formation is unclear. Furthermore, Acp70A KD caused in female flies a non-significant reduction in *ARM* ($p = 0.18$) and *LTM* ($p = 0.49$), a curious finding given that Acp70A is naturally only expressed in male flies (Chen, 1984; Cirera and Aguadé, 1997; Kubli, 2003). A knock down against a gene that does not exist in females, or is at least not transcriptionally active should regularly not result in any observable phenotype. Of note is also that all clearly observable improvements in *LTM* performances (except for Ccha2) are restricted to male flies while females seem to rather show the more logical explainable effect, namely a decrease in *LTM* (Figs. 5.11, 5.14). Beyond that, it also remains unclear why the knock downs of Acp70A and dFmrf resulted in such a drastic increased avoidance behavior of OCT in the sensory acuity tests, especially in male flies (Figs. 5.11c-d and 5.14c-d). This increased avoidance behavior raises the question if the knockdown of these neuropeptides triggers an increase in olfactory sensitivity and therefore underlies the increased performance scores in male *LTM*.

5.6.5. HS-P26-Gal4/ Acp70A-G10 behavioral analysis

Males showed regular *STM* and *LTM* performances which did not change through induction. *MTM* was slightly increased, while *ARM* was slightly decreased after temperature treatment but all changes remained not significant, demonstrating that the overexpression of the male sex-peptide Acp70A did not cause any effects on the learning ability in males (Fig. 5.16a). In females there were no changes observable considering *MTM*, *ARM* and *LTM* no matter if they had previously been induced or not, but *STM* was increased significantly after induction (Fig. 5.16b). Acp70A is regularly not abundant in females (Kubli, 2003; Ueyama and Fuyama, 2003), but is received together with the seminal fluid during mating and is known to be the responsible factor causing behavioral changes after fertilization (Liu and Kubli, 2003; Wigby and Chapman, 2005). Maybe the increased *STM* performances demonstrate a decreased risk behavior or alternatively an increased attention of the females provoked by a mimic of carrying freshly fertilized eggs. Although this effect is not lasting and showed no further influences on the other consolidated or non-consolidated tested memory phases (*MTM*, *ARM* & *LTM*). Acuity controls showed that both sexes avoided all odors with high significance, not depending on being previously induced or not (Fig. 5.15c for males and 5.15d for females). Correct shock reception was proven by high *STM* scores in both sexes.

Taken together these results indicate that none of the targeted neuropeptides alone seems to be the essential molecule responsible for either memory formation or the subsequent consolidation and storage of longer lasting forms of memory. Although some effects were observable no significant results could be detected in *LTM* dependent context in both sexes.

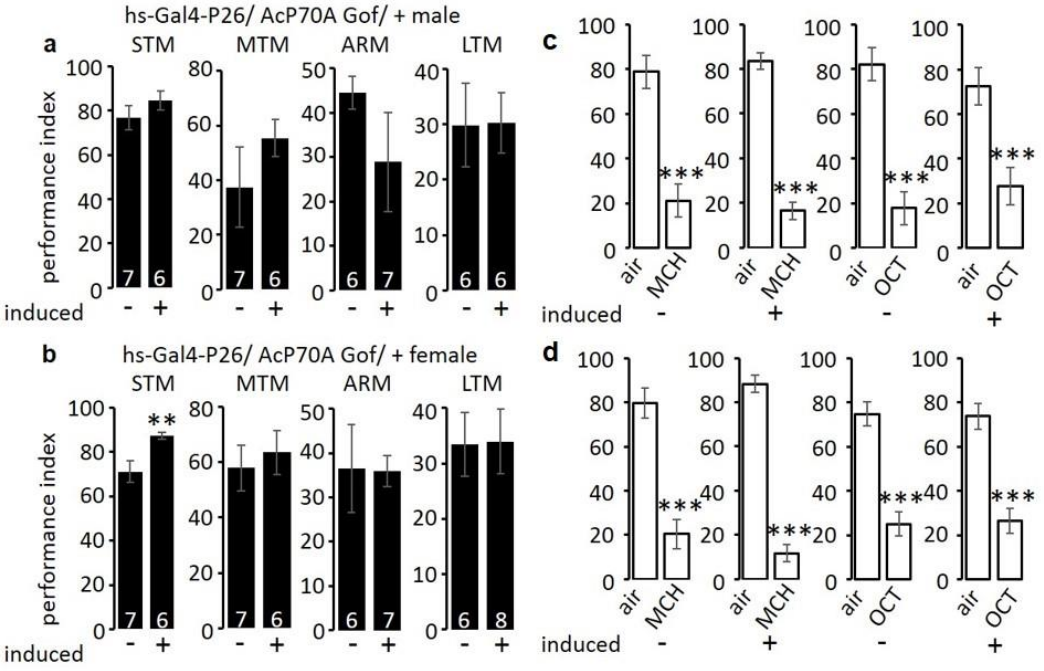


Fig. 5.15. Expression of Acp70A-G10 shows a significant improvement in *STM* formation in females. (**a**) Expression of Acp70A-G10 with hs-P26-Gal4 shows no significant impairment in any tested memory phase in males but increased *STM* in females (**b**). Females show no further impairment in any other tested aversive learning phase. Sensory acuity controls for male (**c**) and female flies (**d**) for the different odors MCH & OCT (CS) in which untrained flies had the choice between one of the odors and air. Note that all flies – induced (+) or untreated (-) avoided both odors with very high significance. Data represent means ± SEM of the indicated number of conditioning experiments in a-b and n=6 in c-d. *p* values from Student's t-Tests between the pairs of data in each panel are indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0,001$).

Still the experiments with previous knock downs against Ccha2 or Acp70A revealed astonishing influences on *LTM* formation especially in male flies (Figs. 5.13a, 5.14a) even if the observed effects are still not clearly explainable. *STM* was only affected in female flies expressing a male specific peptide (Acp70A) and this effect was furthermore not transferred to *MTM* or more consolidated memory forms such as *LTM* or the non-consolidated form of lasting memory (*ARM*) (Fig. 5.15b). The only other observable significant effect was, again in females, the decreased *MTM* performance after Nplp3-RNAi expression. Although the knock

down of the Nplp3-gene in females showed also decreased *ARM* and *LTM* scores after induction the observable effects remained not significant (Fig. 5.12b). Surprisingly male flies expressing Nplp3-RNAi showed contrary effects on their learning ability and got better in their *LTM* performance even if the improvements were also not significant (Fig. 5.12a). Furthermore all crossings in which male flies showed reduced ability to form *LTM* or *ARM* the effects never depended on previous induction, on the contrary induction and therefore knock down of the associated genes mostly resulted in subsequent elevated *LTM* scores.

This observation showed clearly that in those crossings the male flies exhibited no wild type comparable behavior and/or learning and memory capacity, even without previous expression of the corresponding RNAi, indicating that the detected impairments are rather caused by the changes in their underlying genetic background. In females the observable influences of knock downs on *LTM* were more diverse ranging from not detectable (dFmrf, Acp70A-G10) to increased (Ccha2) as well as decreased (Nplp3) memory formation. Nonetheless, as already mentioned, all of these changes in *LTM* and *ARM* performance in females, as well as the influence of knock downs of all tested genes in male flies in general, remained not significant.

5.7 MINOR

As a part of my attended PhD Program (Graduate Program in Translational Neuroscience) hosted by the Collaborative Research Centre 636: Learning and Memory, Implications for Psychopathology (SFB 636) I was expected to do a 'minor' in a field outside of my usual research area (see also: http://www.sfb636.de/sonderforschungsbereich-636.html). In this program doctoral candidates incorporated in projects rooted in basic sciences had to choose a clinical minor and had to visit a clinical unit for an elective. Conversely, postgraduates with a clinical focus had to perform some basic research in a laboratory. For my minor I joined the "Project Group C: Experimental Psychopathology" in the department of Cognitive and Clinical Neuroscience (C01 - Learning and brain plasticity in posttraumatic stress disorder: determinants of risk and the role of cue and context conditioning) hosted by Prof. Dr. Herta Flor at the Central Institute of Mental Health (ZI) in Mannheim, Germany (for additional information see: https://www.zi-mannheim.de/en.html).

The goal of this group was the analysis of learning processes and plastic brain changes in the development and maintenance of posttraumatic stress disorder (PTSD). In a longitudinal study

a high risk sample for PTSD was examined with respect to fear memory and stress sensitivity. Two cross-sectional studies which were focused on emotional/attentional mechanisms related to cue/context processing and the relationship of cued/contextual fear and inhibitory/excitatory mechanisms in extinction and reconsolidation in traumatized persons with and without PTSD were realized. This was achieved using Psychometric, psychophysiological, neuroimaging, electroencephalographic, eye tracking, endocrine and genetic methods (for details see: http://www.sfb636.de/29.html?&L=1).

5.7.1 Subject of minor

Attention, perception, learning and memory processes for affective cue vs. context material in PTSD

5.7.2 Description

The minor part of my thesis was conducted in the department of Cognitive and Clinical Neuroscience under the supervision of Professor Herta Flor, head of the department and Francesca Zidda, PhD candidate. Through this experience I got insights and provided help in the process of collecting data for a project aimed at investigating risk factors and to elucidate mechanisms involved in the development and maintenance of posttraumatic stress disorder (PTSD). Data were collected from PTSD patients and from two control groups, trauma-naïve and trauma-exposed healthy controls that were able to deal with the traumatic experience without developing the disorder. The collection of the data was done with various different cognitive testing procedures shortly described below. Some subtests of the Cambridge Neuropsychological Test Automated Battery (CANTAB) were used in order to assess visuo-spatial memory. The California Verbal Learning Test (CVLT) which measures episodic verbal learning and memory capacities. The Culture Fair Intelligence Test (CFT-20) to measure intelligence through recognizing patterns in different groups of images. The Mehrfachwahl-Wortschatz-Intelligenz-Test (MWT-B) which serves as a tool to measure general intelligence and is based on words. The Kurztest für Allgemeine Intelligenz (KAI), which is the second general intelligence test and is based on letters and numbers. Finally, also the CUBE and PAPER Test (Grundintelligenztest Skala 2) which gives a measure of general intelligence while its focus lies on measuring visuospatial processing in a multiple-choice format without a praxis component.

In addition to the neuropsychological assessment, axis I and II disorder as well as trauma specific diagnostic (in the traumatized groups) were evaluated. For these purposes, the Strukturiertes Klinisches Interview für DSM IV (SKID I; Wittchen et al., 1997 and II; Fydrich et al., 1997) was administered followed by (when applicable) the German version of the Posttraumatic Stress Diagnostic Scale (PDS), the German version of the Clinician-Administered PTSD Scale (CAPS) and the German version of the Childhood Trauma Questionnaire (CTQ; Gast et al., 2001). To assess comorbid depressive symptoms the German version of the Center for Epidemiological Studies Depression Scale (Allgemeine Depressionsskala, ADS-L; Hautzinger and Bailer, 1991) was used. Upon participant consensus in some cases, I was allowed to witness these interviews which helped me getting a deeper understanding of the everyday life problems and clinical consequences traumatized people have to face and how trained psychoterapists/psychologists estimate the severity of the trauma and its sequelae.

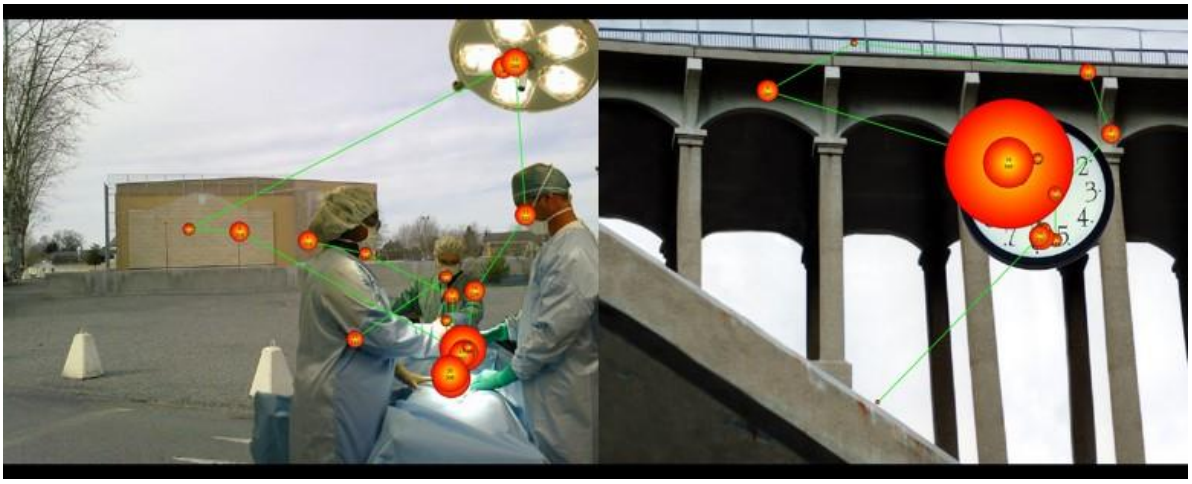


Fig. 5.16. Example pictures from the picture presentation. Pictures with neutral context and a negative, or trauma related cue (left) and a neutral cue (right). The circles are the outcome of the eye tracking data and the size of the circles correlate on the time the subject has looked at these spots during the presentation of the picture.

Participants underwent in a second stage to a simultaneous EEG/Eye-tracker recording while focusing on a visual presentation. The picture set was made of 120 pictures and created using cues from the International Affective Picture System (IAPS) and context found on the internet which were rated as neutral in a separate sample (for example see Fig. 5.16). The 120 pictures were divided into 30 pictures for each affective category (positive, negative, trauma-related

and neutral). Each picture was presented for 6 seconds with a 3 second intertrial interval with a fixation cross to center the eyes before the next picture was presented. During the presentation the movement of the eyes of the subjects have been recorded with an eye tracker (to measure the focus of attention in spatial memory) and in addition an EEG was recorded simultaneously. On the next day a memory test was performed and the participants were asked to decide if the picture was exactly the same as on the day before or if they just recognize the cue or the context but with a different pairing as on the day before.

I personally also joined the control group and participated in the whole procedure as a participant while being still naïve of the purpose of the study.

5.7.3 Objectives

Patients suffering from PTSD regularly show Hippocampal volume reductions (Karl et al., 2006; Kitayama et al., 2005) and functional impairments. They are often described as showing fear responses to trauma reminders outside of contexts in which these cues would reasonably predict danger (see Fig. 5.17). Traumatized people start to generalize their traumatic experience in every type of context which leads to re-experiencing of the eliciting traumatic event, chronic hyperarousal, avoidance behaviors and negative alterations in cognition and mood (Association). Fear learning or aversive associative learning is in this case not anymore a helpful or adaptive behavior because the patients can't differentiate anymore if there is a real threat or not (Acheson et al., 2012). In theory traumatized people have in general problems with attention (if the cue is trauma related), therefore the intelligence and cognitive tests (visual and spatial) were performed to see if their memory is impaired in general or just with trauma-related material. Although many neuropsychological studies have found deficits in verbal declarative memory in patients with PTSD (Buckley et al., 2000; Elzinga and Bremner, 2002; Gilbertson et al., 2001; Gilbertson et al., 2006) the expectation is that patients should perform regularly in non-trauma related tests and should show the same level of intelligence as the control group, but they should show a difference in processing with affective material and an impairment in correctly recognizing safe and dangerous contexts. For example patients could not be able to remember the contextual information related to trauma because they immediately start to avoid the cues so that there is no time for the brain to create a conjunctive contextual memory. This leads to the hypothesis that traumatized patients

suffering from PTSD are significantly impaired in processing contextual information in trauma related material.

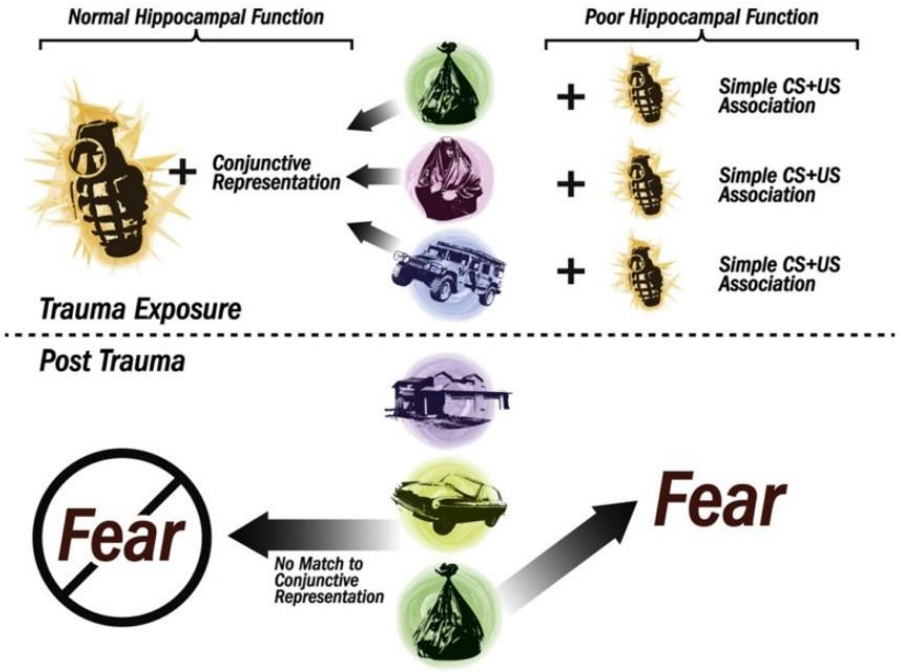


Fig. 5.17. Schematic representation of the role of hippocampal function in context fear memory. Here we suggest that a shift to a predominantly elemental strategy would allow elemental cues to have a much larger role in behavioral responses to the environment, with each discrete cue encoded during trauma able to induce conditioned fear responses across multiple contexts. The left side of the figure demonstrates how normal hippocampal function allows for the formation of a conjunctive context representation consisting of a combination of individual elements. This conjunctive representation is then associated with the traumatic event (in this case an exploding grenade). Upon later exposure to a single element of the original context (in this case the garbage bag), no fear response is triggered. The right side of the figure demonstrates how impaired hippocampal function precludes formation of a conjunctive representation. Instead, each individual element of the context is independently associated with the traumatic event. Due to this single-element association, later exposure to *only* the garbage bag (independent of other contextual elements) is then sufficient to trigger a fear response. (Figure and legend from Acheson et al., 2012)

Finding a method to overcome this lack of contextual information processing might be helpful in drawing novel therapeutic strategies which could lead to overcome the generalization phenomena and therefore to a decrease of PTSD symptoms.

5.7.4 Results

The study is currently ongoing and the datasets have not been finally analyzed up to now, which makes it impossible to draw any conclusions concerning the underlying working

hypothesis. Therefore also no incorporations between the results in my thesis and the data sets created during my minor can be generated.

6. DISCUSSION

6.1 Subsets of mushroom body and *pars intercerebralis* neurons carry a nuclear calcium-dependent *LTM*-trace

Nuclear calcium is one of the universal and evolutionary conserved key molecules in cell signaling which is responsible for a broad spectrum of transcription dependent adaptions (Bengtson and Bading, 2012; Hardingham and Bading, 2010). It is typically involved in orchestrating lasting cellular changes in a use dependent manner (Bading, 2013) by initiating appropriate genomic responses resulting in changes in gene expression crucial for the implementation of stable adjustments. These changes can subsequently lead to the formation of memory and neuronal survival as well as the development of chronic pain or addiction. In these processes, calcium signals induced by synaptic activity diffuse into the nucleus to engage the necessary genomic programs underlying these adaptive processes. In mammalian neurons, nuclear calcium acts primarily through calcium calmodulin-dependent protein kinases (CaMKs) and subsequently controls the activity of CREB/CBP (Chawla et al., 1998; Corcoran and Means, 2001; Hardingham et al., 1999; Hardingham et al., 1997; Impey et al., 2002) and methyl-CpG binding protein 2 (MeCP2) (Buchthal et al., 2012). The nuclear calcium regulated gene pool in mouse hippocampal neurons contains approximately 200 genes, of which many are known or putative CREB/CBP targets. These genes play key roles in acquired neuroprotection, memory consolidation and the development of chronic inflammatory pain (Mauceri et al., 2011; Oliveira et al., 2012; Simonetti et al., 2013; Zhang et al., 2011; Zhang et al., 2007; Zhang et al., 2009). The activation of synaptic NMDA receptors is important for the initiation of the nuclear Ca^{2+}/CaMK-CREB/CBP signaling module after synaptic stimulation (Hardingham et al., 2001; Hardingham and Bading, 2010). Deficits in nuclear calcium signaling may therefore be be a common factor underlying various diseases, including neurodegeneration and cognitive dysfunction (see also 3.3.3).

“The nuclear invasion of calcium transients represents a signaling end point of synaptic activity that — according to the ‘nuclear calcium hypothesis’ (Bading, 2000) — is a common requirement in diverse forms of persistent neuroadaptations. The concept of nuclear calcium signaling offers a beautifully simple process to mirror stimulus strength in the nucleus and

provides the basis for a reliable genomic switch for the progression of adaptations from labile to long-lasting forms." (Bading, 2013)

In *Drosophila melanogaster*, a similar pathway may link nuclear calcium signaling to *LTM* formation. Suppression of NMDA receptor, CaMKII, or CREB function in MB α/β or γ neurons blocks the transcription dependent formation of *LTM* traces in a time and cell specific manner (Akalal et al., 2010; Miyashita et al., 2012; Perazzona et al., 2004; Tully et al., 1994a; Yu et al., 2006). This is consistent with other studies which showed that the activation of NMDA receptors in Kenyon cells generates a calcium signal that, at least in MB γ neurons, may be transmitted into the nuclei to activate a potential nuclear isoform of CaMKII (Griffith et al., 1993). CaMKs target CREB and play a well-documented role in the formation of *LTM* in flies (Akalal et al., 2010; Perazzona et al., 2004; Yin et al., 1994; Yu et al., 2006). Thus, it seems plausible that an evolutionarily conserved nuclear Ca^{2+}/CaMK-CREB signaling module may not only be the primarily responsible pathway for the induction of genetic programs associated with learning in vertebrate but also in invertebrate neurons.

Various recent studies have tracked distinct memory traces in the fly brain to certain subregions of the MB as well as other brain regions. This includes short-lived memory traces located in the antennal lobe (Yu et al., 2004) and the γ neurons of the MB (Blum et al., 2009), middle-term and slightly delayed memory traces in *DPM* neurons and α'/β' mushroom body neurons (Wang et al., 2008; Yu et al., 2005) besides *LTM* traces which have been mapped to the α/β and the γ branches of the mushroom bodies (Akalal et al., 2010, 2011; Blum et al., 2009; Yu et al., 2006). Notwithstanding also other cells, such as the two dorsal-anterior-lateral (*DAL*) neurons (Chen et al., 2012), two pairs of *PPL1* dopaminergic neurons (Plaçais et al., 2012), *crammer* (*cer*)-expressing glial cells surrounding the Kenyon cell bodies (Comas et al., 2004), or NMDA receptor-expressing neurons in the ellipsoid body of the central complex (Wu et al., 2007) have been shown to be required for memory consolidation. Taken together, these findings suggest that different forms of memory and memory phases are encoded in different brain regions and structures (Weislogel et al., 2013).

In the first part of my study we provided evidence that nuclear calcium signaling in γ neurons of the MB is responsible for the transformation of the freshly acquired experiences during spaced olfactory avoidance conditioning into transcription-dependent *LTM.* At the same time,

these signaling pathways to the nucleus were expendable for the formation of transcription independent, short-lasting forms of memory like *STM* and *MTM* and also for the transcription independent but consolidated form of long-lasting memory *ARM* (see Fig. 5.1 B-C and Weislogel et al., 2013). Thus, intact nuclear calcium signaling was needed for the progression of these olfactory memories from short-lived to long-lasting and transcription-dependent forms (see 5.1). Transient decline in nuclear calcium signaling, induced through the nuclear calcium signaling inhibitor CaMBP4, did not lead to permanent damage of the flies' neurons, but rather acutely and transiently impaired the formation of olfactory avoidance *LTM* (see 5.2).

In addition, we identified in the α/β and γ neurons of MB a bisection in the requirements for cAMP signaling and nuclear calcium signaling. For *LTM* formation, α/β neurons (targeted by c739-Gal4) required intact cAMP signaling (Blum et al., 2009) but not nuclear calcium signaling (dark grey bars in Fig. 5.1 D-E). Conversely, in γ neurons of the MB (targeted by 201Y-Gal4), nuclear calcium was essential for the formation of *LTM* (black bars in Fig. 5.1 D-E), whereas cAMP signaling has been reported as dispensable (Blum et al., 2009). The α/β neurons may contribute to *LTM* through a mechanism that involves activation and nuclear translocation of cAMP-dependent protein kinase A and subsequent stimulation of CREB-dependent transcription (Akalal et al., 2010; Yu et al., 2006). In contrast, γ neurons may use nuclear calcium signaling, possibly to activate CaMKs, to control a potentially distinct transcriptional program. This could explain why *LTM* traces that consist of an enhancement of the stimulus-associated calcium influx are detectable 9-24h after spaced training in α/β neurons (Akalal et al., 2011; Wang et al., 2008) and 18-48h after training in γ neurons (Akalal et al., 2010). In summary, it seems that distinct subsets of mushroom body neurons, previously implicated in *LTM*, use distinct different signaling cascades to control the appropriate *LTM*-associated genomic responses (Weislogel et al., 2013).

Although MB γ neurons may represent key cells in nuclear calcium-dependent memory consolidation, it is clear that they are not the only intrinsic component for *LTM* formation, as shown by our Amon-KD experiments and the cAMP dependency in α/β neurons.

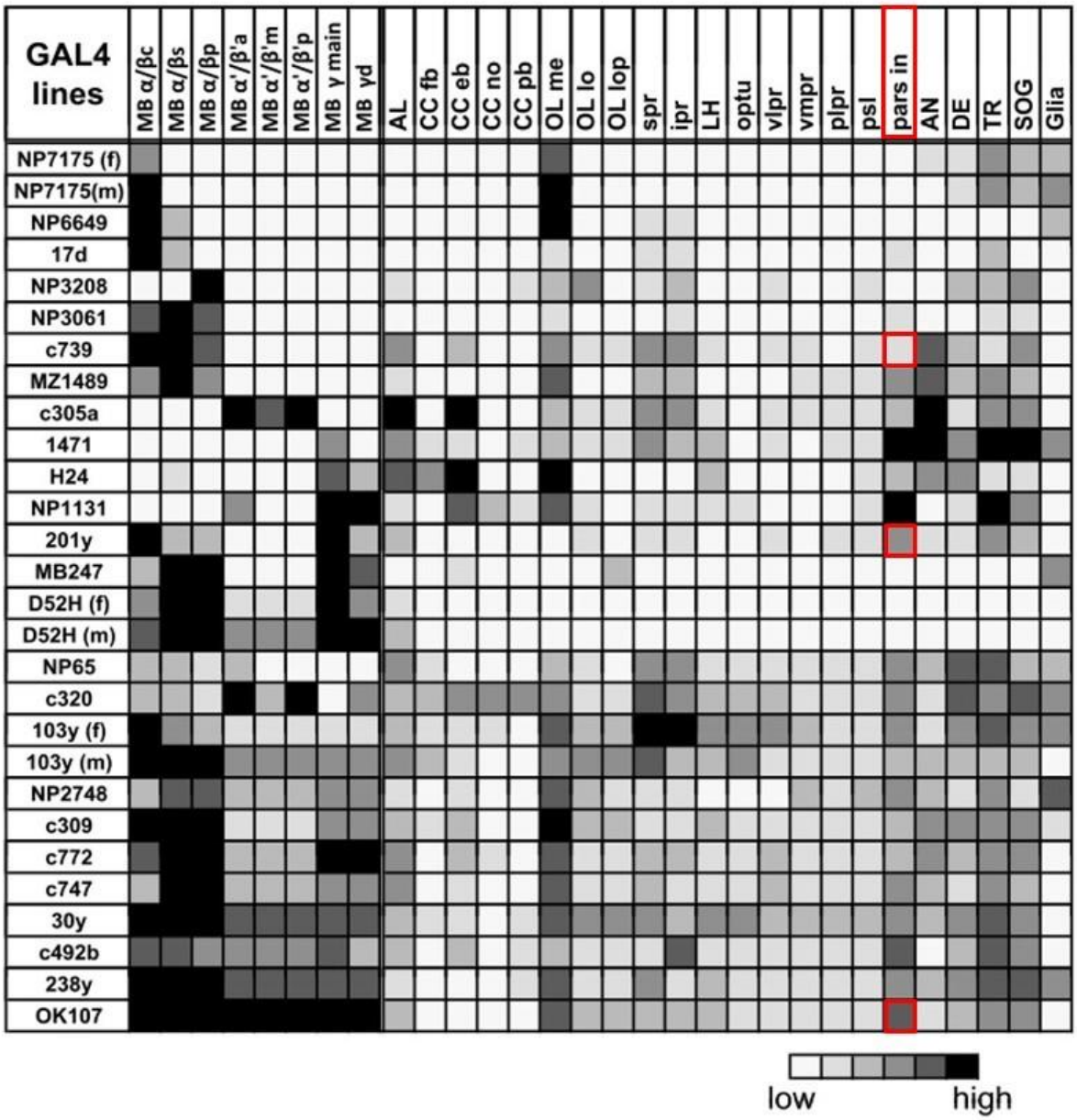


Fig. 6.1. Expression pattern of 25 GAL4 lines. Summary of the expression levels of 25 MB-GAL4s in various brain areas defined by anti-Synapsin immunostaining. Gray scale indicates subjectively evaluated signal intensity. Note that a higher level of fluorescent signals in the certain brain area can result from larger population of GAL4 expressing cells and/or stronger GAL4 expression in each cell. MB, mushroom body; c, core subdivision: s, surface subdivision; p, posterior subdivision; a, anterior subdivision; m, middle subdivision; p, posterior subdivision; d, dorsal subdivision; AL, antennal lobe; CC, central complex; fb, fan-shaped body; eb, ellipsoid body; no, noduli; pb, protocerebral bridge; OL, optic lobe; me, medulla; lo, lobula; lop, lobula plate; spr, superior protocerebrum; ipr, inferior protocerebrum; LH, lateral horn; optu, optic tubercle; vlpr, ventrolateral protocerebrum; plpr, posteriorlateral protocerebrum; vmpr, ventromedial protocerebrum; psl, posterior slope; pars in, *pars intercerebralis*; AN, antennal nerve; DE, deutocerebrum; TR, tritocerebrum; SOG, subesophageal ganglion. Note that OK107 and 201Y drive expression much stronger in *pars intercerebralis* cells then c739. (Figure and legend taken from Aso et al., 2009).

Furthermore, it should be noted that although all utilized Gal4 drivers (OK107, c739 and 201Y), except HS-P26-Gal4, in the MB mapping project were officially declared as ”mushroom body

specific“, recent studies (Aso et al., 2009) revealed that all of these lines drive more or less strong Gal4 expression additionally also in *pars intercerebralis* (*PI*) cells (the master structure of the neuroendocrine system) and elsewhere outside of the MB (see Fig. 6.1). The drivers which showed the most drastic decreased *LTM* learning capacity after CaMBP4 induction (OK107 and 201Y) showed simultaneously strong to very strong expression levels also in *PI* cells. In contrary, the driver which exhibited no defects in the formation of *LTM* after induction (c739) consistently showed nearly no detectable expression in *PI* cells.

The most scattered expression profile was detectable with the heat-shock Gal4 driver line P26 which showed not only observable shock-evoked nuclear calcium transients in small regions distributed brain wide (“random dot pattern”), but in addition also strong deficits in *LTM* formation (Fig. 5.2). Thus, it seems possible that interference with nuclear calcium signaling in regions other than the MB, for example in *PI* cells, may contribute to the *LTM* deficits that we observed.

Our calcium imaging experiments also revealed that the system wide activation of the neuropeptidergic system was associated in part with robust nuclear calcium signaling (Fig. 5.3). Furthermore, suppressed nuclear calcium signaling in different patterns within the neuropeptidergic system impaired all phases of the neuropeptide-dependent memory channel (Fig. 5.5a-b) in a similar manner to that observed in our Amon KD experiments (Fig. 5.7a-c, Fig. 5.8). Since nuclear calcium signaling is typically involved in orchestrating lasting cellular changes in a use dependent manner (Bading, 2013) it seems likely that the US-triggered nuclear calcium activation in neuropeptidergic cells results in appropriate transcriptional and translational changes in these cells to perhaps support or even boost their functional competence during continued stimulation. This would be consistent with the data from our nCounter analysis which proved that suppressed nuclear calcium signaling during conditioning can prohibit or alter normal physiological responses to repeated US exposure. This could explain why an acute blockade of nuclear calcium signaling in amon-Gal4 positive cells resulted in normal *STM* but impaired *LTM* (Fig. 5.5a) perhaps because the affected neurons failed to maintain appropriate neuropeptide signaling during the 10 spaced repeats of the conditioning procedure (see also 6.2). The impairments in *STM/MTM* formation (Fig. 5.5b) in 386Y-Gal4 positive cells on the other hand are an additional hint of the indirect nature of the observed effects under suppressed nuclear calcium signaling conditions. Calcium

signaling to the nucleus and subsequent transcription, translation and alterations in effector protein levels may require several hours suggesting that the US-induced, CaMBP4 sensitive molecular mediators cannot be the cause of the observed phenotypes occurring only minutes after conditioning in *STM* treatment. This underlines the theory that calcium serves a role in assuring the competence of the cells for neuropeptide signaling. However, whether this is indeed the case and which molecules and processes are controlled by US-triggered nuclear calcium signaling in neuropeptidergic cells, as well as the surprising result that in 386Y only *STM/MTM* and not *LTM* formation was affected, needs to be analyzed in future studies. Of note is that the experiments with suppressed nuclear calcium signaling revealed the same effects as all of our Amon-KD experiments, in the sense that *ARM* always remained unaffected, while one or all *ASM* phases (depending on the driver) showed impairments.

In conclusion, we found that, similar to neuroadaptation in vertebrates, synaptic activity-driven calcium transients in the cell nucleus of distinct areas in the fly brain control distinct forms of memory and that the suppression of these transients can influence the formation of all kinds of *ASM* phases (*STM*, *MTM* & *LTM*). If the crucial demand for these transients is restricted to the γ lobes of MB, or *PI* cells, or only *DPM* neurons remains currently unclear. Besides it is also possible that correct nuclear calcium signaling is essential in all of these structures for normal *LTM*. To reveal a deeper insight into this question CaMBP4 has to be expressed selectively in *DPM* neurons, using the highly selective Gal4 driver VT-064246 and in addition, a parallel precise imaging study of OK107-Gal4, 201Y-Gal4, amon-Gal4, c316-Gal4 and 386Y-Gal4 in combination with VT-064246, using the split Gal4 system (see 3.1), needs to be performed to shed light on the circumstance if these lines show deviating or overlapping expression patterns in *DPM* neurons or γ lobes of MB respectively.

[This first part of my Discussion contains excerpts which were also taken for Weislogel et al., 2013, written in collaboration with Prof. Dr. H.Bading, Prof. Dr. C.M.Schuster, Dr. J.M. Weislogel and Dr. C.P.Bengtson]

[The following second part of my Discussion contains parts which were also taken for Hörtzsch et al., 2016 (in revision) written in collaboration with Prof. Dr. C.M.Schuster]

6.2 Neuropeptide signaling from two *DPM* neurons is crucial for the formation of all *ASM* phases in *Drosophila*

6.2.1 Background anatomy and physiology of the neuropeptidergic pathways and their involvement in the formation of all *ASM* phases in *Drosophila*

Aversive olfactory memories form temporally discernable short-, middle- and long-term phases that in *Drosophila* have recently been shown to exist in two parallel channels (Bouzaiane et al., 2015; Isabel et al., 2004a; Plaçais et al., 2012). One of these memory channels is characterized by its resistance to cold anesthesia and the independence of *de novo* protein synthesis of its consolidated phase, *LT-ARM* (Tully et al., 1994a), whereas the other channel requires *de novo* protein synthesis for its consolidated phase, *LTM*. Proven by the fact that feeding cycloheximide, a translation blocker from *Streptomyces griseus*, to wild type flies results in failed *LTM* formation while *ARM* formation remains unaffected (Lagasse et al., 2009).

However, the molecular bases that may differentiate between these parallel aversive memory channels have so far been elusive. In this part of my study we showed that the acquisition and formation of *STM, MTM* and *LTM* strictly depend on the presence of mature neuropeptides in *DPM* neurons whereas all *ARM* phases are apparently independent of neuropeptides. Thus, aversive memory phases are formed in *Drosophila* by a neuropeptide-dependent and a parallel neuropeptide-independent channel (Fig. 6.2). It remains unclear if the observed *LTM* impairments in our blocked nuclear calcium signaling experiments (see 6.1) were caused by a separate γ-lobe specific defect or if it was due to insufficient neuropeptide release from neuropeptidergic cells during spaced conditioning.

Neuropeptides are part of the neuroendocrine signaling system that is generally considered to be involved in evaluating sensory information such that the organism is better prepared for appropriate responses. Consistent with the importance of such an evaluating role of the neuroendocrine system neuropeptide-dependent *LTM* is metabolically more costly (Mery and Kawecki, 2005) and lasts considerably longer than neuropeptide-independent *LT-ARM.* This suggests that long-lasting, perhaps life-long neuropeptide-dependent memories warrant the additional metabolic cost whereas the neuropeptide-independent memories have a more transient and stress-resistant content (Mery and Kawecki, 2005). These higher metabolic costs are directly displayed in the fact that establishment of stable *LTM* memory led to a decreased

life expectancy in male but not in female flies while improved *ARM* led to an increased life expectancy (Lagasse et al., 2012). Furthermore, the capacity to form one of these memory phases is inherited by the next generation, leading to a bias to one of the two memory channels when used as criteria for selection, over a span of several generations (Lagasse et al., 2012). The reason why *ARM* is more stress-resistant is perhaps determined by the circumstances of the massed presentation of the US during its formation, making it meaningful to form a memory that is still capable to perform under such "state of alarm" situations. On the other hand, genesis of stable and stress-labile *LTM* is the result of repeated but spaced US presentation implementing a memory that is ready to react to re-occurring US presentation during the whole remaining life-span of the fly.

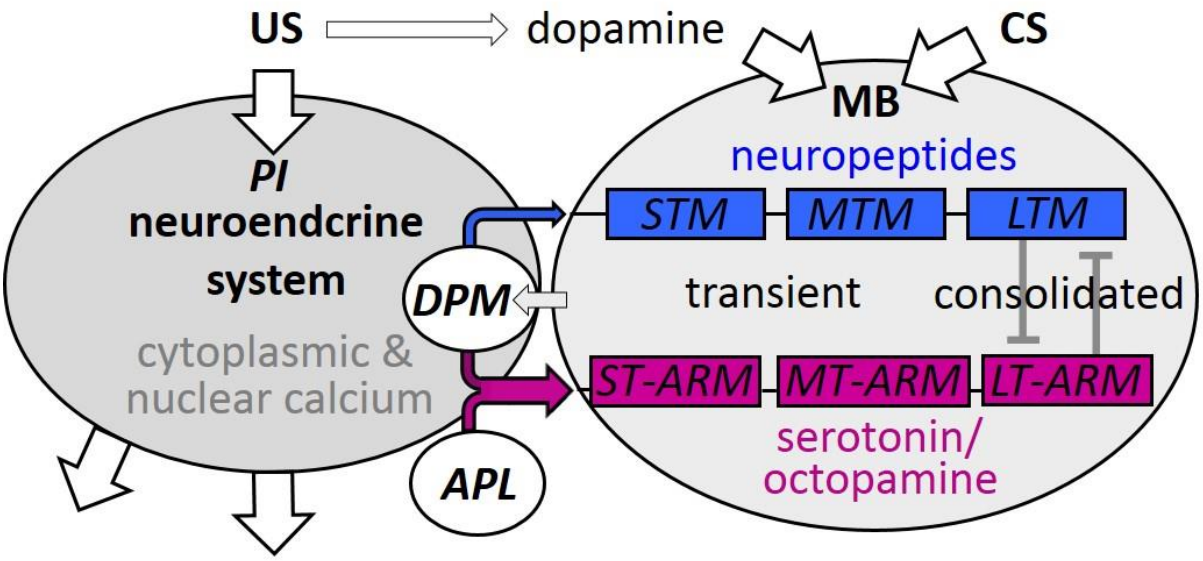


Fig. 6.2. Neuropeptidergic processing of the aversive US and its role in defining a neuropeptide-dependent memory channel. The aversive US elicits a widespread activation of the fly's neuroendocrine system potentially resulting in multiple local signaling events or even systemic responses. One of these addressees of neuropeptidergic signaling is the fly's memory system within the MBs. Two *DPM* neurons receive input from MB neurons and recurrently trigger the formation of a neuropeptide-dependent and a neuropeptide-independent, but serotonin/octopamine-dependent (together with coupled activity from *APL* neurons) memory channel. Both channels store information about the CS and the US in parallel.

How do flies generate two parallel memory channels with similar contents and why should they do that? Recent seminal studies have analyzed in detail the neuronal architecture of the fly's MBs (Aso et al., 2014a) and their output neurons (Aso et al., 2014b). It was subsequently shown that each phase of both memory channels is formed in distinct MB neurons and their

contents are apparently read out by two distinct neuronal output pathways (Bouzaiane et al., 2015). Such functional wiring designs demonstrate that the total memory content exists within MBs in physically segregated states. Our data indicate that neuropeptide signaling from *DPM* neurons is involved in this physical segregation of memory contents already from the time of its acquisition onwards (Fig. 5.9). *DPM* neurons together with their post-synaptic targets, the anterior paired lateral (*APL*) neurons form an exclusive recurrent network (MB-*DPM/APL*-MB) preferentially at the α'/β' lobes of the MB and the anterior peduncle (Fig. 6.3).

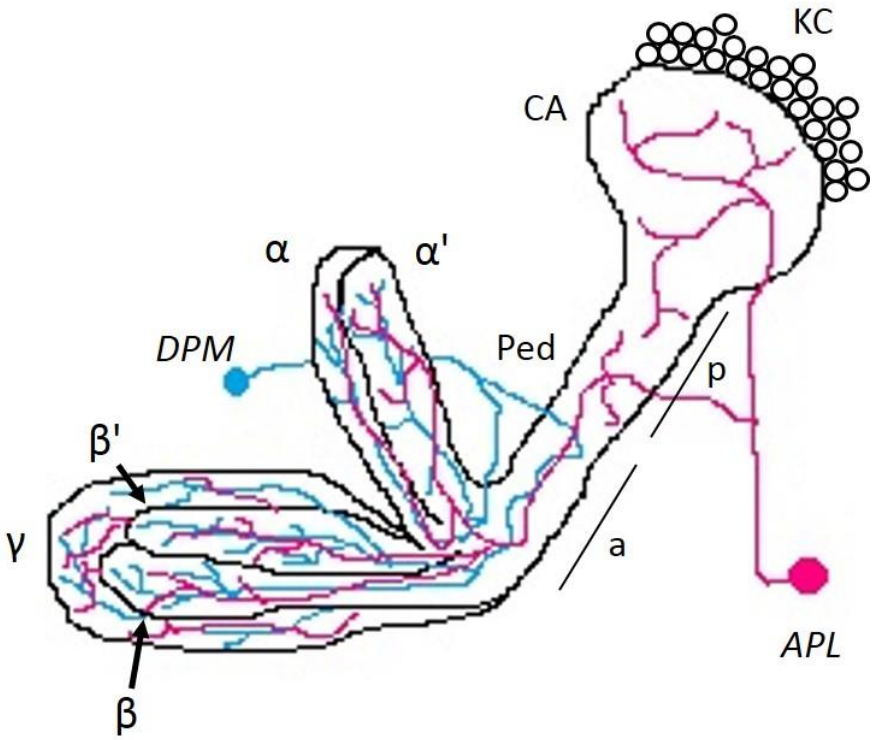


Fig. 6.3. Schematic drawing of the MB-*DMP/APL*-MB network. *APL* neuron and its fibers (red) projecting into the entire mushroom body including calyx (CA) and posterior (p) peduncle (Ped). The *DPM* neuron (blue) innervates all MB lobes (α/β, α'/β', γ) and anterior (a) peduncle building up a recurrent feedback loop with *APL* as well as Kenyon cells (KC) of the MB serving as a small neuronal network to define memory phases.

Thus, *DPM*s communicate with most, if not all, MB neurons. *APL* neurons show calyx-wide dendritic arborizations making them a prime candidate as the responsible address for receiving olfactory signals over projection neurons and subsequently transmit this input to *DPM*s (Wu et al., 2011a).

This signal propagation between *APLs* and *DPMs* is achieved via gap junctions, which in invertebrates are composed of proteins called innexins (inx). Innexins (the invertebrate homologues to vertebrate connexins) form intercellular channels which can be homo- or hetero-oligomeric and thereby exhibit different gating characteristics (Stebbings et al., 2002). In the *Drosophila* genome eight innexine encoding loci have been identified so far (Bruzzone et al., 1996).

It was already shown that the gap junction connection between *DPM* and *APL* is crucial for the formation of *ASM* intermediate-term memory phases, since after a KD of either inx6 in *DPM* neurons or inx7 in *APL* neurons (see also Fig. 6.4) *STM* and primal acquisition, or learning respectively, remained unaffected, while all other *ASM* phases were impaired. Whether these phenomena of impaired memory propagation are also based on an insufficient release of neuropeptides from *DPM* neurons remains currently unclear. *ARM* in contrast was unaffected by inx6/inx7 KD (Wu et al., 2011a). Moreover, *DPM* neurons also seem to play an important role in memory consolidation in a way that they receive input from α'/β' KC, which themselves are activated by spontaneous activity patterns in projection neurons after olfactory conditioning (Keene and Waddell, 2007). Following this activation, the *DPM* neurons subsequently release transmitters, serving as a feedback response onto α'/β' lobes and also forward onto α/β lobes of the KC (Keene and Waddell, 2007). One remaining question is whether the same KCs which generate output onto this feedback loop are also the ones to recurrently receive input from this system. Furthermore, it has been shown that *DPMs* also link this memory consolidation processes to sleep by using two different but parallel signaling systems. First, the *DPMs* release GABA onto wake promoting MB α'/β' neurons, subsequently inhibiting them and therefore act as sleep promoting inhibitory neurons (Haynes et al., 2015). Secondly they simultaneously release the neuropeptide Amnesiac which is suggested to be responsible for sleep onset and maintenance, since the *amnesiac* null mutant amn^{x8} (Moore et al., 1998) exhibits short sleep latency and fragmented sleep patterns (Liu et al., 2008). This is analogous to flies whose neurotransmission from *DPM* neurons to MB neurons was interrupted by cell specific tetanus toxin expression, resulting in the the same increased sleep bout numbers and decreased sleep bout length (Liu et al., 2008)

In addition, this four cell network has been shown to be essential for generating *ARM* phases by means of the serotonergic output of *DPM* neurons acting on α/β KC via d5HT1A serotonin

receptors (Lee et al., 2011). This receptor signaling, in combination with the function of *radish* (Folkers et al., 1993; Folkers et al., 2006) and *bruchpilot* (Knapek et al., 2011) in the α/β KC (Wu et al., 2011b), as well as the KC-V2a cholinergic neuron circuit mediate the formation of *ST-ARM* (Bouzaiane et al., 2015). Secondly the octopaminergic (an invertebrate analog of norepinephrine) output of the *APL* neurons (Leng and Ludwig, 2008; Liu and Davis, 2009) acting on the α'/β' KC via Octβ2R octopamine receptors is together with the α'/β' KC-M6 neuron circuit required for the formation of *LT-ARM* (Bouzaiane et al., 2015) (Fig. 6.4).

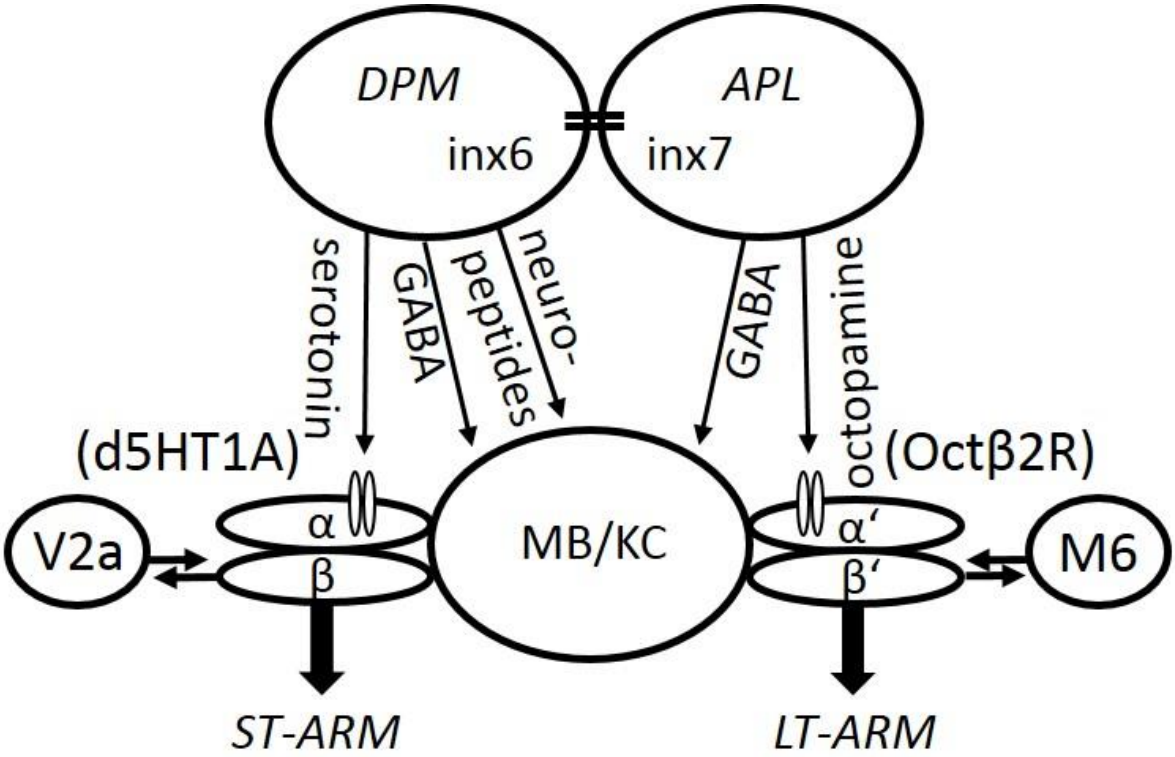


Fig. 6.4. *DPM* and *APL* neurons together with distinct KC form two parallel, transmitter and neuron circuit specific, *ARM* pathways. *DPM* and *APL* neurons are electrically coupled via gap junctions (inx6/inx7) resulting in synchronized activity patterns. *DPM* neurons release serotonin which acts on α/β KCs via d5HT1A receptors (Lee et al., 2011) that together with the KC-V2a cholinergic neuron circuit form *ST-ARM* (Bouzaiane et al., 2015). *APL* neurons release octopamine which acts on α'/β' KC via Octβ2R receptors (Leng and Ludwig, 2008; Liu and Davis, 2009) that together with the α'/β' KC-M6 neuron circuit form *LT-ARM* (Bouzaiane et al., 2015). Additive effects suggest that these two *ARM* pathways exist in the MB in parallel (Wu et al., 2013). Note that GABA release from *DPM* neurons (Haynes et al., 2015) and *APL* neurons (Liu and Davis, 2009) as well as neuropeptide release from *DPMs* (Hörtzsch et al., in revision) and additional activation from the *PI*/widespread neuropeptidergic system targets alternate KC addressees and/or serve in the formation of different memory phases then *ARM*.

Blocking neurotransmission from *APL* neurons after training, but before testing, specifically abolishes *LT-ARM* formation without affecting learning (*STM/ST-ARM*) or generation of stable

LTM. Although *APL* neurons are also GABAergic it has been proven that octopamine is the primary neurotransmitter from *APLs* involved in *LT-ARM* formation (Liu and Davis, 2009).

These findings confirm that through gap-junctional communication, as well as octopaminergic and serotonergic neurotransmission, *APL* and *DPM* neurons modulate together both *ASM* and *ARM* phases (Wu et al., 2013). This underscores the fact that in Drosophila there are not only two different pathways responsible for the formation of peptide dependent *ASM* and peptide independent *ARM*, but also that *ARM* formation itself is suggested to be set up by two distinct anatomical circuits, as revealed by the additive effects of serotonin and octopamine (Wu et al., 2013).

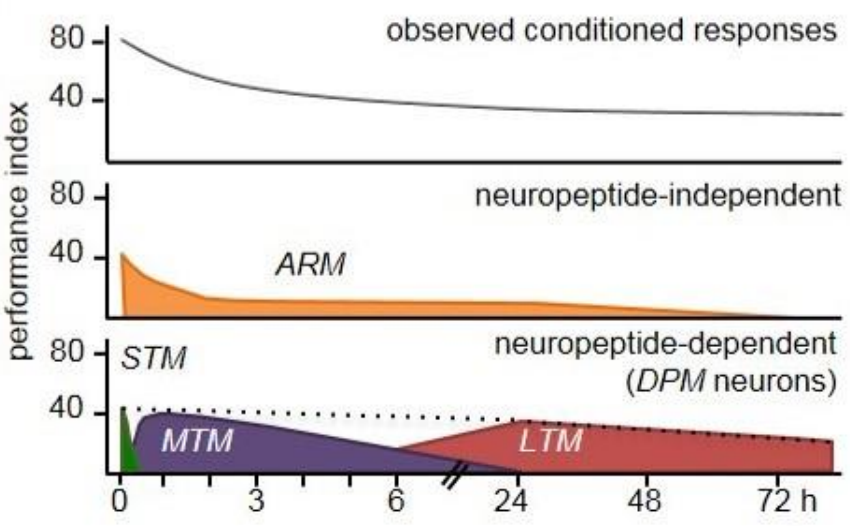


Fig. 6.5. Mechanistic hierarchy of aversive memory phases. Observed conditioned responses of *LTM* trained flies and its decay over time (upper panel). Flies show performance indices as a result of the neuropeptide independent (*ARM*) channel (middle panel) and the neuropeptide dependent (*STM*, *MTM* and *LTM*) channel (lower panel). These parallel pathways are behavioral additive in their unconsolidated phases (first 24h) while at later time points the neuropeptide dependent channel leads to consolidated and permanent *LTM* which actively suppresses the formation of *ARM*. Note that in massed training condition the formation of *LTM* would have failed resulting in consolidated but transient *ARM* (lasting only up to 72h) as the only remaining memory channel (Figure by Schuster, C.M. 2014).

The *DPM* neurons therefore seem to serve a gating function during memory formation. Their serotonergic output (together with octopamine from *APL* neurons) seems to strengthen the neuropeptide-independent *ARM* component and their neuropeptidergic output seems to strengthen the rich, metabolically costly and long lasting *ASM* memory channel (see Fig. 6.2). Since both memory channels are controlled by distinct output signals from the same set of

neurons, it is likely that both memory components are triggered simultaneously resulting in their initial behavioral coexistence (Fig. 6.5 and Fig. 6.6a).

6.2.2 Neuropeptide signaling from two *DPM* neurons is crucial for the formation of all *ASM* phases in *Drosophila*

The assumption that neuropeptidergic signaling from *DPM*s is the key mediator for the formation of all *ASM* phases is consistent with our observations that impaired neuropeptide maturation roughly halved performance scores in *STM* and *MTM* experiments (Fig. 5.7a-c, Fig. 5.8) indicative of a failed *STM/MTM* formation and unaltered *ST-ARM* and *MT-ARM*. It seems therefore plausible that the memory segregation into parallel neuropeptide-dependent and neuropeptide-independent channels serves not only distinct downstream signaling purposes as indicated by their distinct output target neurons (Bouzaiane et al., 2015), but they apparently also converge after single trial conditioning in additive behavior during memory recall. This concept may represent a safety measure to ensure that on a short time-scale of minutes to hours behaviorally relevant information is backed up in parallel and additive memory channels (Fig. 6.5 and Fig. 6.6a).

While the shorter lasting memory phases of both channels appear to coexist there is good evidence that the consolidated phases of both memory channels exclude each other (Bouzaiane et al., 2015; Isabel et al., 2004a; Plaçais et al., 2012). Consistent with this view are our observations that massed conditioning yielded normal *LT-ARM* in all of our Amon KD experiments (Fig. 5.7a-c, Fig. 5.8) whereas spaced conditioning resulted in massively impaired 24h scores particular in those flies with Amon KD selectively in *DPM* neurons (right pair of bars in Fig. 5.8b) indicative of almost absent *LTM* and *LT-ARM*.

How can the same neurons (a single pair of *DPM* neurons perhaps in combination with *APL* neurons) encode two mutually exclusive distinct memory channels depending on whether they receive repeated spaced or massed conditioning? An interesting insight has recently been offered by the finding that the activity levels of a small subset of dopaminergic input neurons to MB neurons, three pairs of protocerebral posterior lateral (*PPL1*)-neurons, correlate with the mode of conditioning and inversely correlate with *ARM* expression (Nassi and Callaway, 2009b; Plaçais et al., 2012). It is currently unclear, what the input of these dopaminergic neurons to MB neurons might mean physiologically.

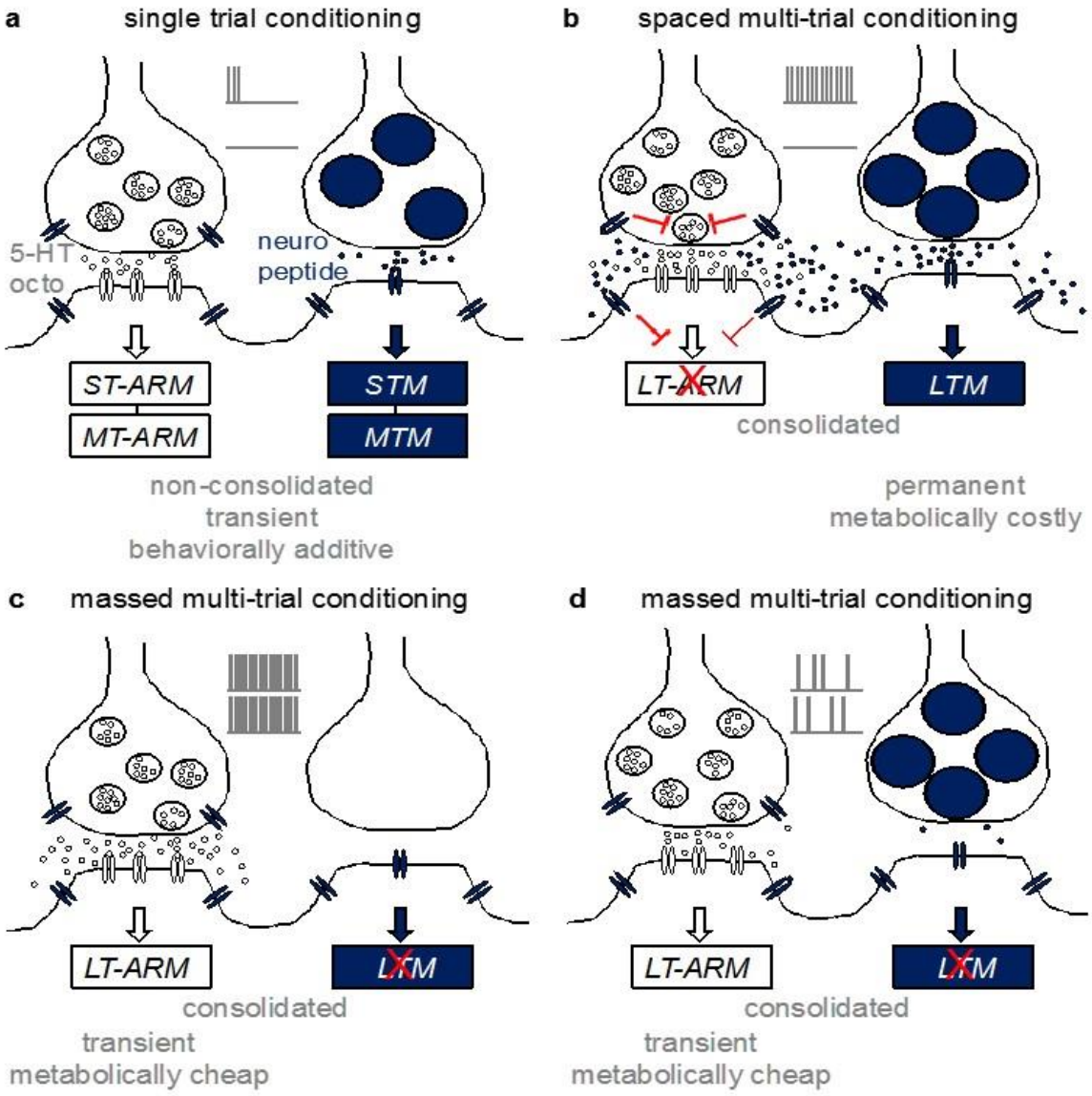


Fig. 6.6. Potential mechanisms underlying the coexistence of the non-consolidated memory phases and the mutual exclusion of consolidated memory phases. (**a**) Single trial conditioning results in a short high frequency activation of *DPM/APL* neurons that release neuropeptides and serotonin (5HT)/octopamine (octo) at distinct terminals within the MB to orchestrate the formation of *STM/MTM* and *ST-ARM/MT-ARM* respectively. (**b**) Spaced multi-trial conditioning results in an extended high frequency activation of *DPM/APL* neurons that is interrupted by pauses. This results in a strong release of neuropeptides that via volume transmission reach nearby terminals to inactivate the release or the postsynaptic effects of serotonin/octopamine. *LTM* forms and excess neuropeptides inhibit the formation of *LT-ARM.* The 15min pauses between each conditioning trial are sufficient to replenish the depleted dense core vesicles from the cell body. (**c**-**d**) Massed multi-trial conditioning suppresses neuropeptide release by either excessive (**c**) or insufficient activation of *DPM* neurons (**d**). The former continuous high frequency activation of *DPM* neurons without pauses depletes neuropeptide stores since their replenishment from the cell body is slow. The latter (**d**) assumes that massed training results in a low firing frequency of *DPM/APL* neurons that is sub-threshold for dense core vesicle release.

However, we hypothesize that it directly or indirectly interferes with the firing activities of the recurrent MB-*DPM/APL*-MB network perhaps resulting in favoring neuropeptide signaling over serotonin signaling from *DPM* neurons or *vice versa*.

A possible mechanism how this could be achieved is based on an emerging property of neuropeptide release that apparently requires high frequency activation to be efficient (Knobloch et al., 2012; Leng and Ludwig, 2008) offering individual neurons the possibility to differentially release classical neurotransmitters and neuropeptides. Enhanced *PPL1* activities during spaced conditioning (Plaçais et al., 2012) could translate into extended high frequency activation of *DPM/APL* neurons during spaced multi-trial conditioning and therefore result in strong neuropeptide release (Fig. 6.6b).

Neuropeptide receptors are often found near other pre- and postssynaptic receptors where they interfere with the signaling of the synaptically released classical neurotransmitters (Ignell et al., 2009; Nässel, 2009). In this way abundantly released neuropeptides during spaced conditioning could, through regional volume transmission (Nässel, 2009), reach nearby terminals to inactivate the release or the postsynaptic effects of serotonin/octopamine and thereby orchestrate the formation of consolidated *LTM* and the inhibition of *LT-ARM.* The rest intervals between the single conditioning trials in this case are sufficient to restock the formerly released neuropeptide carrying dense core vesicles (DCV) from the cytosol of the cell body (Fig. 6.6b).

In contrast, massed, multi-trial conditioning can either lead to a too strong (Fig. 6.6c) or too weak (Fig. 6.6d) *PPL1* triggered activation of the *DPM/APL* neuronal network, resulting in a preference of *ARM* over *LTM* formation. The former hypothesis of uninterrupted high frequency activation of *DPM* neurons (Fig. 6.6c) during massed conditioning leads to a depletion of the presynaptic neuropeptide store, because the replenishment with dense core vesicles from the cell body is slow. It has already been shown that neuroendocrine neuron terminals contain many DCVs and that their capture, which defines consecutively the rate of their turnover, is genetically controlled to determine neuron specific variations in peptidergic function (Bulgari et al., 2014). In type III boutons, which carry an abundant amount of neuropeptides and which are located amongst the neuromuscular junction (NMJ) of *Drosophila* larvae and elsewhere, it has been demonstrated that the half-life of these DCVs averages around 6h and therefore defines the NMJ as a high-throughput system (Bulgari et

al., 2014; Wong et al., 2012). Hence, further examinations regarding the kinetics of the DCV replenishment and their average life span in *DPMs* would be of particular interest to elicit the possible comparability of these two neuronal systems.

The latter potential mechanism (Fig. 6.6d) is relying on the assumption that massed training results in a low *PPL1* triggered firing frequency of the *DPM/APL* neurons which subsequently is sub-threshold for dense core vesicle release. Both of these potential paradigms would permit lasting serotonergic signaling onto MB neurons and therefore would consequently instruct the formation of *LT-ARM* and disable *LTM.* The factors favoring the neuropeptide dependent memory channel over the neuropeptide independent memory channel or *vice versa* are not known. Further of note is that serotonin and octopamine, like neuropeptides, signal via volume transmission and their targets may include neuropeptidergic neurons. Since these transmitters can influence, or trigger, multiple target responses and signaling cascades, entirely divergent explanations underlying the exclusion or the establishment of *LT-ARM* and *LTM* respectively, could be plausible.

It has been well established that neuropeptides can function as hormones when released into the circulation, or as global or regional neuromodulators, or as locally acting co-transmitters to fast neurotransmitters (Nässel, 2009). Although the latter role has not yet been thoroughly investigated in the insect central nervous system our finding that Amon KD in *DPM* neurons impairs the acquisition and formation of *STM* suggests that *DPM* neurons may use neuropeptides, at least in part, as a fast acting co-transmitter or as a yet unknown direct transmitter role, interfering with memory formation already in the first seconds after conditioning. Consistent with this finding of fast acting neuropeptides in flies are results found in the marine annelid *Platynereis dumerilii* in which several neuropeptides, expressed in distinct sensory neurons have been detected. These neuropeptides straightly innervate cilia-based locomotion in terms of altering the beating frequency as well as the resting intervals, resulting in a direct translation from sensory input to locomotor output (Conzelmann et al., 2011). Moreover, based on additional anatomical studies, this simple circuitry is proposed to represent an ancestral state in the evolution of the bilaterian nervous systems, leading to the assumption that neuropeptides play a general role in the regulation of larval locomotion in protostomes as well as deuterostomes. Neuropeptide signaling has even been detected in cnidarians which are regarded as one of the first organisms in animal evolution to possess a

nervous system. Hence, neuropeptides are considered to be the oldest neuronal signaling molecules in animals (Watanabe et al., 2009), indicating that the smaller, simpler and therefore metabolically less costly neurotransmitter molecules have developed later in evolution, setting neuropeptide signaling as the primordial pathway for signal transduction. Many of the *Platynereis* neuropeptides (RYa, DLa, FVMa, FVa, Fla and L11) have close relatives in other species, e.g. marine (*Lottia* & *Aplysia*) and terrestrial (*Achatina*) gastropod mollusks, marine cephalopod mollusks (*Loligo*), terrestrial annelid worms (*Capitella*) and leeches (*Helobdella*) as well as cnidarians (*Podocoryne, Hydractinia* and *Calliactis*) and nematodes (*Caenorhabditis*). This broad array of evolutionary conserved neuropeptides underlines the importance of neuropeptide signaling for a wide array of behavioral adaptions, already in ancient species (Conzelmann et al., 2011). Consistent with this two ionotropic receptors which are FMRFamide dependent and serve as fast responding, depolarizing receptors have recently been identified in different snails (*Helix aspersa*, *Helisoma trivolvis*, *Aplysia californica* and *Lymnaea stagnalis*) (Lingueglia et al., 2006). This is exceptional since FMRFamide and its related peptides typically affect the slower responding G protein-coupled receptors (GPCR) which are thought to be the vast majority of all neuropeptide related receptors in *Drosophila* (Hewes and Taghert, 2001). The *Drosophila* genome harbors 45 neuropeptide related precursor genes which encode for approximately 75 neuropeptides who are associated to 45 GPCRs (Nässel, 2009; Nassel and Winther, 2010). If our observed fast neuropeptide signaling component in *Drosophila* is exclusively GPCR dependent and if this wide array of neuropeptides exists also in higher animals, e.g. mammals or even humans still remains unknown to date. Notwithstanding it is commonly accepted that in a wide array of species a large number of orphan receptors remain whose associated ligand is currently unknown (O'Malley, 1989; O'Malley and Conneely, 1992). Therefore, natural and synthetic ligands are presently used in broad reverse endocrinology approaches in different species, to identify target receptors and dissect their biological determinations (Civelli, 2012; Kliewer et al., 1999).

In addition to the functional evidence of fast neuropeptide signaling we found that a large fraction of the fly's neuropeptidergic system is instantaneously activated with each aversive US (Fig. 5.3). Such a rapid and system wide activation of neuropeptidergic cells might indicate that fast neuropeptide signaling is involved in evaluating the aversive US on various levels of the system with multiple parallel neuronal targets resulting in potential systemic effects. One

of these addressees of this system is obviously the fly's association center, the MBs, and their relevant extrinsic cells, such as the *PPL1* and the *DPM* neurons.

Neuropeptide signaling is initiated by the aversive US in an almost system wide manner (Fig. 5.3) and is, therefore, apart from its role in defining the neuropeptide-dependent channel of aversive olfactory memories, likely involved in several further processes. These likely include those addressees that are involved in executing aversive behavior and transferring the whole system to a state of alarm, preparing it for "fight or flight" responses, such as the control of the heart rate, locomotor activity or fight and aggression (Davis et al., 2014; Nassel and Winther, 2010). Furthermore, the *PI* and the downstream neuropeptidergic signaling systems have been implicated to play a role in the regulation of various behaviors and processes such as sleep (Crocker and Sehgal, 2010; Crocker et al., 2010; Foltenyi et al., 2007), sexual behavior (Belgacem and Martin, 2002; Gatti et al., 2000), metabolism (Broughton et al., 2005), circadian behavior (Cavanaugh et al., 2014), and development and ecdysis (Ewer, 2005). Of note is the apparent involvement of neuroendocrine signaling in olfactory information processing at the level of local interneurons in the antennal lobes where neuropeptide signaling seems to adjust the dynamic ranges and sensitivities towards certain odors (Carlsson et al., 2010; Ignell et al., 2009; Nassel and Winther, 2010)

Given that each of these bodily functions are known to be controlled by distinct neuropeptides (Nassel and Winther, 2010) it seems likely that the US triggers the parallel release of multiple process-specific neuropeptides whose identities depend on the cellular and functional context. Activation of US associated memory formation to possibly avoid similar future experiences appears logical. In the context of neuropeptide-dependent memory phases several distinct processes might be distinguished, such as the primary acquisition of an aversive stimulus combination, the subsequent formation of two transient memory phases (*STM* and *MTM*) and the establishment of consolidated life-long *LTM*. Is each of these neuropeptide-dependent memory-related processes mediated by its specific neuropeptide signature or are they all governed by the same peptide? In support of the former hypothesis is the finding that the original mutant of the putative PACAP-like neuropeptide gene *amnesiac* amn^{1} shows impaired *MTM* with only weak or no effects on *STM* and *ARM* (Feany and Quinn, 1995b; Quinn et al., 1979). Amnesiac has therefore been considered to function as an *MTM*-specific neuropeptide (Dubnau and Tully, 1998; Tully and Gergen, 1986) indicating that the

other memory phases might require additional or different neuropeptides. However, the *bona fide* null mutant *amn*^{x8} (Moore et al., 1998) indeed shows a pronounced *STM* impairment in addition to the *MTM* defect (DeZazzo et al., 1999) (Fig. 5.9d) that are indistinguishable from our acute Amon KD in *DPM* neurons (Fig. 5.9c). Although we can't rule out that potential developmental defects in the chronic *amn*^{x8} mutant (DeZazzo et al., 1999) are responsible for the stronger *STM* impairment, the similarity to our Amon KD results might indicate that Amnesiac is the neuropeptide that controls acquisition and formation of *STM* and *MTM*. The KD of Amon was moreover the only experiment showing consistent results when we interfered with neuropeptides. Nplp3, dFmrf, Ccha2 and Acp70A, targeted in our RNAi experiments revealed no clear conclusions, indicating that neuropeptide dependent memory formation is either mediated by the neuropeptide Amnesiac alone or it interferes with additional peptides and therefore demands a distinct signature in this sequence. At the time the RNAi experiments were set up, we had not started our examination of the *amnesiac* mutant. Therefore, it was still unclear that Amnesiac would, despite the former assumption that it only plays an important role in *MTM* formation, turn out to be also a prime candidate for the examination of *LTM* formation. Referring to our nCounter data we chose the genes which elicited the most drastic alterations, following spaced conditioning, to serve as candidates for KD experiments. Taken together the whole "single gene KD assay" (see 5.5) showed that either we had targeted the wrong neuropeptides or the combinatorics were incorrect (therefore this whole set of data should rather be considered as preliminary). However, if this is indeed the case and whether the same peptide or distinct peptide signatures are responsible for the formation of all neuropeptide-dependent memory phases remains unknown.

Despite the lack of clear conclusions from our nCounter/single gene KD study, it is already clear that the formation of memory and its different phases in *Drosophila* is a wide and complex field including multiple signaling cascades and mechanisms (see also Fig. 6.2, Fig. 6.4 and Fig. 6.6). Despite the already mentioned neuropeptide mutant *amnesiac* a whole group of mutants which reveal learning and memory defects is known today. For example learning and acquisition is blocked in *latheo*, which plays a central role in regulating Ca^{2+} and activity-dependent synaptic plasticity (Boynton and Tully, 1992) and *linotte,* which is disrupted in a gene that shows high similarities to receptor tyrosine kinases (Dura et al., 1993; Dura et al., 1995) and seems to be crucial for brain development (Davis, 1996). Both *latheo* and *linotte*

mutants show structural brain defects and undersized mushroom bodies (Moreau-Fauvarque et al., 1998; Pinto et al., 1999; Simon et al., 1998). The same effects of disrupted learning and *STM* is observable in the classical cAMP/PKA signaling cascade impaired learning mutants *dunce* and *rutabaga* (see 3.3.4). In *radish,* whose molecular nature is still unknown (Davis, 1996; Folkers et al., 1993; Folkers et al., 2006) and *bruchpilot*, which encodes for a ubiquitous presynaptic active zone protein crucial for facilitating efficient vesicle release at low stimulation frequencies (Fouquet et al., 2009; Kittel et al., 2006Wagh, 2006 #1415), formation of *ARM* is disrupted, while *LTM* forms normal (Knapek et al., 2011). However, to date not a single additional neuropeptide apart from Amnesiac has been reported to play a role in memory formation.

6.3 Summary

6.3.1 Nuclear calcium signaling

We found that, similar to neuroadaptation in vertebrates, synaptic activity-driven calcium transients in the cell nucleus of distinct areas in the fly brain control distinct forms of memory and that the suppression of these transients can influence the formation of all kinds of *ASM* phases (Weislogel et al., 2013).

6.3.2 Neuropeptides

This study underscores the importance of neuropeptide signaling in orchestrating higher order brain functions. We found that in *Drosophila* a single pair of neurons, the *DPM* neurons, is responsible for gating the here defined neuropeptide-dependent and neuropeptide-independent memory channels. Together with the fact that these neurons form a well-defined recurrent network with MB output neurons (Wu et al., 2011a) and that their neuropeptidergic output is already crucial during the phase of acquisition and onwards, provides a promising starting point for deciphering the detailed cellular and physiological roles of neuropeptide signaling in guiding memory and sleep related phenomena (Haynes et al., 2015; Liu et al., 2008).

Taken together our results set the starting point for future surveys that could potentially offer principal mechanistic insights into how neuropeptide signaling affects memory formation (embedding in this context also the need for sleep), also in mammalian systems (Borbély et

al., 2013) and what their roles might be in mental disorders (Borbély et al., 2013; Scheich et al., 2016). The finding that suppressed neuropeptide release in determined time windows, shortly after a traumatic experience, may avoid the generation of undesired life-long aversive memory, could lead to the development of novel therapeutic approaches and drugs. In the long run these results may be contributive to the improvement of treating patients, suffering from anxiety disorders and PTSD, as well as insomnia or general sleep related disorders.

6.4 Outlook

6.4.1 The *amnesiac* gene product and its role in memory formation

Drosophila UAS effector lines carrying an RNAi construct against the *amnesiac* gene have been ordered, to elicit if a *DPM* neuron selective KD of the *amn* gene product is capable to mimic the observed phenotypes in this study. This should help to shed light onto the question if Amnesiac is the only neuropeptide responsible for the formation of all *ASM* phases, or if creation and consolidation of memory is rather based on a neuropeptidergic code.

6.4.2 Neuropeptide signaling and its role in sleep and sleep related memory formation

Null mutants of the *amnesiac* gene (amn^{x8}) show disrupted sleep patterns comparable to flies who express tetanustoxin in *DPM* neurons (6.2). However, in preliminary sleep experiments, we observed that *DPM* selective Amon KD resulted in a severe increase of sleep (intense increase in the length of sleep bouts and decrease in activity bout numbers), compared to amn^{x8} but also wild type flies. Further studies are necessary to reveal if there are other neuropeptides in the fly which promote wakefulness and which role sleep behavior plays in the establishment of *ASM* and *ARM* phases.

6.4.3 Is extinction encoded in a whole new memory channel?

Recent studies in our laboratory (Khouaja et al., unpublished data) revealed the possibility for the existence of a third memory channel, which seems to play a key role in extincition phenomena (see 3.2.3), whose underlying physiological adaptions remain so far elusive. Nonetheless, we could observe, or actively evoke, phenomena similar to those observed in human psychopathology (renewal, reinstatement and reconsolidation) which are supposed to rely on this channel. Moreover, we could show that this third channel is characterized by its

nuclear calcium signaling-dependency and simultaneous neuropeptide signaling-independency. If extinction indeed forms a discrete third memory channel or if it displays a variation of *ARM*, since it is not life-long lasting (perhaps because it lacks neuropeptide signaling) needs to be examined in future studies.

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8. APPENDIX

Nuclear calcium regulated

Reference genes mean2	mean3	SEM2	SEM3	% (mean3/mean2)*100	% (SEM3/mean2)*100	T-test *p* of all 4 values of wt-trained and gt-trained	genes
0.610499	0.606407	0.016955	0.165378	99.32971729	27.08900158	0.981160514	14-3-3zeta
0.435303	0.606481	0.030674	0.16331	139.3239607	37.51638628	0.34265736	Act5C
0.471743	0.601341	0.027106	0.165603	127.4721201	35.10456451	0.469253618	betaTub56D
0.603261	0.619458	0.025753	0.158968	102.684951	26.35151713	0.923161653	dlg
0.572824	0.645229	0.060331	0.141823	112.640039	24.75853135	0.65509187	eIF-2alpha
0.581778	0.665328	0.045166	0.150131	114.3610536	25.80545838	0.613241392	Gapdh1
0.448168	0.596324	0.026146	0.16246	133.057971	36.24982261	0.402617071	RpII140
0.680849	0.69575	0.099202	0.12942	102.1885061	19.00860266	0.93016773	Rpl32
0.587221	0.583671	0.038213	0.164251	99.3955095	27.97087765	0.983888905	Tbp

Candidate genes mean2	mean3	SEM2	SEM3	% (mean3/mean2)*100	% (SEM3/mean2)*100	T-test p of all 4 values of wt-trained and gt-trained	genes	
13089.48	9662.858	728.4372	605.7326	73.82152739	4.627627826	0.011137874	amontillado	DOWNREGULATED BY BLOCKED CALCIUM
37141.1	26890.56	2524.251	1723.014	72.40107453	4.639102501	0.01534375	7b2	
5111.973	3738.541	286.3224	110.9499	73.13304649	2.17039368	0.004225056	atf3	
9867	6610.5	587.1946	85.41321	66.99604743	0.865645194	0.001531772	kayak (cFos)	
204.4915	117.9454	11.15194	29.49062	57.67739561	14.4214431	0.033513382	dFMRF	
4327.67	2063.368	248.8695	174.1058	47.6785006	4.023083795	0.000300302	AstB	
12786.54	8027.671	1023.912	673.7577	62.7821783	5.269271549	0.008146425	AstC	
1501.557	1128.023	95.47953	82.15994	75.12357847	5.471650704	0.025105651	CAPA	
4108.366	2644.51	329.6716	46.56366	64.36890175	1.133386359	0.004584643	CRZ	
3078.092	2193.741	174.8164	78.84903	71.26950619	2.561620396	0.00364817	DH44	
250.1099	156.9928	18.79697	7.585751	62.76952193	3.032967014	0.003715936	DILP1	
296.3158	149.0689	31.35058	32.3341	50.30746227	10.91204297	0.017046044	DILP2	
144.0334	49.62966	14.22691	3.090296	34.45705677	2.145541569	0.001038186	DILP3	
17091.42	11093.91	1077.659	225.633	64.90925523	1.320153658	0.00159157	DMS	
1023.121	696.9494	45.05114	73.73686	68.11993571	7.207051484	0.009237753	dNR2	
1887.276	1181.915	151.9382	90.00475	62.62541977	4.769028439	0.00716605	DTK	
7113.117	5868.745	270.3472	71.50063	82.50595536	1.005194143	0.004329767	Fur1	
7820.16	6539.409	154.7796	213.5424	83.62244436	2.730666019	0.002833839	Fur2	
5382.763	3915.942	310.4482	228.8811	72.74965821	4.252112011	0.008936299	Hiw	
4129.885	1775.56	320.9016	139.673	42.99297568	3.382008275	0.000525058	Hugin	
6652.75	4430.75	208.1255	121.7692	66.60027808	1.830358325	9.21408E-05	IPT	
4816.997	2878.756	466.1913	358.5326	59.76245703	7.443073667	0.016496481	NPLP1	
3141.368	2749.249	150.1933	79.26464	87.51755691	2.523252305	0.060355821	Wnd	
503.8045	996.4163	82.79945	22.25491	197.7783736	4.417369298	0.00120929	DILP3	UPREGULATED BY BLOCKED CALCIUM
2079.106	3445.908	283.8271	171.9795	165.7398844	8.271797474	0.006226416	DILP6	
3931.611	5497.778	163.9973	176.7127	139.835241	4.494663386	0.00063315	homer	
10657.32	15320.46	357.3648	395.0233	143.7553464	3.706593121	0.000123036	staufen	
1772.025	2551.673	358.4832	291.8176	143.9975831	16.46803146	0.142640568	CCH2	NOT AFFECTED
67731.96	53430.85	7205.624	2823.159	78.88572222	4.16813349	0.114115226	NPLP3	
62.78162		7.307823		0	0	0.084229199	Sex-peptide	
6565.923	6944.203	360.837	381.7375	105.7612519	5.813920241	0.498521187	activin	
2396.433	2709.15	494.1233	483.6831	113.0492627	20.18345949	0.666972154	AKH1	
5322.112	5146.371	137.4648	164.4308	96.69791723	3.089577318	0.443572843	Akt	
3.894062	1.103588	2.894062	0.103588	28.34029078	2.660164768	0.372463925	Amnesiac	
2521.745	2142.834	210.1008	215.5036	84.9742518	8.545811151	0.254809312	Apis-ITG-like	
2638.561	2089.86	281.4498	208.7554	79.20455167	7.911712327	0.168424233	AstA	
17.83537	1.810765	14.84501	0.810765	10.15266782	4.545830021	0.322513925	burs	
25137.73	21117.07	1352.001	1246.85	84.00548073	4.96007393	0.071455082	CamKII	
8.770403	30.34378	4.675089	18.26011	345.9792923	208.2014626	0.295998705	CCAP	
106.4636	71.53764	22.90962	19.40647	67.19448985	18.22827473	0.288892809	CCM1	
9509.884	8698.286	1024.908	1223.089	91.46573469	12.86124327	0.629184875	DH31	
9046.662	7222.582	575.2539	610.8443	79.83698345	6.752152079	0.072668418	DILP2	
24.41205	4.103588	7.877659	2.967078	16.80968164	12.15415291	0.052392888	DILP4	
1547.09	1480.376	230.2538	249.861	95.68775379	16.15038176	0.850819089	DILP5	
31.14407	22.42853	7.807176	4.002527	72.01540462	12.85164942	0.358869543	DILP7	
1248.925	971.2707	110.6256	116.0419	77.7685257	9.291341266	0.134014538	dNR1	
11144.5	9843	914.5739	1206.52	88.32159361	10.82614739	0.42299146	DSK	
13.765	40.7922	7.870699	25.00886	296.3471576	181.6844031	0.342357942	EH	
98.91518	84.90886	15.4481	10.5381	85.84006937	10.65367619	0.482179998	ETH	
1579.827	1602.558	74.59321	117.4634	101.4388263	7.435205941	0.875598739	GPB5	
92.72116	84.43402	18.55465	11.74139	91.06230005	12.66311646	0.718854101	LK	
702.1711	571.7563	95.04211	54.39013	81.42691952	7.745994514	0.278646316	NPF	
413397.9	499390.2	48203.36	142533	120.8013239	34.47840422	0.588403059	NPLP2	
156.0949	150.6421	13.6999	21.07953	96.50675	13.50430352	0.835477602	NPLP4	
1	1.103588	0	0.103588	110.3588462	10.35884624	0.355917684	pburs	
843.5098	692.2673	158.5367	37.39477	82.0698617	4.433235182	0.388969746	PDF	
6122.501	6079.662	563.5088	530.7863	99.30030993	8.669435844	0.957666112	Proctolin	
15.7338	2.853588	14.7338	1.718248	18.13667364	10.92074411	0.418596181	SIFamide	
8082	6456.75	510.2604	571.9705	79.8904974	7.07709081	0.078244104	sNPF	

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