Stress activated protein kinase: Central mediator of stress- and infection-induced changes in sensory processing, learning and memory in honeybee (*Apis mellifera*)

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Von

Javaid Iqbal

Saarbrücken

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Dekan:	Prof. Dr. -Ing. Stefan Diebels
Prüfungsvorsitzender:	Prof. Dr. Jörn Walter
Gutachter:	Prof. Dr. Uli Mueller
Gutachter:	Prof. Dr. Petra Bauer
Gutachter:	Prof. Dr. Bernd Grünewald
Akademischer Mitarbeiter:	Dr. Helmut Kallenborn

In the name of Allah,
The most beneficient,
The most merciful

DEDICATION

This dissertation is dedicated to my Grand mother (Zaneeb Bibi) and late Grand father (Muhammad Siddique) who has been a great source of motivation and inspiration. They raised me since my childhood and I can never forget their role in my character and career building. I am highly grateful from core of my heart and soul for their unconditional love, guidance and support in each and every moment of my life.

I also dedicate this dissertation to my late mother (Nusrat Bagum) to whom I always miss in my life and my father (Zafar Iqbal) who always stands with me to protect in all difficult situations and instilled the importance of hardworking and higher education.

Special feelings of gratitude and dedication to my beloved wife (Sara Javaid) and cute daughter (Eiman Javaid) who gave me courage, happiness and moral support to achieve this milestone.

CONTENTS

1 INTRODUCTION

1.1 Learning and memory: Overview

Learning and memory is one of the most dynamic topic in neuroscience and closely associated with our daily life and experiences. Learning is the acquisition of new knowledge from the environment that leads to development of new skills, performances and adaptive behaviour according to particular experience. Memory is the process that stores the acquired information. In general, learning and memory ranges from simple to complex forms, including non-conscious or implicit memory (reflexes and motor skills) to conscious or explicit memory (goal oriented learning). The process of memory formation and the underlying mechanism is evolutionary highly conserved and consists of different temporal and highly dynamic phases. We remember some information for years while other information is only available for minutes to hours. These phases are dissectable into long-term memory (LTM) and short-term memory (STM) that are based on different mechanisms and independent from each other (Figure 1.1).

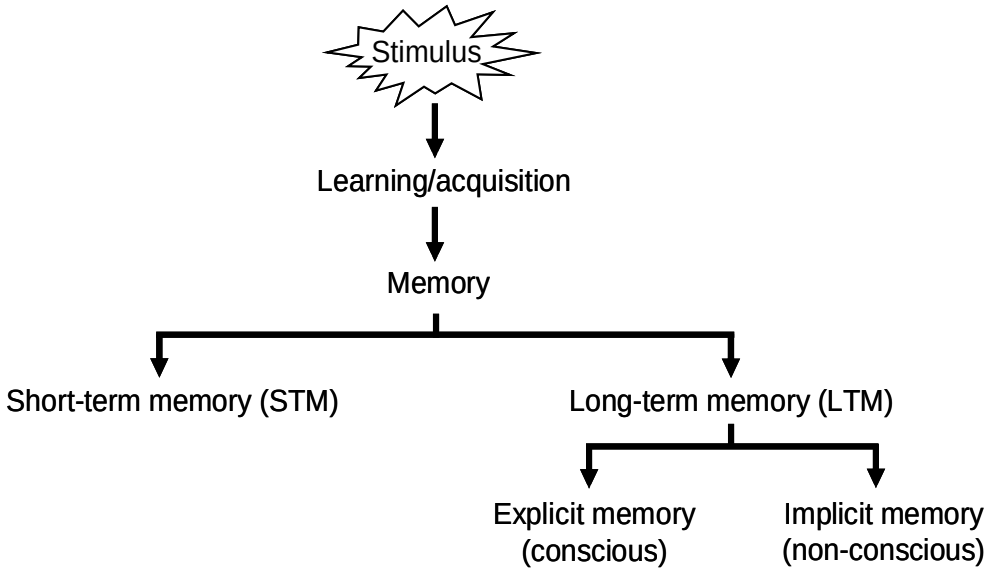


Figure 1.1: Phases of learning and memory

Investigations using different model system including molluscs, insects and mammals have shown that learning and memory leads to changes at the molecular, cellular and network level. All these processes are summarized by the term neural plasticity and share some common mechanistic features [47, 57, 78, 158, 162, 189, 193, 196, 258]. Due to the relatively

simple nervous system and a variety of behavioural paradigms, insects are favourable models to search for the mechanism underlying learning and memory formation.

1.2 Forms of learning

1.2.1 Non-associative learning

Habituation and sensitisation are two forms of non-associative learning [158]. Habituation is a process in which the behavioural response of an animal to a stimulus is decreased after repetition of stimulus over duration of time. In contrast to adaptation that occurs at the level of sensory system, habituation is a process located in the central nervous system. The learning underlying habituation is a fundamental process of biological systems and does not require any consciousness or motivation. For example, if the dog hears a sound, it will respond by turning its head towards the sound but if the sound is coming repeatedly without any pleasant or unpleasant situation, then the dog will simply ignore and cease to respond it.

Sensitisation is the increase in animal's reflex response as a result of presentation of strong or noxious stimulus. In contrast to habituation, sensitisation arouses the animal to pay attention to different stimuli because they are potentially followed by reward or painful consequences. For example, the gentle touching of siphon in *Aplysia* resulted in withdraw of its gills for short time. After an electric shock to its tail however, the same gentle touch to the siphon will elicit a longer withdrawal of gills [158].

1.2.2 Associative learning

Associative learning is a complex form of learning and based on associations between different events i.e. a particular stimulus and a particular response. The classical Pavlovian conditioning and the operant or instrumental learning are two forms of associative learning paradigms used to study the mechanisms underlying learning and memory formation.

In **classical conditioning**, a naturally occurring stimulus (CS; conditioned stimulus) is paired with an appetitive or aversive stimulus (US; unconditioned stimulus). After conditioning, the animals behave as CS predicts the US and produces the conditioned response (CR). The best known example of this associative form of learning has been described by Ivan P. Pavlov in dogs [222] where bell ringing (CS) was associated with food (US). CS alone provokes no response in the organism. After presenting CS repeatedly together with US, the dog eventually learns to associate between CS and US and started to secret saliva (CR; conditioned response) after presenting CS alone (Figure 1.2). Factors like the frequency and

the timing of stimulus presentation are important and affect the conditioning. Eliciting CR after presenting the CS hours after conditioning is referred as memory retrieval. Moreover, if the animal faces CS many times without any food, the CR can be gradually eliminated, a process known as memory extinction.

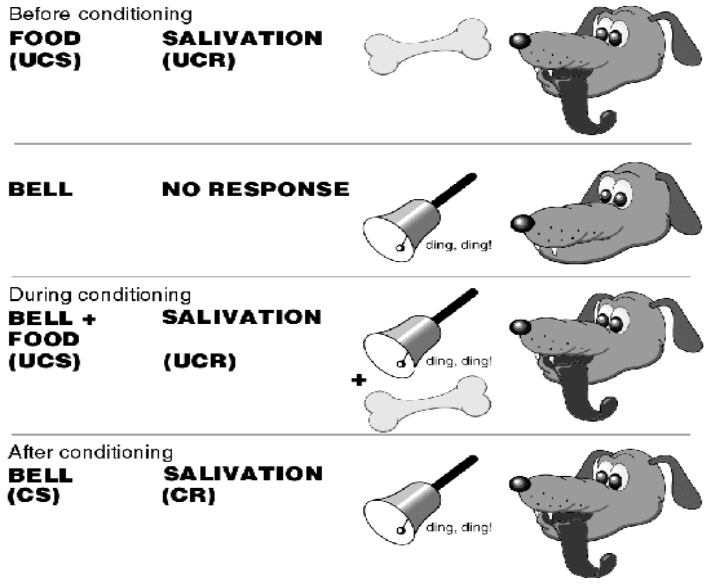


Figure 1.2: Pavlovian classical conditioning
(UCS; un-conditioned stimulus, UCR; un-conditioned response, CS; conditioned stimulus, CR: conditioned response).
(Ref: http://www.northern.ac.uk/NCMaterials/psychology/lifespan%20folder/Learningtheories.htm)

The **operant or instrumental conditioning** has been invented by B.F Skinner to explain the effects of consequences of a particular behaviour on the future occurrence of that behaviour. It is also regarded as trial and error conditioning, in which animal response (R) is associated with a stimulus (S) that follows consequences (reward or punishment). Like classical conditioning, the stimulus can be either appetitive or aversive. The animal learns either to participate or to avoid the stimulus. The animal learns to behave (e.g. press a lever) in order to either obtain a reward (food) or to avoid punishment (electric shock) (Figure 1.3). Positive and negative reinforcement strengthens the behaviour positively and negatively either by experiencing a favourable condition (pleasant stimulus) or avoiding a negative condition (aversive and unpleasant stimulus) respectively. In contrast, punishment and extinction weakened the behaviour either by experiencing a negative condition or not experiencing a positive or negative condition. Although other more complex forms of learning exist (e.g. imprinting learning), they are not easily accessible for neuronal and cellular analysis.

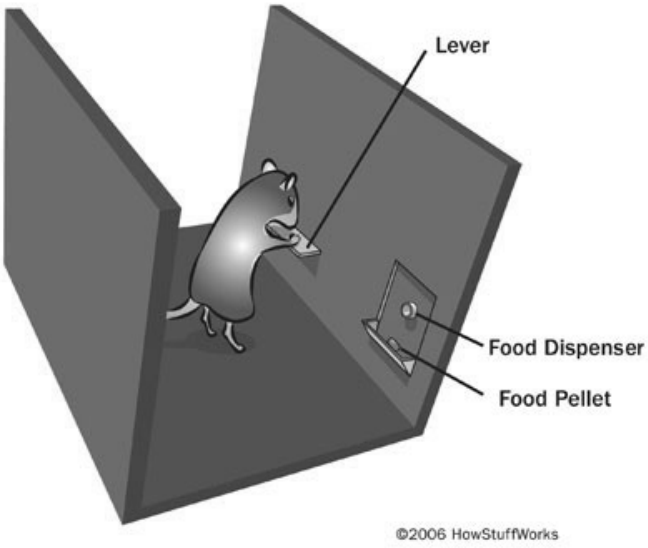


Figure 1.3: Skinner basic operant conditioning box
(Ref: http://static.howstuffworks.com/gif/dog-training-19.jpg)

1.3 Cellular and molecular mechanism of learning and memory

In vertebrates and invertebrates, different neuronal structures within the brain (hippocampus, amygdala and mushroom bodies) have been involved in specific types of learning and memory such as spatial, explicit, emotional and olfactory memory respectively. Despite the different brain structures and neural networks, the neuronal activity (e.g. induced by learning) leads to changes in synaptic connections as the neural substrate of behavioural plasticity and underlie highly conserved mechanism in different species [71, 78, 158, 189, 193, 207].

Throughout animal kingdom, short-term memory (STM) mechanistically differs from long-term memory (LTM). STM does not require the synthesis of protein or RNA. It is supported by transient changes in neuronal communication including the regulation of transmitters release [158]. In *Aplysia*, serotonin released after a single tail shock elevates cyclic AMP by activation of the adenylyl cyclase (AC). Cyclic AMP activates the cAMP-dependent protein kinase (PKA) that leads to the phosphorylation of pre-existing proteins. In case of sensitisation in *Aplysia*, PKA phosphorylates specific K^+ currents causing a prolonged depolarization and thus increased Ca^{2+} influx into the presynaptic terminal [165]. In addition to cAMP, the second messenger Ca^{2+} also plays a central role in the modulation of neuronal activity and the connectivity between neurons [211]. For long term memory, the activated PKA recruits the mitogen- activated protein kinase (MAPK). Both are then translocated to the nucleus and phosphorylate the cAMP response element binding protein (CREB). The transcriptional factor CREB in turn activates several immediate early genes necessary to trigger gene expression required for the growth of new synaptic connections (Figure 1.4).

Thus, the synaptic changes underlying LTM require activation of gene expression, new protein synthesis and formation of new synaptic connections.

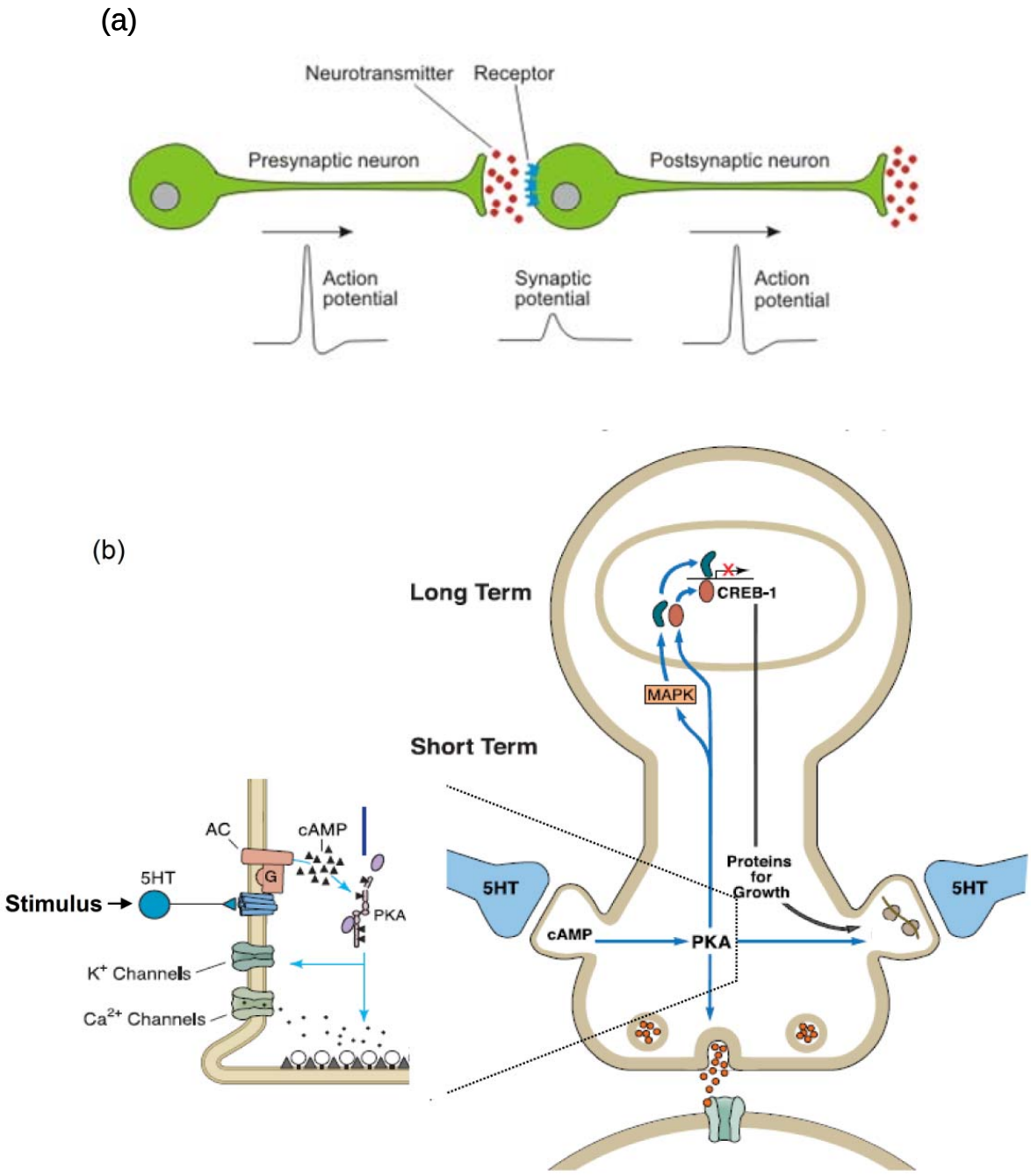


Figure 1.4: Schematic diagrams of mechanisms underlying learning and memory
(a) Scheme illustrating the synaptic connection, the major target of processes underlying learning and memory formation **(b)** Schematic diagram showing the cellular and the molecular mechanism of learning and memory formation in *Aplysia*. Release of the neurotransmitter serotonin (5HT; hydroxytryptamine) from the presynaptic interneuron elevates cAMP levels by activation of adenylyl cyclase (AC). Cyclic AMP triggers PKA and thus leads to phosphorylation of target proteins involved in the rapid synaptic action underlying STM. Strong activation of PKA also triggers MAPKs that leads to the translocation of the kinase to the nucleus and to the activation of transcription factor CREB that regulates gene expression and protein synthesis required for LTM (Modified from [157]).

1.4 Factors modulating behaviour

Learning is influenced by a variety of internal and external factors including the current internal physiological state (hunger, fatigue), emotional state (happiness, sadness, fear of harm, anxiety), earlier memories of similar situations, hormones, diseases and many more (Figure 1.5). These factors interact with each other and may interfere directly or indirectly with a variety of physiological functions that in turn drastically affect the performance of animals in a particular manner. Especially the impact of infections and environmental stress on behaviour and thus the identification of the involved signalling cascades are timely topics in neuroscience.

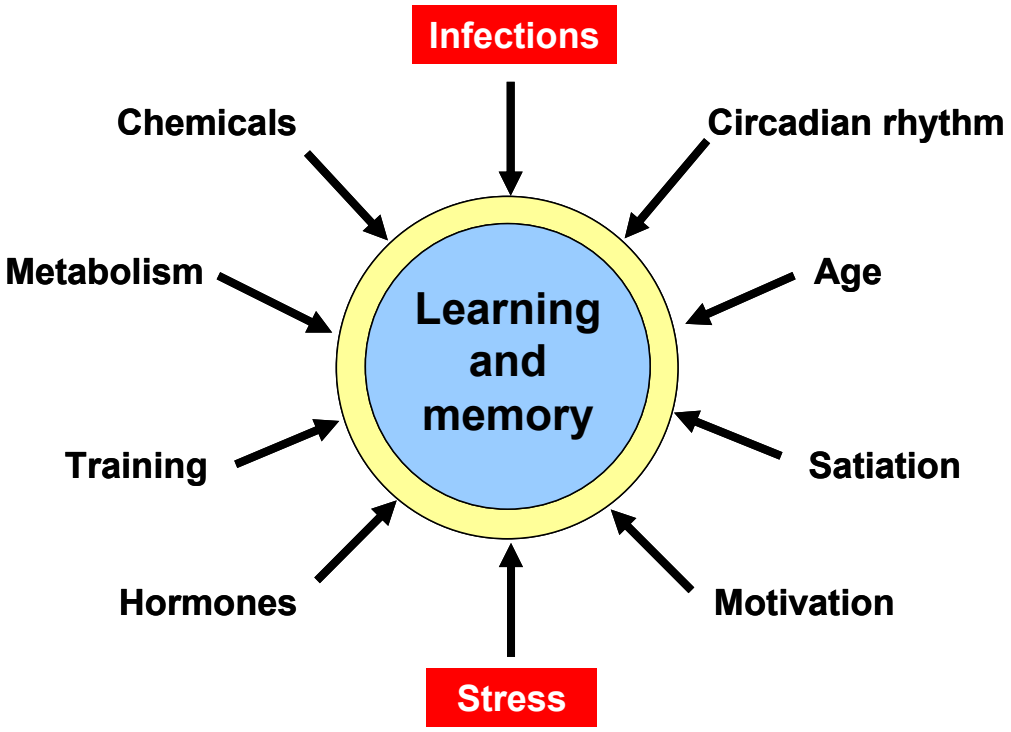


Figure 1.5: Internal and external factors modulating behaviour

1.4.1 Infection and behaviour

In vertebrates, bacterial and viral infections can cause defects in the morphology and in the function of the nervous system including a wide range of impairments in cognitive and motor function, but also social behaviour [27, 290]. In mice, the *Pneumococcal meningitis* bacteria causes deficits in learning and spatial memory [309]. In human brains, HIV infection selectively damages the cortex and affects brain regions that control language, sensory and motor functions [286]. Viral infection in mice causes neurobiological impairments and

alterations in aggressive behaviour, cognitive ability, locomotor activity and deficits in spatial reference memory [27, 156, 165]. Even offspring of mice infected by the influenza virus show deficiencies in exploratory behaviour and social interaction [259].

Observations from a variety of insect species also reveal evidence for effects of viral infection on developmental processes, locomotor activity, feeding, mating and other behaviours [49, 155, 225, 298]. Dengue virus infection prolongs feeding activity in mosquitoes and increases the probability of host infection by extending probing time or feeding on additional hosts [225]. Interestingly, viral infections seem to cause specific behavioural impairments rather than global defects.

Honeybee and virus infection

The sudden collapse of honeybee (*Apis mellifera* L.) hives is very common and as recently reported from USA, a major problem now a days. Beekeepers lost 50 to 90% of their colonies from "Colony Collapse Disorder" about five times more than the normal winter losses. The increased mortality reduces the pollination efficiency and drastically affects agricultural profits in the last years. In addition to other potential reasons, viral infections have been added to this list due to their mysterious role in sudden collapse of honeybee colonies in USA [73]. It is unclear whether they interfere with the immune system or facilitate infections from other sources.

Honeybee is infected with more than 18 different viruses including Kashmir bee virus (KBV), Sacbrood bee virus (SBV), Black queen cell virus (BQCV), Acute bee paralysis virus (ABPV) and Deformed wing virus (DWV). Most of these viruses are small icosahedral particles about 30 nm in diameter [13, 21]. The viral infections usually show no apparent symptoms in honeybees with an exception for SBV and DWV that show clear clinical symptoms identifiable for bee keepers at larval and adult stages respectively. Deformed wing virus (DWV) is very abundant in Europe, Africa, and Asia and can be detected in up to 90% of colonies [28, 282] with infection in different life stages and body parts of honeybee [61, 97, 325]. The DWV infection in early development leads to deformation of wings, paralysis, and mortality of the emerging bees [170]. Although, the infection of adult bees do not show strong effects, DWV infected colonies show weakness, depopulation and sudden collapse [28]. The development of highly sensitive, specific and rapid molecular methods has provided a powerful tool for diagnosis of bee viruses [110]. The fact that virus infection persists in honeybee makes it feasible that virus infection may impair sensory processing and behaviour by interfering with neuronal functions. Besides the morphological malfunctioning of DWV

and its diagnosis, the interference of DWV infection in neuronal plasticity of honeybee is not known.

1.4.2 Stress stimuli and behaviour

Stress is the physiological and emotional reaction to distinct internal and external events (environmental stress). The organism response to stress is a complex process of physiological and behavioural changes that protect the organism from stress. It includes an alarm state, hormone production, short term resistance as coping mechanism and exhaustion. In mammals, the body responses to stress by activating the nervous system (hypothalamus) that promotes the adrenal glands to produce more stress hormones (adrenaline and cortisol) and release them into the blood stream [46]. These hormones speed up the heart beat, breathing rate, blood pressure and metabolism and regarded as stress or survival response (Figure 1.6). The stress response is activated rapidly when needed but is terminated afterwards. If it is inadequate, excessive or prolonged then animal may experience psychological as well as physical damage [79, 261].

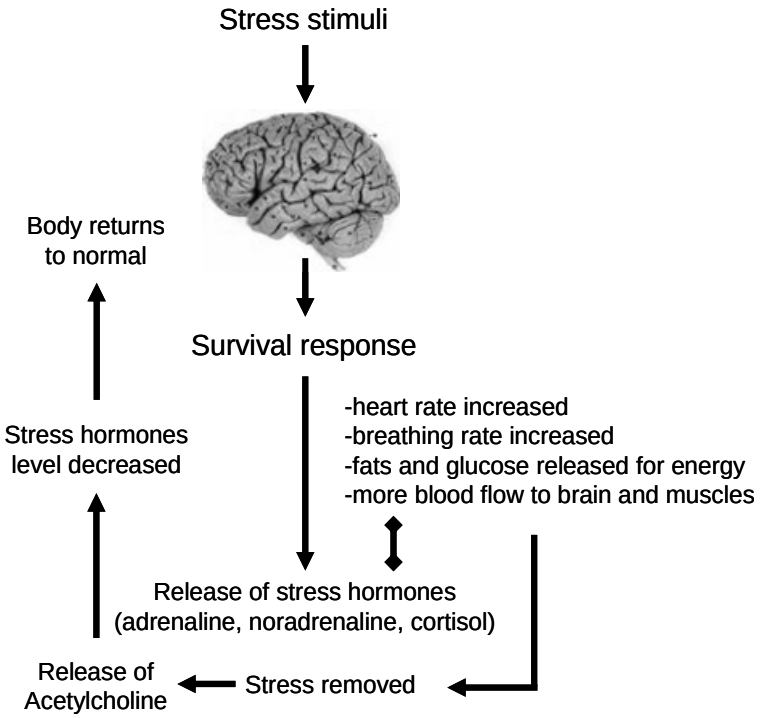


Figure 1.6: Natural defence mechanism against stress stimuli
Hypothalamus responses to stress and promotes adrenal gland to release stress hormones. These hormones make the heart beat faster and raise the metabolism. The animal natural response is to run away or fight back and thus known as fight or flight system.
(Modified from: www.gladeanamcmahon.com/stress_guide.gif)

Stress affects learning and memory in both positive and negative ways [145]. Positively, it can help to compel us to action and may result in new awareness and a new excited perspective while negatively, it may result in psychological, behavioural, or emotional effects [68, 80]. Animal studies have shown that different brain regions (hippocampus, amygdala and prefrontal cortex) important for cognition and emotions are vulnerable to stress stimuli [46, 187, 188]. Besides morphological effects, chronic and oxidative stress stimuli have profound effect on cognitive, motor skills, anxiety behaviour and spatial memory of adult rat and mice [103, 143, 221, 261]. Moreover, different aspects of stress (source, duration, intensity and timing) and learning type are proposed to interact with learning and memory in distinct form [245]. In *Drosophila*, starvation, mechanical and oxidative stress affects the dopaminergic transmission along with sexual receptivity of female and ovarian development [215].

Honeybee and stress stimuli

Various factors like physical activity, insecticides, circadian rhythm, parasites and satiation have been reported to influence the performance and distinct forms of behaviour of honeybee [1, 87, 166, 169, 263, 327]. In addition, few examples show the correlation between stress and cognitive behaviour. In this case, the oxidative stress induced by the injection of ferrous ammonium citrate (FAC) into antennal lobes and mianserin induced disruption of octopamine receptor in antennal lobes cause inhibitory effect on olfactory behaviour of honeybee in dose and time dependent manner [94]. In stressed bees, the brain levels of biogenic amines such as octopamine and dopamine are decreased and cause latency in the reward behaviour of honeybee that takes longer time to fly between hive to feeder [60]. The alarm pheromone (isopentyl acetate) provokes an immediate early gene (IEG) expression in antennal lobes along with a slow behavioural response in honeybee [10]. In a recent study, learning and memory of honeybee is unaffected in nutritionally stressed bees [184] while pathogen induced energetic stress can affect the honeybee performance [185]. Despite of these diverse findings, little is known that how stress is mediated at the molecular level and how it affects the learning and memory of honeybee.

1.4.3 Cellular pathways underlying stress

The action of stress is mediated by hormones and by signalling pathways. Stress induces the release of stress hormones that can influence behaviour and cognitive performance [175, 218, 237, 316]. These hormones are lipophilic and can enter the brain to modulate specific

functions. The hormones bind to intercellular mineralocorticoid receptors (MR) or glucocorticoid receptors (GR). These activated receptors regulate gene expression either directly via glucocorticoid response elements (GRE) or by interacting with other transcriptional factors (c-Jun, AP1, NF-kB and CREB). In addition to activation of hormonal pathway, stress also activates specific neural circuits by the release of neurotransmitters that in turn trigger many intracellular signalling cascades through specific receptors [79, 174, 234, 244]. These intracellular cascades transmit the signal into the nucleus to activate different transcriptional factors (Figure 1.7).

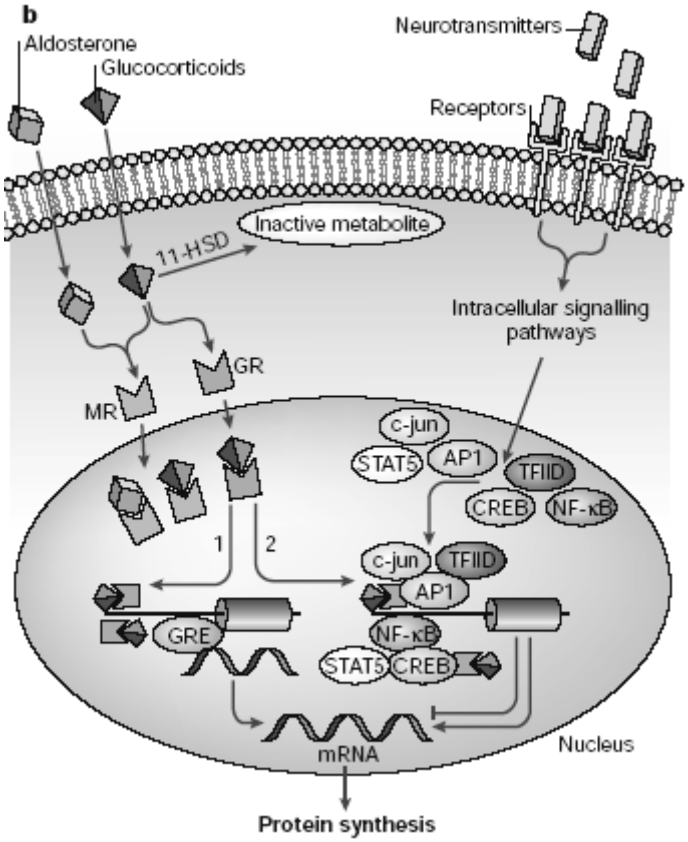


Figure 1.7: Cellular mechanism of stress response
The stress hormones operate through mineralocorticoid and glucocorticoid receptors (MRs and GRs) [244]. These receptors either enhance or inhibit the expression of target genes by direct interaction with glucocorticoid response elements (GRE) or with transcription factors. Moreover, the neural circuits triggered by stress release neurotransmitters that control the transcription factors via activated intracellular signalling pathways.

1.5 Mitogen activated protein kinase (MAPK) pathways

Among the intracellular signalling pathways, mitogen-activated protein kinases (MAPKs) are one of the most ancient and evolutionary highly conserved signalling pathways [85, 144, 311]. MAPKs are serine/threonine-specific protein kinases that mediate the signal transduction from cell surface receptor to downstream transcription factors. MAPKs have an important role in regulation of various cellular processes like proliferation, gene expression, mitosis, cell growth, cell survival, and apoptosis. MAPKs are also necessary for cellular differentiation, development, learning and memory [146]. These are activated by diverse stimuli ranging from environmental stress, cytokines, neurotransmitters, hormones, cellular stress and growth factors etc [85]. Moreover, infections by virus, bacteria, parasites and fungi can also activate these pathways to mediate gene expression [139] and are the potential targets for immune response [173].

It has been shown that MAPK pathways also interact with other signalling pathways to create a complex network that ultimately determine the cellular process. There are three distinct sub-families of MAPKs (extra cellular regulated kinases; ERKs, p38 MAP kinase and Jun-N-terminal kinase (JNK) [56, 77, 250]. The activation of specific MAPKs involves highly regulated and modulated cascades of phosphorylation events mediated by sequential and concerted activation of upstream kinases. The immediate upstream of MAPKs are dual specificity enzymes that can phosphorylate serine/threonine and tyrosine residues in their MAPKs substrates (Figure 1.8). The substrate specificity of MAPKK is very narrow and each phosphorylates only one or a few of the MAPKs [223]. In addition, MAPKs themselves are also regulated by complex feedback and crosstalk mechanism [152]. The activated MAPKs mediate the phosphorylation of transcription factors (ATF2, ELK1, and AP1) which then bind to DNA response elements and regulate gene expression to control large number of cellular processes.

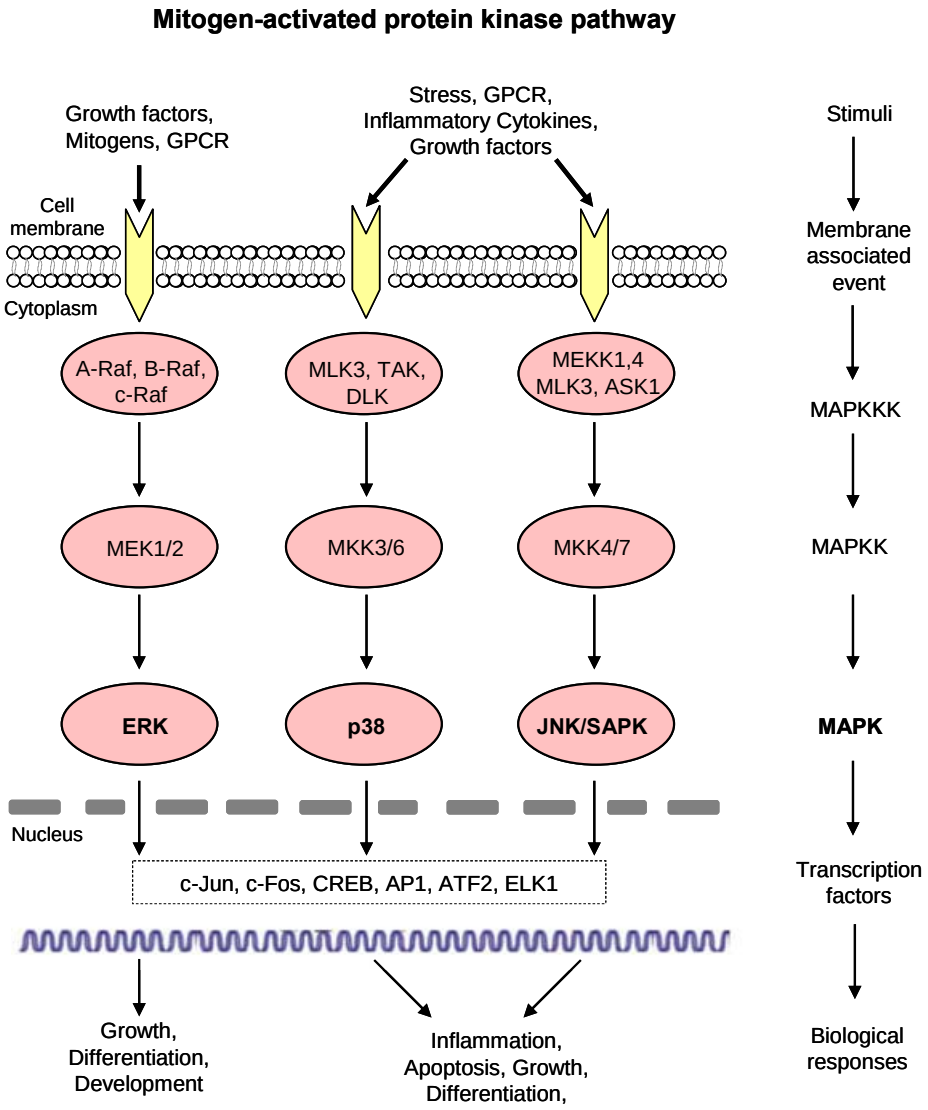


Figure 1.8: Schematic diagram of mitogen-activated protein kinase (MAPK) pathway
Extracellular stimuli (growth factors, inflammatory cytokines, G-protein coupled receptors and stress) activate the MAPK pathways through GTPases. The activated MAPKKKs (RAF, MEKK, TAK) phosphorylate MAPKKs at two serine residues that leads to their activation and subsequent phosphorylation of MAPKs (ERK, JNK, P38) on both threonine and tyrosine residues. The activated MAPKs can translocate to the nucleus to phosphorylate transcription factors such as ATF2, ELK1, AP1(c-Jun, c-Fos) and thus change gene transcription. Growth factors activate the ERK pathway while stress and inflammatory cytokines preferentially activate JNK and p38 pathways.

1.5.1 MAPKs: Implication in learning and memory formation

It is clear that MAPKs are highly expressed in brain and play an important role in various forms of synaptic plasticity and memory in different species ranging from invertebrates to mammals [258, 281, 284, 285]. MAPKs are required for consolidation of LTM, regulating the

functions of different neurotransmitters and control the activation state of nuclear transcription factors [50].

Among MAPKs families, the role of ERK and p38 in synaptic plasticity and memory is characterized in more detail as compared to JNK which is mainly studied for its stress response. Both (ERK and p38) are implicated in hippocampal long-term potentiation (LTP) and in long-term memory (LTM), and together with other kinases are activated after associative learning task in different organisms [7, 88, 160, 202]. For example, ERK activation is necessary for LTM consolidation in fear conditioning of rat and crab *chasmagnathus* [19, 95]. In *Aplysia*, a shock produces short-term memory (STM) for sensitisation of tail-elicited siphon withdrawal reflex. While, five repeated tail-shocks trigger two distinct phases of memory, an intermediate-term and a long-term memory, and activate MAPKs via second messenger cAMP. The intermediate-term memory requires protein synthesis without transcription whereas long-term memory needs the activation of ERK and p38 for new synaptic connections, protein synthesis and transcription [158, 258]. The p38 MAPK in hippocampus of rats is involved in fear memory extinction, short- and long-term memory for inhibitory avoidance [14, 239] while both ERK and p38 are activated in associated eye blinking conditioning in rabbit [329].

1.5.2 Jun-N-terminal kinase (JNK)/ Stress activated protein kinase (SAPK)

Jun-N-terminal kinase (JNK) also known as stress activated protein kinase (SAPK) is a central mediator of stress and contains dual phosphorylation motifs at threonine and tyrosine. It is activated by cytokines, lipopolysaccharides and a variety of stress stimuli like heat shock, osmotic shock, free radicals and UV light etc. JNK is essential for the regulation of physiological as well as pathological processes via transcription factors and gene expression [39, 77, 147, 302]. Active JNK dimers can translocate to the nucleus and regulate transcription via c-Jun, ATF-2 and other transcription factors (Figure 1.9). The conservation of the MAPK pathways in insects, molluscs, and mammals points to a conserved function of these MAPKs in mediating extracellular stimuli (stress and infection) across different animal species. The JNK has been linked to stress response and immune response in both insects and mammals where insects exhibits homologue of mammalian JNK [85, 172, 203, 266, 279]. The studies show the JNK-dependent immune response where LPS activates JNK in wax moth larvae (*Galleria mellonella*) and *Drosophila* cell culture [266, 315]. In *Drosophila* cell line SL2, JNK can control the cytoskeletal gene expression after LPS treatment [42]. In *Rhodnius prolixus* the specific JNK-inhibitor SP600125 blocks the glutamine-dependent

increase in MT fluid (malphigian tubules) and reveals the role of JNK in fluid secretion in insects [129]. In fibroblasts, JNK can be activated by inflammatory cytokines and double stranded viral RNA [69]. Like other insects, bioinformatics screening in honeybee also show the orthologue of JNK pathway in association with immunity [92].

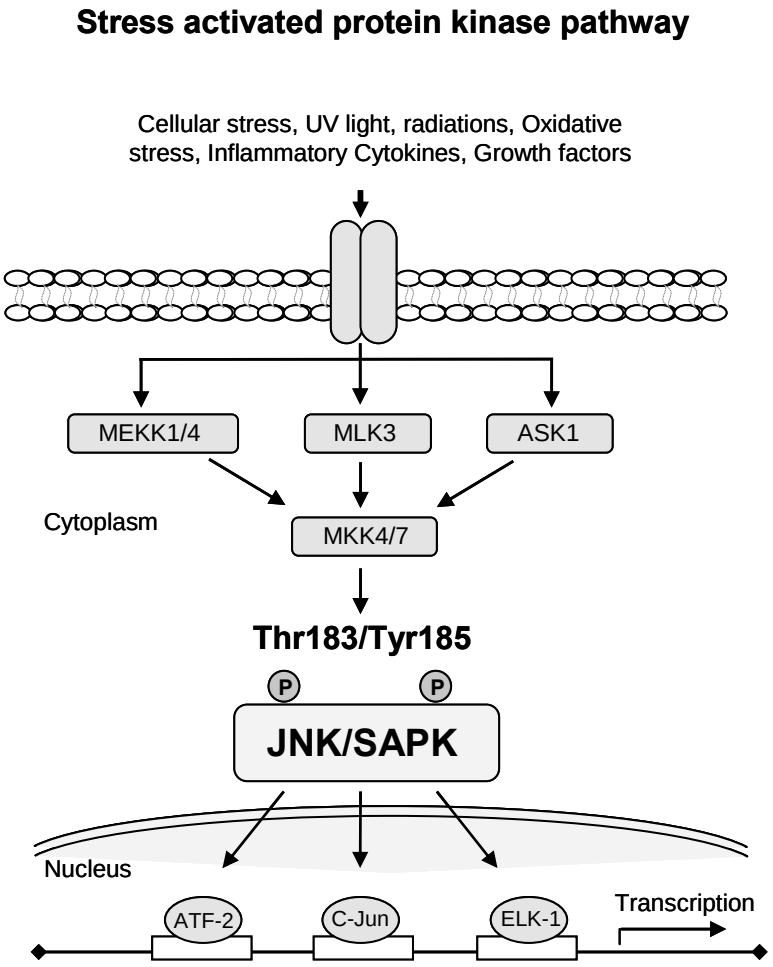


Figure 1.9: Jun-N-terminal kinase/ Stress activated protein kinase
A variety of extracellular stimuli can phosphorylate the JNK. JNK is dually phosphorylated and therefore fully activated by MEK4 and MEK7 at two phosphorylation sites threonine and tyrosine (Thr183/Tyr185) within the activation loop. After translocation into the nucleus, JNK regulates the transcription through its effect on c-Jun, ATF-2 and other transcription factors.

1.5.3 JNK and neuronal plasticity

Although the function of JNK is well investigated with respect to stress and immune response, the specific contribution of this pathway in neuronal plasticity and memory formation is barely known [50]. There are only few examples showing the contribution of JNK in plasticity. The interleukin-1β induced inhibitory effect on hippocampal LTP is associated with

increase in JNK activity [299] while no activation is observed after eye-blink conditioning in rabbit [329]. Studies using specific JNK-inhibitor SP600125 show that the inhibition of hippocampal JNK enhances the STM but suppress the LTM formation and extinction of an inhibitory avoidance task in rats [31, 32]. JNK activation also causes reduction in neurotransmitter release in *Drosophila* neurons while oxidative stress increases acetylcholinesterase expression via transcriptional activation through JNK [91, 326]. The JNK have also been reported to regulate hippocampal synaptic plasticity during transforming early LTP to late LTP [58].

While all these findings are based on the hypothesis that JNK only acts via transcription factors, a recent investigation provides clear experimental evidence for non-transcriptional activation of JNK. In this case, JNK regulates the AMPA-R (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) trafficking following changes in neuronal activity by a rapid phosphorylation [285]. Aside from these findings, little else is known about role of JNK in neuronal plasticity and mechanism underlying learning and memory formation.

1.6 Honeybees: a suitable system to investigate the function of JNK in learning

1.6.1 PER conditioning of honeybee

The simply structured nervous system and striking cognitive capabilities of honeybee make them an important insect model in neurobiology. Learning odours, colours and flowers shape is essential for bees and reveals many characteristics of associative learning in vertebrates. The proboscis extension response (PER), which is elicited by an appetitive stimulus to antennae or proboscis is used as a monitor to test for sensory capabilities as well as different forms of learning [196]. The associative olfactory learning paradigm comprises the pairing of a conditioned stimulus (CS, odour) and an unconditioned stimulus (US, sucrose). During conditioning, CS is paired with a subsequent sucrose reward and the animal forms an association between these two stimuli (Figure 1.10 a) and extend its proboscis when stimulated with CS alone. The process of associating the odour with sucrose is fast and follows the rules of classical conditioning [33]. The appropriate timing of CS and US during conditioning trial is an essential requirement for PER conditioning [124].

The number of learning trials induces different memories which exhibit different properties. The memory after a single learning trial is less stable than the memory after multiple trials

that requires translation and transcription and is long-lasting [118, 211]. A single conditioning trial produces a pronounced behaviour change and bees show a memory that decays within days. Multiple learning trials facilitate consolidation into LTM that can last for several months. Thus, as in other species, memory formation in honeybees is a dynamic process and shows different phases which are induced and maintained by different mechanisms. These memory phases are either independent or dependent on protein synthesis and defined as short-term memory, mid-term memory and long-term memory respectively (Figure 1.10 b). The features of memory phases and the underlying molecular processes in honeybees are very similar to the other investigated species and demonstrate a high conservation of learning and memory in animals.

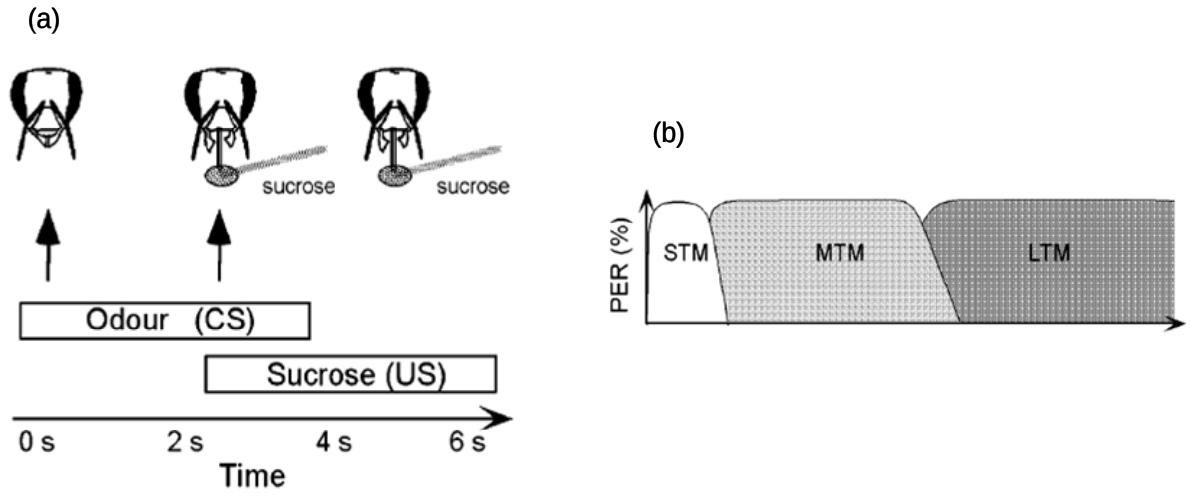


Figure 1.10: Olfactory conditioning of PER in honeybee
(a) Honeybees are conditioned by pairing an odour stimulus (CS) with a sucrose reward (US) to the antenna and proboscis. Memory retention was tested different times after training with odour alone. **(b)** Memory phases: short-term memory (STM) and mid-term memory (MTM) are independent of protein synthesis while long-term memory requires protein synthesis [211].

1.6.2 Neural basis implicated in learning and memory of honeybee

The brain areas and neural circuits implicated in olfactory learning are well characterized in honeybee [211]. The chemosensory receptors of the antenna receive olfactory information (CS) and convey it into the glomeruli of antennal lobes. Glomeruli comprises of dense synaptic connections between different sensory neurons, interneurons and projection neurons. The olfactory information is shifted to the calyx of the mushroom bodies (MBs) and the

lateral protocerebrum via projection neurons. The projection neurons terminate on MB intrinsic neurons (Kenyon cells) in lip region of the calyx. The Kenyon cells process input from different sensory modalities and their output is connected with other brain areas via α and β lobes of the mushroom bodies. In parallel, the un-conditioned stimulus (US) from antenna is mediated by the VUMmx1 (ventral unpaired median neuron maxillare 1) that converges with the CS pathways in the antennal lobes, the lateral protocerebrum and the MBs [123] (Figure 1.11).

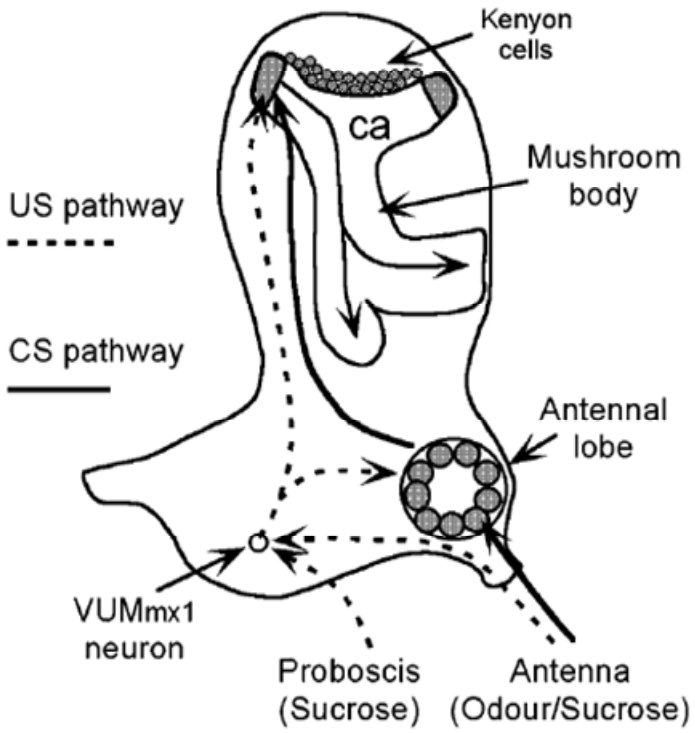


Figure 1.11: Neuronal circuits involved in olfactory learning of honeybee
The antennal lobes and the mushroom bodies are convergence sites of the CS and US pathways. The CS and US information is transferred from antennal lobes to the Kenyon cells of the mushroom bodies either directly or via VUMmx1 respectively [211].

1.7 Goal of this work

The highly conserved molecular processes underlying learning prompted me to study the impact of external stimuli (infection and environmental stress) on the well investigated behavioural repertoire of honeybee. In both vertebrates and invertebrates, the role of JNK in mechanism of learning and memory formation is not well understood and demands for a thorough investigation, especially *in vivo*. Therefore, I aim to address the following topics:

- Investigate the impact of viral infections and external stress stimuli on honeybee behaviour. This requires to set up new procedures for controlled viral infections and stress stimulations in the lab in order to assay their effects on a variety of behavioural paradigms.
- Analyse the role of JNK as a potential mediator between stress and behaviour. To address this question, it is necessary to establish techniques to localize, measure and manipulate JNK in the honeybee brain.
- Do all JNK mediated effects on behaviour require transcription and is there a difference between the applied stress stimuli?
- Does stress directly affect the action of neurotransmitters?

This project will present the first detailed basis for the future analysis of the molecular pathways (especially JNK) affected by viral infections and other environmental stress stimuli. In addition, it will also provide information to identify the molecular targets of the JNK pathway interacting with cognitive functions.

2 MATERIALS AND METHODS

2.1 METHODS

2.1.1 Experimental animals and molecular diagnostic tools

Collection and screening of virus infected bees

Sluggish and apparently sick bees were collected from hives owned by beekeepers in the vicinity of Saarbrücken, Germany (spring to winter 2006/07). From each target colony, dead bees were also tested for possible deformed wing infection (DWV). At the collection site, bees were immediately frozen and stored in liquid nitrogen until subsequent RNA extraction and analysis. After screening with RT-PCR, a winter dead colony was selected as positive candidate for viral source for artificial infection.

Preservation of bees for RNA extraction

Since RNA is degraded very quickly, different preservation conditions were tested to get a good quality of RNA from honeybee samples. The honeybee samples were preserved in 70% and 95% ethanol at different temperatures (room temperature, -20°C and -40°C) for a time range from 1-14 days. RT-PCR was used to amplify and test the quality of extracted RNA after different preservation parameters. The following table (Table 2.1) summarizes all preservation conditions and indicates the importance of preservation conditions of the honeybee samples because the performance of RT-PCR is directly dependent on the quality of RNA. These data demonstrate that preserving the RNA at lower temperature in higher ethanol percentage leads to longer stability of RNA than the other preserving conditions.

Ethanol (%)	Preservation temp.	Days of preservation	RT-PCR signal
70%	room temperature	1	No band
	-20°C	1 6	Visible band Weak band
	-40°C	1 6 7	Visible band Visible band Visible weak band
95%	-40°C	4 6 11 14	Visible band Visible band Visible band Visible weak band

Table 2.1: Storage and preservation conditions of RNA
The table indicates that the stability of RNA is higher, if stored at lower temperature in higher percentage of ethanol. The RNA is degraded very quickly, if stored at higher temperature**.**

RNA extraction

Homogenization

Five adult bees from each colony were pooled and homogenized in liquid nitrogen by using mortar and pestle. The homogenized tissues (80-100 mg) were taken and mixed with 1 ml Trizol reagent (Invitrogen, Germany) followed by the protocol as written below. The mixtures were incubated for 5-10 min at room temperature (RT) to complete the dissociation of nucleoprotein followed by centrifugation at 12000 x g at 4°C for 10 min to remove the tissues.

Phase separation

The supernatants were collected in new tubes and 200 µl chloroform: isoamyl alcohol (chloroform-IAA, 24:1) was added per 1 ml Trizol reagent. After vigorous shaking with hand for 15 seconds, the samples were incubated for 2-3 min at RT. After centrifugation at 12000 x g for 15 min at 4°C, the mixture was separated into a lower red, phenol chloroform phase, an intermediate phase, and a colourless upper aqueous phase (RNA was in upper aqueous phase). The upper aqueous phase (approx. 0.6 ml) was transferred to a fresh tube and the rest was discarded. Equal volume (sample's volume) of chloroform-IAA was added and centrifuged at 12000 x g for 15 min at 4°C. The upper aqueous phase was transferred to new tube and the above step was repeated again.

RNA precipitation

To reduce the level of contamination with proteoglycans and polysaccharides, RNA was precipitated from aqueous phase using 0.25 volume isopropanol (250 µl / 0.5 ml sample) and 0.25 volume of buffer (250 µl / 0.5 ml sample) containing 1.2 M NaCl, 0.8 M sodium citrate.$15H_2O$ [67]. The mixture was incubated at -20°C for 10-20 min and centrifuged at 12000 x g for 10 min at 4°C. The supernatant was decanted by dumping the liquid (RNA remains attached to the walls of eppendorf tube).

RNA wash

The RNA pellet was washed with 1 ml 75% ethanol (EtOH) per 1 ml Trizol reagent used for the initial homogenization. The sample was mixed and centrifuged at 7500 x g for 5 min at 4°C. Except pellet, rest was discarded and washing step with EtOH was repeated again followed by centrifugation. The supernatant (EtOH) was carefully removed and the visible pellet was let to dry for 5-10 min. Care was taken that the pellet was not complete dried.

Re-suspension of RNA

The RNA pellet was resuspended in DEPC (di-ethyl-propyl carbonate) treated water containing 0.1 mM EDTA (pH 8). If the pellet did not dissolve, the mixture was incubated at 55-60°C in a thermo-block for 5-10 min. The RNA concentration was quantified by a spectrophotometer [243] and RNA samples were stored at -80^{o}C in the presence of ribonuclease inhibitor (Fermentas, Germany) for subsequent use. For safety reason, the whole protocol was performed in the fume hood because Trizol (phenol) and chloroform are toxic by contact, ingestion and inhalation.

DNA extraction

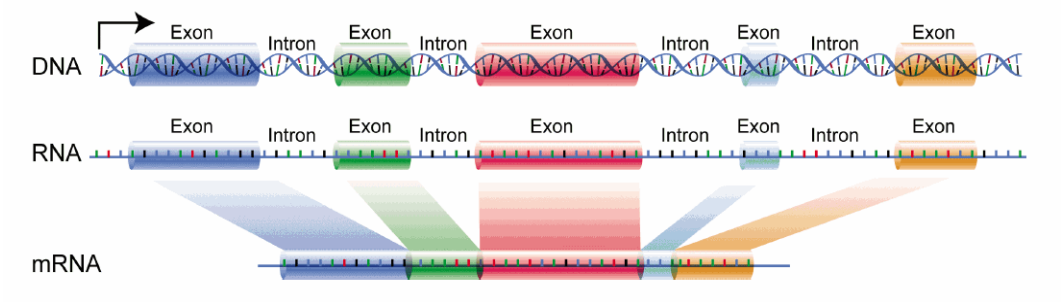
The DNA was extracted with phenol either from fresh bee or bees preserved in 75% EtOH. The adult honeybee was cut into small pieces by using a sharp razor and mixed with 1 ml DNA extraction buffer (50 mM Tris-Cl, 120 mM NaCl, 1% SDS, 20 mM (pH 8) EDTA, 0.2 mg/ml or 200 µg/ml Proteinase K) followed by incubation at 55°C for overnight with continuous agitation. Next day, equal volume of phenol (1 ml) was slowly added and mixed by hand for 15-20 min (don't vortex the solution) followed by centrifugation for 5 min at maximum speed at RT. The phenol precipitated all the protein from sample. The supernatant was collected into separate tubes by avoiding to take the ring between two phases of solution and equal volume 1 ml, chloroform: IAA (24:1) was added and mixed slowly by hand for 10 min. The mixture was centrifuged at maximum speed for 5 min at RT. The supernatant was taken out in a new tube and the above step was repeated followed by centrifugation again. After collecting the supernatant in a new tube, 70% isopropanol (equal volume of solution) was mixed slowly by hand to see the floating DNA in the tube (DNA is white in colour and it precipitates). After centrifugation for 30 min at maximum speed at 4°C, all solution except DNA pellet was discarded. DNA pellet was washed two times with 70% EtOH at maximum centrifugation speed for 10 min at 4°C. After removing all EtOH, the pellet was let to dry for 5-10 min and DNA pellet was re-dissolved in 100-150 µl of DNA storage buffer (10 mM Tris-Cl (pH 8), 0.1 mM EDTA). DNA concentration was quantified with spectrophotometer by making 1:100 times dilution and the quality of DNA was checked by loading it directly on the agarose gel.

Designing of oligonucleotide primers

The oligonucleotide primers were designed from available NCBI (National Centre for Biotechnology Information) sequences of honeybee (NC_007084, NC_007085), Deformed

wing virus (DWV: NC_004830), Kashmir bee virus (KBV: NC_004807), Sacbrood virus (SBV: NC_002066) and Acute bee paralysis virus (ABPV: NC_002548) with the 'pDRAW32' DNA analysis software (http://www.acaclone.com/) and 'Oligonucleotide Properties Calculator' (http://www.basic.northwestern.edu/biotools/oligocalc.html). The tubulin and actin primers were used as positive control in optimization of PCR and RT-PCR. Since in reverse transcription, the introns are exempted, therefore both primers were designed on separate exons (Figure 2.1) and leads to two different fragments of higher and lower sizes (bp) with PCR and RT-PCR respectively.

(a)



(b)

Tubulin primer

NC_007084.2

LOC410614: Similar to Tubulin alpha-6 chain (Alpha-tubulin 6) (Alpha-tubulin isotype M-alpha-6)[*Apis mellifera*]; **Chromosome:** LG15, **GeneID:** 410614, **Annotation:** NC_007084.2 (6388072...6394071), **XM_394092.3 (length:** 1629 bp**, exons:** 8)

EXON: 1244-1396 (153 bp)

ATGTTGTCGATCGATCCACGCACCGGAGCTTACACCAGTTGTTGTTTACTCTATCGTGGAGATGTGTCC
GCAAATGACGTT**AACAGAACGATCGCGTCACTG**AAAGGGGCAAAGTCTATTCGTTTCGTCACTTGGG
CTCCGACCGGGTTCAAA

Tubulin Forward primer: (AAC AGA ACG ATC GCG TCA CTG)

INTRON: 1397-1760 (364 bp)

GTACGTATTATTATTTACGATTTCGCTTATTTACGATTACGCATATATTTTTCCTTCCTTTTTTTCTTTCG
CGTATAATTTTCCATTTTGAAACGACAAATAGTACGCGAAGATGGAAATATATTGAAATGATGCAGCA
AAATTGCTAGATTTCCGTGTTGATATATTTCCAACACTGTGGAAAAAAGTGGCGGTACGTTGAACCGT
CGAGCAGATAATGTAAGTTTTTCTCTTCTCGAACGAGTTCAACTGTTTAGTCTTACGTTATGAAAATAA
ACGATTCTCCGTGTAAACAGAACTTGATACGAAATAGTTTTAGCAATTTATTGGAAAAAAGAAAAAAG
AGAAACAAATGCATTCGTAG

EXON: 1761-2005 (245 bp)

GTAGGTATTAATTATCAGCCAACCACGACCGTCCCTGGCGGTGATCTGGCAAAATCGAACAGGACAGT
GGTGATGATCAGCAACAACACAGCTATGAGACACAGATGGTCGATGCTCACCCGGAAGTACGACTTG
ATGTACCAGAAAAGAGCTTTCTTTCATCATTACATAGGGGAGGGCATGGAGGAGGATTTTTTCAAAGA
GGCGCGG**GAGGACATGATGACTCTGATC**AATGATTACAAAGA

Tubulin Reverse primer (GAT CAG AGT CAT CAT GTC CTC)

Actin primer

NC_007085.2

LOC552637 :Similar to Actin-5C [*Apis mellifera*]; **Chromosome**: LG16, **GeneID**: 552637, **Annotation**: NC_007085.2 (535802...538049, complement), **XM_625012.1(length**: 1131 bp, **exons**: 4)

EXON: 493-871 (379bp)

GGAATTATGGTTGGTATGGGACAAAAGGATAGTTATGTGGGAGATGAAGCACAAAGTAAAAGAGGTG
TTTTAACAGTTAAATATCCAATTGAACATGGTATTGTAACAAATTGGGATGATATGGAAAAAATATGG
CATCATACATTCTATAATGAGTTAAGGATTGCTCCGGAAGAACACCCCGTTCTTCTGACTGAAGCTCCT
TTGAATCCTAAAGCTAATCGAGAGAAGATGACACAAATTATGTTTGAAACTTTTAATTCTCCAGCAAT
GTATGTCGCCATACAAGCTGTTTTATCGCTTTACGCTT**CAGGACGCACTACAGGCAT**TGTTCTAGATA
GCGGAGATGGAGTTTCACACACAGTACCAATTTACGAGG

Actin Forward primer: (CAG GAC GCA CTA CAG GCA T)

INTRON: 872-1460 (589bp)

GTACGTGCCGTTTTATTTACCATTTCAATCAAAAAATTATTTTTTTCGAATCACATTCTTTTGTTTAAAA
TTCATATAATTTATATTATAAGATTAATACTTTTTACTAATATAGTACATAATTTTTATTGTAAATTTTT
ATATTAATTTACTTTCCTTTATGATATTAAAAAAATTATGATTATTTTTGTTACAAAAATTATATATTTT
TATAATTAACATCGTGTAACTTTTTATCGCAACTTATAGAGATATTTTTACATACTACACGAAAATATT
ATTCTTTATTTTTTTTATTTGAAATATTTAATCATCCAAATTTTATTCAATTTTTATTTTTTAATTTAAAT
AAAAATTGAGAAAAAATTGAGAAAAAAATTTATCTTATTTACAAATATAGTAAACATTTTTCTTACAA
ATTTTTTGTTAAAATATTGCAATATAAAAATTATTTTTTTAATGATTATTTAAAGTTTAATATTTCTAT
CAAAATTCTTGTAAATTTTTATACAATTTTTTTGTTTAAAGAATGAATACATACATTAATCTTATCATTT
CAAATACAATGAAAAATTCTATTTGATCCAG

EXON: 1461-1696 (236bp)

GATATGCATTGCCGCATGCAATTTTACGTATGGATTTGGCTGGTCGTGATTTAACGGATTATCTTATGA
AAATTCTAACGGAAAGAGGATATTCTTTT**ACCACTACCGCAGAGCGTG**AAATCGTTCGAGATATTAA
AGAAAAATTGTGTTATGTGGCTTTGGACTTTCAGCAAGAAATGGCTACGGCTGCTGGTTCAAGTGCTTT
GGAAAAAAGCTATGAATTACCAGATGGTCAG

Actin Reverse primer: (CAC GCT CTG CGG TAG TGG T)

Figure 2.1: Designing of oligonucleotide primers against tubulin and actin gene
(a) During transcription, introns are omitted and give product of different band size in PCR and RT-PCR. (Ref: http://upload.wikimedia.org/wikipedia/commons/1/12/DNA_exons_introns.gif). **(b)** The tubulin and actin primers were designed on two different exons from honeybee sequences (NC_007084, NC_007085). The underline protein sequence represents the position of primers.

Detail summary of primers

The following table provides the specific salt concentration ($MgCl_2$), annealing temperature and fragment size (bp) of each primer in PCR and RT-PCR (Table 2.2).

Name	*F *R	Primer DNA sequence *F=Forward primer *R=Reverse primer	Salt concentration and Annealing temp.	Fragment size and Reference
Tubulin	F R	AACAGAACGATCGCGTCACTG GAGGACATGATGACTCTGATC	2 mM, 58°C	646 bp (PCR) 262 bp (RT-PCR)
Actin	F R	CAGGACGCACTACAGGCAT CACGCTCTGCGGTAGTGGT	2 mM, 58°C or 1 mM, 55°C	738 bp (PCR) 149 bp (RT-PCR)
DWV	F R	CCTGCTAATCAACAAGGACCTGG CAGAACCAATGTCTAACGCTAACCC	2 mM, 58°C	355 bp (RT-PCR) (Genersch, 2005)
KBV	F R	GAT GAA CGT CGA CCT ATT GA TGT GGG TTG GCT ATG AGT CA	2 mM, 58°C or 1 mM, 55°C	410 bp (RT-PCR) (Stoltz et al., 1995)
SBV	F R	ACCAACCGATTCCTCAGTAG CCTTGGAACTCTGCTGTGTA	2 mM, 58°C	450 bp (RT-PCR) (Grabensteiner et al. 2001)
ABPV	F R	CATATTGGCGAGCCACTATG CCACTTCCACACAACTATCG	2 mM, 58°C	400 bp (RT-PCR) (Bakonyi et al. 2002)

Table 2.2: Detailed summary of primers
The table indicates the primer sequences, salt concentration, annealing temperature, PCR and RT-PCR product sizes and references if applicable [22, 109, 114, 278]. Here the abbreviations represent deformed wing virus (DWV), kashmir bee virus (KBV), sacbrood bee virus (SBV) and acute bee paralysis virus (ABPV).

Reverse transcription and PCR amplification

RNA purification with DNase treatment

To remove DNA contamination, 1 µg extracted RNA was treated with DNase I (RNase-free) in presence of RNase-inhibitor (Fermentas, Germany) at 37°C for 30 min followed by incubation at 65°C for 10 min to deactivate the DNase according to manufacture protocol.

RT-PCR

A two step RT-PCR protocol was used for the diagnosis of virus infection from extracted RNA of honeybee. Reverse transcription was carried out with an average of 1 µg RNA, random hexamer primer/ gene specific reverse primer and M-MuLV reverse transcriptase (Fermentas, Germany) according to manufacture protocol. PCR amplification was performed with 5 µl cDNA, specific virus primers, actin/tubulin specific primer, Taq polymerase and

1 mM-2 mM $MgCl_2$ (Fermentas, Germany). The mixture was heated at 95°C for 10 min, followed by 35 amplification cycles under following conditions; 95°C for 30 sec, 58 °C for 1 min, and 72 °C for 1 min followed by 72 °C for 10 min for the final extension and to complete the polymerization. A negative (water) and positive virus-infected controls were also included in each RT-PCR. PCR products were analysed by 1% agarose gel electrophoresis.

RT-PCR: Protocol

DNase treatment

- RNA = 7 µl (200 ng/µl)
- 10 x buffer with $MgCl_2$ = 1 µl
- RNase-inhibitor = 1 µl
- DNase (RNase-free) = 1 µl
- Incubate at 37°C for 30 min
- 25 mM EDTA = 1 µl

Total volume of mixture 11 µl

- Incubate at 65°C for 10 min (to deactivate DNase)

* In case of less volume of RNA solution, the total volume (11 µl) was achieved by adding RNase free water

cDNA synthesis without DNase treatment

- RNA = 7 µl
- Specific primer (antisense)/ Hexamer primer = 1 µl

12 µl

- Incubate at 70°C for 5 min
- Chill on ice

cDNA synthesis after DNase treatment

- Above RNA mix = 11 µl
- Specific primer (antisense)/ Hexamer primer = 1 µl

12 µl

- Incubate at 70°C for 5 min
- Chill on ice

Add the following in the order indicated for RNA with or without DNase treatment

- 5x reaction buffer =4 µl
- 10 mM 4dNTPs mix =2 µl
- Ribonuclease inhibitor =0.2 µl
- DEPC-treated water =1.5 µl
- **For hexamer primer**: Incubate at 25^{0}C for 5 min
- **For gene specific primer:** Incubate at 37^{0}C for 5 min
- M-MuLV =0.8 µl

20 µl

- **For Hexamer primer**: Incubate at 25°C for 10 min followed by incubation at 37°C for 60 min
- **For Specific primer**: Incubate at 37°C for 60 min
- Stop the reaction by heating at 70°C for 10 min and chill on ice

PCR PROTOCOL

cDNA	= 5 µl	**DNA**	= 1 µl
Taq buffer+ KCl	= 2 µl	Taq buffer+ KCl	= 2 µl
dNTPs (10 mM)	= 0.5 µl	dNTPs (10 mM)	= 0.5 µl
primer 1	= 1 µl	primer 1	= 1 µl
primer 1	= 1 µl	primer 1	= 1 µl
Taq polymerase	= 0.5 µl	Taq polymerase	= 0.5 µl
	10 µl		6.0 µl

$MgCl_2$	Water conc.	$MgCl_2$	Water conc.
1. 0.8 µl (1 mM) +	9.2 µl	1. 0.8 µl (1 mM) +	13.2 µl
2. 1.6 µl (2 mM) +	8.4 µl	2. 1.6 µl (2 mM) +	12.4 µl
3. 3.2 µl (4 mM +	6.8 µl	3. 3.2 µl (4 mM +	10.8 µl
Total amount	20 µl	Total amount	20 µl

Protocol 2.1: Protocol of RT-PCR

2.1.2 Artificial infection of DWV

Animal collection for controlled infection and behavioural analysis

Adult honeybees were caught from the hive a day before the experiment. The hives were tested with RT-PCR to confirm the absence of virus infection. The bees from same hive were used for each single experiment to deal with bees of same group. All experiments were performed at room temperature. After immobilization on ice, the bees were mounted in small metal tubes with a strip of tape between head and thorax allowing the free movement of antenna and proboscis for hemolymph infection and behavioural analysis. The animals were fed with sucrose (1 M) each evening until satiation and kept in darkness in a plastic container at relative humidity of 70% and at 20-25°C during the infection and behavioural test [212]. The behavioural experiments were performed in summer (2006) while for *in vitro* molecular experiments, bees were also caught in winter (2007) from the artificially bee rearing room with controlled humidity and temperature. In case of oral infection, the caught bees were cooled down and transferred directly to wooden small cages where they can move and fly

easily. Every day, the fresh sucrose paste (1 M) and water was provided in the cages in ample quantity.

Crude bee homogenate for artificial infection

Five bees from each group (DWV-infected or non-infected) were homogenized separately in liquid nitrogen and mixed with 5 ml phosphate-buffered saline (PBS, pH 7.4). The samples were centrifuged at 3000 x g for 30 min at 4°C to get rid of all tissues [232]. The supernatant was removed and stored in small aliquots at -20°C for future artificial infection. Later on, different dilutions were made with PBS and also stored at -20°C. Before extracting crude homogenate, bees from both groups were confirmed with RT-PCR indicating high level of virus in DWV-infected bees while no virus was detected in non-infected bees. The crude homogenate of DWV-infected bees (DWV-lysate) was used for artificial virus infection while crude homogenate of non-infected bees (control-lysate) was used as control.

Pharmacological and control injections

In case of systemic injections (drugs / virus infection / control) into the hemplymph of honeybee, a small hole was poked into the thorax with a sharp syringe needle and 2-4 µl of solution was injected into the thorax using a micro-capillary, as described previously [212]. A specific JNK-inhibitor; SP600125 (Sigma, Germany) was used for inhibition of JNK kinase while a transcription blocker; actinomycin-D (AppliChem, Germany) was used to block the transcription process. In parallel, the control groups were injected with appropriate controls (10-20% DMSO, PBS, control-lysate) to evaluate the effect of drug on animals.

DWV injection in hemolymph

For systemic injections into the hemolymph (2-4 µl), different dilutions of crude homogenate (1:10, 1:100 and 1:1000) were tested to get optimized dilution and amount for behavioural analysis. Finally, 2 µl of 1:1000 diluted DWV-lysate and control-lysate was selected as an appropriate injection. RT-PCR was used to demonstrate the time window of infection with different concentrations and to monitor the level of DWV infection at different days after hemolymph injection. As a prerequisite for controls injection, two groups were injected with control-lysate and PBS (phosphate buffered saline) to monitor any side effect of injection on mortality and motor activity of honeybee. After confirming that both show no difference, control-lysate was used as an appropriate control.

DWV oral infection

For oral infection of DWV, the sucrose was contaminated with DWV-lysate and control-lysate. The honeybees were kept in small wooden cages separately (Figure 2.2) provided with sucrose paste containing either DWV-lysate or control-lysate at a dilution of 1:100 and water was changed daily. At different time points from 2nd to 7th days of infection, RT-PCR was used to monitor the level of DWV-infection. Both, alive bees and bees dying during this time were included in RT-PCR to monitor the level of infection.

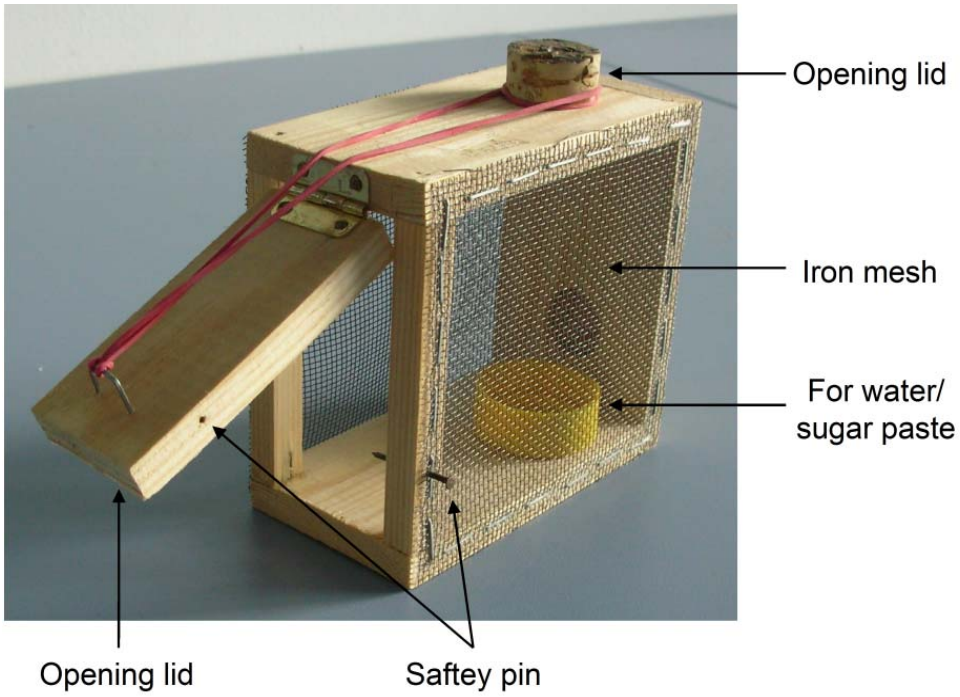


Figure 2.2: Wooden cage for oral infection of DWV

2.1.3 Exposure of external stress stimuli

The honeybees were exposed to two types of external stress stimuli

1. Ultraviolet light (UV light)
2. Shaking with light (SL)

The adult forager honeybees were caught a day before the experiment and were harnessed in the tubes. The bees were fed with 1 M sucrose solution until satiation with or without a window in head of bee for ELISA (Enzyme-linked immunosorbent assay) and behavioural analysis respectively.

Exposure of UV light

The harnessed bees were either exposed to UV light or white light in a small wooden box with internally fixed UV lamp (λ=365 nm, Figure 2.3) or white light lamp for different periods of time (5, 15 and 30 min). Finally, 30 min was used as appropriate time to get an effect on behaviour and for activation of stress activated protein kinase.

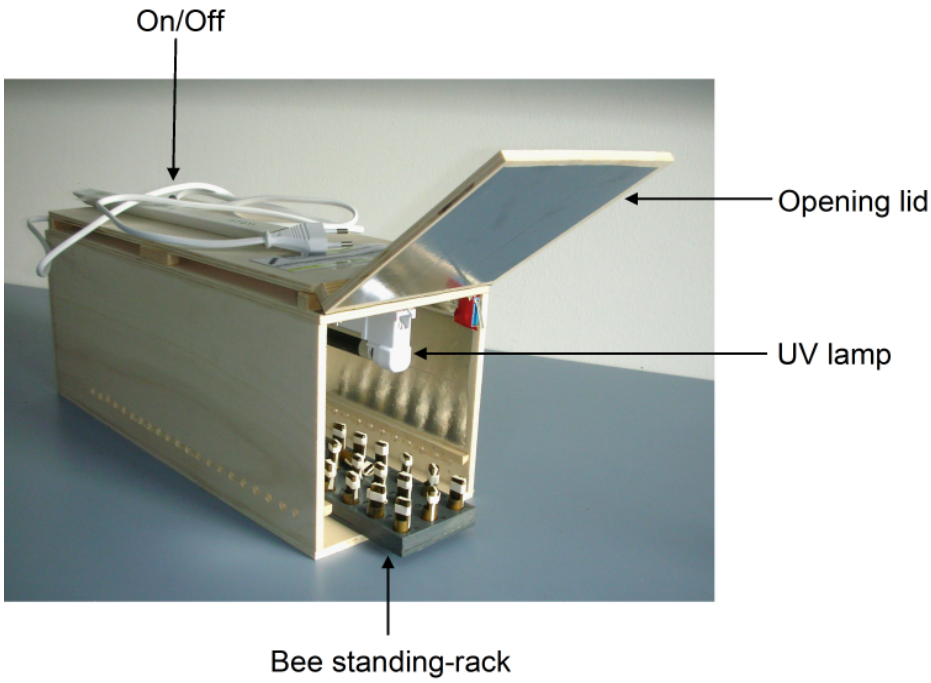
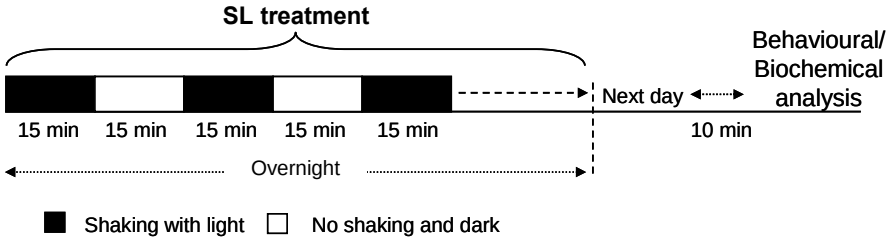


Figure 2.3: UV light lamp
The bees were exposed to UV light for different duration of time (5, 15 and 30 min).

Exposure of shaking with light (SL)

The harnessed bees were kept overnight in a plastic box at room temperature either in complete dark without shaking (control: bees without any stress) or in white light with continuous shaking (SL: bees under shaking and light stress). The treatment comprises of 15 min shaking with light followed by 15 min complete rest (no shaking and dark). Next day, bees were placed at experimental place 10 min before the actual experiment.

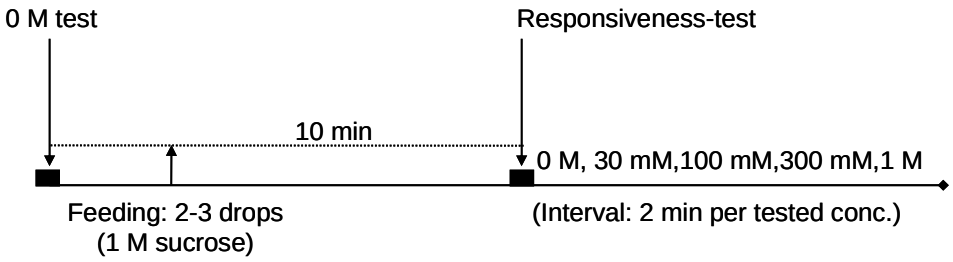


Protocol 2.2: SL stress treatment

2.1.4 Behavioural analysis

Responsiveness to appetitive stimuli

Sucrose responsiveness of the bees to appetitive stimuli was tested using the proboscis extension response (PER). The 0 M responsiveness test was performed 10 min before each experiment by applying a single stimulus (0 M, water) to an antenna. If the bees were more responsive to 0 M (>20%), all bees in experiment were fed with 2-3 drops of 1 M sucrose 10 min before the actual experiment to reduce their responsiveness. After that, an antenna was stimulated by a series (inter-trial interval, 2 min) of defined stimuli with gradually increasing sucrose concentrations (0 M, 30 mM, 100 mM, 300 mM and 1 M). For each sucrose stimuli, the PER was monitored and used as measurement of sucrose responsiveness for each bee.

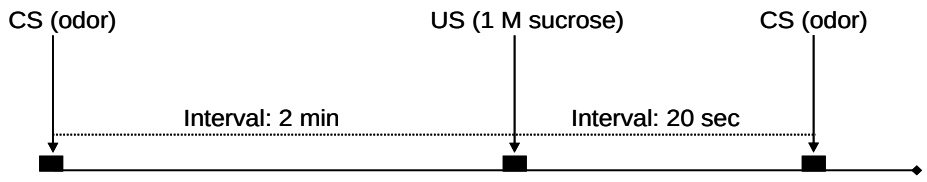


Protocol 2.3: Sucrose responsiveness

Non-associative learning tests

Sensitisation

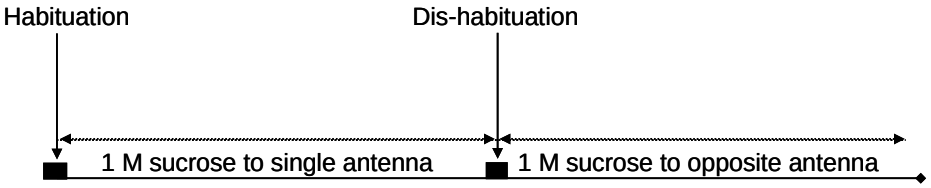
The bees were first presented CS (odor) and after 2 min the bees were sensitised by antennal stimulation with sucrose (1 M). After 20 sec, the second odor stimulus was presented to test for sensitisation of experimental groups. The percentage animals showing a positive response (PER) to second odor stimulus was used for statistical comparison.



Protocol 2.4: Sensitisation (Non-associative learning)

Habituation

Habituation of bees was tested by repeated stimulation of an antenna with 1 M sucrose at an inter-stimulus interval of one second. The number of PER occurring before five consecutive response failures defines the habituation criterion [212]. To show that the bee was only habituated to one antenna and was still able to respond to a sucrose stimulus, the opposite antenna was stimulated with a single sucrose stimulation (dis-habituation). The number of applied stimuli to habituate honeybee was used to compare the two groups. The PER of both groups were normalized with respect to PER of control group and used for the graphical presentation and statistical comparison



Protocol 2.5: Habituation (Non-associative learning)

Associative olfactory learning

PER was implicated to investigate any alteration in associative learning mechanism of virus infected or stress exposed bees (UV/SL). Before conditioning, each bee was placed into the experimental situation in front of an air exhauster. Associative conditioning was performed as described earlier [211]. A conditioning trial comprises the pairing of an odor stimulus (clove) (conditioned stimulus, CS) with a sucrose reward (1 M) (unconditioned stimulus, US). After the animals received three successive conditioning trials at an inter-trial interval of 2 min, memory tests were performed 2 and 24 h after training by stimulating the bees with odor only (Figure 2.4). The animals responding with PER were calculated from each group and were used for statistical comparison.The bees which did not extend their proboscis after US were excluded from the experiment.

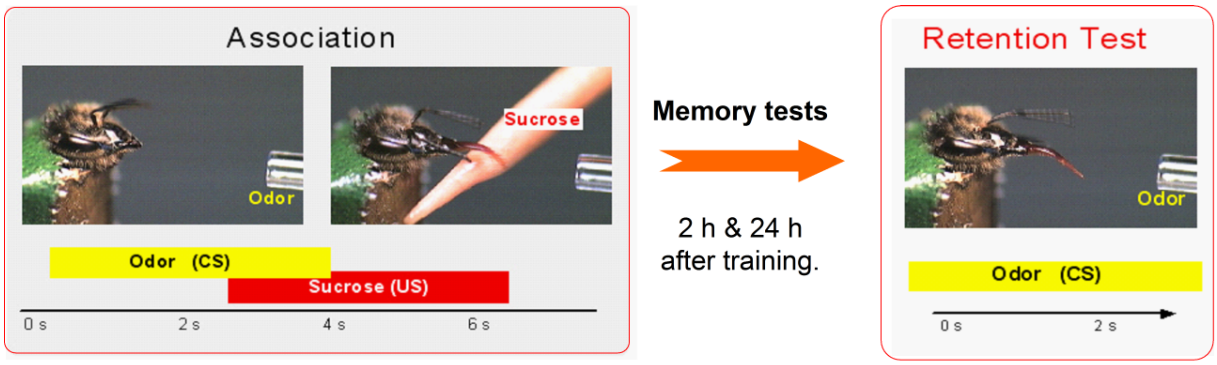


Figure 2.4: Schematic diagram of olfactory conditioning in honeybee
The diagram shows the paring of CS and US in time dependent manner (Ref: U. Mueller).

Protein blast analysis

The protein sequence blast was used to compare the honeybee JNK (XP_392806) with human JNK (AAI30571; P45983.2, P45984.2, P53779.2), mouse JNK (NP_057909.1) and *Drosophila* JNK homologue (NP_723541) (NCBI; National Centre for Biotechnology Information). Following online free web tools were used to determine the similarity between amino acid sequences and to computationally predict the target phosphorylation sites in honeybee amino acid sequences.

1. Multalin interface page (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html)
2. NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/NetPhos/)
3. Kinase-Phos (http://kinasephos.mbc.nctu.edu.tw/)

The mammalian based JNK antibody (Cell signaling Technology Inc. Danvers MA) against two phosphorylation sites i.e. Theronine 183 and Tyrosine 185 (Thr183/Tyr185) were used for molecular analysis of JNK pathway in honeybee.

2.1.5 SDS-PAGE and Western blotting

Sample preparation

The honeybee was immobilized on ice and its head was cut off with a sharp razor. The head was mounted on a melted wax and immediately cooled on ice. The glands, trachea and ocelli were gently removed with fine forceps. The central brain of honeybee was dissected and homogenized in a glass douncer homogenizer with 300 µl of homogenizing buffer (PBS, 1 mM EDTA, 4 M Urea).

In case of *Drosophila*, whole heads of 15 *Drosophila* were removed and homogenized in 300 µl of homogenizing buffer. For mouse, very small piece of frozen mouse brain in liquid nitrogen was thawed and homogenized in 200 µl of homogenizing buffer. After homogenization, the samples were transferred to eppendorf tube and sonification was performed with HTU SONI 130 sonifier (Schwäbisch Gmünd, Germany) at 40% amplitude for 5 to 10 times (one second duration). Sample loading buffer was added in a ratio of 1:4. After that, the samples were incubated at 95°C for 5 min in thermo block and were loaded on a SDS gel.

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis)

The protein samples were separated by using 10% SDS-polyacryamid gel at 20 mA per gel for approximately 40 min according to standard protocol. The protein sample will separate according to their electrophoretic mobility (molecular weight of protein). The two glass plates (10x10 cm) were assembled with a spacer (0.5 mm thick) between the plates. The two plates were sealed from sides with agarose (0.5% containing 1% SDS) and resolving gel buffer (10% acrylamid, pH 8.8) was poured into the glass plates by leaving about 2 cm space for stacking gel. Few drops of 1-butanol were added on the surface of buffer between the plates. After 30 minutes, the gel was polymerized completely. After that, the butanol was rinsed out completely with distilled water. The stacking gel (4% acrylamid, pH 6.6) was poured above the polymerized resolving gel. The comb was inserted with care not to get any air bubbles stuck underneath and leave the gel for 30 min for complete polymerization. The comb was removed and the wells were flushed thoroughly with running buffer. The glass plates were clamped in electrophoresis apparatus and the chambers were filled with running buffer according to the instruction of electrophoresis apparatus.

20 µl of each probe per lane was loaded into the wells on stacking gel for electrophoresis. The pre-stained SDS molecular weight standard marker (pre-stained marker, Sigma, Germany) was also loaded in one lane for determination of protein size in loaded probes. A current of 20 mA was applied for running of gel until the blue dye front reaches the bottom.

Western blotting

After separation of the samples on SDS-PAGE, the proteins were transferred to nitrocellulose membranes. The gels and whatman filter papers were soaked in blotting buffer for impregnation. The gel, membrane and filter paper were arranged in the following order; 3 layers of filter paper, nitrocellulose membrane, gel, 3 layers of filter paper. A falcon tube was

scrolled over to remove air bubbles between them. Protein transfer from gel to nitrocellulose membrane was performed in the blot chamber proceeded at 20 V, 0.4 A and 8 W for the duration of 40 min (U=20 V, I=0.4 A, P=8 W, T=40 min). For more gels, the current is increased by 0.4 A for each additional gel.

Membranes were blocked for 1 h with blocking buffer (2% BSA in PBS and 0.1% TWEEN 20) with gentle shaking at room temperature. After blocking, membranes were incubated overnight at 4°C with JNK antibody (1:8000) or Phospho-JNK (1:500) in blocking buffer (with traces of sodium acid). Next day, the primary antibodies were removed for recycling and the blots were washed (3x5 min) in PBS. The blots were incubated for 1 h at room temperature with anti-rabbit IgG labeled with peroxidase (1:10,000 in blocking buffer). After that, blots were again washed (3x5 min) with PBS. Signals were visualized using the western lightening chemiluminescence plus kit (Perkin-Elmer, Belgium, Zaventem). The blots were immersed in chemiluminescence reagent solution for 2 min and were covered with a plastic bag. The blots were exposed to X-ray film for different exposing times (50 sec, 1 min to 5 min).The X-ray films were developed in dark room with standard reagents (developer and fixer) and dried with warm air.

2.1.6 Enzyme-linked Immunosorbent Assay (ELISA)

The ELISA was used to quantify the relative JNK activity in honeybee brain after different experimental treatments (infection, UV/SL exposure, inhibition of JNK and transcription). ELISA enables the large scale rapid analysis of MAPKs and substitutes the western blotting and gel-based kinase assays [300]. This novel technique is also used in many other studies to monitor the activity of JNK [26, 134, 141]. The 96 wells microtiter plates (F96 Maxisorp, NUNC-IMMUNO plate, Denmark) were used for ELISA analysis. The relative JNK activity in each sample represents the ratio of the phosphorylation state of JNK at positions Thr183/Tyr185 and the total amount of JNK, as determined by ELISA using antibodies directed against the phospho-domain and total JNK. The relative amounts of the antigens in each sample were measured by ELISA as described below.

Sample preparation

After catching the bees, a window was cut in the cuticle of honeybee head for easy excess to the brain (Figure 2.5). After the treatments as indicated in results, bees were immediately immersed in 50% cooled EtOH (-20^{o}C) and incubated at ice for 30 min to conserve the state

of JNK phosphorylation. After that, the bees were decapitated and mounted on wax to dissect the brains. The glands, tracheae and ocelli were removed from the brain and central brain was dissected for homogenization. Each brain was homogenized and sonified 4-5 times (amplitude 40%) with a HTU SONI sonifier in 500 µl homogenizing ELISA buffer (PBS, 1 mM EDTA). The samples were diluted with a ratio of 1:1 with homogenizing buffer to reduce the excess of antigen amount.

Figure 2.5: Dissection of window in head cuticle of honeybee.

Coating

During coating, two identical ELISA plates were used for JNK and phospho-JNK antibody respectively. All ELISA plates were pre-filled with 50 µl of homogenizing ELISA buffer and 50 µl of diluted samples were added to the wells of 1st and 7th column. Starting with 50 µl, the samples were diluted within a row of 6 wells. 6th and 12th column were left blank as a control while the rest remained in pipette was discarded. Now the final volume in each well was 50 µl and again 50 µl of homogenizing ELISA buffer was loaded in each well giving the final volume of 100 µl per well. The plates were incubated for 1 h at room temperature (RT) with gentle shaking.

Blocking and primary antibody

After coating of antigen, the plates were incubated with 400 µl of blocking buffer (PBS containing 0.5% BSA) for 1 h at RT with gentle shaking to block the remaining binding sites of wells. After removing blocking buffer, the plates were incubated with primary antibodies overnight at 4°C with gentle shaking. One plate was incubated with 100 µl antibodies against total JNK (1:5000 in PBS, 0.5% BSA, traces of Na-acid) and the other plate with antibodies against phosphorylated JNK (1:1000 in PBS, 0.5% BSA, traces of Na-acid).

Secondary antibody and staining

Next day, primary antibodies were removed for recycling and the plates were washed with PBS (3x3 min) followed by incubation with 100 µl of anti-rabbit IgG labeled antibody with alkaline phosphatase (1:4000 in PBS, 0.5% BSA, traces of Na-acid) for 1 h at RT with gentle shaking. After that, secondary antibody was removed for recycling and plates were washed again with PBS (3x3 min). 200 µl of staining buffer (*o*-nitro phenyl phosphate in RxN buffer) were added per well and incubated at 37°C for complete staining.

Data measurement and analysis

The conversion of the phosphatases substrate *o*-nitro phenyl phosphate was measured with a micro-plate reader (Safire, Tecan) at 405 nm using 620 nm as background. The relative concentration of the antigen is reflected by the slope of the optical densities calculated from the dilution series of each sample. Since only measurements performed on the same ELISA plate are comparable, all slopes on a particular plate were normalized to the mean value of the slope calculated for the control group on this plate and the *t*-test was used to evaluate the significance between control and experimental groups of bees.

2.1.7 Immunohistochemistry

The honeybee with a window in head capsule (Figure 2.5) was immersed in 4% para-formaldehyde for 30 min at room temperature. Bee brains were dissected and again incubated in 4% para-formaldehyde for 3.5 hours at 4^{o}C followed by three times washing with 0.1 M phosphate buffer for 15 min. The brains were dehydrated in increasing grades of isopropanol (60%, 90%, and 100%) for 1 h each at RT followed by incubation for 1 h in 100% isopropanol at 45^{o}C. After that, the brains were incubated in paraffin-isopropanol mixture (1:1) at 70°C for 1 h and then in paraffin at 60°C for overnight. Next day, paraffin was changed and tissues were again incubated for 2 h at 60°C. Then, the paraffin embedded brains

were cooled down in cold water and sectioned into 7 µm thin slices with a Microtome (Microtome 2040, Autocut, R.Jung GmbH, Nußloch Germany). Sections were mounted on poly-L-lysine coated slides with distilled water and dried on heat plate at 40°C and incubated overnight at 45°C to let them dry completely.

The paraffin was removed by rinsing the slices in Xylol (2x5min) and rehydrated in successive steps of decreasing ethanol (96%, 90%, 80%, 70% and 50%) for 2 min each. The tissues were de-masked by cooking them in 0.1 M sodium citrate buffer (pH 6) for 1 min and then incubated for 9 min below boiling temperature. After 20 min cooling, slides were washed in distilled water for 5 min and blocked with blocking buffer (0.5% BSA in PBS + 0.5% Triton X-100). Later, the slides were incubated overnight at 4°C with primary antibodies of total JNK (1:400) and phosphorylated JNK (1:500) in blocking buffer. Next day, after three times washing with PBS-Tx (0.1% (v/v) Triton-X100, 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4) for 5 min, slides were incubated with secondary anti-rabbit IgG alkaline phosphates (1:400) in blocking buffer for 1.5 h at room temperature. After three times washing with PBS-Tx for 5 min, tissues were incubated with staining buffer (1 mg NBT in 10 ml RxN buffer (0.1 M Tris, 1 mM Mg^{2+}, pH 8.8) + 2 mg BCIP in 50 µl DMSO). After successful staining, slides were again washed with PBS-Tx (3x5 min). To conserve staining, the slides were washed with distilled water for 2 min followed by washing with increasing concentrations of ethanol (50%, 70%, 80%, 96% and 100%) for 2 min each. At the end, slides were incubated with Rotihistol for 5 min and a drop of Rotihistokitt was used with a cover slip to cover the slides. The images were recorded with a microscope and an attached camera [209].

2.1.8 Calcium imaging

The Fluo-4 (chemical fluorescent indicator) and cameleon (genetically encoded fluorescent indicator) were used to determine the acetylcholine (ACh) induced calcium signal (Ca^{2+} dependent fluorescence) in Kenyon cells of honeybee (adult) and *Drosophila* (larvae) respectively. Each indicator shows the fluorescence only when calcium is bound and thus demonstrates the excitation spectra of the cells. The fluorescence intensity was measured with fluorescence microscope and reflects the intracellular calcium concentration. Since both species reveal the similarities in ACh induced calcium dependent fluorescence signal. Therefore, the genetically manipulated *Drosophila* larvae (201y-GAL4, UAS-cameleon 2.1) were used frequently (Figure 2.6). In this case, the cameleon fluorescence is selectively

expressed in the mushroom bodies and does not need to be stained with chemical fluorescence indicators.

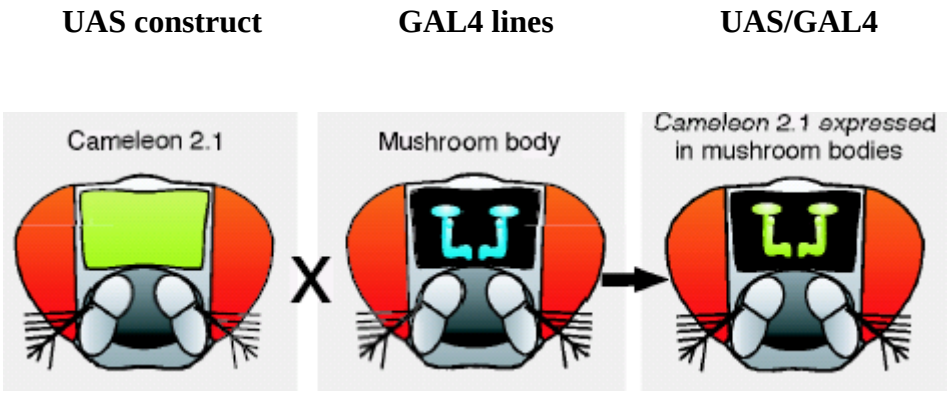


Figure 2.6: Specific expression of cameleon in mushroom bodies of transgenic *Drosophila*
The crossing of UAS cameleon 2.1 with specific Gal4 line leads to restricted cameleon expression in mushroom bodies [96].

Dissection and fixing of neural cells

The glass slides were washed with ethanol and after drying, small round circles (6-8 per slide) were marked on it. The boundary of marked circles was mounted with wax and the surface of circles was coated with poly-L-lysine. An adult brain of honeybee or 14 larval brains of *Drosophila* were dissected under the microscope in honeybee / *Drosophila* ringer solution (without Ca^{2+}). The brains were incubated in 50 µl collagenase enzyme at 34°C for 30 min and were stirred with pipette frequently. After the centrifugation at 1600 rpm for 3.5 min, the supernatant was discarded and the pellet was immersed in 200 µl of honeybee / TC100 *Drosophila* medium (cell nutrition). After that, 20 µl of each sample were loaded on each marked circle on poly-L-lysine coated glass cover slide and incubated for overnight at RT in dark to fix the cells on glass slide.

Staining and measurement of Ca^{2+} signals

After fixing, the glass slides were washed with PBS. After that, 20 µl *Drosophila* ringer (with Ca^{2+}) (*Drosophila* cells) and 20 µl of honeybee ringer (with Ca^{2+} and Fluo-4) (honeybee cells) were loaded per circle followed by incubation for 30/40 min at RT in dark respectively. For treatment, the *Drosophila* cells on the slides were exposed to UV light (UV lamp; Figure 2.3) for different periods of time including 5 min as an appropriate time point.

For inhibition of JNK and transcription, 2 µl of 200 µM JNK-inhibitor and actinomycin-D (final conc. 20 µM per circle) were applied on different slides. The DMSO concentration in all samples was 0.1%. After adding JNK-inhibitor or Act-D, slides were incubated for 30 min at RT followed by UV treatment as explained above. In parallel, a slide without any drug treatment and UV light exposure were included as positive control.

The changes in Ca^{2+} signals were measured with calcium imaging microscope (Figure 2.7), when cells were stimulated with 2 µl of 200 µM ACh or control (*Drosophila* / honeybee ringer with Ca^{2+}). The final concentration of ACh or control was 20 µM per circle. The percentage of stimulated cells was used to calculate the significant changes in ACh induced calcium signal.

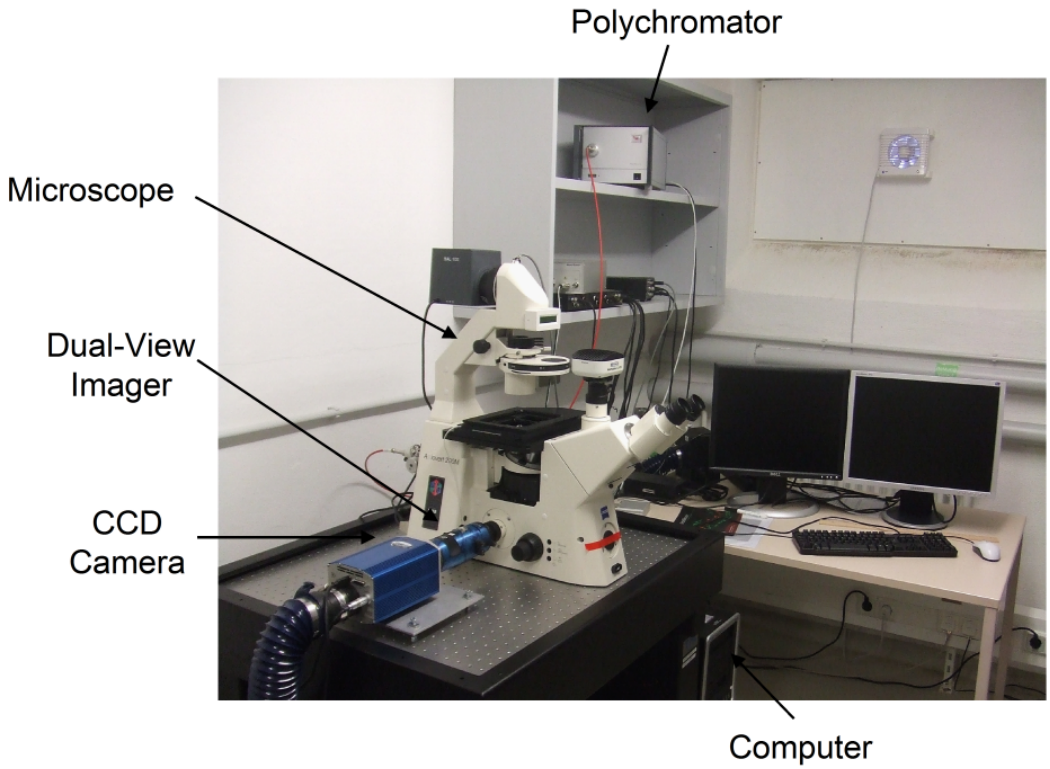


Figure 2.7: Calcium imaging microscope

2.2 MATERIAL

2.2.1 Chemicals

1-Butnaol	(Central chemical depot, Saarland University)	C.C.D
2-Mercaptoethanol		Merck
Acetylcholine chloride		Sigma
Agarose		C.C.D
APS (Ammonium peroxodisulfate)		C.C.D
BCIP (5-Brom-4-Chlor-3-Indolylphosphate)		Appli Chem
BSA (Bovine serum albumin)		Roth
$CaCl_2$ (Calcium chloride)		C.C.D
Chemiluminescence plus kit		Perkin-Elmer, Belgium, Zaventem
Chloroform		C.C.D
Clove oil (Nelkenöl)		Pharmacy
Collagenase / Dispase		Roche
DEPC (di-ethyl-propyl carbonate)		C.C.D
DMSO (Dimethyl sulfoxide)		C.C.D
DNase I (RNase free)		Fermentas
dNTPS mix		Fermentas
E 124 (food color)		C.C.D
EDTA (Ethylenedinitrilotetraacetic acid)		C.C.D
Ethanol		C.C.D
Fluo-4-AM (Fluorescence color dye)		Molecular probes
Glucose		C.C.D
Glycerin		C.C.D
Glycin		Roth
Hexamer primer		Fermentas
Isoamyl alcohol		Acros Organics
Isopropanol		C.C.D
KCl (Potassium chloride)		Merck
KH_2PO_4 (Potassium di-hydrophosphate)		Merck
L-15 Leibovitz medium		Invitrogen
Liquid nitrogen		C.C.D

Methanol	C.C.D
$MgCl_2$	Fermentas
$MgCl_2.6H_2O$ (Magnesium chloride)	Merck
M-MuLV reverse transcriptase	Fermentas
$Na_2HPO_4.2H_2O$ (di-Sodium hydrogen phosphate-2-hydrate)	C.C.D
NaCl (Sodium chloride)	C.C.D
$NaHCO_3$ (Sodium hydrogen carbonate)	C.C.D
NaOH (Sodium hydroxide)	Z-Chem
NBT (Nitro blue tetrazolium chloride)	Appli Chem
Paraffin wax	Fluka
Para-formaldehyde	C.C.D
Phenol	Sigma-Aldrich
Pipes	Appli Chem
p-NPP (para-Nitrophenylphosphat-disodium-hexahydrate)	Appli Chem
Poly-L-Lysine	Sigma-Aldrich
Proline	Appli Chem
Proteinase K enzyme	Sigma-Aldrich
Ribonuclease inhibitor	Fermentas
RNase inhibitor	Fermentas
Roti-Histokitt	Roth
Rotihistol	Roth
Rotiphorese Gel 30 (0.8% Bisacrylamid, 30% Acrylamid)	Roth
SDS (Sodium dodecyl sulfate)	Roth
Sucrose	Supermarket
Taq polymerase	Fermentas
TC 100 insect media (E15-856)	PAA
Temed (N,N,N',N'-Tetramethylethylenediamine)	Appli Chem
Tris (Tris-(hydroxymethyl)-aminomethane)	Sigma
Triton X-100 (Polyethylenglycol-tert-octyl-phenylether)	Fluka
Trizol reagent (Cat. No 15596-026)	Invitrogen
Tween 20 (Polyoxyethylensorbitanmonolaurat)	Appli Chem
Urea	C.C.D
Xylol	C.C.D

2.2.2 Antibodies and pharmacological drugs

Primary antibodies

Phospho-SAPK/JNK (Thr183/Tyr185) antibody (Cat. No. 9251)	Cell signaling
SAPK/JNK antibody (Cat. no. 9252)	Cell signaling

Secondary antibodies

Anti-Rabbit IgG peroxidase conjugate (Western blotting)	Sigma
Anti-Rabbit IgG (whole molecule) Alkaline phosphatase antibody (ELISA: product no. A3687)	Sigma
Anti-Rabbit IgG Alkaline phosphatase conjugate (Immunohistochemistry)	Sigma

Pharmacological injections

SP600125: JNK-inhibitor (Cat. No. S5567)	Sigma
Actinomycin D: transcription blocker (CAS no. 50-76-0)	AppliChem

Molecular weight marker

SeeBlue Plus2 Pre-Stained (western blotting)	Invitrogen
MassRuler DNA ladder, Low range (for PCR)	Fermentas

2.2.3 Apparatus and miscellaneous material

Apparatus

Binocular Leitz DMRB microscope	Leica
Binocular S6 D	Leica
Blotting apparatus	Owl
Calcium imaging microscope	Zeiss
Cold light lamp KL 1500 LCD	Schott
Digital shaker KS 501	IKA Labortechnik
DNA electrophoresis gel	Owl
ELISA-reader Safire	Tecan
Eppendorf centrifuge 5804 R	Eppendorf
Eppendorf master cycler personal (PCR devices)	Eppendorf

Flaming/Brown micropipette puller	Sutter Instrument corp. USA
Folio packing device	Severin
HTU SONI 130 Sonifier	Schwaebisch Gmünd Germany
Membrane vacuum pump	Vacuubrand GmbH
Micro-pipetten puller model	P-87 Sutter instrument Co.
Microtom autocut 2040	Reichert-Jung
pH-meter (inolab pH 730)	WTW
Power station 300 plus	Labnet
Shaker and heating device IKA RCT bass	IKA labortecnik
Spectrafuge 24 D centrifuge device	Labnet
Spectrophotometer- Biomate	Thermo Electron corp.
Switching power DPS-4005 PFC	Voltcraft
Thermo heating block	Labnet
UV light lamp (Nu-8 KL)	Benda
UV light lamp	Self-made
Vortex mixer device VX100	Labnet
Water bath	Labnet
Weight balance CP3202S and CP225D	Sartorius
White light lamp	Self-made

Miscellaneous material

384 wells ELISA plates (black)	Nunc
96 wells ELISA plates (NUNC-IMMUNO plate)	Thermo Labsystems
Bee tubes standing Rack	Self-made
Blot combs	Owl
Capilettor stik (1-5 µl)	Selzer
Capilettor tips Glass capillary (1-5 µl)	Selzer
Dental wax (medium)	Ubert
Developing film BioMax X-ray Film	Kodak
Eppendorf tubes (1.5 ml)	Eppendorf
Glass homogenizer with glass pestle	Braun
Honeybee harnessed tubes	Self-made
Honeybee mini cages	Self-made
Injection syringes	Braun
Mortar and pestle	C.C.D

Nitrocellulose-membrane (Optitran BA-S 85)	Schleicher and Schuell
Silicon (Baysilone-paste mittelviskos)	Bayer
Tooth picks	Super market
Whatman-filter paper	Schleicher and Schuell

2.2.4 Buffer and solutions

PBS (Phosphate buffer saline)

for 10x PBS

2.7 mM KCl	10 g/5l
1.8 mM KH_2PO_4	12.5 g/5l
137 mM NaCl	400 g/5l
10.1 mM Na_2HPO_4	90 g/5l

Make up the required volume with H_2O

SDS-Page

Homogenizing buffer

1 mM EDTA
4 M Urea
in PBS

Sample loading buffer

5x stock solution
10 mM 2-Mercaptoethanol
0.05% Bromphenolblau
20% Glycerin
10% SDS
0.2 M Tris
pH 6.8 with HCl

Resolving gel buffer 10% (4 ml)

1.617 ml Aqua dest.
0.020 ml 10% APS
1.333 ml Rotiphorese gel 30 (10% Bis-acrylamid)
1.000 ml Resolving buffer
0.020 ml 20% SDS
0.010 Temed

Resolving buffer

1.5 M Tris (pH 8.8 with HCl)

Stacking gel

4% (2 ml): 1.193 ml Aqua dest.
0.010 ml 10% APS
0.267 ml Rotiphorese gel 30 (10% Bis-acrylamid)
0.500 ml stacking buffer
0.025 ml 20% SDS
0.005 ml Temed

Stacking buffer

0.5 M Tris (pH 6.8 with HCl)

Running buffer (10 x stock solution) ***for 10x buffer***

2 M Glycin	300.28 g/2l
1% SDS	20 g/2l
0.25 M Tris	144.2 g/2l

Make up the required volume with H_2O

Western blotting

Blotting-buffer ***1x buffer***

0.2 M Glycin	28.8 g/2l
20% Methanol	400 ml/2l
0.25 M Tris	60.6 g/2l

Make up the required volume with H_2O

Blocking buffer 2% BSA 0.1% Tween 20 in PBS

ELISA

Homogenizing ELISA buffer 1 mM EDTA in PBS

Blocking buffer 0.5% BSA in PBS

RxN-buffer 1 mM $MgCl6H_2O$
0.1 M Tris-HCl pH 8.7

Staining solution 1 mM p-NPP
in RxN-buffer

Immunohistochemistry

Fixation buffer 50 ml 8% Para-formaldehyde
50 ml 0.2 M Phosphate buffer
with 1 M NaOH

Phosphate buffer

Stock solution A 0.2 M KH_2PO_4

Stock solution B 0.2 M $NA_2HPO_4.\ 2H_2O$

2 part stock solution A + 8 part Stock solution B

De-masking buffer 0.1 M Sodium citrate buffer (pH 6)

Blocking buffer-Tx 0.5% BSA 0.1% Triton X-100 in PBS

Staining buffer 1 mg NBT in 10 ml RxN buffer
2 mg BCIP in 50 µl DMSO

Washing buffer PBS-Tx 0.1% v/v Triton-X100
137 mM NaCl
2.7 mM KCl

10.1 mM Na_2HPO_4
1.8 mM KH_2PO_4

Calcium imaging

Drosophila ringer solution (Without Ca^{2+})

130 mM NaCl
5 mM KCl
2 mM $MgCl_2$
36 mM Sucrose
5 mM Herpes
in 100 ml distilled water
pH 7 adjusted with NaOH

Drosophila ringer solution (With Ca^{2+})

130 mM NaCl
5 mM KCl
2 mM $MgCl_2$
2 mM $CaCl_2$ x 2 H_2O
36 mM Sucrose
5 mM Herpes
in 100 ml distilled water
pH 7 adjusted with NaOH

Drosophila medium

TC 100 insect medium (PAA E15-856)
10% FCS (Foetal cell serum)
1% P/S (Penicillin / Streptomycin)

Honeybee ringer solution (With Ca^{2+})

130 mM NaCl
6 mM KCl
5 mM $CaCl_2$ x 2 H_2O
4 mM $MgCl_2$
10 mM Herpes
23 mM Glucose
117 mM Sucrose
in 100 ml distilled water
pH 7 adjusted with NaOH

Honeybee ringer solution (Without Ca^{2+})

130 mM NaCl
6 mM KCl
4 mM $MgCl_2$
10 mM Herpes
23 mM Glucose
117 mM Sucrose
in 100 ml distilled water
pH 7 adjusted with NaOH

Collagenase/Dispase-stock solution

10 mg Collagenase/Dispase
in 100 µl dH_2O

Collagenase/Dispase

100 µl Coll./Dis.-Stock solution
in 9.9 ml PBS

Bee ringer + Fluo-4 solution

50 µg Fluo-4 +50 µl DMSO (Stock solution)
1 µl stock solution + 500 µl bee ringer with Ca^{2+}
(20 µl per circle)

Bee medium

Primary medium (PM)

1 g Sucrose
0.625 g Fructose
0.825 g Proline
10 g Saccharose = Sucrose
in 70 ml Leibovitz's L-15 medium
(GIBCO: 11415-064)
Sterilize and add 180 ml L-15 medium

Bee medium (BM-3)

0.125 g Pipes (pH 6.7-6.8) in 20ml of 166.5 ml PM. Sterilize and mix in rest PM
Add 25 ml FCS/sterile and
2 ml Yeastolat/sterile (Gibco 18200-048)

3 RESULTS

3.1 VIRUS INFECTION AND JNK: ROLE IN LEARNING AND MEMORY OF HONEYBEE

3.1.1 Diagnosis of honeybee viruses

Two major genetic diagnostic tools (PCR and RT-PCR) were used for the diagnosis of honeybee viruses. Primers against bee viruses like Kashmir bee virus (KBV), acute bee paralysis virus (ABPV), deformed wing virus (DWV) and Sacbrood virus (SBV) were designed from available nucleotide sequences by using computer based software tools. Since each virus requires a defined set of primers, the conditions for each of these primers were optimized before the screening procedure. As optimization can only be carried out with samples containing the viruses, positive infected samples were obtained from the "Institut für Virologie, Justus Liebig Universität Giessen". Repeated RT-PCR experiments for each primer were performed to optimize conditions and quality of amplification (Figure 3.1)

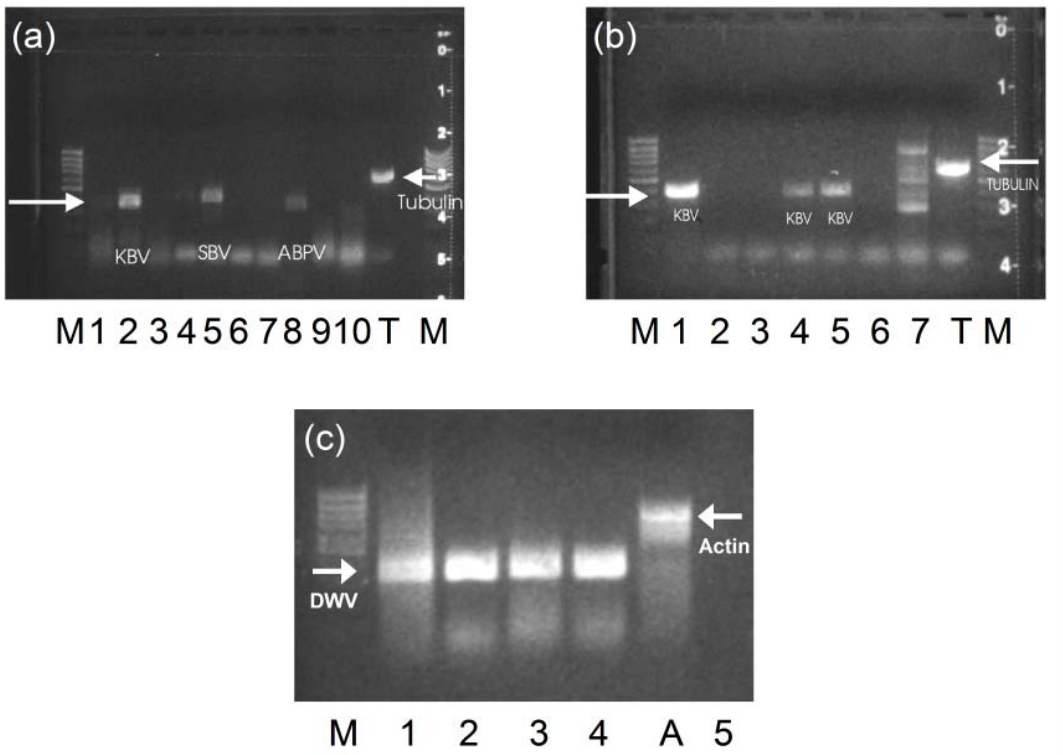


Figure 3.1: Diagnosis of honeybee viruses with RT-PCR
M, marker; T, Tubulin positive control (646 bp); A, Actin control (738 bp) **(a)** lane 1-3, KBV amplification with different salt concentrations (410 bp); lane 4-6, SBV amplification with different salt concentrations (450 bp); lane 7-9, ABPV amplification with different salt concentrations (400 bp); lane 10, negative control (water) **(b)** Diagnosis of KBV: lane 1-7, KBV amplification with different temperature and salt concentrations (410 bp **(c)** Diagnosis of DWV: lane 1-4, RT-PCR with different infected samples (355 bp); lane 5, negative control (water).

3.1.2 Screening and characterization of DWV infection

To investigate the persistency and natural infection of honeybee viruses in the vicinity of Saarbrücken, a screening procedure was started by collecting honeybee samples from beekeepers in surrounding areas of Saarbrücken. Different storage techniques like ethanol (75%, 99%) and liquid nitrogen were used to avoid sample degradation. Finally, liquid nitrogen was used to transport and store bee samples until their analysis in the laboratory. Each collected sample was subjected to several RT-PCRs for diagnosis of different viruses. Care was taken to process all collected samples immediately after collection in liquid nitrogen to avoid any degradation of RNA.

RT-PCR-based screening of honeybee samples revealed strong DWV signals (Figure 3.2 a). Samples were also checked repeatedly for infection with other bee viruses but in these bees, I found no evidence for infections by other viruses (ABPV, SBV and KBV) as tested by RT-PCR. Despite the strong DWV infection, there were no symptoms for wing deformation. This demonstrates that DWV can persist in adult bees without showing any symptoms. This screening resulted in detection of naturally occurring infection with DWV and provided a first candidate to investigate the artificial virus infection and learning in honeybees in more detail. In order to localize the DWV in different body region of honeybee, the honeybee was divided in to three body parts: head, thorax and abdomen. The repeated RT-PCR experiments show that the DWV signal was high in the abdomen and gradually decreases in the thorax and head (Figure 3.2 b).

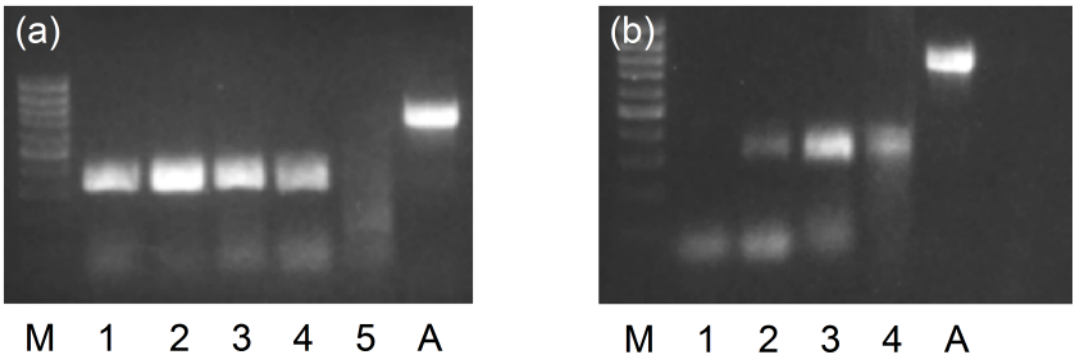


Figure 3.2: Localization of DWV in honeybee
M, marker; A, Actin positive control (738 bp) **(a)** DWV infection: lane 1-4, RT-PCR from different infected samples; lane 5, negative control (water). **(b)** Localization of DWV in body regions of honeybee: lane 1, head; lane 2, thorax; lane 3, abdomen; lane 4, DWV-positive control (355 bp).

3.1.3 Artificial virus infection

For the planned behavioural experiments, it was necessary to artificially infect groups of bees with virus while parallel handled control groups were not infected. In a first approach, the crude homogenate of DWV-infected bees (DWV-lysate) containing high level of virus and crude homogenate of non-infected bees (control-lysate) was used for artificial infection of honeybees under laboratory conditions. The RT-PCR diagnosis confirmed high virus signal in virus infected and no detectable signal in non-infected bees respectively.

Two different controls i.e. control-lysate and PBS were also injected in honeybee to compare the effect of control injections on the survival of honeybees. No difference was observed between the survivals of both groups of honeybees (Figure 3.3). This demonstrates that control-lysate can be used as proper control injection in further experiments.

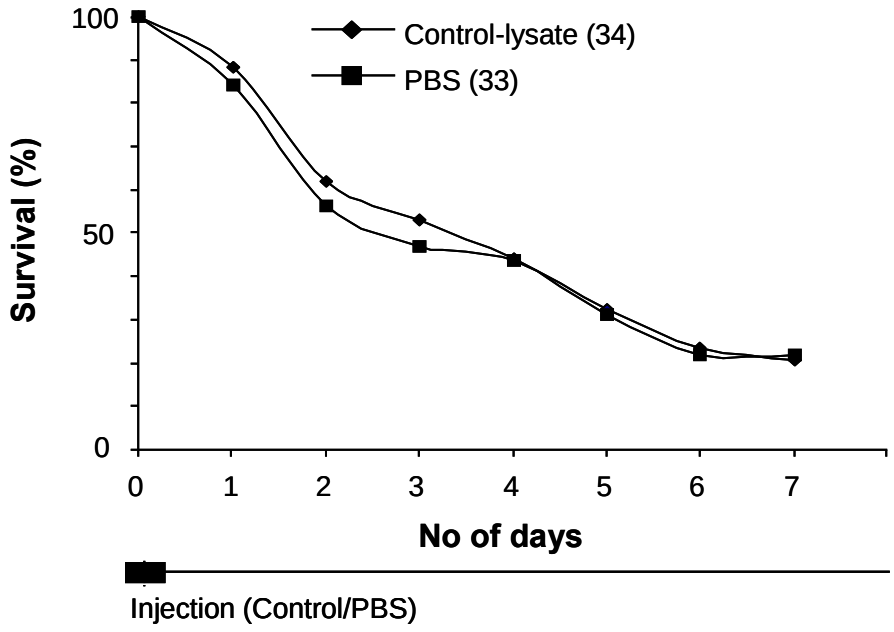


Figure 3.3: Survival of bees injected with control injections
Both solutions were injected into two different groups of honeybees at day 0 and survival was monitored for 7 days. The bees were fed with 1 M sucrose 3-4 times per days. The percentage of surviving bees remains same in both groups of bees.

3.1.3.1 DWV infection via food (oral application)

To test for a possible transfer of DWV via food, groups of bees were fed with sucrose alone or sucrose contaminated with control-lysate or DWV-lysate. The DWV infection was monitored

at different time periods after oral feeding of DWV using RT-PCR. The bees were kept in small wooden cages (Figure 2.2) and were fed with contaminated sucrose. As shown in Figure 3.4, these feeding experiments did not lead to a detectable infection by DWV within the tested time window of 7 days. Thus, oral application either does not lead to a DWV infection at all, or it takes much longer to reach the threshold level for DWV infection.

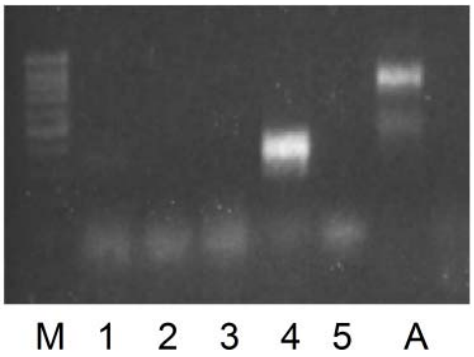


Figure 3.4: Oral application of DWV
M, marker; A, Actin positive control (738 bp) : lane 1, seventh day after continuous oral application of DWV-lysate in sucrose; lane 2, seventh day after continuous oral application of control-lysate in sucrose; lane 3, seventh day after continuous oral application of sucrose; lane 4, DWV-positive control (355 bp); lane 5, negative control (water).

3.1.3.2 DWV infection via hemolymph

Injection of DWV-lysate directly into the hemolymph of bees causes a strong RT-PCR signal when compared with the control groups injected with control-lysate or PBS. A signal was visible 3 days after injection and the signal increases gradually from 3 to 6 days (Figure 3.5). Thus injection in hemolymph can be used as an appropriate method for artificial infection of DWV in honeybees.

The DWV-lysate injected group shows very high mortality after 2-3 days of infection. The control-lysate injected group showed higher survival than infected group (Figure 3.6). Moreover, virus infected bees show a decrease in motor activity like slow antennal movement and slow extension of proboscis after sucrose stimulation while no difference in motor activity is observed in the control group.

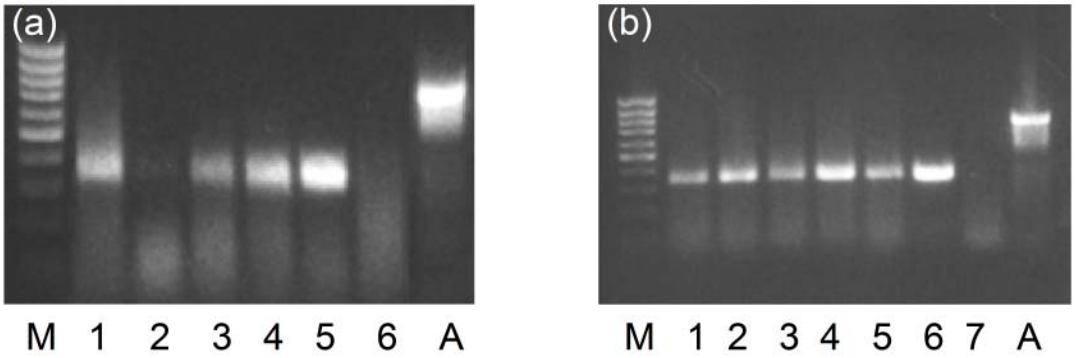


Figure 3.5: Time course of DWV hemolymph infection
M, marker; A, actin positive control (738 bp) **(a)** lane 1, DWV-positive control; lane 2-5, represent 2nd to 5th day after DWV lysate injection, respectively; lane 6, negative control (water). **(b)** lane 1-4, represent 3rd to 6th day after DWV-lysate injection, respectively; lane 5-6, DWV-positive control; lane 7, negative control (water).

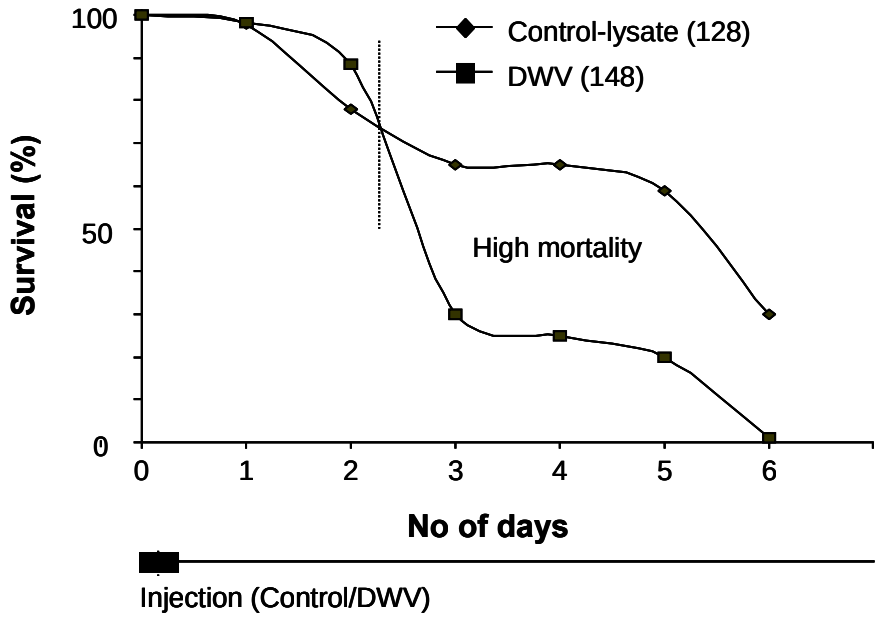


Figure 3.6: Survival of bees after DWV-lysate or control-lysate injection
Solutions were injected in two different groups of honeybees and survival percentage was monitored for consecutive 4 days. The bees were fed with 1 M sucrose 3-4 times per days. The survival percentage in DWV-lysate injected group decreased very rapidly after 2 days.

3.1.4 DWV and behavioural analysis

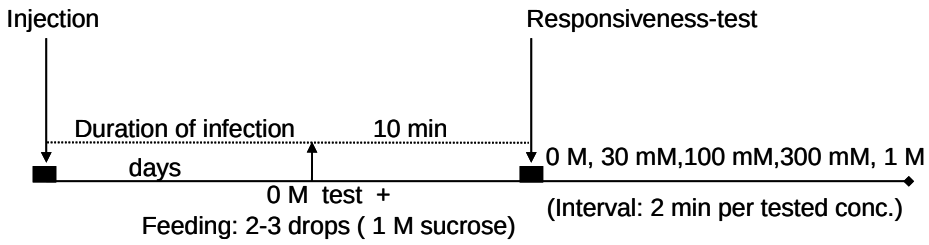
Associative learning is an essential component of the foraging behaviour of honeybee and of dance communication. Honeybees provide the opportunity to study non-associative learning

such as sensitisation and habituation as well as associative olfactory learning with single animals. These learning paradigms are based on the proboscis extension response (PER) elicited by appetitive stimuli like sucrose and thus it is necessary to test whether processing of this appetitive stimuli is affected by DWV infection.

3.1.4.1 DWV infection affects responsiveness to appetitive stimuli

Honeybees respond to sucrose concentration by protruding their proboscis. Since sucrose is used as sensory stimuli and reward in associative learning, it is necessary to test whether the responsiveness is changed after DWV infection. Based on the results derived from RT-PCR measurement (Figure 3.5 ab), I tested the responsiveness of bees at two time points during DWV infection: the first and the fourth day after injection. At these time points, I verified the level of DWV infection with RT-PCR and tested whether an antennal stimulation with sucrose elicits the PER.

Gradually increasing concentrations of sucrose (0 M, 30 mM, 100 mM, 300 mM, and 1 M) were used to test the responsiveness of control and infected honeybees. On the first day after injection, the responsiveness of DWV infected bees does not differ from that of the control group (Figure 3.7 a). However, on the fourth day after injection, the responsiveness of the DWV infected group to water and low sucrose concentration is strongly increased when compared with the control group (Figure 3.7 b). This data clearly indicates a principle effect of viral infections on sucrose responsiveness. The fact that sensory processing is also implicated in sucrose-rewarded learning makes an impairment of associative learning very likely. Since there were no differences in the responsiveness to high sucrose concentrations between DWV infected and control bees, I used 1 M sucrose as appetitive stimuli in the behavioural analysis of associative and non-associative learning.



Protocol 3.1: DWV infection and sucrose responsiveness

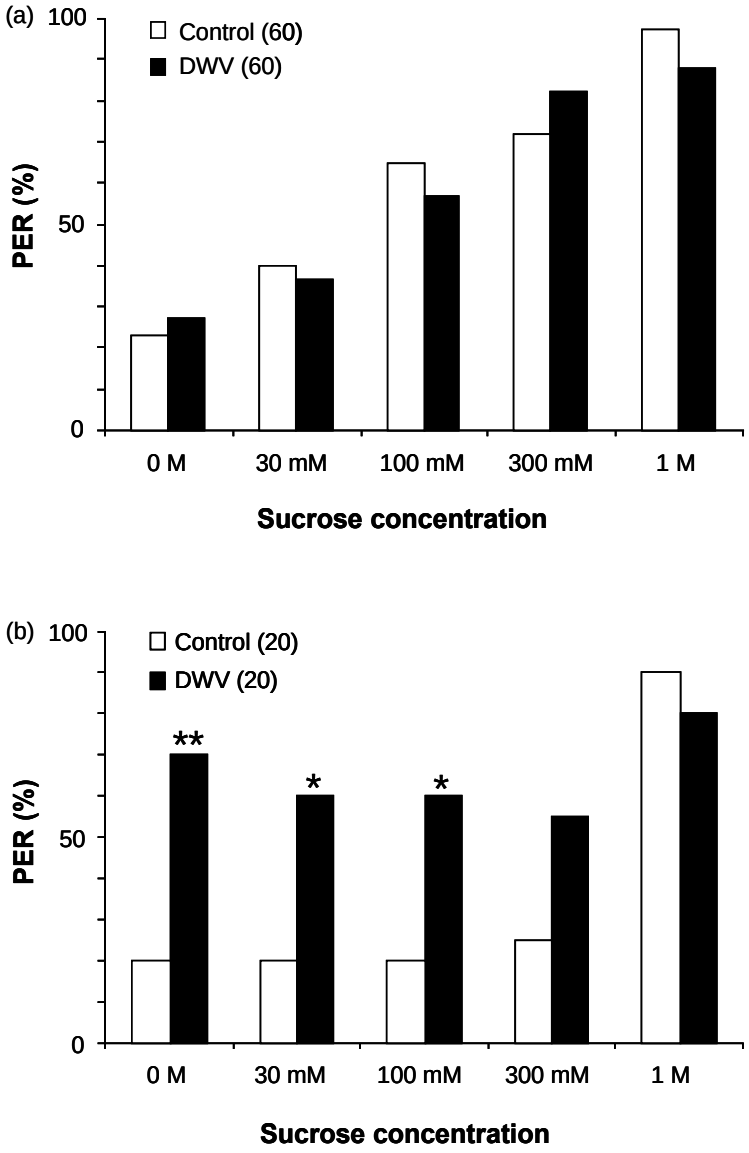


Figure 3.7: DWV infection and sucrose responsiveness
Sucrose responsiveness of bees was tested on the **(a)** first and **(b)** fourth day after artificial hemolymph infection with control-lysate and DWV-lysate. After each stimulation of an antenna with successive single stimuli of increasing sucrose concentrations (0-1 M), the PER (proboscis extension response) was recorded. The data show the mean responsiveness indicated by the percentage of PER. The asterisks indicate significant difference between the groups (χ^2 test / Fisher exact test; $^{**}p < 0.01$,$^{*}p < 0.05$).

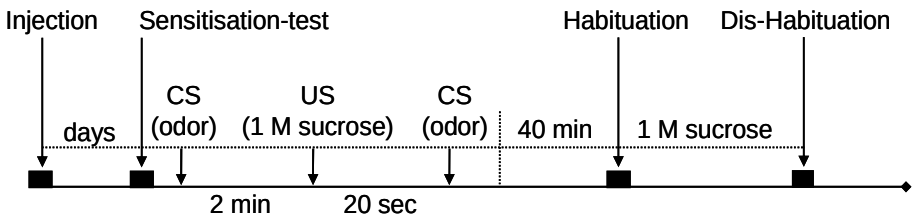
3.1.4.2 DWV infection and non-associative learning

Sensitisation and habituation of PER

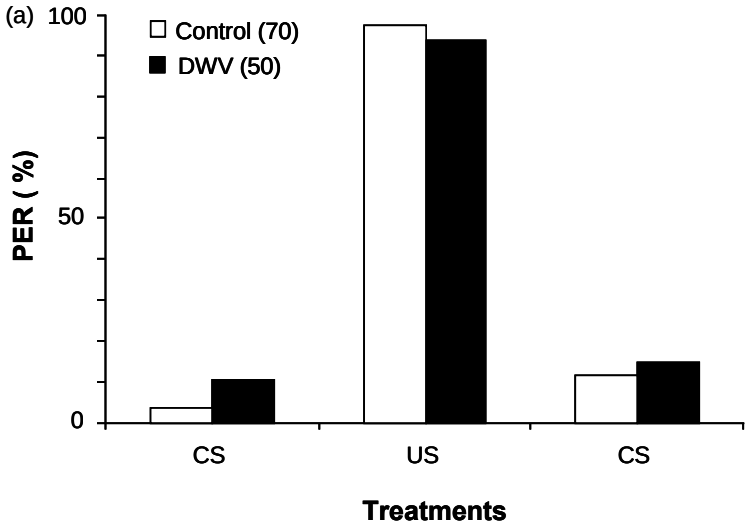
Sensitisation is the increased responsiveness to a neutral sensory stimulus (odor) shortly after application of a stimulus (sucrose) that arouses the animal. This non-associative learning paradigm was used to test any potential impairment in the processing of the sensory stimulus (odor). After testing the responsiveness to an odor stimulus, the honey bee was sensitised by

antennal stimulation with sucrose (1 M) that elicit the same responsiveness in DWV and non-infected bees (Figure 3.7). The second odor stimulus was immediately presented after 20 sec. As illustrated in Figure 3.8 a, DWV infected bees do not differ from control bees indicating that DWV infection does not affect processes underlying sensitisation using high sucrose concentrations (1 M). In both groups, the first odor stimulus elicits PER only in a few bees (less than 10%), while the arousing stimuli (1 M sucrose) leads to high levels of PER (more than 90%). Odor stimulation immediately after arousal also triggers PER in only a few animals (less than 20%) and indicating that there is no effect on odor processing.

Habituation represents the gradual decrease in responsiveness during a continuous series of repeated stimulations. When the PER is habituated, sucrose stimuli will no longer elicit PER. The observed difference in sucrose responsiveness between DWV and control (Figure 3.7) is diminished by using a high sucrose concentration (1 M) for repeated stimulation. Testing habituation from the first day until the fourth day after injection did not reveal any difference between the DWV infected group and the control group (Figure 3.8 b). Since DWV infection in both groups has been verified by RT-PCR, these results show that habituation is not affected by DWV. This together with the results on sensitisation shows that DWV infection does not affect non-associative behaviour.



Protocol 3.2: DWV infection and non-associative learning of honeybee



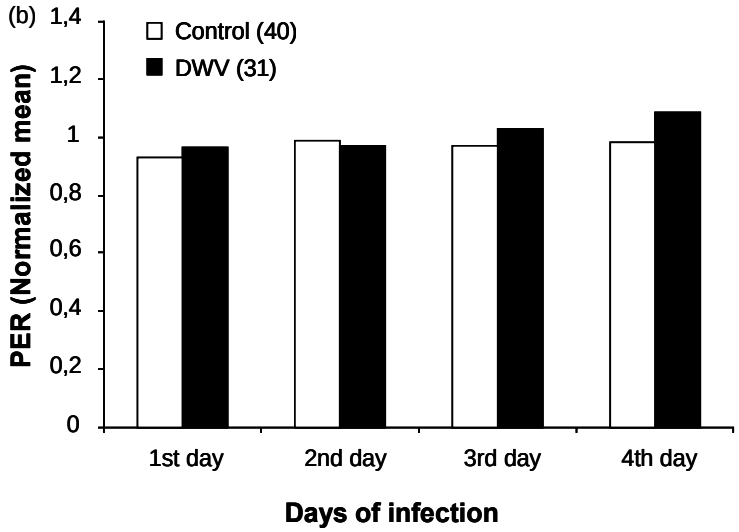
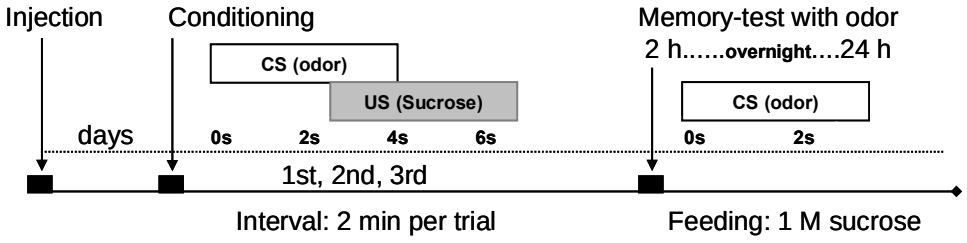


Figure 3.8: DWV and non-associative learning of honeybee
(a) Sensitisation of PER: The data shows mean of PER of control-lysate and DWV-lysate injected animals. The sensitisation of PER is unaffected with the DWV infection in honeybees. **(b)** Habituation of bees to sucrose: The data shows mean of normalized value of PER of control-lysate and DWV-lysate injected animals. The habituation of PER is also unaffected with the DWV infection in honeybees.

3.1.4.3 DWV and associative olfactory learning

Associative learning is a central prerequisite of foraging and social behaviour. The well-established associative olfactory conditioning paradigm consists of the pairing of an odor stimulus (CS) with a sucrose reward (US). Figure 3.9 shows that DWV infected bees show a significantly reduced acquisition when compared with control bees. Moreover, memory retention as tested 2 and 24 h after conditioning is also low in DWV infected bees. Thus, DWV infection seems to have specific effects on neuronal signalling processes because DWV infection only impairs associative learning without affecting non-associative processes.



Protocol 3.3: DWV infection and associative learning and memory formation

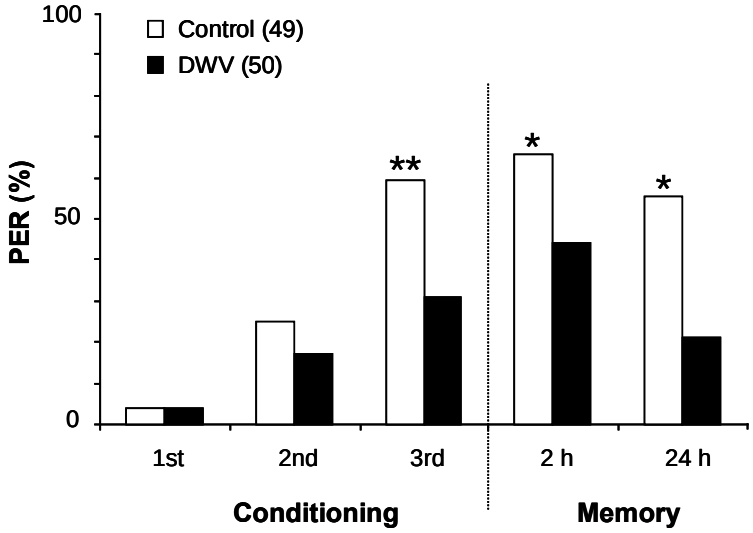


Figure 3.9: DWV infection and associative learning and memory
Olfactory conditioning of PER on the third day after infection with control-lysate and DWV-lysate. The animals were conditioned by pairing an odor stimulus (CS) with a subsequent sucrose reward (US) to the antenna and proboscis. Memory retention was tested on the fourth day after infection by stimulation with odor alone. The data show the percentage of PER elicited after CS stimulation in the control-lysate and DWV-lysate injected group. The asterisks indicate significant differences between the groups (Fisher exact test; $^{**}p < 0.01$,$^{*}p < 0.05$).

3.1.5 DWV infection and stress activated protein kinase

Viral infections as well as infections from other sources induce an immune response and it is feasible that DWV infection triggers the immune system of honeybee as described for viral infections in mammalian system. Among these pathways, JNK (Jun-N-terminal kinase) is a cascade that plays a major role in triggering immune response after infections in mammals.
The protein sequences were compared in vertebrates and the honeybee by using the sequence data bank. The comparison of human JNK (AAI30571; P45983.2, P45984.2, P53779.2), mouse JNK (NP_057909.1), *Drosophila* JNK homologue (NP_723541) and the honeybee JNK homologue (XP_392806) reveals a high identity at the level of the amino acid sequence (Figure 3.10 a). Especially the phospho-domain (Thr183/Tyr185) critical for the regulation of JNK activity is identical between insects and mammals and points to conserved functions of the JNK signalling cascade (Figure 3.10 b). This opened the possibility to use commercially available mammalian antibodies to detect and quantify honeybee JNK and thus to determine the contribution of JNK in infection triggered signalling pathways in mechanism underlying learning and memory formation. Since JNK has been hardly described in invertebrates, I first characterized the JNK system in honeybees and established techniques to measure and manipulate the JNK activity. Based on the available information of the peptide sequence, I

tested several antibodies for their specificity for honeybee JNK. By using western blotting and ELISA (Enzyme-Linked ImmunoSorbent Assay), I could verify the specificity of antibodies as tools to detect honeybee JNK.

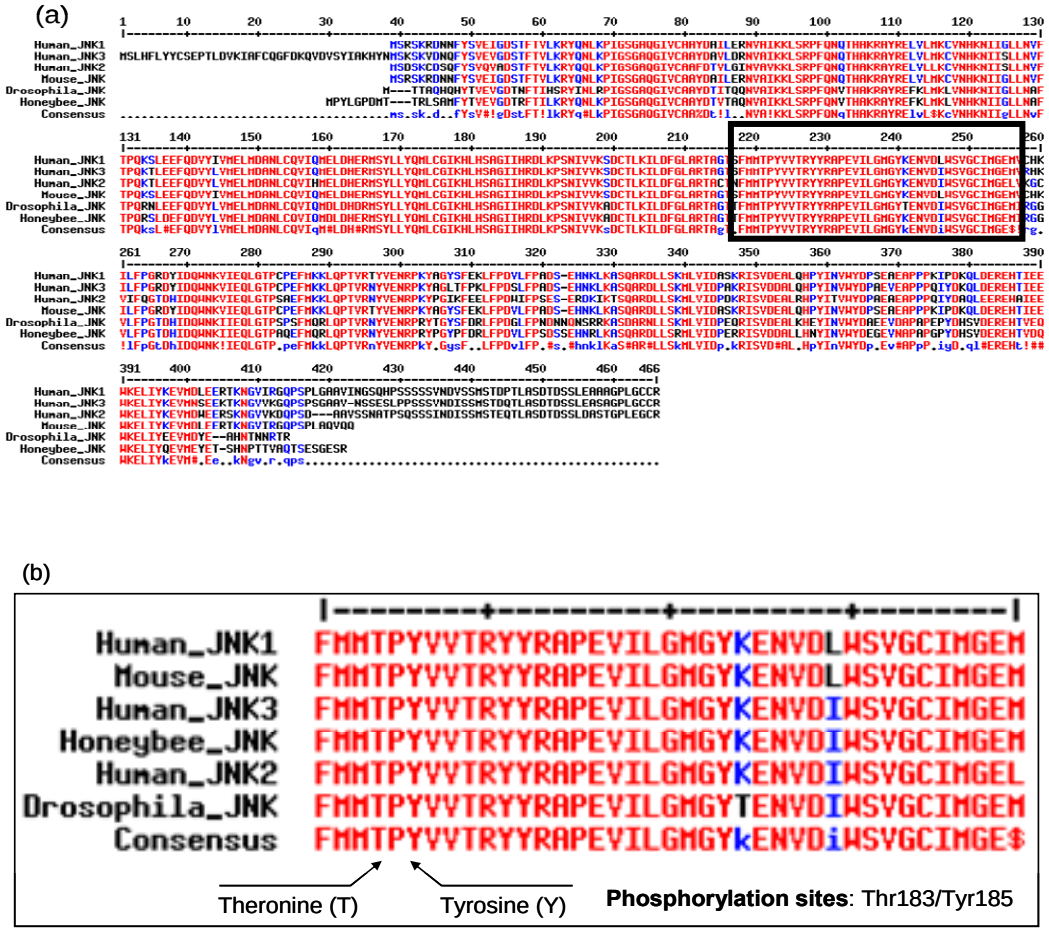


Figure 3.10: Protein blast analysis of JNK
(a) Comparison of the protein sequences of human JNK (AAI30571; P45983.2, P45984.2, P53779.2), *Drosophila* JNK homologue (NP_723541) and the honeybee JNK homologue (XP_392806) reveals a high identity at the level of the amino acid sequence. **(b)** Highly similarity of two phosphorylation sites (Thr183/Tyr185) between insects and mammals pointing towards the conservation of JNK signalling cascade.

3.1.5.1 Mammalian antibody detects JNK in honeybee brain

To determine the specificity, localization and the relative JNK activity in the brain of honeybee, I selected antibodies that detect JNK regardless of the state of phosphorylation at position (Thr183/Tyr185) and a phosphospecific antibody that detects only the phosphorylated phospho-domain (Thr183/Tyr185). After optimizing the handling protocol and buffers, I was able to establish a procedure that enables the conservation of the state of

phosphorylation and thus the state of JNK activity throughout the time of dissection, western blotting and ELISA.

Western blotting

As expected from identical amino acid sequence between honeybee, *Drosophila* and mouse (Figure 3.10 b), the western blot with JNK antibody show the detection of similar molecular weight of JNK in honeybee brain (46 kDa) as in *Drosophila* and mouse brain (Figure 3.11). In parallel, phospho-JNK antibody reveals the identical results. Thus mammalian based antibodies can detect the JNK with or without phosphorylation and was used for further protein analysis.

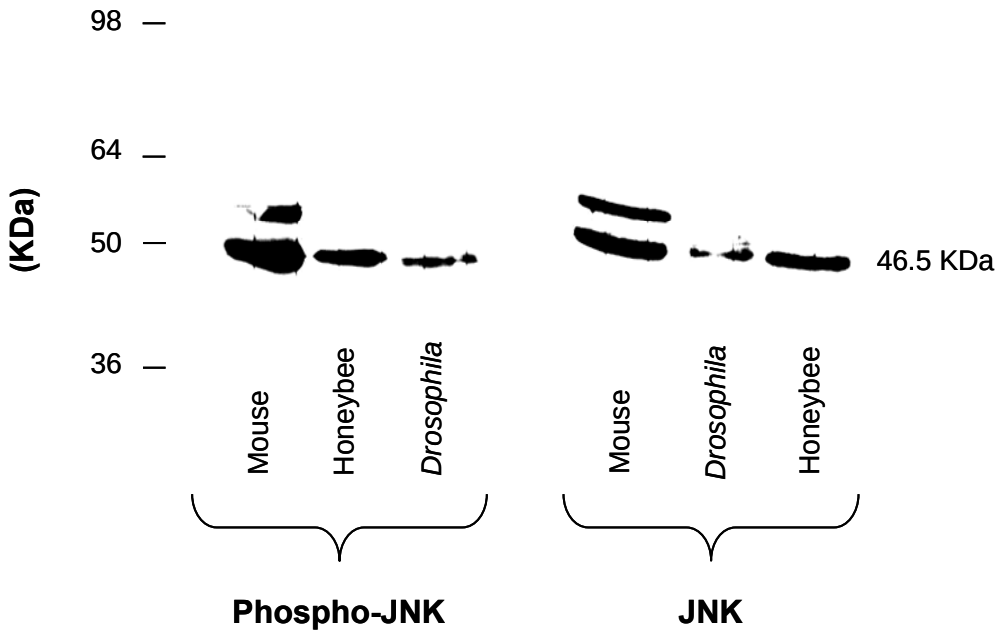


Figure 3.11: Immunological detection of JNK in honeybee brain
The western blotting shows that antibodies against JNK (Thr183/Tyr185) recognize similar corresponding proteins in the brain homogenate of honeybee as in *Drosophila* and mouse. Molecular weight (46.5 kDa) is indicated on the right side of the picture. Proteins from all target samples are situated at the same attitude. Testing of phospho-JNK antibody also reveals the identical results.

Immunohistochemistry

Using antibodies against JNK in immunohistochemistry demonstrates homogenous labeling throughout the honeybee brain. (Figure 3.12 ab). All brain areas are labeled and no apparent difference in labeling intensity is detectable between soma and neuropil. In contrast, the antibodies against the phosphorylated phospho-domain (Thr183/Tyr185) indicate enzyme

activity in a very characteristic and restricted labeling pattern. While most brain areas are labeled very weak, the α-lobes and the peduncle (Pe) of the mushroom bodies (MB) as well as the large Kenyon cells show a strong immunolabeling (Figure 3.12 cd). Although the used fixation procedure was optimized for tissue integrity and not to preserve the phosphorylation state, the strong labeling suggests a high JNK activity in the MBs.

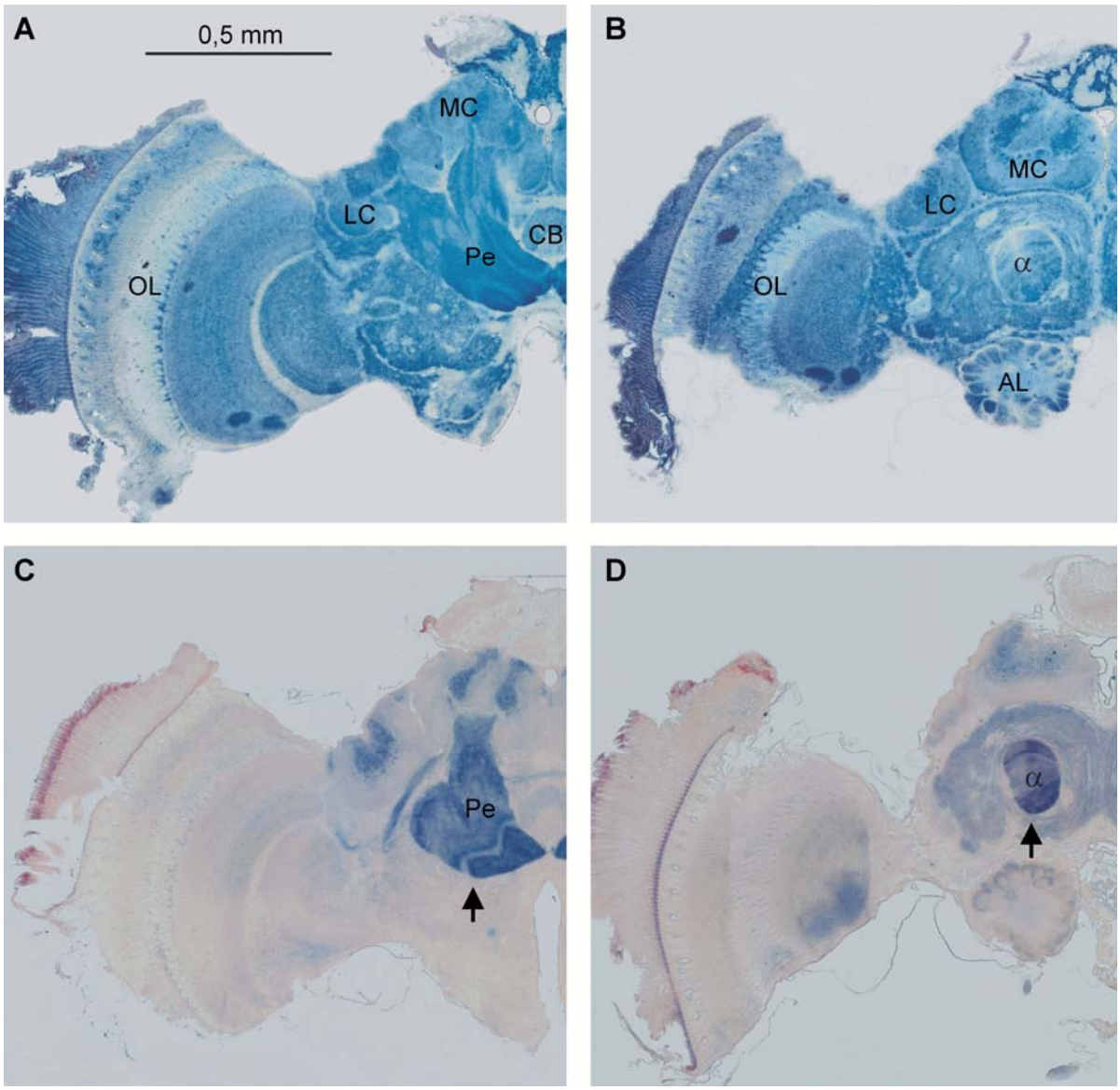


Figure 3.12: Immunohistological localization of JNK activity in honeybee brain
The JNK antibody shows the distribution of JNK in overall honeybee brain (A and B) while the phospho-JNK antibody reveals strong staining of peduncles (Pe) and α-lobes (α) of mushroom bodies that are indicated by arrows (C and D). The immunoreactivity is barely detectable in antennal lobe (AL), optical lobe (OL), lateral calyx (LC), median calyx (MC), central body (CB) and remaining parts of brain.

3.1.5.2 Quantification of JNK activity in honeybee brain

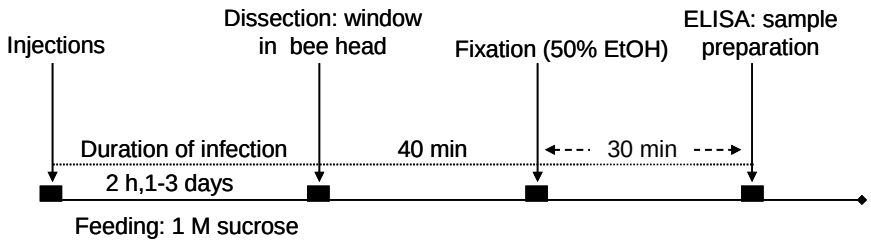
To test whether JNK in honeybees is activated by virus infection, ELISA was used to measure the relative JNK activity in honeybee central brain (excluding optical lobes). The relative JNK activity is defined as the value of the relative phosphorylation at position Thr183/Tyr185, determined by the phospho-specific JNK antibody, divided by the value of JNK, determined by the JNK antibody in the same sample.

$$\text{Phosphorylation level} = \frac{\text{Phospho-JNK}}{\text{JNK}}$$

Since phosphorylation is easily degradable, the question was how to prevent the protein denaturation and fix protein phosphorylation in its actual state. Pre-tests showed that immersing and incubating the whole honeybees in 50% cooled (-20 ^{0}C) ethanol with a window in head (Figure 2.5) for 30 min at ice preserves the state of JNK phosphorylation in the brain tissue of honeybee.

DWV activates the JNK in brain of honeybee

The control experiments with solutions (10-20% DMSO, PBS and control-lysate) reveal identical level of relative JNK activity (Figure 3.13 a). In contrast, the injection of DWV-lysate into honeybee hemolymph significantly activates the JNK in honeybee central brain in time dependent manner. DWV significantly up-regulates the JNK activity 2 h and 3 days after infection except after 1 day where JNK is down-regulated (Figure 3.13 b). At later time point (3 days), DWV impairs the behaviour of honeybee where RT-PCR also reveals the high virus signal (Figure 3.5).



Protocol 3.4: Fixation and sample preparation for ELISA

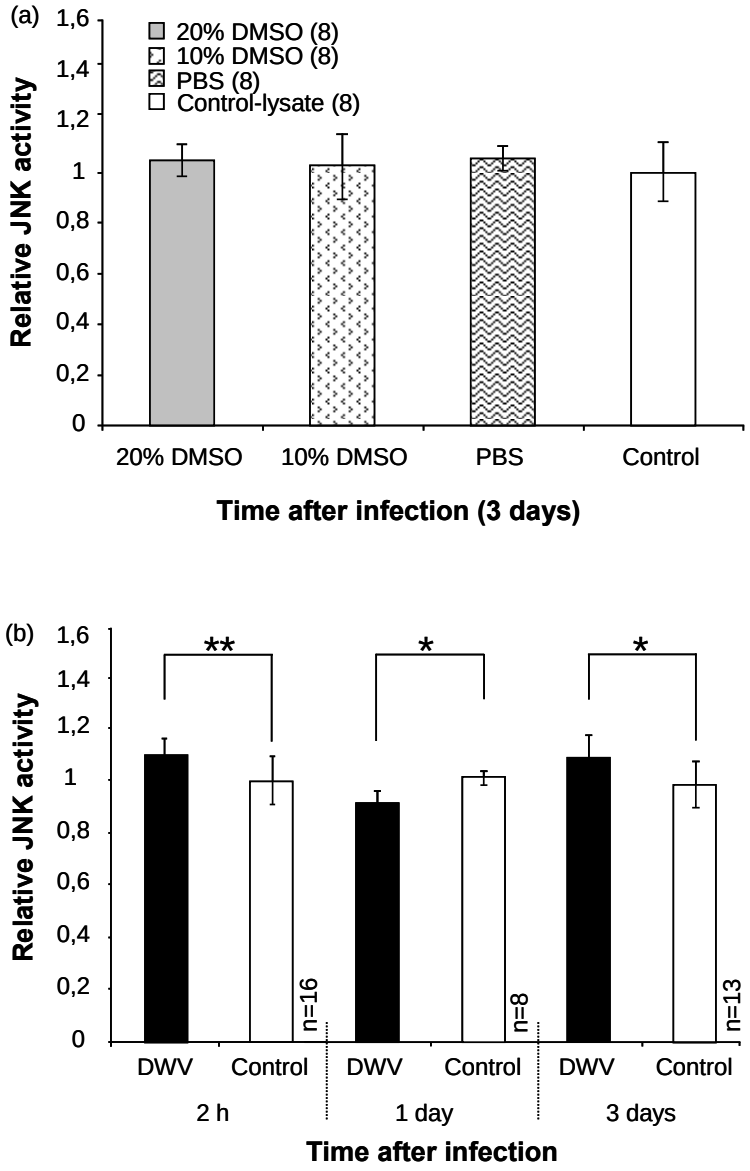
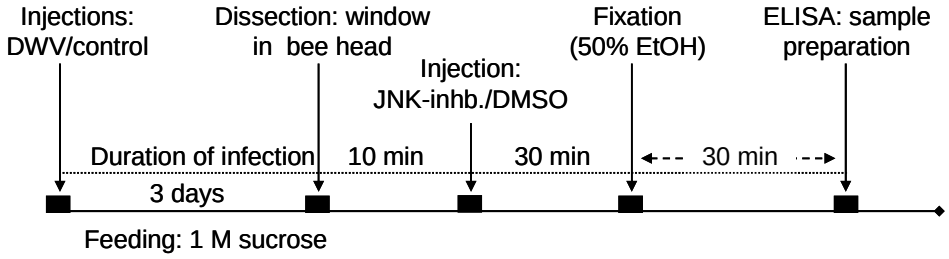


Figure 3.13: Quantification of relative JNK activity in honeybee brain
(a) Control injections (DMSO 10-20%, PBS and control-lysate) lead to identical relative JNK activity. **(b)** The level of JNK activity is increased significantly after 2 h, decreased after 1 day and again increased after 3 days of infection in DWV-lysate injected animals as compared to control-lysate injected animals. The asterisks indicate a significant difference between the two groups (τ-test; $^{**}p < 0.01$, $^{*}p < 0.05$).

3.1.5.3 Inhibition of JNK rescues DWV induced elevation in JNK activity

A specific JNK-inhibitor (SP600125) was used to investigate whether the virus induced activation of JNK in the honeybee brain can be rescued by its injection into the hemolymph. Two groups of honeybees were injected with DWV-lysate while in parallel another group was injected with control-lysate 3 days after infection, JNK-inhibitor (in 10% DMSO) was injected 30 min before the ELISA in one of the DWV infected groups. In addition, other

groups were also injected with 10% DMSO for equal handling in all groups of animals. As shown in Figure 3.14, the systemic inhibition of JNK with JNK-inhibitor during virus infection prevents the elevation in JNK activity. This provides strong evidence that virus infection induces JNK activation in the honeybee brain *in vivo*.



Protocol 3.5: Inhibition of JNK and ELISA

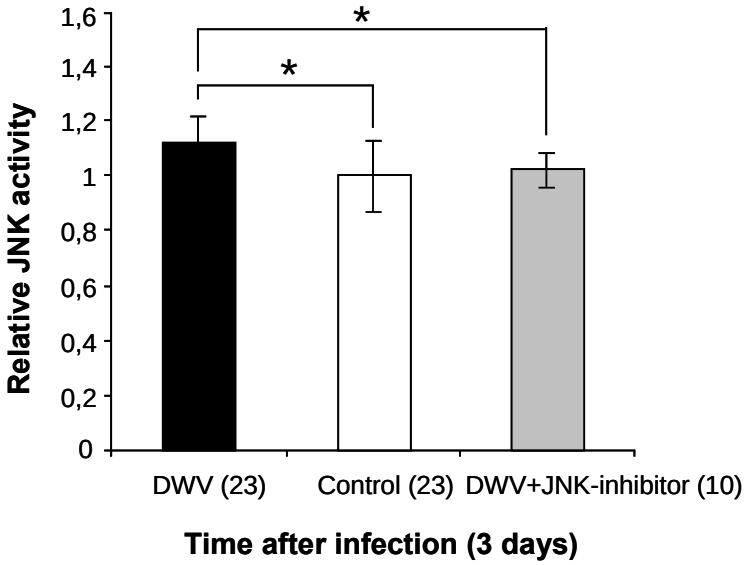


Figure 3.14: Inhibition of JNK and quantification of JNK activity
3 days after virus infection with or without JNK-inhibitor, the level of JNK activity is decreased in JNK-inhibitor injected animals as compared to control animals. For each experiment, values were normalized to the mean value of control samples. The asterisks indicate a significant difference between the two groups (τ-test; **p* <0.05)

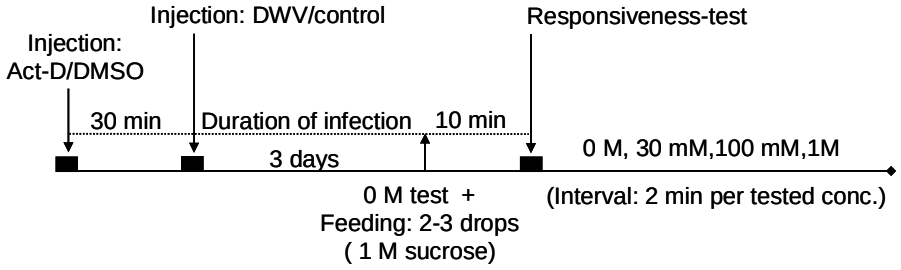
3.1.6 DWV infection acts via transcription

Transcription blocking rescues DWV induced impairments in behaviour

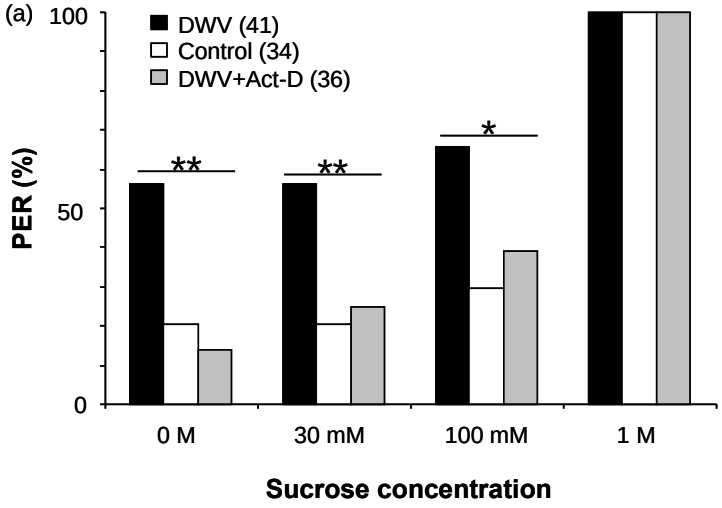
The JNK pathway regulates many transcriptional factors that are responsible for the regulation of transcription and gene expression. The previous studies reveal that JNK acts

exclusively via transcription processes and this prompted me to test whether blocking transcription rescues the DWV induced impairments in sensory processing, learning and memory formation of honeybee. A transcriptional blocker (actinomycin-D; Act-D) was used to block complete transcriptional machinery. Three separate groups of honeybees were injected with DWV, DWV with Act-D and control-lysate respectively. In addition, 20% DMSO was injected in groups without Act-D injection for equal handling of all animals. It is found that Act-D prevents the virus induced effects on sucrose responsiveness. Responsiveness of animals from the control group does not differ from responsiveness in animals injected with DWV in presence of transcription blocker (Figure 3.15 a). Moreover, transcription blocking shortly before virus infection partially rescue learning and complete rescues the impairment in memory formation (Figure 3.15 b). These data clearly demonstrate that JNK partially acts via transcription.

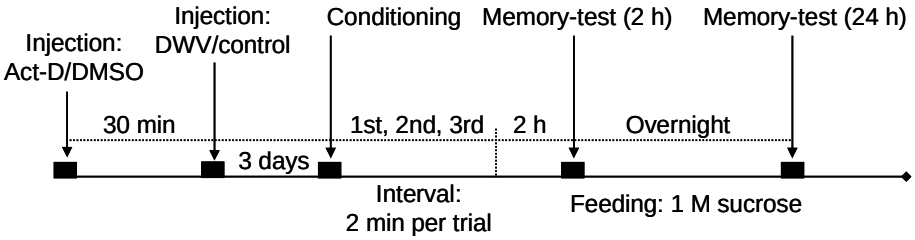
Responsiveness



Protocol 3.6: Transcription blocking during DWV infection and sucrose responsiveness



Associative learning



Protocol 3.7: Transcription blocking during DWV infection and associative learning

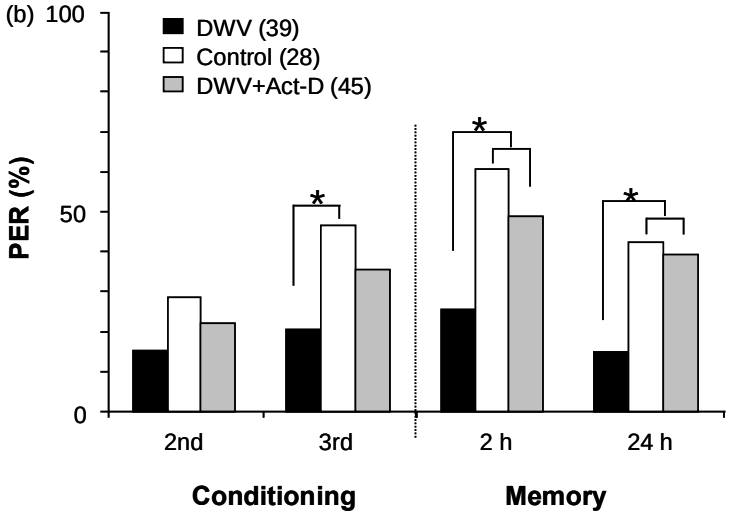


Figure 3.15: Transcription blocking rescues DWV induced behaviour impairments
(a) Injection of actinomycin-D rescues the virus induced changes in sucrose responsiveness. The data shows the PER of DWV, DWV + Act-D and control injected animals **(b)** Transcription blocking with actinomycin-D only rescues the virus induced impairment in memory formation of honeybee. The asterisks indicate a significant difference between two groups (Fisher exact test; $^{**}p < 0.01$,$^{*}p < 0.05$).

3.2 EXTERNAL STRESS STIMULI AND JNK: ROLE IN LEARNING AND MEMORY OF HONEYBEE.

The mammalian JNK is also reported to be triggered by many environmental stress stimuli and has an important function in well described mammalian immune response. Despite of the JNK contribution in immune response, very few studies in mammals reveal its involvement in learning and memory. Therefore, I investigated the potential effect of external stress on learning and memory via stress activated protein pathways by using behavioural paradigm in honeybee.

Screening of stress stimuli

In invertebrates, little is known about stress parameters and how stress influences behaviour. Therefore, I started different screening procedure to identify physiological relevant stimuli to study the implication of JNK in learning and memory of honeybee. Two types of stress stimuli were utilized in subsequent experiments.

1. **Short exposure to ultraviolet light; UV (acute stress stimulus):**

The honeybees were exposed to UV light for different period of times (5, 15 and 30 min) with UV lamp (Figure 2.3). 30 min was used as an optimal time point for UV exposure in molecular and behavioural analysis.

2. **Overnight shaking with light; SL (chronic stress stimulus)**

The bees were kept for the whole night in a plastic box and kept on a shaker with alternate cycles (15 min) of shaking with light and complete rest (no shaking with dark).

3.2.1 External stress: Quantification of JNK activity in honeybee brain

3.2.1.1 UV light exposure activates JNK

Like DWV infection, the relative activity of JNK was measured in brain of honeybee with ELISA after UV light exposure. Comparing DWV infection (3 days after infection) with UV light exposure (30 min), both stimuli (infection and stress) significantly increase the JNK activity in the honeybee brain as compared to control-lysate and white light (control) respectively (Figure 3.16 a). This shows that besides virus infection, UV light exposure is also an appropriate external stimulus to activate JNK in honeybee.

During time course of UV light exposures, the elevation of JNK activity is already detectable after 15 min of UV light exposure while no elevation was detected after 5 min UV light exposure when compared with the corresponding white light control (Figure 3.16 b). Moreover, UV light induced JNK activity is back to baseline after a period of time (1-2 h)

which does not differ from the control group (data not shown here) and suggests a transient UV light induced activation of JNK.

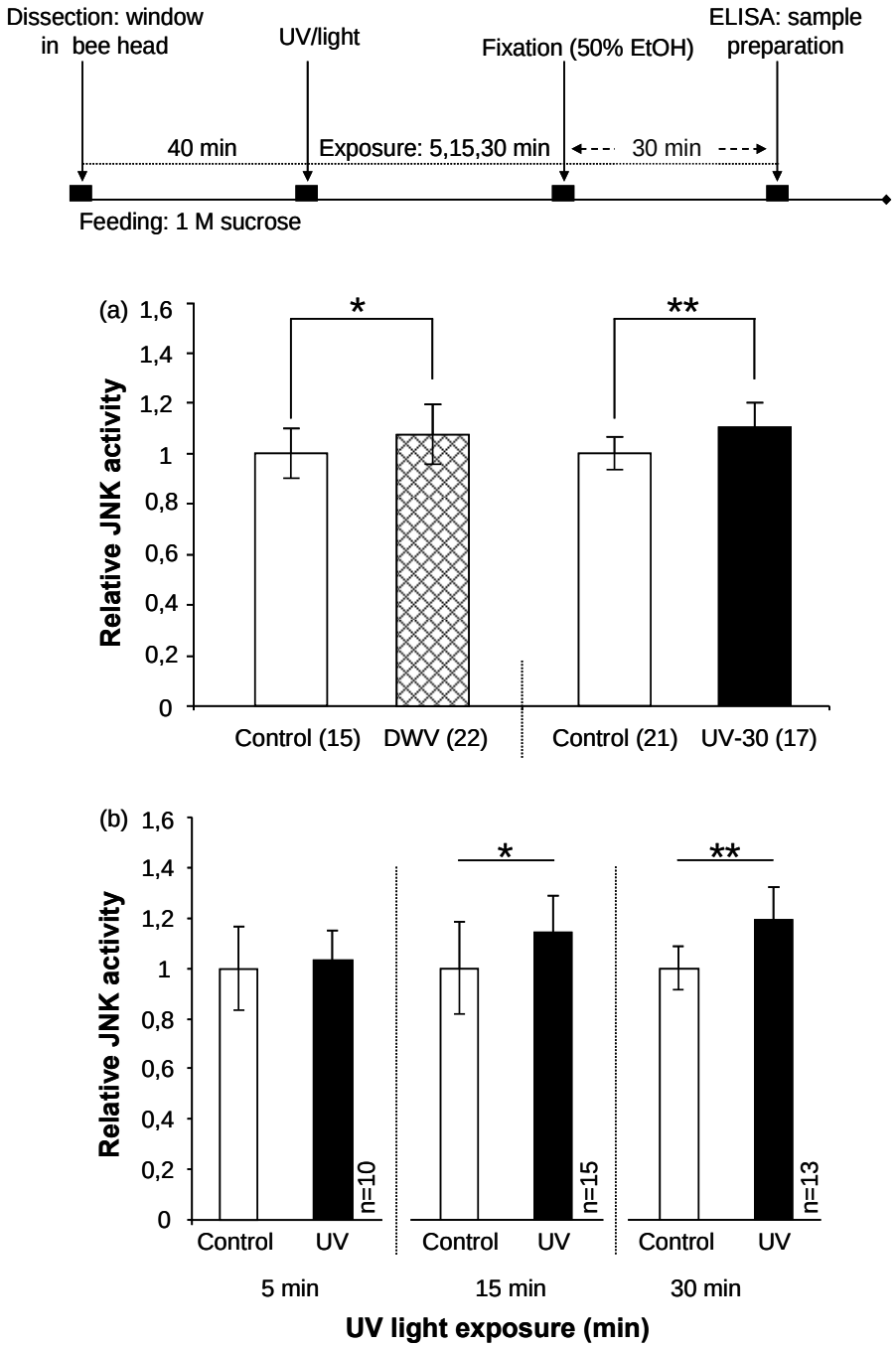


Figure 3.16: Quantification and time course of JNK activity after UV light exposure
(a) Quantification of relative JNK activity in brain of honeybee after DWV infection (3 days after infection) or control infection and after 30 min exposure to UV light or white light (control). The level of JNK activity is increased in DWV infected and UV light exposed animals as compared to control animals. **(b)** Time course of UV light exposure and quantification of relative JNK activity in brain of honeybee. The level of JNK activity is increased in UV light exposed animals (15 min and 30 min) as compared to control animals in contrast to 5 min UV light exposure where no difference is observed between two groups. For each experiment, values were normalized to the mean value of control samples. The asterisks indicate a significant difference between the two groups (τ–test; $^{**}p < 0.01$,$^{*}p < 0.05$)

3.2.1.2 Shaking with light (SL) activates JNK

To test whether the second external mechanical stress stimulus i.e. overnight shaking with light (SL) also triggers the JNK, I measured the relative JNK activity in honeybee central brain in comparison to animals exposed to UV light (30 min). SL also significantly increases the JNK activity in honeybee brain when compared with control animals (dark without shaking). This elevation is identical with JNK activity in animals exposed to 30 min UV light (Figure 3.17). Thus two different stimuli (UV and SL) are able to activate the JNK pathway.

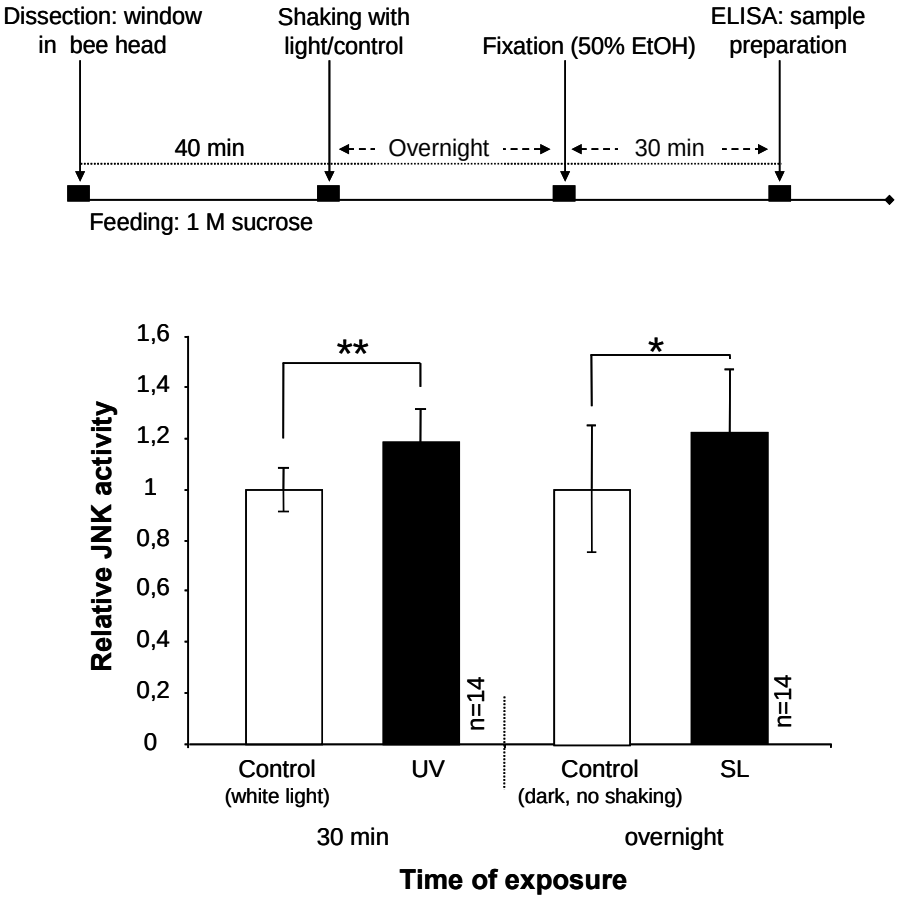


Figure 3.17: JNK activity after UV light and SL exposure
Quantification of relative JNK activity in brain of honeybee after overnight shaking with light or control (dark without shaking) and 30 min exposure to UV light or white light (control). For each experiment, values were normalized to the mean value of control samples. The level of JNK activity is increased in SL and UV light exposed animals as compared to control animals. The asterisks indicate a significant difference between the two groups (τ–test; $^{**}p < 0.01$,$^{*}p < 0.05$)

3.2.2 Stress and behavioural analysis

The similarity between JNK activation with virus infection and stress stimuli (UV and SL) suggested that activation of JNK by stress stimuli may also lead to similar deficits in the

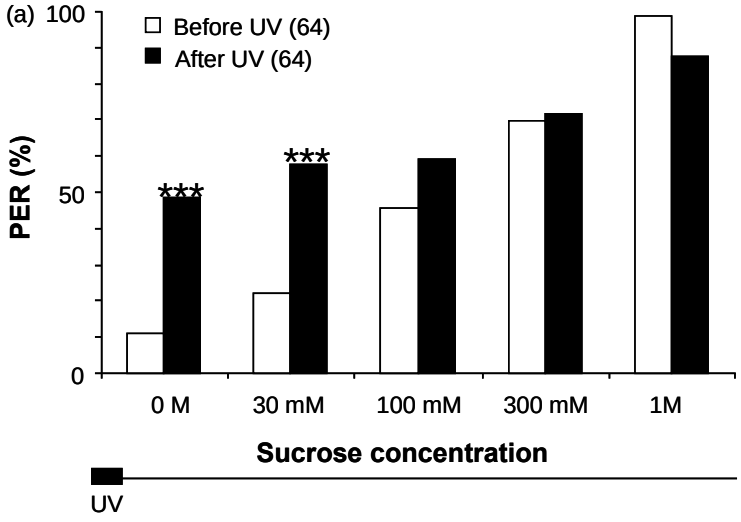
behaviour of honeybee as in case of DWV infection .Therefore, I used these two stress stimuli (UV and SL) to investigate their effect on responsiveness to appetitive stimuli and associative conditioning of honeybee.

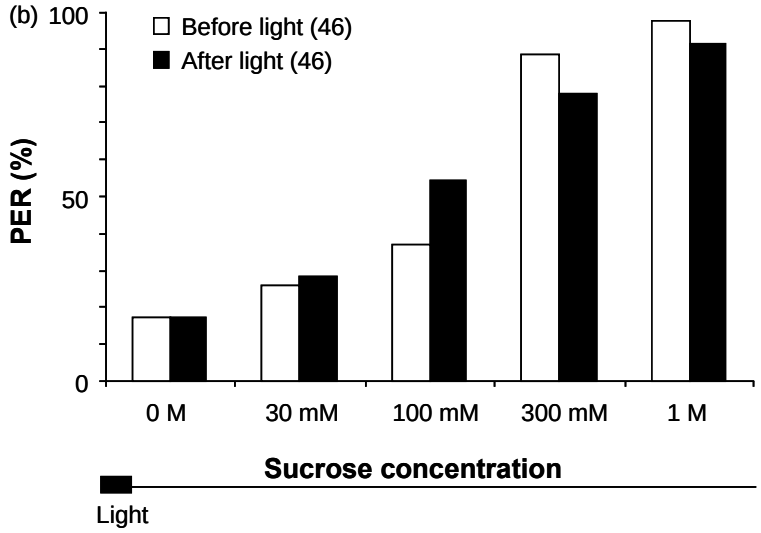
3.2.2.1 Stress and responsiveness to appetitive stimuli

UV light and SL exposure affects responsiveness

The responsiveness of animals was measured before and 30 min after exposure to UV light and control (white light). Gradually increasing concentrations of sucrose (0 M, 30 mM, 100 mM, 300 mM, and 1M) were used to test the responsiveness of both UV light and normal-light exposed honeybees. Honeybees exposed for 5 min to either UV light or white light do not differ in their responsiveness. However, when animals are exposed for 30 min to UV light, responsiveness to water and low sucrose concentrations is significantly increased as compared to animals treated with white light (Figure 3.18 a). In the light exposed control group responsiveness before and 30 min after light exposure is indistinguishable (Figure 3.18 b). The UV light exposure for 15 min already causes a significant but intermediate effect, indicating a dose-dependent action of UV light on the neuronal processing of sensory stimuli *in vivo*. The response to high sucrose concentrations is not affected by UV light exposure.
Interestingly, honeybees exposed to SL show a significant increase in the responsiveness as compared to control animals (Figure 3.18 c) as observed in infection and UV light exposure. Thus, these different stimuli have the same principle effect on sucrose responsiveness of honeybee as in DWV infection.

UV light and responsiveness





SL and responsiveness

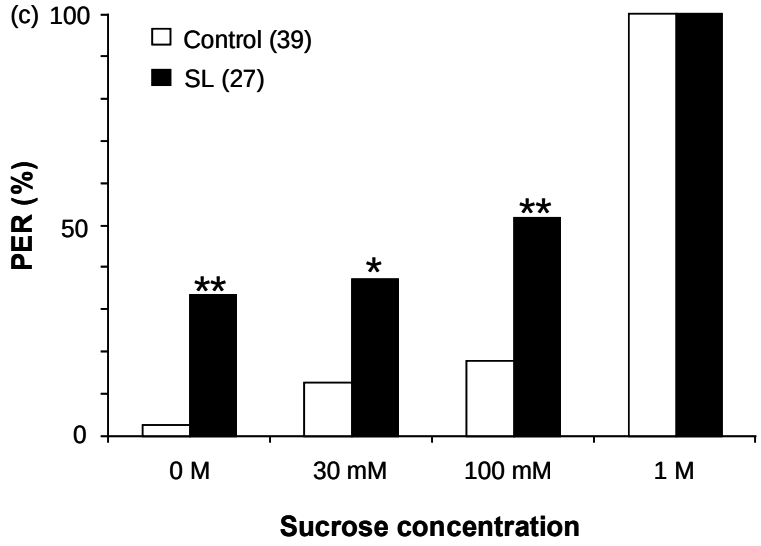
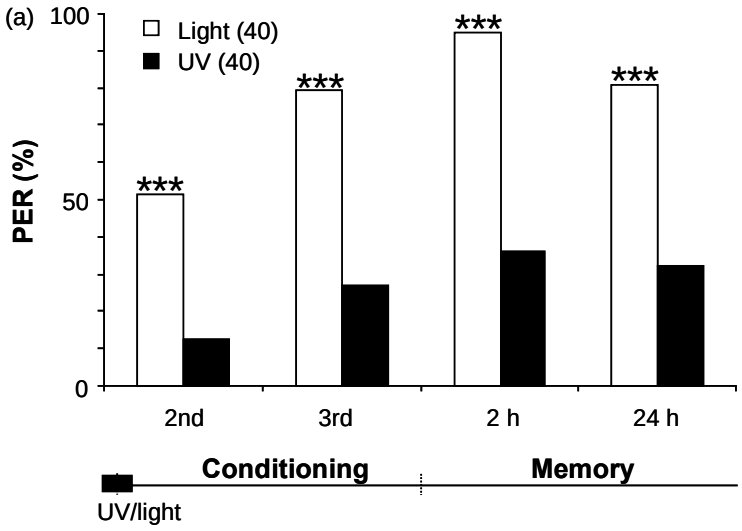


Figure 3.18: UV and SL exposure impairs sucrose responsiveness
(a) UV light exposure increases the responsiveness to low sucrose concentrations. The data shows the PER of animals before and after 30 min UV light exposure **(b)** White light (control) exposure shows no change in sucrose responsiveness of honeybee before and after exposure. The data shows the mean of PER of animals before and after white light exposure. **(c)** Shaking with light increases the honeybee responsiveness to low sucrose concentration. The data shows the PER of animals exposed to shaking with light and control animals exposed to dark without shaking. The asterisks indicate significant difference between the groups (χ^2 test / Fisher exact test; ***p <0.001,**p <0.01,*p <0.05).

3.2.2.2 Stress stimuli: associative learning and memory formation

The increase of responsiveness to water and low sucrose concentrations after stress stimuli (UV and SL) exposure implies a possible effect on appetitive associative learning in honeybees. To address this question, groups of honeybees were exposed to either UV or white light for 30 min just before associative olfactory conditioning. To avoid potential effects of UV light on sucrose responsiveness on learning and thus on US processing, I used a high sucrose concentration (1 M) as US stimulus that reliably elicits PER independent of the light exposure (Figure 3.18). Additional experiments show that the spontaneous reactivity to the CS stimulus does not differ between UV and white light exposed honeybees. Even though the basic responses to the CS stimulus or US stimulus used for conditioning are apparently not influenced by UV light, UV light exposure (30 min) immediately before associative conditioning causes a severe impairment in acquisition and memory formation (Figure 3.19 a). Honeybees of the control group exposed to white light show a normal acquisition and memory formation. To test whether the low memory retrieval measured at 2 h and 24 h is due to the impaired acquisition, the UV light exposure was shifted to a time window after acquisition. Leaving acquisition intact, UV light exposure after acquisition still causes an impairment of memory formation (Figure 3.19 b). The non-associative form of learning in honeybee i.e. habituation and sensitisation remains unaffected after exposure of these stress stimuli (data not shown here). These findings demonstrate, that UV light influences both, processes underlying acquisition and processes required for memory formation.

UV light and associative learning



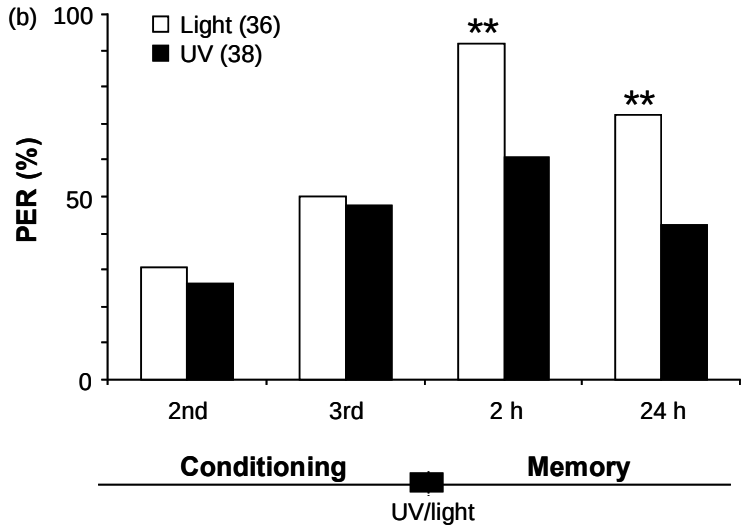


Figure 3.19: UV induced impairment in associative learning and memory
(a) Exposure of UV light before training shows impaired acquisition and memory formation 2 h and 24 h after training as compared to control animals. The data shows the mean of PER of UV light and white light (control) exposed animals. **(b)** UV light exposure after training also shows impaired memory formation 2 h and 24 h after training as compared to control animals. The data shows the PER of UV light and white light (control) exposed animals. The asterisks indicate significant difference between the groups (Fisher exact test; ***p <0.001,**p <0.01).

Like UV light (Figure 3.19 a), exposure to SL also impairs learning and memory formation as compared to control animals with the exception that 2 h memory formation remains intact (Figure 3.20). This data shows that different external stimuli have a critical role on mechanism underlying associative learning and memory formation of honeybee.

SL and associative learning

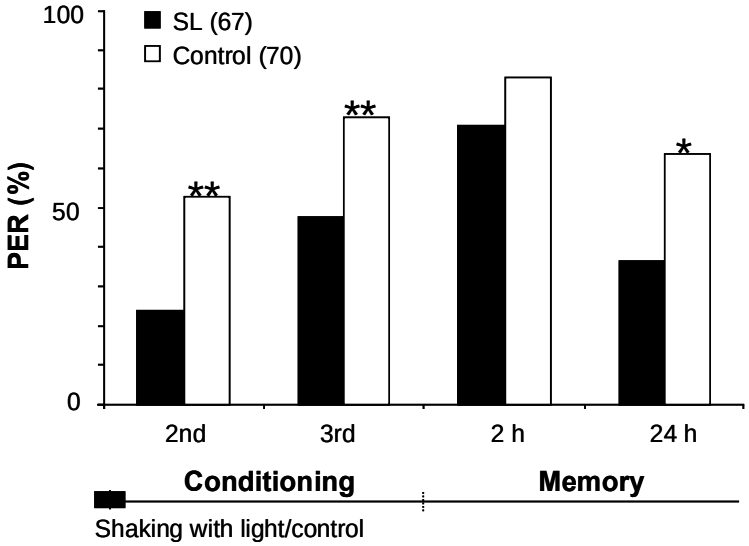


Figure 3.20: SL induced impairment in associative learning and memory
Overnight shaking with light before training shows impaired acquisition and memory formation 24 h after training as compared to control animals while 2 h memory remains intact. The data shows the

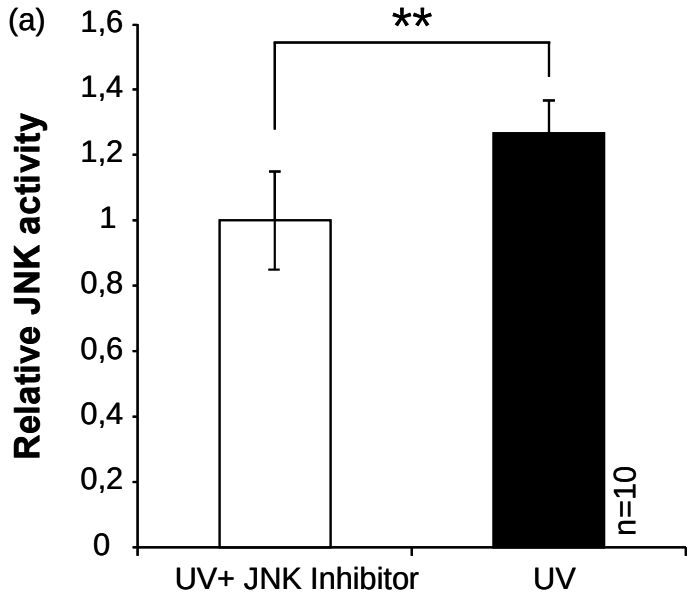
mean of PER of SL exposed and control animals. The asterisks indicate significant difference between the groups (χ^2 test; $^{**}p < 0.01$,$^{*}p < 0.05$).

3.2.3 Inhibition of JNK and rescue of stress elevated JNK activity

The finding that both stress stimuli (UV and SL) affect the JNK activity in the brain (Figure 3.17) and cause an impairment in behaviour (Figure 3.18, 3.19 and 3.20) points to a potential role of JNK in mediating these effects. Since JNK interacts with other MAPKs (mitogen activated protein kinases) that may also have a role in the stress-induced deficit in learning and memory, it is necessary to confirm whether the JNK pathway is really responsible for mediating the deficits in learning and memory caused by the stress stimuli. Thus I tested whether injection of SP600125, a selective inhibitor of JNK [26] during exposure to stress stimuli (UV and SL) prevents the stress induced JNK activity and rescues the impairment in behaviour.

The systemic injection of JNK-inhibitor is carried out at two time points depending on the type of stress stimuli. For short exposure of acute stress (UV light, 30 min), it is injected before UV exposure while for long exposure of chronic stress (SL, overnight), it is injected after the SL exposure. As shown in Figure 3.21 a, the systemic inhibition of JNK before UV light exposure significantly prevents the UV light induced elevation in relative JNK activity. In parallel, injection of JNK-inhibitor after SL stress also significantly decreases the JNK activity as compared to SL exposed group (Figure 3.21 b).

This clearly indicates the down-regulation of stress activated JNK by specific JNK-inhibitor *in vivo*.



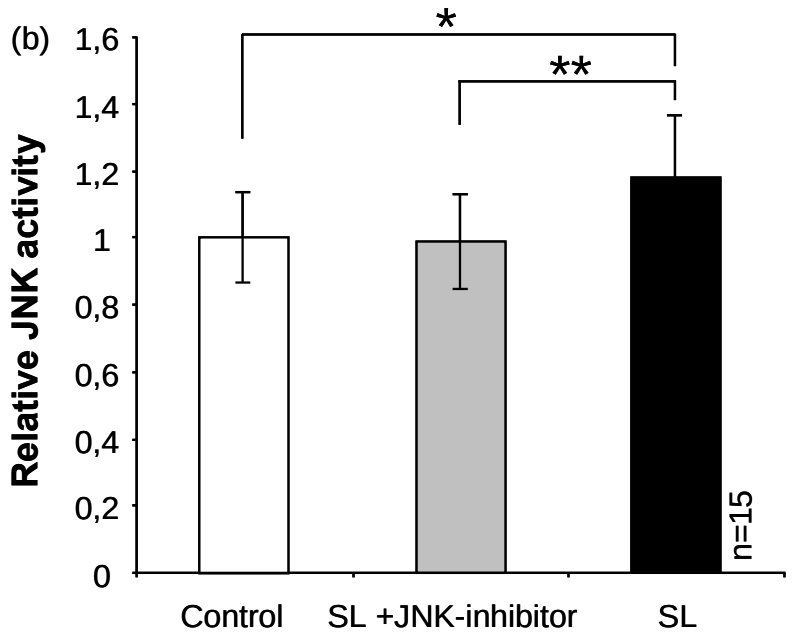


Figure 3.21: Inhibition of JNK prevents UV/SL induced elevation in JNK activity
(a) In presence of UV light exposure, the level of JNK activity is decreased significantly in JNK-inhibitor injected animals **(b)** The level of JNK activity is decreased significantly in JNK-inhibitor injected group as compared to SL exposed animals. For each experiment, values were normalized to the mean value of control samples. The asterisks indicate a significant difference between the two groups (τ–test; $^{**}p < 0.01$,$^{*}p < 0.05$)

3.2.4 UV/SL: Behavioural analysis with JNK-inhibitor

To demonstrate whether the stress (UV and SL) induced impairment in behaviour are mediated by JNK, behavioural analysis was carried out after inhibition of JNK with JNK-inhibitor.

Sucrose Responsiveness and JNK-inhibitor

As shown in Figure 3.22, the SL and UV light exposure in presence of a JNK-inhibitor reduces the responsiveness to water and low sucrose concentrations as compared to the control animals (dark without shaking or white light). Since the application of JNK-inhibitor SP600125 alone does not affect responsiveness (data not shown), the results demonstrate a critical role of the JNK pathway as a mediator of stress induced effects on sensory processing of honeybee.

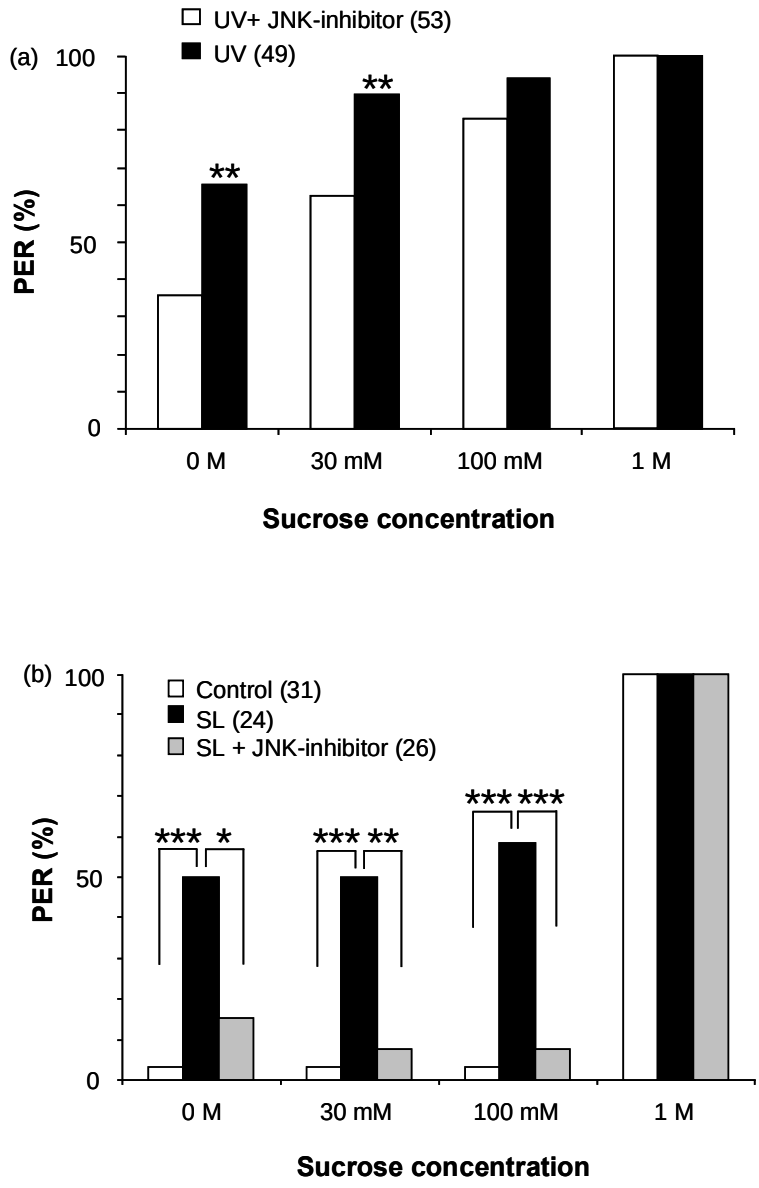


Figure 3.22: JNK-inhibitor rescues the UV/SL impaired sucrose responsiveness
(a) Injection of JNK-inhibitor significantly rescues the UV light induced changes in sucrose responsiveness. The data show means of responsiveness of UV exposed groups with or without JNK-inhibitor injection. **(b)** Injection of JNK-inhibitor also significantly rescues the SL induced changes in sucrose responsiveness. The data show means of responsiveness of SL exposed animals with or without JNK-inhibitor injection and control animal (dark without shaking). The asterisks indicate a significant difference between two groups (χ^2 test / Fisher Exact test; $^{**}p < 0.01$,$^{***}p < 0.001$,$^{*}p < 0.05$)

Associative learning and JNK-inhibitor

The inhibition of JNK activity during SL and UV light exposure also rescues the stress induced impairment in learning and memory formation in honeybee in a distinct way (Figure 3.23). Since 2 h memory does not seem to be affected by SL itself (Figure 3.20), therefore, it also remains intact after JNK inhibition. These findings provide clear evidence for a critical

inhibitory role of JNK in mechanisms underlying sensory processing, olfactory learning and memory formation.

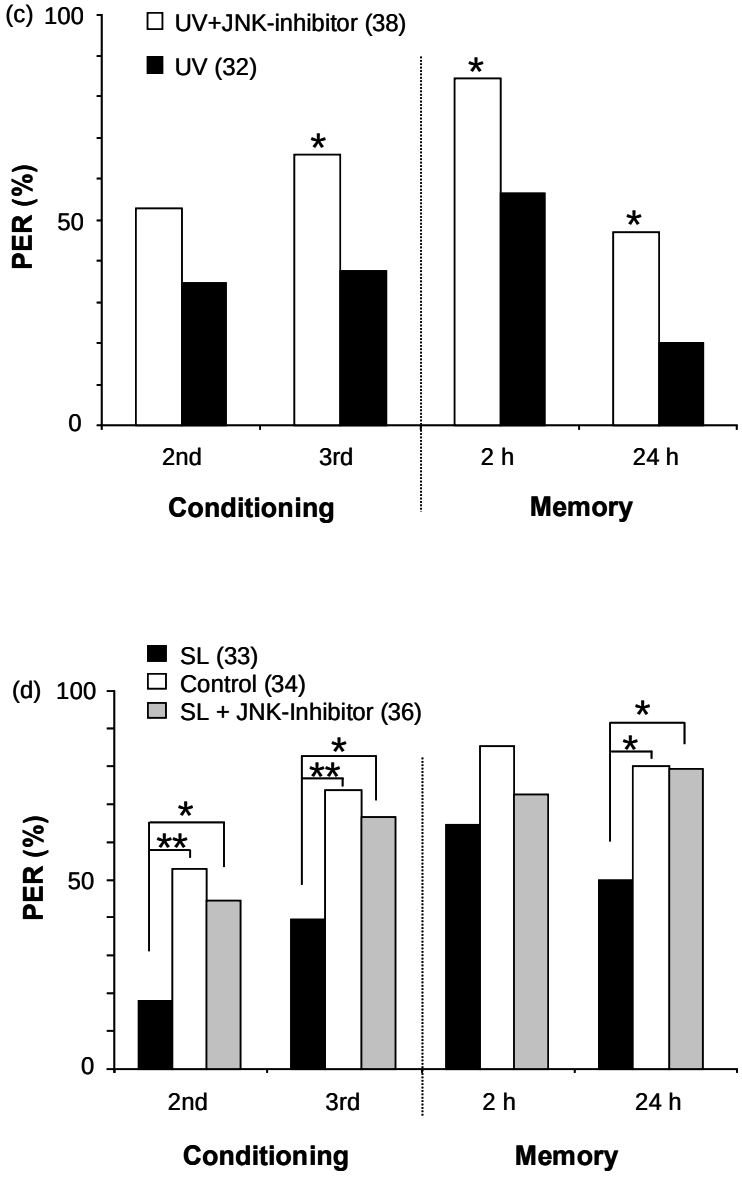


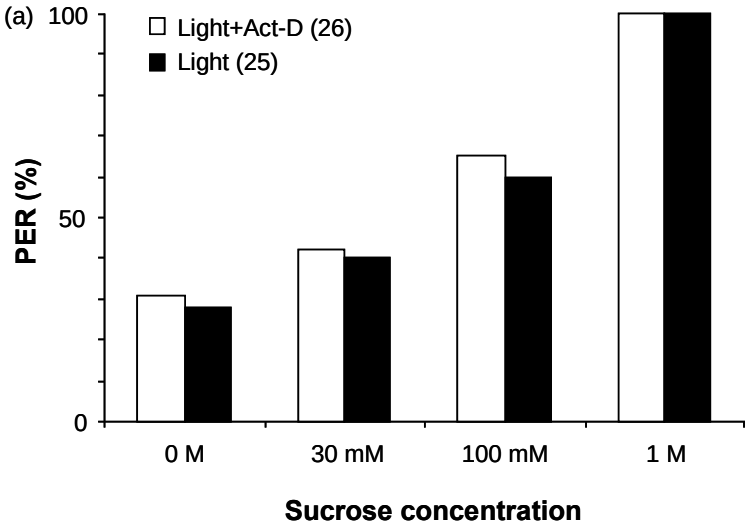
Figure 3.23: JNK-inhibitor rescues UV/SL impaired associative learning
(a) Injection of JNK-inhibitor rescues UV light induced impairment in associative olfactory learning and memory formation. The data show the mean PER of UV exposed groups with or without JNK-inhibitor injection. **(b)** Injection of JNK-inhibitor rescues SL induced impairment in learning and 24 h memory formation. The data shows mean PER of SL exposed animals with or without JNK-inhibitor injection and control animal. The asterisks indicate a significant difference between two groups (Fisher Exact test; ** $p < 0.01$, * $p < 0.05$)

3.2.5 Are stress induced changes in behaviour of honeybee transcription dependent?

Many studies reveal that JNK acts directly via transcription in stress response, but little is known about the transcriptional dependent or independent action of JNK pathway in cognitive functions. Therefore, Act-D was used to investigate whether the stress induced changes in behaviour (responsiveness and associative learning) depends on transcription. Moreover, does the transcription blocking during stress exposure mimic the effect of the JNK inhibition.

The investigation shows that systemic injection of Act-D before white light exposure (control) does not show any affect on responsiveness (Figure 3.24 a). In contrast, Act-D injection before UV light exposure prevents the UV induced increase in responsiveness of honeybees (Figure 3.24 b). This shows that changes in sensory processing caused by UV light exposure dependents on transcription. Thus it provides evidence that the fast UV light induced effects on responsiveness are mediated by JNK action on transcriptional processes. In contrast, injection of Act-D before SL treatment does not prevent the SL induced effects on responsiveness and demonstrates that changes in sensory processing caused by SL do not require transcription. (Figure 3.24 c).

Responsiveness



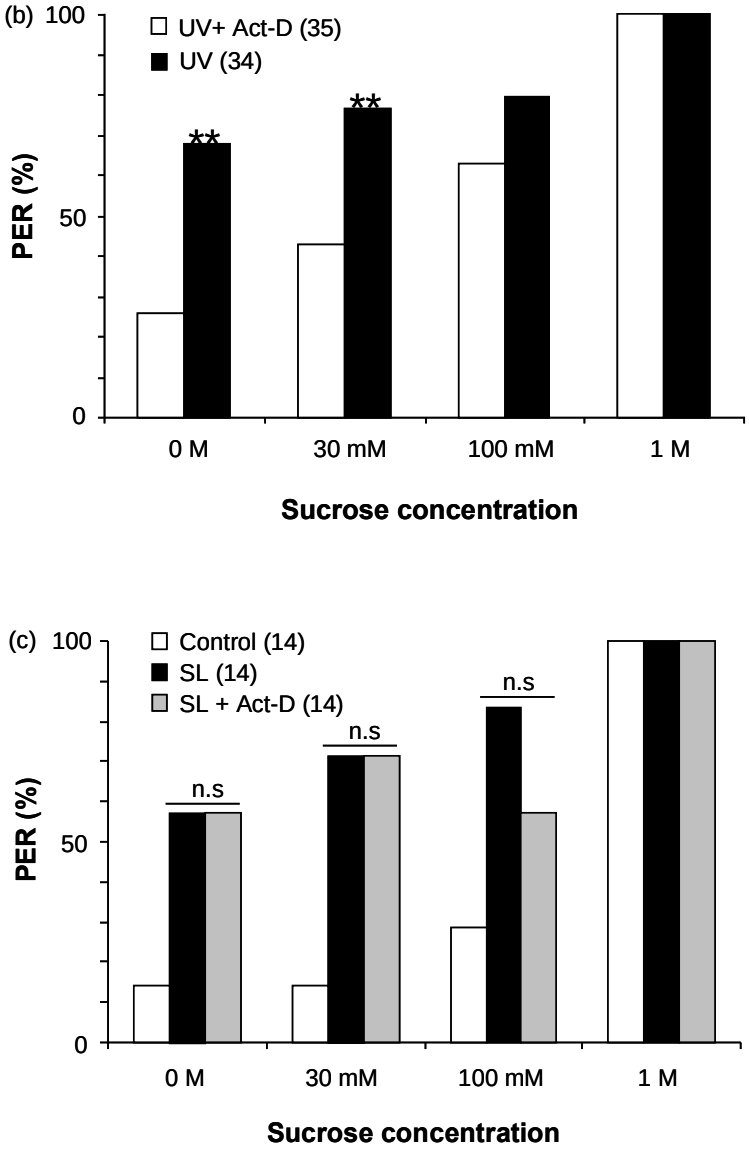


Figure 3.24: Stress can change responsiveness with or without transcription
(a) The sucrose responsiveness remains unchanged with or without Act-D after exposure to white light. **(b)** The Act-D rescues the UV light induced changes in sucrose responsiveness. The data shows the PER of UV exposed animals with or without Act-D injection. **(c)** The Act-D does not rescue the SL induced changes in sucrose responsiveness. The data shows the PER of SL exposed animals with or without Act-D injection and control animals. In all experiments, the n.s indicates the non-significance while asterisks indicate a significant difference between two groups (Fisher exact test; $^{**}p < 0.01$,$^{*}p < 0.05$)

My findings that stress can affect the sensory processing with or without transcription promoted me to explore whether associative learning and memory share the similar mechanism. Blocking transcription during UV light exposure rescues the acquisition and memory formation as compared to the UV light exposed groups (Figure 3.25 a). In contrast, no rescue is observed in case of animals exposed to SL in presence of Act-D (Figure 3.25 b).

Taking together, although the effect of UV/SL on behaviour requires JNK activity, they differ in their transcription dependency.

Associative learning

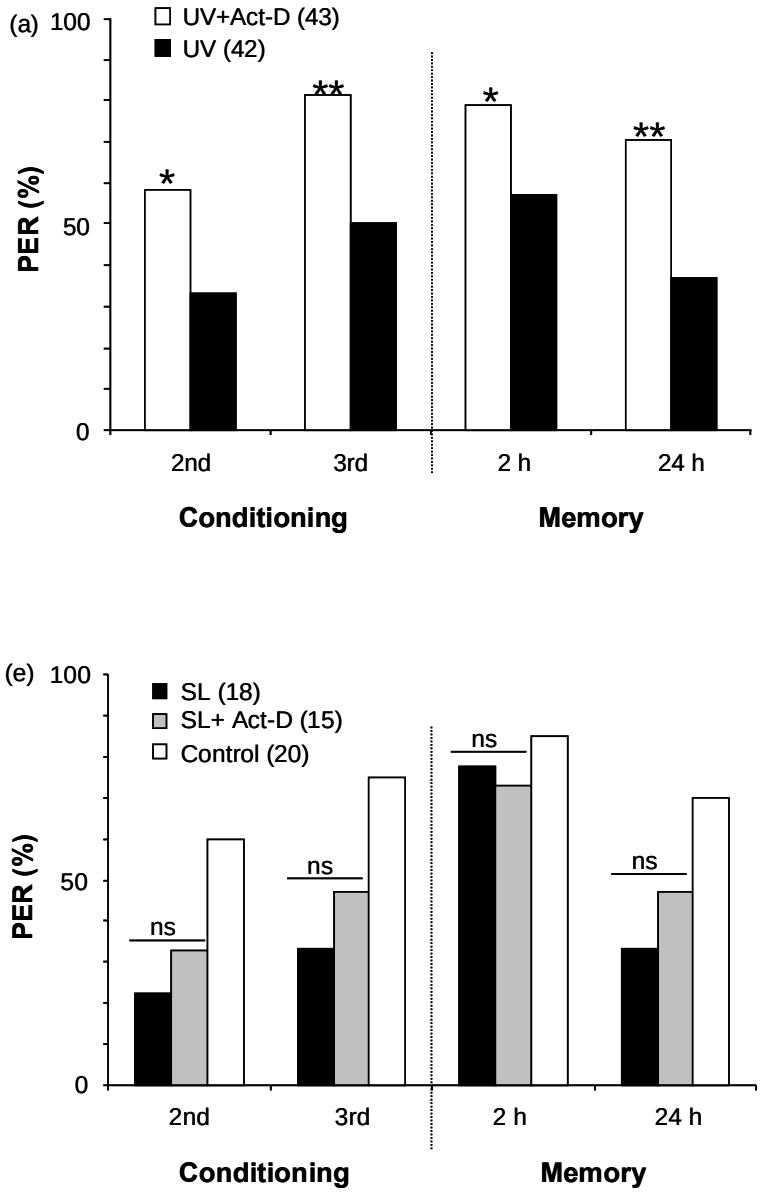


Figure 3.25: Stress can change associative learning and memory with or without transcription
(a) Transcription blocking with Act-D rescues the UV light induced impairment in learning and memory formation of honeybee. The data shows the PER of UV exposed animals with or without Act -D injection. **(b)** Transcription blocking does not rescue the SL induced impairment in learning and memory formation of honeybee. The data shows the PER of SL exposed animals with or without Act - D injection and control animals. In all experiments, the asterisks indicate a significant difference between two groups (Fisher exact test; $^{**}p < 0.01$,$^{*}p < 0.05$)

3.3 UV LIGHT AFFECTS ACETYLCHOLINE (ACh) TRANSMISSION IN THE KENYON CELLS OF MUSHROOM BODIES

My immunohistological analysis in honeybee reveal that JNK activity is mainly detected in Kenyon cells of the mushroom bodies (Figure 3.12) and JNK has a critical role in modulating behaviour when activated by UV light. This promoted me to investigate the hypothesis that UV light may also have a direct impact on the acetylcholine cholinergic neurotransmission. Acetylcholine (ACh) induces depolarization that leads to the influx of the second messenger Ca^{2+} that in turn stimulates biochemical cascades and changes in gene expression. Thus intracellular calcium represents a valuable indicator of neuronal activity [96] and its dynamics can be accessed by imaging using calcium sensitive dyes.

The honeybee (adult) and *Drosophila* (larvae) brain were used to address this hypothesis. From Kenyon cells, ACh action (ACh induced Ca^{2+} dependent fluorescence / calcium signal) was measured by a chemical fluorescent indicator (Fluo-4) or a genetically encoded fluorescent indicator (cameleon protein) in honeybee and *Drosophila* (201y-GAL4; UAS-Cameleon 2.1) respectively.

Cameleon protein in *Drosophila*

In *Drosophila,* cameleon is associated with two fluorescent proteins (EYFP; enhanced yellow fluorescent protein and ECFP; enhanced cyan fluorescent protein) which are internally connected with calmodulin, a Ca^{2+} binding protein (CaM) and M13, a calmodulin binding-peptide (Figure 3.26). On calcium binding, fluorescence resonance energy transfer (FRET) occurs from ECFP to EYFP by emitting light at 485 nm and 535 nm respectively. The ratio between EYFP and ECFP emission reflects the changes in intracellular calcium concentration [96] and used to determine the effect of UV light on ACh action.

$$\textbf{Ratio} = \frac{\text{EYFP}}{\text{ECFP}}$$

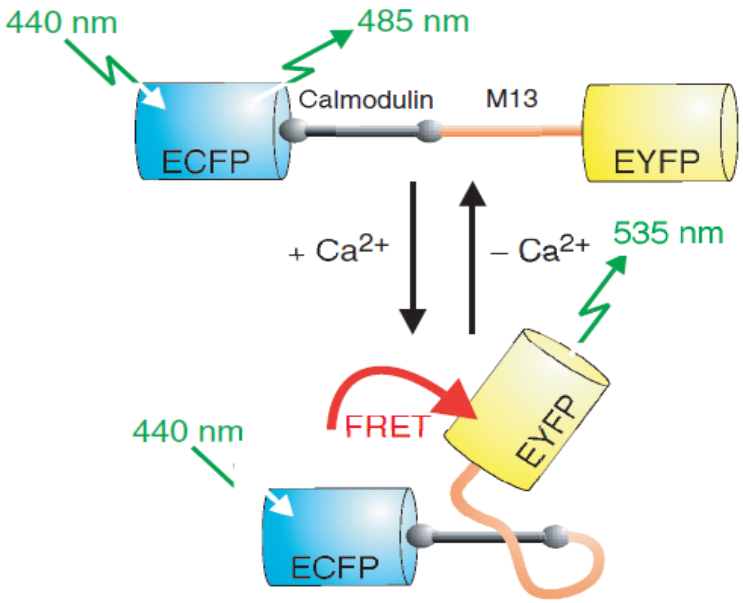


Figure 3.26: Schematic diagram of cameleon protein in *Drosophila*
The binding and releasing of Ca^{2+} changes the fluorescence resonance energy transfer (FRET) from ECFP (enhanced cyan fluorescent protein) to EYFP (enhanced yellow fluorescent protein). Calcium influx can be detected by simultaneous shift in the emission intensities of both fluorophores [96]

3.3.1 Acetylcholine stimulates the Kenyon cells of honeybee and *Drosophila*

After preparation of neuron, the viability of cells was tested by stimulation with 2 µl of 200 µM ACh or control (*Drosophila* / honeybee ringer). The damaged neurons will not react to ACh. The gradual increase in fluorescence and colour of Kenyon cells with time scale (0-20 sec) is clearly visible after ACh application (Figure 3.27).

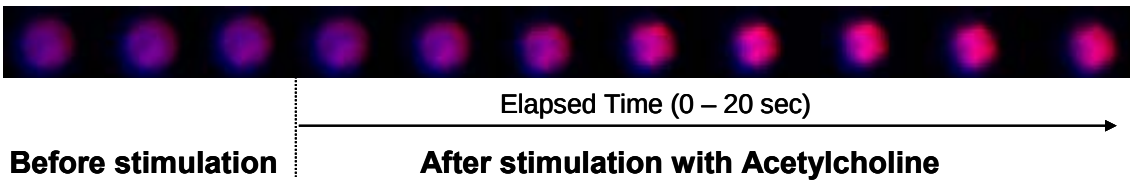


Figure 3.27: Stimulation of Kenyon cells with ACh
Visible change of the emitted fluorescence of Kenyon cells before and after ACh application. The readings were taken before and 0, 2, 4, 6, 8,10,12,20 seconds after addition of ACh.

Figure 3.28 shows the time dependent increase of fluorescence intensity after application of ACh solution on Kenyon cells of *Drosophila* and honeybee. In comparison, control (ringer) does not show any excitability in both species. The ratio between EYFP and ECFP emission in *Drosophila* and the changes in Fluo-4 emission in honeybee were normalized to calculate the percentage increase in fluorescence intensity.

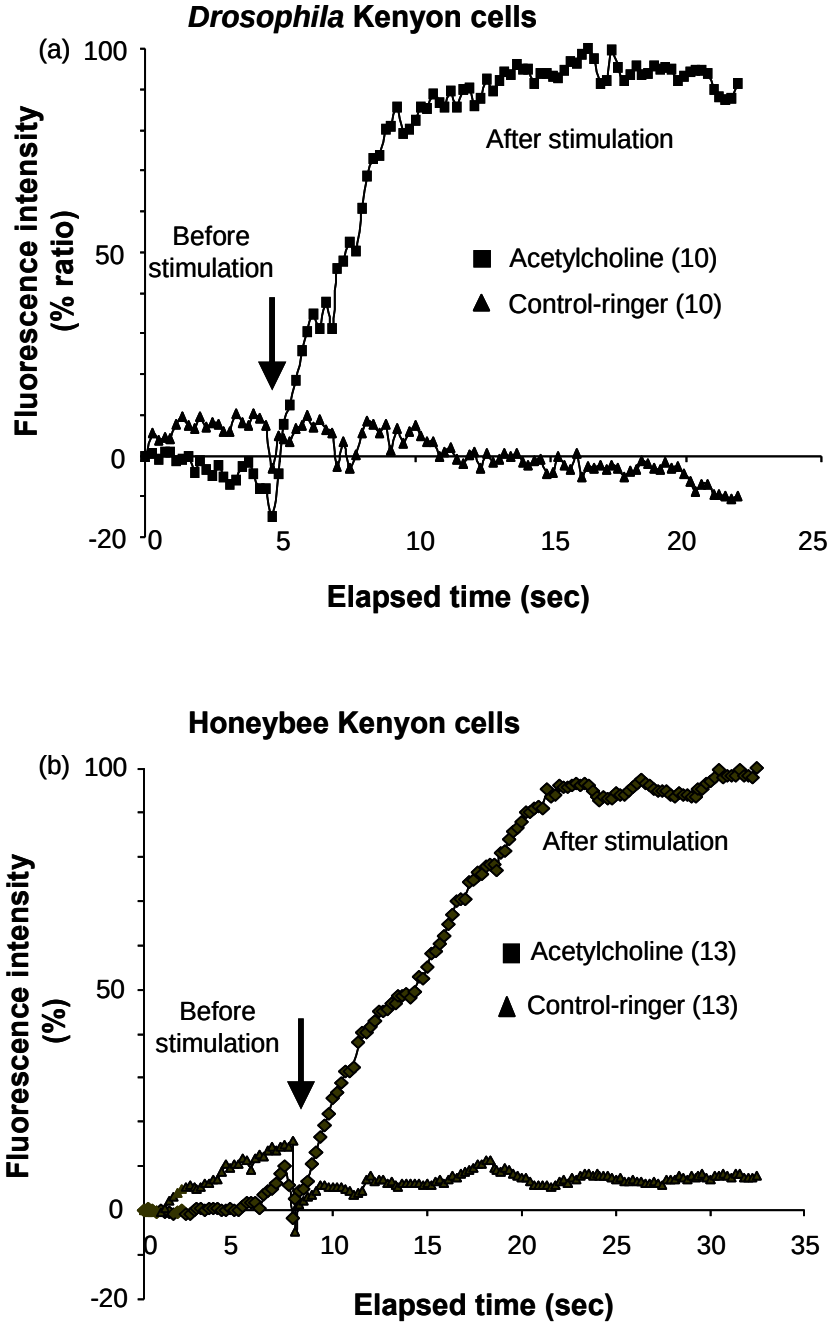


Figure 3.28: Change in fluorescence intensity of ACh stimulated Kenyon cells (a; *Drosophila*, b; honeybee) The application of 20 µM ACh shows increasing fluorescence intensity of *Drosophila* and honeybee Kenyon cells as compared to control (ringer) where no excitability is observed. The arrows indicate the application time of ACh/ringer

ACh significantly increases the calcium dependent fluorescence signal from Kenyon cells of *Drosophila* and honeybee as compared to control (Figure 3.29). Since, I aim to address the qualitative effect, therefore a simplified procedure was formulated to distinguish between stimulated and non-stimulated cells. Here the ratio of each cell before and after stimulation with ACh / ringer defines the status of cells.

$$\textbf{Ratio} = \frac{\text{Values after stimulation}}{\text{Values before stiumulation}} = \begin{cases} \text{Non-stimulated cell} \\ \text{Stimulated cell} \end{cases}$$

Stimulation criteria: After many experiments, a reliable threshold ratio (TR) was formulated to declare the cells as stimulated. The total number of stimulated cells was taken to calculate the percentage of stimulated cells in both groups.

Threshold ratio: For *Drosophila* cells = > 1.01

For honeybee cells = > 1.068

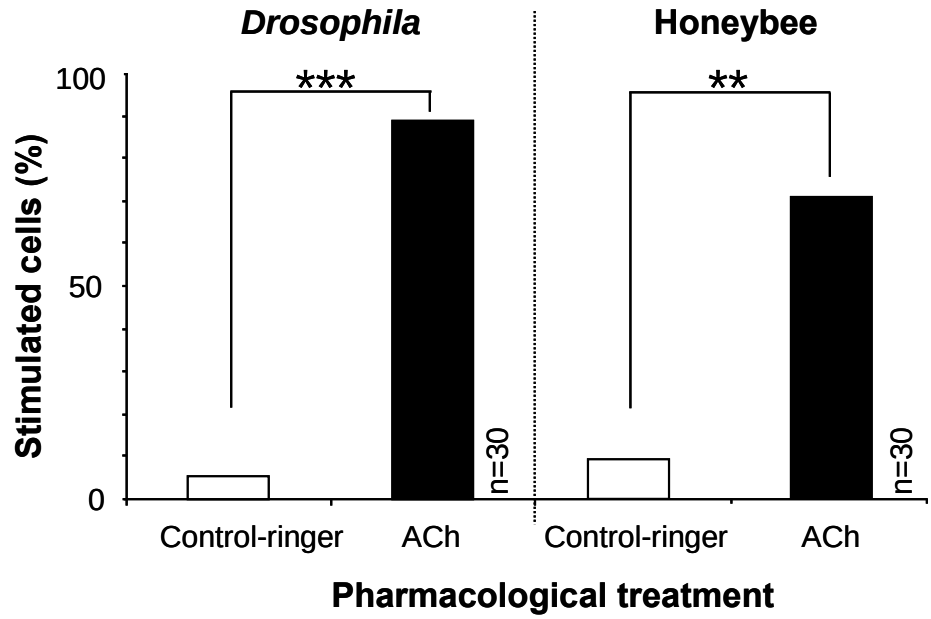


Figure 3.29: Percentage of stimulated *Drosophila* and honeybee Kenyon cells
As compared to control-ringer, ACh (20 µM) leads to significant increase in the percentage of stimulated *Drosophila* and honeybee Kenyon cells. The asterisks indicate a significant difference between two groups (Fisher exact test; ***p <0.001,**p <0.01).

3.3.2 UV light exposure affects ACh action in Kenyon cells

UV light was used as external stimuli to test its impact on ACh action in *Drosophila* Kenyon cells. The cells were exposed for different times to UV light (5, 15 and 30 min) followed by stimulation with ACh. The other group of cells stimulated only with ACh was used as control. As shown in Figure 3.30, the percentage of ACh stimulated cells decreases with different duration of UV light exposure. In parallel, a control group without UV light exposure shows significantly higher stimulation with ACh. Since the minimum UV light exposure (5 min) already gives the significant difference (high stimulation) and to avoid lethal effect of UV light, I used this short exposure time for my further experiments.

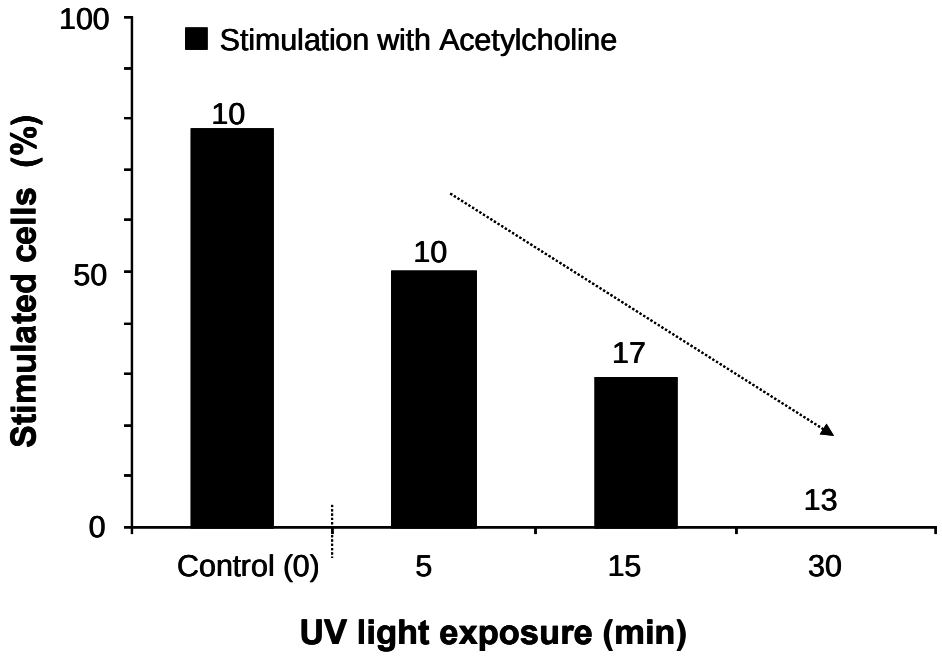


Figure 3.30: The calcium dependent fluorescence decreases with UV light
Percentage of stimulated cells with ACh (20 µM) after UV light exposure decreased gradually with respect to exposure time (5, 15 and 30 min).

Since cameleon is associated with two fluorescent proteins (EYFP and ECFP). Therefore, I investigated whether 5 min UV light exposure has any effects on the fluorescence itself. As shown in Figure 3.31, no difference in fluorescence intensity is observed before and after UV light exposure. This indicates that the observed change in FRET signal (Figure 3.30) is not due to UV effect on the fluorescence, and also reveals the 5 min as appropriated UV light exposure duration.

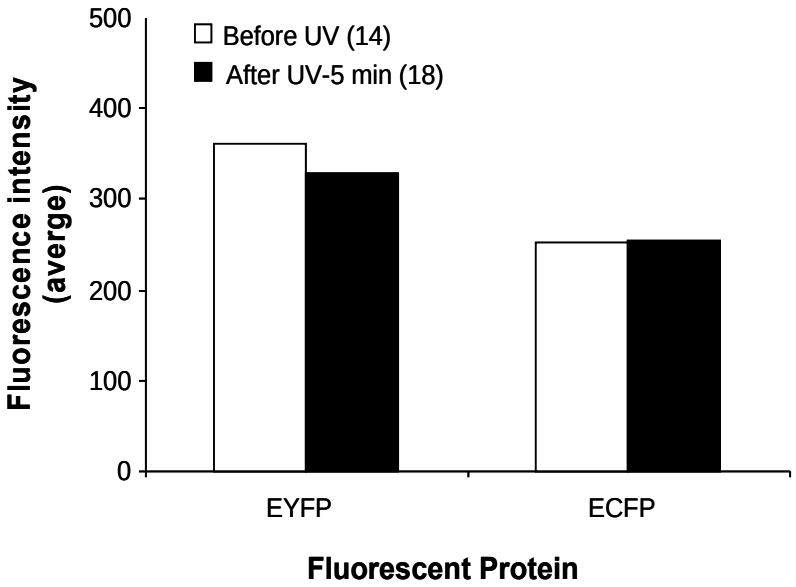


Figure 3.31: UV light does not affect fluorescence
The mean fluorescence intensity of EYFP and ECFP reveals no difference before and after exposure of UV light (5 min) on the Kenyon cells of *Drosophila* and thus UV light is an appropriate stress stimulus to be used in calcium imaging

Using 5 min UV light exposure in a new experiment reconfirms the initial results by reducing the number of ACh stimulated cells (Figure 3.32). The other groups were stimulated either with ACh or ringer solution and did not receive any UV exposure treatment. Taken together, this supports my hypothesis that UV light has a direct impact on ACh action (ACh induced Ca^{2+} signal) in neural plasticity of honeybee.

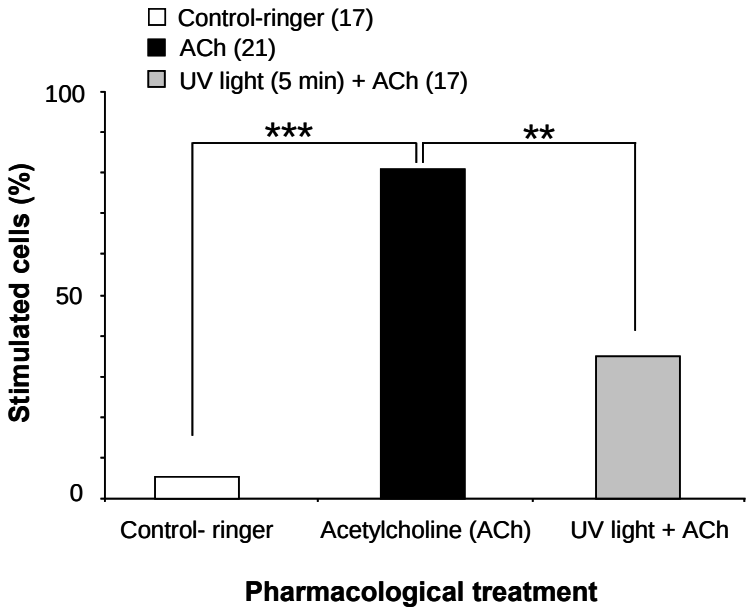
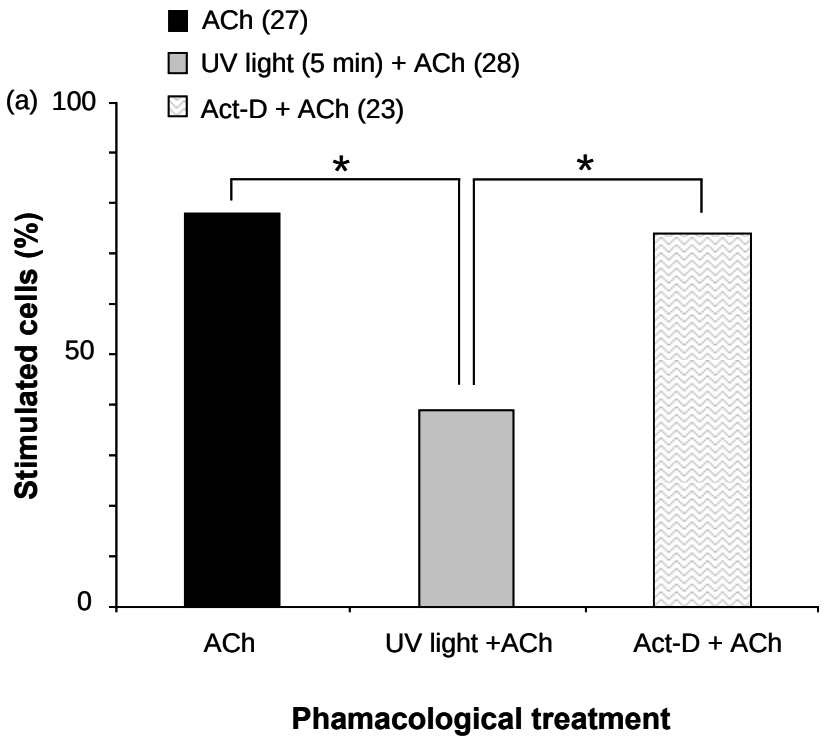


Figure 3.32: UV light decreases the number of ACh stimulated cells
The percentage of stimulated cells is significantly reduced after exposure of UV light as compared to group without UV exposure. The groups stimulated with ACh and ringer also shows a significant difference. The asterisks indicate a significant difference between two groups (Fisher exact test; ***p <0.001, **p <0.01).

3.3.3 UV light mediated changes in ACh action depends on transcription

Since my investigation has shown that UV light impairs the behaviour via transcription and via JNK pathway, it is feasible that this also applies on the observed UV effect on ACh action in Kenyon cells. Specific JNK-inhibitor and Act-D (transcription blocker) were applied on *Drosophila* Kenyon cells to address this question. JNK was inhibited before UV light exposure of Kenyon cells that were then stimulated with ACh. Unfortunately, JNK-inhibitor is very sensitive to UV light and crystallizes leading to a complete damage of the Kenyon cells. Thus testing the JNK inhibition is not feasible in this *in vitro* experiment.

To investigate whether UV light induced changes in calcium signal implicate transcription processes, Act-D was applied 30 min before the ACh application. In a control experiment, the cells with or without Act-D show the identical results and demonstrating that Act-D does not disturb the stimulation itself. In parallel, the UV light exposed group without Act-D shows a significant decrease in stimulation (Figure 3.33 a). As shown in Figure 3.33 b, the application of Act-D (30 min before UV light) significantly increases the number of ACh stimulated cells as compared to UV exposed group without Act-D. Thus transcription blocking restores the UV light induced effects on ACh action to normal level. Taken together, this data confirms that the effect of UV light on ACh action is transcription dependent.



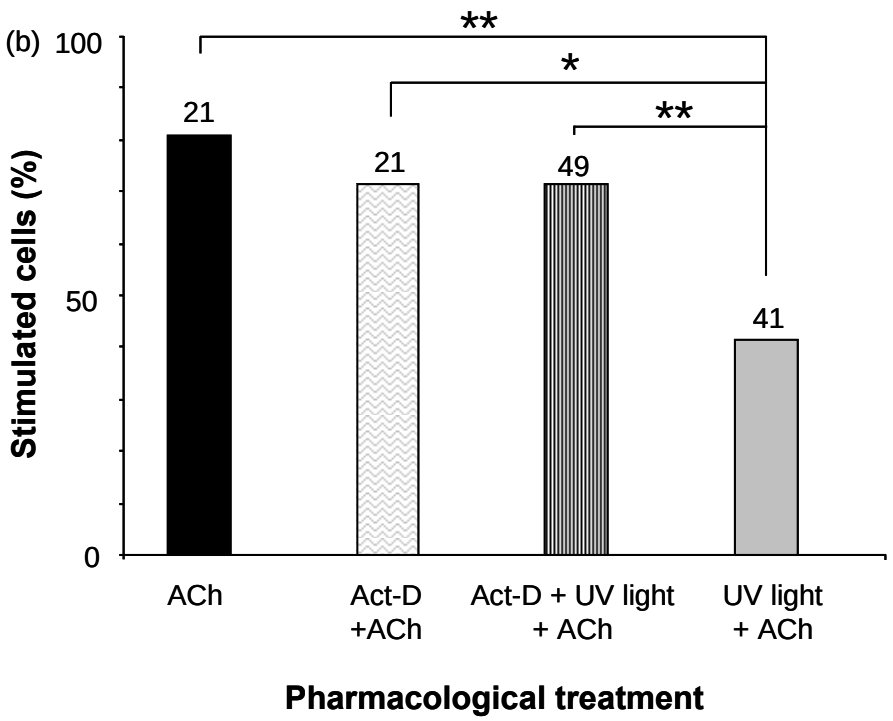


Figure 3.33: UV light mediated changes in ACh action are via transcription
(a) Application of Act-D on Kenyon cells does not affect the calcium signal. It gives the similar stimulation level as observed in ACh group **(b)** The application of Act-D 30 min before UV light exposure significantly brings back the UV light induced decrease in calcium signal as compared to UV light exposed group. No significance between stimulation of all other groups was detected. The asterisks indicate a significant difference between two groups (Fisher Exact test; $^{**}p < 0.01$,$^{*}\ p < 0.05$).

3.4 Summary of results

My results demonstrate that virus infection and stress stimuli induce changes in sensory processing and associative learning and memory formation of honeybee. These changes in behaviour are modulated by JNK mainly localized in peduncles and α-lobes of mushroom bodies. My results provide evidence for transcriptional (for DWV and UV light) as well as non-transcriptional (for SL) routes of JNK action in modulation of behaviour. Moreover, initial results point to the involvement of acetylcholine neurotransmission in UV induced physiological changes. In summary, all this data supports my notion for the critical role of JNK in mechanisms linking external stress stimuli with underlying learning and memory of honeybee (Figure 3.34).

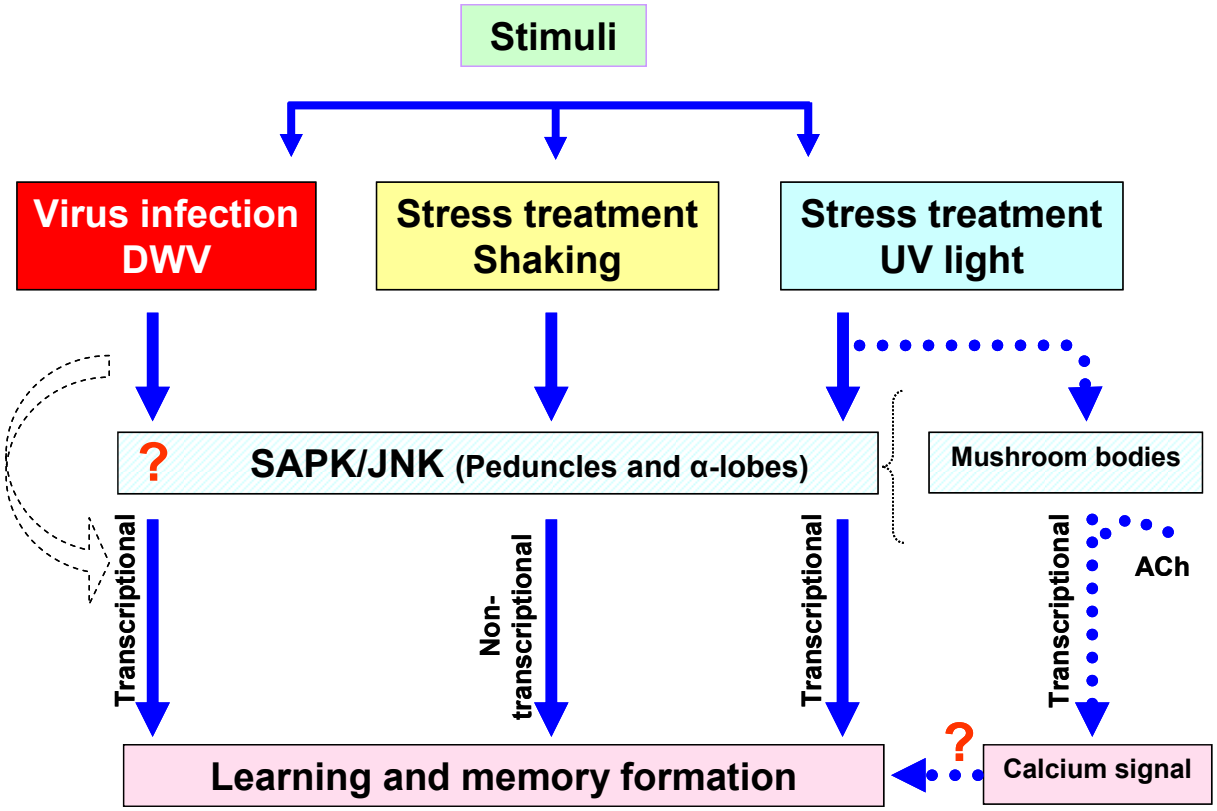


Figure 3.34: Schematic diagram of summarized results

4 DISCUSSION

4.1 Behavioural analysis after DWV infection

The honeybee is an essential component in agriculture for their role in pollination. Approximately one third of our food requires honeybee pollination [53, 101]. The poorly understood phenomena of colony collapse disorder (CCD) cause drastic honeybee loses (50 to 90%) that are five times higher than normal winter loss [73]. Recently, an extensive survey of beekeepers reveals a total of 35.8% colonies loss due to CCD [297]. Besides other threats, virus infections are also considered as potential reason due to their mysterious role in CCD [73]. My work provides the first experimental proof for an important role of DWV in changing the learning capabilities of honeybee [140]. It will open a new field where cognitive abilities can be investigated in conjunction with CCD as mentioned in the recent review by Stankus [274].

4.1.1 DWV infection via food and hemolymph

My data shows that only direct hemolymph infection by DWV is effective and leads to a strong replication of the virus, while oral application is ineffective, which is in agreement with other studies [21]. Another study suggests that the oral transmission route is due to presence of virus in digestive tract and feces of queen [62]. In my experiments no signal is detected in adult foragers when fed with virus contaminated food. However, since I tested only up to 7 days of continuous oral application, I cannot exclude that a long-lasting oral uptake of DWV-contaminated food during hibernation may be a source of DWV infection. Another reason for delayed infection may be the evolved mechanism of bee viruses to depress the host response. It allows them to survive for long periods in honeybee without any drastic effects but later on cause latent infections [230]. In addition, a number of non immunological factors in midgut (gut juice, peritrophic membrane etc) may also contribute to modify and control the outcome of viral infections. The peritrophic membrane limits the virus infection due to its impermeable or hardly permeable barrier [112].

In agreement with results on queens and drones [62], my RT-PCR measurements of DWV hemolymph infection in foragers showed a maximal signal in the abdomen, followed by the thorax and head of the adult honeybee and indicate the abdominal tissues as major accumulation site for DWV [63]. This is an agreement with a study where high DWV signal is detected in all body regions including head of deformed adults and infected asymptomatic honeybees [170]. Thus under natural conditions, hemolymph infection via parasites like

Varroa destructor is the most likely scenario for DWV transmission and collapse of a honeybee colony [21, 43, 180]. Like mite infection, artificial injection of DWV into the hemolymph easily enters the body to infect different organs [97] which may not the case after oral infection. My data is in agreement with the *Drosophila* C virus (DCV) infection in *Drosophila* adult flies: the injection of DCV (single stranded RNA virus; ssRNA) causes infection and mortality within 3-10 days [66, 86], while ingestion of DCV does not cause illness or substantial immune response [241].

4.1.2 DWV infection and behaviour of honeybee

Many studies reveal that a variety of infection in insects modifies the behavioural performance. Female crickets (*Acheta domesticus*) lay more eggs after bacterial and parasitic infection [3]. Feeding behaviour of tobacco hornworm (*Manduca sexta*) is suppressed after parasitic infection [4]. Dengue virus infects the nervous system of mosquito and prolongs the blood feeding [225]. Nucleopolyhedrovirus infected larvae *Mamestra brassicae* L. (Lepidoptera: Noctuidae) exhibit changes in larval mobility and feeding rates [298]. Female moth (*Helicoverpa zea*) show changes in reproductive behaviour and pheromone production when infected with insect virus Hz-2v [49]. Baculovirus infection results in enhanced locomotry activity of silkworm (*Bombyx mori*) [155]. Although these studies report various modification of animal performance but no study deals with the effect of infection on neural plasticity. For the first time, I have shown that DWV infection of adult forager bees leads to specific behaviour impairments. The unique combination of enhanced sucrose responsiveness, normal non-associative learning and defects in associative learning caused by DWV infection has not yet been observed. These observations contradict all present studies, where increased sucrose responsiveness is always combined with an improved associative learning and changes in non-associative learning.

The genotype, the role of the bees in foraging, the satiation level and many other parameters affect sucrose responsiveness and in parallel very defined features of associative learning [102, 253, 254]. These strongly linked physiological processes depend to a great extent on molecular processes mediated by the biogenic amine. DWV infection might disrupt the level of biogenic amines in the hemolymph of honeybee. The biogenic amines are considered as modulator of behaviour and nervous function [90, 93]. Among them, octopamine (OA) and serotonin may be involved in immune-nervous system communication [5] and also found in brain of honeybee [197, 257]. Early studies have shown that infection by West Nile Virus (ssRNA virus) decreases the serotonin content in the brain of infected mice [20]. This virus is

well known to spread through mosquito and cause neuroinvasive disease (encephalitis) in variety of animals [54, 128]. In Lepidopterous insects, bacterial infection can increase the level of octopamine in the hemolymph that alters physiological functions [4]. Increased octopamine (OA) in hemolymph is reported to disrupt the neural network for swallowing in the frontal ganglion of *Manduca sexta* [200]. The injection of octopamine elevates sucrose responsiveness and enhances acquisition, inhibition of octopaminergic transmission decreases responsiveness and impairs acquisition [125, 195, 198, 255]. All this is in contrast to the demonstrated effects of DWV infection behaviour and points towards the involvement of yet unknown molecular mechanism. My finding that DWV infection does not interfere with habituation supports this notion. Processes that affect sucrose responsiveness and associative learning also affect habituation [44, 212, 253]. The discrepancy between the current knowledge and the behavioural effects after DWV infection demands for new explanations.

Although the transmitted material (virus, bacteria, etc.) has not been specified, a report demonstrates that foragers infested by *Varroa destructor* are affected in non-associative forms of learning [166]. Recently, enhanced sucrose responsiveness of honeybee after fungal infection (*Nosema ceranae)* is reported and supports my findings [185]. DWV infection together with other infecting agent (e.g. *Varroa* mite) can also cause immunosupression and increase the susceptibility to various microbial pathogens [116, 321] that leads to combined disease symptoms (parasitic mite syndrome) at colony level [260]. Thus my data together with previous findings suggest a combined role of different factors including DWV in honeybee colony collapse disorder.

Infection and immune response

Although DWV infection is quite weak in the head as compared to other body regions, it may interfere with molecular mechanisms underlying learning at different levels. It is feasible that viral infection may interfere with the molecular network via changes in gene expression induced by the immune system. This is supported by a study where parasitic mite infection in the gut of bumble bee impairs monitoring of floral resources and economic foraging decisions [107]. Thus it is most likely that virus infection indirectly affects the central nervous system via implication of immune response. This idea is strengthen by findings that the injection of non-pathogenic lipopolysaccharide (LPS) triggers immune response and affects olfactory conditioning but not sensitization in honeybees and bumble bees [12, 178, 233]. These studies are also in agreement with my findings where DWV infection impairs associating learning 3 days after infection. Interestingly, LPS injection and viral infections also cause learning

deficits in mammals [270, 308]. Since the effects on behaviour are observed days after infection, it is feasible that viral infections (possibly also LPS injections) lead to changes in the gene expression pattern, which has been reported in mice [17]. A recent paper reveals that bacterial infection (*Escherichia coli*) also influence the gene expression in honeybee brain 8 h after infection [252] and supports the hypothesis that DWV infection affects the gene expression that has been shown to play an important role in caste differentiation and other behaviour [310].

Behavioural fever and hygienic behaviour

Like other insects, honeybee also has behavioural fever [275]. The honeybees maintain high temperature inside the hive which is lethal to pathogens but not to bees [219]. The insects prefer high temperature during infection because it can enhance the immune function and their survival chance during infection [204, 235]. The fact that DWV does not lead to any behavioural effect until 3 days after infection, might be due to behavioural fever as a response to infection. This may explain why DWV persist long time in non-symptomatic bees without doing any harm. Another possible reason of delayed DWV effect may be the production and release of interferons (INFs) like substances from virus infected cells within few hours after infection. These glycoproteins can tolerate heat and extreme pH, and may produce translation inhibitory protein that in turn block the virus replication [112].

Honeybee also exhibits a defined antiseptic or hygienic behaviour by which bees detect and remove infected animals from the hive [313]. In case of bacterial (*Paenibacillus larvae*) and fungal (*Ascosphaera apis*) infection, the infected brood is either removed from the hive immediately after infection or after their death [240, 317]. Honeybees also remove pupae that are parasitized with mite and use the hygienic behaviour as defence against parasitic mite *Varroa destructor* [38, 272]. In case of fungal infections, the hygienic line bees exhibit increased sensitivity to odor of the infected brood and remove them quickly [182, 183, 273, 313]. In contrast, artificial DWV infection does not affect the odor sensitivity and habituation and thus a different mechanism may apply.

4.2 Behavioural analysis after stress treatment

In mammals, stress can affect the performance of animal in two ways: either it improves or it impairs learning and memory performance. It is proposed that stress released transmitters and hormones can shift the stress response in both directions depending on the timing and localization of their respective actions [145]. In mammals, stress has been shown to affect

locomotor activity, spatial memory, anxiety, exploration and sexual behaviour [103, 221, 261]**.** The stress induced morphological and behaviour changes also depend on age, genotype, type of stress and indicate the complexity of stress response pathways. Even individuals of the same population can respond differentially to the same stressor [117, 312]. Like mammals, insects must also respond to changes in their environment that may affect their performance. The behavioural plasticity of honeybees is affected by many factors such as circadian rhythm, parasites, satiation level and insecticides [2, 83, 87, 166, 169, 327]. In *Drosophila*, a recent study revealed the role of mushroom bodies in the modulation of behavioural responses to the environmental stressors such as starvation and oxidative stress [214]. Despite of the well defined behavioural repertoire, no connection between stress and behavioural plasticity has yet been demonstrated in bees. My work provides the experimental proof that two stress stimuli (UV light; UV and shaking with light; SL) enhance sucrose responsiveness and impair learning and memory formation of honeybee.

4.2.1 UV light and reactive oxygen species (ROS)

My findings are in agreement with a recent study where iron-induced oxidative stress in antennal lobe of honeybee also shows inhibitory effects on olfactory behaviour of honeybee in a dose and time dependent manner [94]. A variety of factors producing oxidative stress can damage the nervous system by several interacting mechanism including changes in intracellular Ca^{2+} and release of excitatory amino acids [122]. It seems feasible that the UV light induced behavioural changes might be due to oxidative stress created in the brain of honeybee. Moreover, high doses of UV light can also produce excessive free radicals [191] that cause the increased intracellular level of reactive oxygen species (ROS) in animals [55]. The production of these highly reactive molecules like oxygen ions, free radicals, peroxides [98] can effect important intra- and extracellular structures that may change the cognitive performance. In mammals, UV light is also involved in apoptosis, cancer, clustering and internalization of cell surface receptors [304]. In *Drosophila*, UV irradiations induces apoptosis at different developmental stages of embryo [330].

Although the UV light exposure was shorter (>15 min) than DWV infection (>3 days), both treatments lead to the same impairment in behaviour, pointing towards a common mechanism underlying these behavioural changes. Recent experimental evidence claims that short term stress can also impair cellular communication in brain areas associated with learning and memory [59]. In this study, novel processes of acute stress activated selective molecules

(corticotrophin releasing hormone) disrupt the dendritic spines of hippocampal neurons. This in turn limits the ability of synapses to process information [295]. Thus it is feasible that UV light might interrupt the basic process of neuronal transmission to affects the behaviour of honeybee.

4.2.2 SL stress treatment and behaviour

It is well know that vertebrates as well as invertebrates have various forms of sleep, rest and wakefulness [150, 154, 288]. In mammals, any disruption in sleep cycles or internal rhythm may adversely affect the physiology and performance [292]. Adult honeybee has their own developed endogenous circadian rhythms which is an important component of its social behaviour [37, 280, 289]. Honeybee also exhibits sleep like states which is demonstrated by comparing the amount and episodes of antennal immobility during night [248, 249]. Although the antennal movements have not been measured, SL induced changes in behaviour might be due to an imbalance between normal circadian cycle and disturbed sleep pattern or both. SL stress treatment enhances the sensory responsiveness which matches with an increased sensory responsiveness of sleep deprived animals. [249, 262, 332]. My results are in agreement with studies in mammals where sleep deprivation suppresses the performance of learned tasks [303].

Various studies have shown that loss of sleep prior to training differentially affect the synaptic plasticity and subsequent behaviour [100, 176]. In rat, inadequate sleep produces several molecular and cellular alterations that impair spatial learning and hippocampal long-term potentiation [186, 242]. In human, lack of sleep causes marked deficits in hippocampal activity during episodic memory encoding that results in worse subsequent retention [324]. In *Drosophila,* mushroom bodies (MBs) can regulate the phenomenon of sleep via cyclic-AMP-dependent protein kinase [115, 150] pointing towards the implication of signalling pathways in the MBs in mechanism underlying stress induced behavioural impairments. It is known that neuronal pattern and consequently the release of neurotransmitter change significantly during sleep and wake cycle [70]. Some of these speculative aspects might also be involved in the modified behaviour after SL stress treatment in honeybee.

Role of immunity and biogenic amines during stress treatment

The majority of early research indicated that both minor and major stressful events affect a variety of immunological mechanisms. It is clear that stress can both enhance and suppress immune system function [84, 99]. Stress induced changes in immune function are also

reported in molluscs and insects [6, 168]. These stress associated immune de-regulations are consequential for health [111, 220] and might be the reason for changes in behaviour. Another explanation is that stress can modulate many biological factors other than immunity (blood flow, level of hormones, body temperature etc) [177] that can affect the performance of animals.

Insect nervous system has high concentration of biogenic amines that play a major role in regulation of various physiological and behavioural processes [93, 136, 198, 224]. It is feasible that stress treatment (UV and SL) might change the level of these amines that leads to change in performance of honeybee. This idea is supported by different studies in insects. In honeybee, stress treatments (CO_2, chilling and vertical spin) can depress the level of octopamine and dopamine and these stressed bees need longer time to fly from hive to feeder [60]. Changes in biogenic amine (especially OA) in honeybee, *Drosophila, Schistocerca gregaraia, Perilaneta americana* are also triggered by external stress stimuli such as mechanical, chemical, extreme temperature, heat and handling [65, 75, 126, 127, 137]. In addition to stressful events, the level of biogenic amines is also modified by different colonies, age and different seasons. The combination of both factors might be the reason for different outcomes on behavioural levels.

4.3 JNK Pathway mediates the behaviour of honeybee

In literature, JNK is mainly associated with immune and stress response but the function of JNK in neural plasticity is poorly understood [50]. Although a JNK homologue is proposed in computational screening of honeybee genome [92], no experimental investigation has been done so far. My data provides the first experimental proof for the presence of JNK in honeybee brain. Western blotting confirms the predicted molecular weight of JNK (46.5 kDa) as in other animal species [45, 167, 199, 203, 291, 301]. The mammalian JNK comprises three closely related genes (JNK1, JNK2 and JNK3) and are either widely expressed or restricted only to brain and neuronal tissue. The alternative splicing of these genes results in 10 isoforms of JNK with short form (46 kDa) and long form (54 kDa) that differ in their substrate affinities [23, 147, 223]. Each JNK may be expressed as either in its long form or C-terminally truncated short form [77]. Although these genes are not fully described in insects, my data provides evidence for the short form of JNK1 (46 kDa) in honeybee brain tissues that is higly identical to DJNK / basket in *Drosophila* [217]. However, the possibility of variant forms could not be ruled out.

Immunohistological data reveals high phosphorylation of JNK in regions (peduncle and alpha lobes of mushroom bodies) implicated in honeybee learning and memory formation. In mammals, various studies show that JNK1 is widely expressed at high level in all brain regions and exhibits a constitutive activity in brain [34, 45]. Moreover, expression of mammalian JNK1 in the dendrites and axons of neurons [108, 171] supports my findings in honeybee where major JNK activity is detected in non-nucleus areas of neurons (dendrites and axons) within the mushroom bodies. This JNK expression is an indication for a potential role of JNK in synaptic plasticity which is also reported in mammals [58, 331] and *Drosophila* [246]. Previous studies provide evidence for the involvement of JNK in basal synaptic transmission in hippocampus and its role in regulation of neurotransmitter release in hippocampal CA1 area [72]. Taken together, JNK pathway in honeybee brain is very likely implicated in multiple signaling modes.

4.3.1 DWV infection and stress treatments activate JNK

DWV infection: I have shown that DWV infection activates the JNK in the brain of honeybee in a distinct time pattern. Studies in other insects and mammals also have shown that JNK can be activated by virus infection, lipopolysacchride, inflammatory cytokines and viral RNA [42, 69, 85, 203, 266, 302]. DWV infection induces an up-regulation of JNK activity after 2 h followed by down-regulation after one day but again up-regulation after three days of infection. In addition, no activation of other cascades like p38 and ERK (p44/46) were observed during this time window. This indicates that virus infection leads to JNK activation within few hours which is not accompanied by changes in behaviour (visible > 3 days after infection). Here the self-defence system of insects can not be ignored which is supported by various studies. In *Drosophila,* it is proposed that any infection (virus, parasites, bacteria and fungi) activates different pathways (NF-kB, JAK-STAT and JNK etc) in fat bodies and haemocytes. This leads to the production of antimicrobial peptides that together with phagocytosis of microorganism (encapsulation) by blood cells are essential for insect's self-defence [89, 133, 138, 139].

Like in mammals, LPS triggers immune system by activation of JNK pathways in insects and suggest that JNK is an evolutionary conserved component in immune responses [56, 77]. In *Drosophila* cells and wax moth larvae (*Galleria mellonella*), LPS activates the JNK pathway within 5 min to 240 min in a dose dependent manner [265, 266, 315]. The late activation of JNK (5 days after infection) is also shown in female mice *in vivo*, where injection of influenza

A virus into the olfactory bulb activates JNK and leads to lethal encephalitis disease [205]. DWV infected bees might consume more energy as in case of LPS and *Nosema* infection [185, 294] and causes energy shortage that can activate JNK as observed in mammals [216, 268]. Conclusively, my data provides a unique JNK activation pattern which may be involved in triggering changes in sensory processing and associative learning but not in non-associative learning.

UV and SL stress treatment: My results demonstrated that like virus infection, environmental changes (UV and SL) also activate the JNK and change the animal performance. This points to a common signalling mechanism underlying all these behaviour modifications. The UV irradiation activates the JNK in the brain of honeybees depending on exposure times (>15 min). This is in agreement with other animal or cell culture studies where JNK is well known to respond to extracellular stimuli including UV irradiation in the same time range [25, 82, 135, 199, 291]. In mammalian Leukemia cells, UV radiation leads to initial changes in cell membrane and activation of JNK pathway [304]. In Hela cells and mouse fibroblast cells, UV light induces ROS [328] that strongly activates JNK and cause induction of many target genes [9, 238]. Besides my findings of increased JNK activity after different exposure times of UV light, JNK activity is also reported to be sensitive to different intensity / doses of UV light [8, 82, 268]. This might be the reason for weak elevation of JNK activity after minimum UV light exposure (5 min) in honeybee.

In addition to short and defined stimuli, my findings also show that long lasting procedure like SL treatment also activates JNK. In this case, the potential interruption of normal sleep pattern of honeybee by SL treatment can not be ruled out to activate JNK. Although no study is available for JNK implication in this scenario, various cascades important in learning and immune response (cAMP-dependent kinase, CREB and NF-kB) are reported to be activated in brain tissues of mammals and insects after sleep deprivation [64, 132, 332]. It is also feasible that SL treatment might increase the physical activity of honeybee to consume more energy that lead to glucose deprivation which is known to activate JNK in mammalian cells [216, 268].

Since different external treatments (infection and stress stimuli) leads to activation of JNK and changes in behaviour, my results suggest a principle link between all these components. Interestingly, these effects are unique because only distinct forms of behaviour such as sensory processing and associative learning are effected while non-associative learning remains unaffected. In this case, the potential cross talk between different signalling pathways and other unknown non-specific mechanism can not be ruled out.

4.3.2 Inhibition of JNK restores stress induced JNK activity and behavioural changes

My findings arouse a critical question whether the induced changes in behaviour are really modulated via JNK pathway *in vivo*. By employing the specific JNK-inhibitor (SP600125), my results convincingly show the down-regulation of virus and stress induced JNK activation. Generally, SP600125 is well known to inhibit JNK and its specificity has been verified in many studies where it is also used as an important tool to treatment of various diseases [18, 26, 31, 181, 302, 306]. My study demonstrates that JNK-inhibitor successfully rescues the stress (UV and SL) induced changes in sensory processing, learning and memory formation. In case of DWV infection, the reversible action of JNK-inhibitor did not allow to address the role of JNK due to the long lasting behavioural analysis. Nevertheless, my findings provide clear evidence for a critical inhibitory role of JNK in mechanism underlying behavioural plasticity.

There are only a few mammalian examples describing the role of JNK in memory formation and the involvement of JNK in sensory processing has been absolutely unknown so far. One report in rats demonstrated that in a taste aversion paradigm, presentation of conditioned stimulus differentially activates the ERK and JNK in the insular cortex [29]. Two other studies in rat passive avoidance paradigm propose an opposite role of JNK-inhibitor in short-term memory (STM; 2 h) and long-term memory (LTM; 24 h) where intra hippocampal injection of JNK-inhibitor enhances STM but blocks LTM without affecting the locomotory / exploratory activity of the animal [31, 32]. None of these studies demonstrate a link between stress induced JNK activation and behaviour, and thus does not allow a direct comparison with my data. However, my findings that increased JNK activity impairs LTM in honeybee show parallels to studies addressing cellular mechanisms of plasticity in long term-potentiation (LTP). LTP is widely studied as a physiological correlate of memory formation in mammalian brain [71]. The early phase of LTP does not require protein synthesis and involves covalent modification of pre-existing proteins, as other froms of short term plasticity and short-term memory. The late phase of LTP requires both translation and transcription and thus resembles the features of long-term facilitation in *Aplysia* and other forms of long lasting plasticity [158]. Thus both vertebrates and invertebrates share the common conserved mechanism underlying learning and memory formation.

One study in rat demonstrated the inhibitory effect of oxidative stress (IL-1β) on hippocampal LTP in association with an increase in JNK activity [299]. Although this study did not investigate the inhibitory role of JNK, it supports my results that stress induced activation of

JNK interferes with long-term memory formation. In addition, my findings that impaired LTM can be rescued with JNK-inhibitor are supported by previous studies showing a crucial role of JNK in long-term potentiation. In this case, Aβ (beta-amyloid peptide) activates JNK and impairs the LTP: JNK-inhibitor inhibits JNK, enhances synaptic transmission, and blocks the Aβ-mediated attenuation of LTP in hippocampal CA1 area *in vitro* [72, 305]. In another study, application of JNK-inhibitor resulted in complete reversal of cytokine-mediated inhibition of LTP in rat hippocampus [74].

In my results, stress induced JNK activation affects early sensory processing that usually depends on changes in neuronal transmission. But in the literature, only few studies are available to demonstrate that JNK can influence the mechanism of synaptic transmission. In mammals, it is reported that JNK regulates the synaptic plasticity in hippocampus [58, 331]. In rat, JNK directly regulates the AMPA-R trafficking following changes in neuronal activity by rapid phosphorylation [285]. These studies provide enough evidence to postulate that similar mechanism may be true in honeybee and would enable a fast modulation of synaptic transmission.

Recently, a study demonstrated that serotonin receptors (5-HT_{1A}) activate JNK within 10 min [293]. Although, it remains unclear how this relates to stress, 5-HT_{1A} receptors are very important in central nervous system due to their inhibitory effect on learning, memory and neuronal activity [48, 52, 142, 159, 192, 206, 227, 256, 322]. These receptors are also known to inhibit adenylyl cyclase (AC) and cAMP production in VUM neurons of *Drosophila* and in ganglia of *Aplysia* [16, 35, 148, 247, 287]. Since cellular components are likely to affect the coupling of receptors to specific signalling pathways [190], it is feasible that virus infection as well as stress stimuli might activate JNK via 5-HT receptors.

4.4 Transcription dependence of stress modulated behaviour

Generally, activation of JNK pathway is associated with stimulation of down-stream transcription factors (c-Jun/AP-1) in the nucleus where it regulates gene expression [39, 56]. In *Drosophila,* the early transcription factor AP-1 functions upstream of CREB (cyclic AMP response-element binding protein) to control the synaptic plasticity [246]. Thus, it is possible that JNK activates CREB which is a central component in LTM formation in *Drosophila*, honeybee, rodents, and long-term facilitation in *Aplysia* [76, 153, 210, 264, 323]. Since the pattern of gene expression is directly correlated with behavioural plasticity in honeybee [310], it is possible that the stress induced changes in behaviour might involve the transcriptional machinery. This question is addressed by injection of actinomycin-D (Act-D) during virus

infection and exposure of stress treatments. Act-D is a widely used transcription inhibitor that binds non-covalently to DNA, blocks RNA polymerase, prevents RNA synthesis and inhibits replication of many RNA and DNA viruses [30, 104, 149, 231, 276, 318].

4.4.1 Transcription inhibition restores DWV induced behavioural changes

In case of DWV infection, the transcription inhibition completely rescues the changes in sensory processing and memory formation of honeybee. Thus it is assumed that virus induced behavioural changes acts via transcription. Although DWV belongs to the group of positive single stranded RNA virus (+ssRNA) that mainly replicates in cytoplasm, many studies suggest that +ssRNA virus acts via transcription and gene expression. The injection of *Drosophila* C virus (DCV; +ssRNA virus) into adult *Drosophila* flies induces a substantial change in gene expression profile 24 h and 48 h after injection [86, 130]. In mice, the influenza virus (RNA virus) causes long-term behavioural changes in anxiety and spatial learning as well as alterations in gene expression in certain regions of the brain [27]. Recently, *Varroa* infection is reported to change gene expression [213] which is also a potential transmitting agent for DWV in honeybees. Thus it is more likely, that combination of different factors including DWV can change the behaviour via transcription that may be a potential reason for colony collapse disorder. Although, deregulation in neurotransmitter and bacterial infection are known to influence the gene expression in honeybee [36, 252], none of these studies showed the rescue of virus induced behavioural changes with inhibition of transcription.

Since Act-D is also known to inhibit virus replication, it remains unclear if Act-D inhibits virus or host transcription. In early studies, Act-D has been reported for its dose dependent inhibitory effect on the replication of +ssRNA viruses in Hela and hamster kidney cells [226, 251]. The dose and experimental condition is very critical for the effectiveness of Act-D [24] and low dose of Act-D does not inhibit +ssRNA virus [276]. Based on this, it is feasible that Act-D may block the synthesis of components crucial for replication of DWV virus. Moreover, it is also possible that inhibition of both transcription and replication occurs in parallel and thus limit the infection either individually or together to rescue the behavioural changes.

4.4.2 Transcription inhibition restores UV induced behavioural changes

In case of UV light, my experiments confirm that by blocking transcription with Act-D, JNK modulated behavioural changes are fully restored. This suggests that UV mediated effects on behaviour are mediated via JNK action on the transcription process. Although the neural targets of JNK are not fully investigated, there is convincing evidence that UV radiation immediately activates different transcription factors (AP-1; c-Fos, c-Jun, ATF) by JNK pathway [229, 238, 314]. In mouse, UV radiation has been demonstrated to increase the transcript levels of c-Fos and c-Jun *in vivo* [15, 307]. In Hela cells, UV radiation rapidly (within 30-60 min) triggers the transcription activity of c-Jun most likely via JNK pathway [277]. In another study, JNK activation in Hela cells and human MRC5 cells is correlated with transcriptional activity of c-Jun after short UV radiation (15-30 min) [201]. Taking together, these reports support my findings that UV light (>15 min) rapidly activates the JNK pathway that most likely acts via transcription. In honeybee, the inhibition of transcription during learning specifically effects LTM (≥3 days) but not memory until 3 days [318]. The fact that Act-D immediately rescues UV induced effects on sensory processing, learning and early memory formation (0-1 day) clearly argues for transcription processes different from that reported for memory formation. Thus, my data describes a special role of Act-D in improvement of UV light induced behavioural changes in honeybee.

Moreover, it is feasible that UV light might result in a release of alarm pheromone in honeybee that in turns affects gene expression and behaviour. The release of alarm pheromone in rats increases defensive behaviour, decreases exploratory and grooming behaviour and increases the expression of transcription factor [163, 164]. In honeybee, alarm pheromone causes a dose-dependent decrease of honeybee learning performance [296] and induces immediate early gene expression and slow behavioural response [10]. Based on these reports, it is hypothesized that UV increased release of alarm pheromone might be one of the reason for change in gene expression via JNK pathway.

4.4.3 SL induced behavioural changes are non-transcriptional

Mechanical stress induced behavioural changes in sensory processing, learning and memory formation remains totally unaffected after transcription inhibition. Thus my data clearly demonstrates that this particular JNK induced behavioural changes is not mediated via transcription (Figure 4.1). At that time, all JNK actions were reported to regulate transcription while non transcriptional aspect of JNK was poorly known. Later on, my results are supported by a recent study showing that JNK phosphorylates and dephosphorylates AMPA receptors

(AMPA-R) within minutes resulting in decrease and increase in neuronal activity respectively [285]. The AMPA-R is a glutamate regulated cation channels that mediate fast synaptic transmission in the central nervous system. The dynamic regulation of these receptors is crucial for short and long-term modification of synaptic efficiency [267].

With regard to synaptic activity, JNK activation is also reported to disrupt transmitter release and alter presynaptic composition in *Drosophila* neurons within time range of 6 h [91, 215]. In rat, oxidative stress induced activation of JNK causes a decrease in glutamate release and attenuate both early and late components of LTP while its inhibition could enhance glutamate release to facilitate short term mnemonic trace [161, 299]. All these observations are sufficient to formulate the idea that SL induced JNK activation might down-regulate the neuronal activity for short duration that in turns modifies the behaviour transiently.

Although many other studies can be connected to the observed SL induced behavioural changes, none of these studies implicate JNK. So, it is possible that SL treatment increases the physical activity and causes energy deprivation. This in turn may be the reason for enhancement in sucrose responsiveness of the bees in my experiments. In *Drosophila,* nutritional deficiency is reported to cause impairment in associative learning and memory [121, 320]. Another possible reason for behavioural changes might be deprived sleep during SL treatment which is reported to impair subsequent performance on various task, both in animals and in humans [179, 186, 242, 292]. All these stressful events may collectively alter other mechanism in brain (temperatures, hormone concentration and metabolism) that are sufficient to change the behaviour independent of transcription. But again very similar effects of different stress stimuli on behaviour irrespective to transcription argue for a common conserved process.

4.5 Acetylcholine action in Kenyon cells: a potential target of UV light

The presence of JNK in mushroom bodies (a structure important for learning) promoted me to investigate whether UV light can also have effect on basic neurotransmission in Kenyon cells of honeybee. Insect olfactory pathway contains central neuropile of mushroom bodies that receive cholinergic input from the antennal lobe and are involved in olfactory learning and memory formation [131, 194]. Studies have shown that major parts of the olfactory pathway in insects brain are cholinergic where nicotinic acetylcholine receptors (nAChRs) mediate the fast action of ACh at cholinergic synapses [11, 151, 236, 283].

Since action of acetylcholine (ACh) is important in olfactory signal processing, it is feasible that any change may deteriorate the signal processing. My data proofs this hypothesis by

measuring the ACh stimulated intracellular Ca^{2+} signal which is considered as a indicator of neuronal activity [96, 333]. In both *Drosophila* and honeybee ACh stimulates Kenyon cells in a similar way that is in agreement with the conserved function of ACh in insects. The transgenic *Drosophila* (20ly-GAL4; UAS-Cameleon 2.1) with genetic fluorescence indicator (cameleon) provides an easy tool to address the action of ACh in Kenyon cells [96]. My data demonstrates a UV dependent decrease in ACh action which can successfully be restored by Act-D (shortly before UV light) *in vitro*. However, the role of JNK remains unclear since the JNK-inhibitor is highly sensitive to UV light and does not allow to address this question *in vitro*. Nevertheless, my data indicates a transcription based mechanism which is consistent with my findings that Act-D rescues the UV induced behavioural changes.

In vertebrates and invertebrates, acetylcholinesterase (AChE) is expressed in nervous system and muscles where it plays an essential role in degradation of acetylcholine in the synaptic cleft [269]. UV light may increase its expression that in turn will hydrolyze ACh very quickly and thus terminate its action. A recent study in mammalian cells is available to support this idea where H_2O_2 (oxidative stress) can induce AChE expression by JNK pathway via transcription. In this case, inhibition of JNK can suppress the stress induced AChE expression [326]. The correlation between up-regulation of AChE and JNK activation is also reported in cancer cell line where JNK-inhibitor can block these up-regulations [81]. In astroglia, acute oxidative stress causes up-regulation and release of AChE followed by rapid and sustained changes in mRNA expression of AChE [40, 41]. The direct comparison of my results with these studies is limited because AChE mainly acts outside the cells whereas, I already have isolated Kenyon cells for *in vitro* analysis. Therefore, it is most likely that this phenomenon only applies for *in vivo* conditions.

Since ACh binds to AChRs, it is feasible that UV light may affect the AChRs that in turn will reduce the ACh action. In honeybee, inhibition of nAChRs before and after training has a distinct inhibitory effect on acquisition and memory phases *in vivo* [106] while their inhibition is also reported *in vitro* [113, 319]. Different mammalian studies give supporting reference for the proposed inhibitory effect of UV light on nAChRs via transcription. Oxidative stress is reported to influence the expression of nAChRs, modify their gene expression and affect learning and memory formation [105, 119, 120, 208, 228, 271]. A recent paper demonstrates that oxidative stress inactivates neuronal nAChRs in a use-dependent manner and reduces the effect of ACh at synapses junctions [51]. Although, it is clear that stress can impair cholinergic signalling mediated mechanisms at synapses, none of these studies implicate Act-D to rescue this impairment.

4.6 Schematic illustration of JNK modulated behaviour

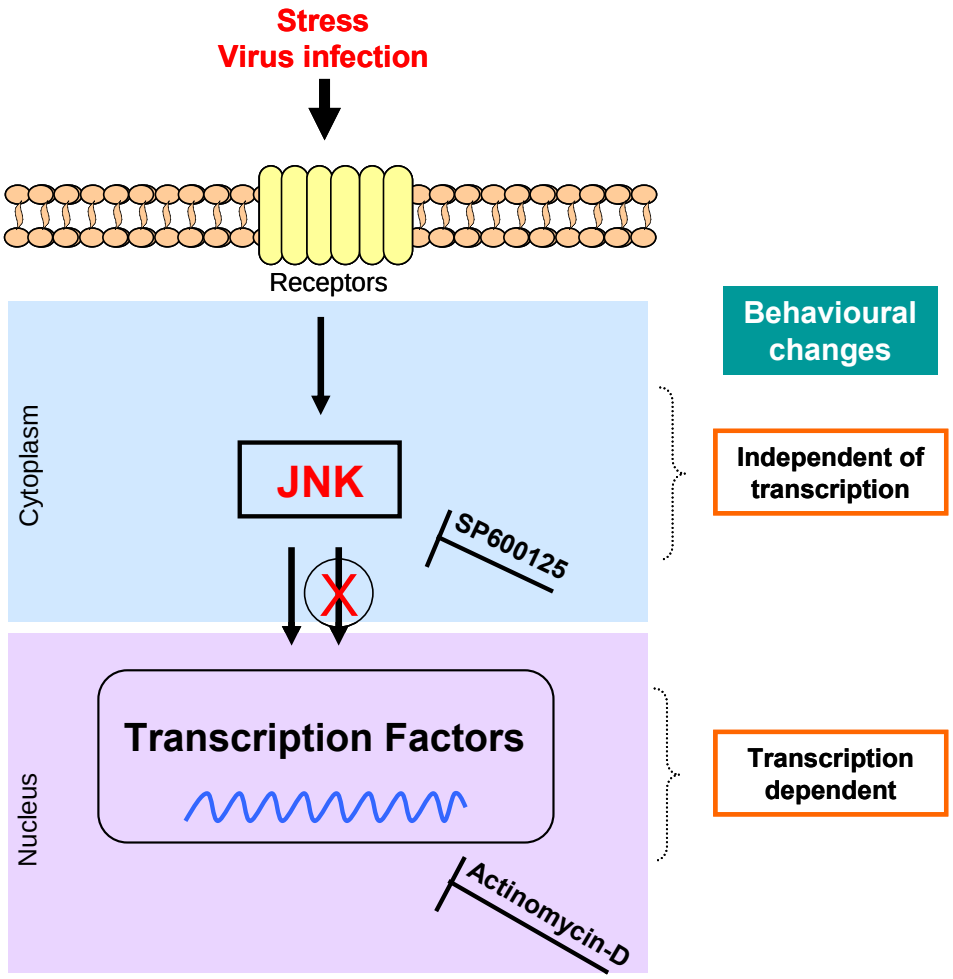


Figure 4.1: Schematic illustration of JNK modulated behaviour
Different external stress stimuli and virus infection causes very similar behavioural changes in sensory processing, associative learning and memory formation while non-associated learning remains unaffected. The stress induced behavioural changes are modulated by the JNK pathway and can be rescued by the JNK-inhibitor (SP600125). Using actinomycin-D, it is demonstrated that JNK can act via two different routes: transcriptional as well as non-transcriptional. Both routes leading to similar behavioural changes suggest a common mechanism.

5 REFERENCES

[1] Abramson, C.I., I.S. Aquino, F.S. Ramalho, and J.M. Price, The effect of insecticides on learning in the Africanized honey bee (*Apis mellifera* L.). Arch Environ Contam Toxicol, 1999. 37(4): 529-35.

[2] Abramson, C.I., P.A. Wanderley, M.J. Wanderley, J.C. Silva, and L.M. Michaluk, The effect of essential oils of sweet fennel and pignut on mortality and learning in africanized honeybees (*Apis mellifera* L.) (Hymenoptera: Apidae). Neotrop Entomol, 2007. 36(6): 828-35.

[3] Adamo, S.A., Evidence for adaptive changes in egg laying in crickets exposed to bacteria and parasites. Anim Behav, 1999. 57(1): 117-124.

[4] Adamo, S.A., Parasitic suppression of feeding in the tobacco hornworm, *Manduca sexta*: parallels with feeding depression after an immune challenge. Arch Insect Biochem Physiol, 2005. 60(4): 185-97.

[5] Adamo, S.A., Comparative psychoneuroimmunology: evidence from the insects. Behav Cogn Neurosci Rev, 2006. 5(3): 128-40.

[6] Adamo, S.A. and N.M. Parsons, The emergency life-history stage and immunity in the cricket, *Gryllus texensis*. Animal behaviour, 2006. 72(1): 235-244.

[7] Adams, J.P. and J.D. Sweatt, Molecular psychology: roles for the ERK MAP kinase cascade in memory. Annu Rev Pharmacol Toxicol, 2002. 42: 135-63.

[8] Adler, V., S.Y. Fuchs, J. Kim, A. Kraft, M.P. King, J. Pelling, and Z. Ronai, jun-NH2-terminal kinase activation mediated by UV-induced DNA lesions in melanoma and fibroblast cells. Cell Growth Differ, 1995. 6(11): 1437-46.

[9] Adler, V., A. Schaffer, J. Kim, L. Dolan, and Z. Ronai, UV irradiation and heat shock mediate JNK activation via alternate pathways. J Biol Chem, 1995. 270(44): 26071-7.

[10] Alaux, C. and G.E. Robinson, Alarm pheromone induces immediate-early gene expression and slow behavioral response in honey bees. J Chem Ecol, 2007. 33(7): 1346-50.

[11] Albert, J.L. and C.J. Lingle, Activation of nicotinic acetylcholine receptors on cultured *Drosophila* and other insect neurones. J Physiol, 1993. 463: 605-30.

[12] Alghamdi, A., L. Dalton, A. Phillis, E. Rosato, and E.B. Mallon, Immune response impairs learning in free-flying bumble-bees. Biol Lett, 2008. 4(5): 479-81.

[13] Allen, M. and B. Ball, The incidence and world distribution of honeybee viruses. Bee world, 1996. 77(3): 141-62.

[14] Alonso, M., L.R. Bevilaqua, I. Izquierdo, J.H. Medina, and M. Cammarota, Memory formation requires p38MAPK activity in the rat hippocampus. Neuroreport, 2003. 14(15): 1989-92.

[15] Amstad, P.A., G. Krupitza, and P.A. Cerutti, Mechanism of c-fos induction by active oxygen. Cancer Res, 1992. 52(14): 3952-60.

[16] Angers, A., M.V. Storozhuk, T. Duchaine, V.F. Castellucci, and L. DesGroseillers, Cloning and functional expression of an *Aplysia* 5-HT receptor negatively coupled to adenylate cyclase. J Neurosci, 1998. 18(15): 5586-93.

[17] Asp, L., S. Beraki, F. Aronsson, L. Rosvall, S.O. Ogren, K. Kristensson, and H. Karlsson, Gene expression changes in brains of mice exposed to a maternal virus infection. Neuroreport, 2005. 16(10): 1111-5.

[18] Assi, K., R. Pillai, A. Gomez-Munoz, D. Owen, and B. Salh, The specific JNK inhibitor SP600125 targets tumour necrosis factor-alpha production and epithelial cell apoptosis in acute murine colitis. Immunology, 2006. 118(1): 112-21.

[19] Atkins, C.M., J.C. Selcher, J.J. Petraitis, J.M. Trzaskos, and J.D. Sweatt, The MAPK cascade is required for mammalian associative learning. Nat Neurosci, 1998. 1(7): 602-9.

[20] Ayat, M. and E. Kamar, Effect of West Nile Virus on serotonin concentration of mouse brain MIRCN J Microbiol Biotechnol, 1989. 5(4): 543-545.

[21] Bailey, L. and B.V. Ball, Honeybee pathology. 2nd ed. 1991: London, UK Academic press.

[22] Bakonyi, R., A. Farkas, M. Szendroi, Dobos-Kovács, and M. Rusvai, Detection of acute bee paralysis virus by RT-PCR in honey bee and *Varroa destructor* samples: Rapid screening of representative Hungarian apiaries. Apidologie, 2002. 33: 29-40.

[23] Barr, R.K. and M.A. Bogoyevitch, The c-Jun N-terminal protein kinase family of mitogen-activated protein kinases (JNK MAPKs). Int J Biochem Cell Biol, 2001. 33(11): 1047-63.

[24] Barry, R.D., The Effects of Actinomycin D and Ultraviolet Irradiation on the Production of Fowl Plague Virus. Virology, 1964. 24: 563-9.

[25] Bender, K., C. Blattner, A. Knebel, M. Iordanov, P. Herrlich, and H.J. Rahmsdorf, UV-induced signal transduction. J Photochem Photobiol B, 1997. 37(1-2): 1-17.

[26] Bennett, B.L., D.T. Sasaki, B.W. Murray, E.C. O'Leary, S.T. Sakata, W. Xu, J.C. Leisten, A. Motiwala, S. Pierce, Y. Satoh, S.S. Bhagwat, A.M. Manning, and D.W. Anderson, SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci U S A, 2001. 98(24): 13681-6.

[27] Beraki, S., F. Aronsson, H. Karlsson, S.Ö. gren, and K. Kristensson, Influenza A virus infection causes alterations in expression of synaptic regulatory genes combined with changes in cognitive and emotional behaviors in mice. Molecular Psychiatry, 2005. 10: 299-308.

[28] Berenyi, O., T. Bakonyi, I. Derakhshifar, H. Koglberger, and N. Nowotny, Occurrence of six honeybee viruses in diseased Austrian apiaries. Appl Environ Microbiol, 2006. 72(4): 2414-20.

[29] Berman, D.E., S. Hazvi, K. Rosenblum, R. Seger, and Y. Dudai, Specific and differential activation of mitogen-activated protein kinase cascades by unfamiliar taste in the insular cortex of the behaving rat. J Neurosci, 1998. 18(23): 10037-44.

[30] Betzel, C., R. Rachev, P. Dolashka, and N. Genov, Actinomycins as proteinase inhibitors. Biochim Biophys Acta, 1993. 1161(1): 47-51.

[31] Bevilaqua, L.R., D.S. Kerr, J.H. Medina, I. Izquierdo, and M. Cammarota, Inhibition of hippocampal Jun N-terminal kinase enhances short-term memory but blocks long-term memory formation and retrieval of an inhibitory avoidance task. Eur J Neurosci, 2003. 17(4): 897-902.

[32] Bevilaqua, L.R., J.I. Rossato, J.H. Clarke, J.H. Medina, I. Izquierdo, and M. Cammarota, Inhibition of c-Jun N-terminal kinase in the CA1 region of the dorsal hippocampus blocks extinction of inhibitory avoidance memory. Behav Pharmacol, 2007. 18(5-6): 483-9.

[33] Bitterman, M., R. Menzel, A. Fietz, and S. Schäfer, Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). J Comp Psychol, 1983. 97(2): 107-119.

[34] Bjorkblom, B., N. Ostman, V. Hongisto, V. Komarovski, J.J. Filen, T.A. Nyman, T. Kallunki, M.J. Courtney, and E.T. Coffey, Constitutively active cytoplasmic c-Jun N-terminal kinase 1 is a dominant regulator of dendritic architecture: role of microtubule-associated protein 2 as an effector. J Neurosci, 2005. 25(27): 6350-61.

[35] Blenau, W. and A. Baumann, Molecular and pharmacological properties of insect biogenic amine receptors: lessons from *Drosophila melanogaster* and *Apis mellifera.* Arch Insect Biochem Physiol, 2001. 48(1): 13-38.

[36] Bloch, G. and A. Meshi, Influences of octopamine and juvenile hormone on locomotor behavior and period gene expression in the honeybee, *Apis mellifera.* J Comp Physiol A Neuroethol Sens Neural Behav Physiol, 2007. 193(2): 181-99.

[37] Bloch, G. and G.E. Robinson, Chronobiology. Reversal of honeybee behavioural rhythms. Nature, 2001. 410(6832): 1048.

[38] Boecking, O. and W. Drescher, The removal response of *Apis mellifera* L. colonies to brood in wax and plastic cells after artificial and natural infestation with V*arroa jacobsoni* Oud. and to freeze-killed brood. Exp Appl Acarol, 1992. 16: 321–329.

[39] Bogoyevitch, M.A. and B. Kobe, Uses for JNK: the many and varied substrates of the c-Jun N-terminal kinases. Microbiol Mol Biol Rev, 2006. 70(4): 1061-95.

[40] Bond, C.E. and S.A. Greenfield, Multiple cascade effects of oxidative stress on astroglia. Glia, 2007. 55(13): 1348-61.

[41] Bond, C.E., P. Patel, L. Crouch, N. Tetlow, T. Day, S. Abu-Hayyeh, C. Williamson, and S.A. Greenfield, Astroglia up-regulate transcription and secretion of 'readthrough' acetylcholinesterase following oxidative stress. Eur J Neurosci, 2006. 24(2): 381-6.

[42] Boutros, M., H. Agaisse, and N. Perrimon, Sequential activation of signaling pathways during innate immune responses in *Drosophila*. Dev Cell, 2002. 3(5): 711-22.

[43] Bowen-Walker, P.L., S.J. Martin, and A. Gunn, The transmission of deformed wing virus between honeybees (*Apis mellifera* L.) by the ectoparasitic mite *varroa jacobsoni* Oud. J Invertebr Pathol, 1999. 73(1): 101-6.

[44] Braun, G. and G. Bicker, Habituation of an appetitive reflex in the honeybee. J Neurophysiol, 1992. 67(3): 588-98.

[45] Brecht, S., R. Kirchhof, A. Chromik, M. Willesen, T. Nicolaus, G. Raivich, J. Wessig, V. Waetzig, M. Goetz, M. Claussen, D. Pearse, C.Y. Kuan, E. Vaudano, A. Behrens, E. Wagner, R.A. Flavell, R.J. Davis, and T. Herdegen, Specific pathophysiological functions of JNK isoforms in the brain. Eur J Neurosci, 2005. 21(2): 363-77.

[46] Bremner, J.D., Does stress damage the brain? Biol Psychiatry, 1999. 45(7): 797-805.

[47] Brennan, P.A. and E.B. Keverne, Neural mechanisms of mammalian olfactory learning. Prog Neurobiol, 1997. 51(4): 457-81.

[48] Buhot, M.C., S. Martin, and L. Segu, Role of serotonin in memory impairment. Ann Med, 2000. 32(3): 210-21.

[49] Burand, J.P., W. Tan, W. Kim, S. Nojima, and W. Roelofs, Infection with the insect virus Hz-2v alters mating behavior and pheromone production in female *Helicoverpa zea* moths. J Insect Sci, 2005. 5: 6.

[50] Cammarota, M., L.R. Bevilaqua, J.H. Medina, and I. Izquierdo, ERK1/2 and CaMKII-mediated events in memory formation: is 5HT regulation involved? Behav Brain Res, 2008. 195(1): 120-8.

[51] Campanucci, V.A., A. Krishnaswamy, and E. Cooper, Mitochondrial reactive oxygen species inactivate neuronal nicotinic acetylcholine receptors and induce long-term depression of fast nicotinic synaptic transmission. J Neurosci, 2008. 28(7): 1733-1744.

[52] Carli, M., E. Tatarczynska, L. Cervo, and R. Samanin, Stimulation of hippocampal 5-HT1A receptors causes amnesia and anxiolytic-like but not antidepressant-like effects in the rat. Eur J Pharmacol, 1993. 234(2-3): 215-21.

[53] Carreck, N.L. and I.H. Williams, The economic value of bees in the UK. Bee World, 1998. 79(3): 115-123.

[54] CDC, West Nile virus update--United States, January 1-August 19, 2008., in MMWR. http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5733a3.htm. 2008, August 22, Centers for Disease Control and Prevention (CDC). Washington, DC. 57 (33): 899-900.

[55] Chan, W.H., C.C. Wu, and J.S. Yu, Curcumin inhibits UV irradiation-induced oxidative stress and apoptotic biochemical changes in human epidermoid carcinoma A431 cells. J Cell Biochem, 2003. 90(2): 327-38.

[56] Chang, L. and M. Karin, Mammalian MAP kinase signalling cascades. Nature, 2001. 410(6824): 37-40.

[57] Chen, C. and S. Tonegawa, Molecular genetic analysis of synaptic plasticity, activity-dependent neural development, learning, and memory in the mammalian brain. Annu Rev Neurosci, 1997. 20: 157-84.

[58] Chen, J.T., D.H. Lu, C.P. Chia, D.Y. Ruan, K. Sabapathy, and Z.C. Xiao, Impaired long-term potentiation in c-Jun N-terminal kinase 2-deficient mice. J Neurochem, 2005. 93(2): 463-73.

[59] Chen, Y., C.M. Dube, C.J. Rice, and T.Z. Baram, Rapid loss of dendritic spines after stress involves derangement of spine dynamics by corticotropin-releasing hormone. J Neurosci, 2008. 28(11): 2903-11.

[60] Chen, Y.L., Y.S. Hung, and E.C. Yang, Biogenic amine levels change in the brains of stressed honeybees. Arch Insect Biochem Physiol, 2008. 68(4): 241-50.

[61] Chen, Y.P., J.A. Higgins, and M.F. Feldlaufer, Quantitative real-time reverse transcription-PCR analysis of deformed wing virus infection in the honeybee (*Apis mellifera* L.). Appl Environ Microbiol, 2005. 71(1): 436-41.

[62] Chen, Y.P., J.S. Pettis, A. Collins, and M.F. Feldlaufer, Prevalence and transmission of honeybee viruses. Appl Environ Microbiol, 2006. 72(1): 606-11.

[63] Chen, Y.P. and R. Siede, Honey bee viruses, In: Advances in Virus Research, K. Maramorosch, A.J. Shatkin, and F.A. Murphy, Editors. 2007, Academic Press. 51-54.

[64] Chen, Z., J. Gardi, T. Kushikata, J. Fang, and J.M. Krueger, Nuclear factor-kappaB-like activity increases in murine cerebral cortex after sleep deprivation. Am J Physiol, 1999. 276(6 Pt 2): R1812-8.

[65] Chentsova, N.A., N.E. Gruntenko, E.V. Bogomolova, N.V. Adonyeva, E.K. Karpova, and I.Y. Rauschenbach, Stress response in *Drosophila melanogaster* strain inactive with decreased tyramine and octopamine contents. J Comp Physiol [B], 2002. 172(7): 643-50.

[66] Cherry, S. and N. Perrimon, Entry is a rate-limiting step for viral infection in a *Drosophila melanogaster* model of pathogenesis. Nat Immunol, 2004. 5(1): 81-7.

[67] Chomczynski, P. and K. Mackey, Short technical reports. Modification of the TRI reagent procedure for isolation of RNA from polysaccharide- and proteoglycan-rich sources. Biotechniques, 1995. 19(6): 942-5.

[68] Christianson, S.A., Emotional stress and eyewitness memory: a critical review. Psychol Bull, 1992. 112(2): 284-309.

[69] Chu, W.M., D. Ostertag, Z.W. Li, L. Chang, Y. Chen, Y. Hu, B. Williams, J. Perrault, and M. Karin, JNK2 and IKKbeta are required for activating the innate response to viral infection. Immunity, 1999. 11(6): 721-31.

[70] Cirelli, C. and G. Tononi, Gene expression in the brain across the sleep–waking cycle. Brain Research, 2000. 885: 303–321.

[71] Cooke, S.F. and T.V. Bliss, Plasticity in the human central nervous system. Brain, 2006. 129(Pt 7): 1659-73.

[72] Costello, D.A. and C.E. Herron, The role of c-Jun N-terminal kinase in the A beta-mediated impairment of LTP and regulation of synaptic transmission in the hippocampus. Neuropharmacology, 2004. 46(5): 655-62.

[73] Cox-Foster, D.L., S. Conlan, E.C. Holmes, G. Palacios, J.D. Evans, N.A. Moran, P.L. Quan, T. Briese, M. Hornig, D.M. Geiser, V. Martinson, D. vanEngelsdorp, A.L. Kalkstein, A. Drysdale, J. Hui, J. Zhai, L. Cui, S.K. Hutchison, J.F. Simons, M. Egholm, J.S. Pettis, and W.I. Lipkin, A metagenomic survey of microbes in honey bee colony collapse disorder. Science, 2007. 318(5848): 283-7.

[74] Curran, B.P., H.J. Murray, and J.J. O'Connor, A role for c-Jun N-terminal kinase in the inhibition of long-term potentiation by interleukin-1beta and long-term depression in the rat dentate gyrus in vitro. Neuroscience, 2003. 118(2): 347-57.

[75] Davenport, A.P. and P.D. Evans, Stress-induced changes in the octopamine levels of insect haemolymph. Insect Biochem, 1984. 14: 135-143.

[76] Davis, G.W., C.M. Schuster, and C.S. Goodman, Genetic dissection of structural and functional components of synaptic plasticity. III. CREB is necessary for presynaptic functional plasticity. Neuron, 1996. 17(4): 669-79.

[77] Davis, R.J., Signal transduction by the JNK group of MAP kinases. Cell, 2000. 103(2): 239-52.

[78] Davis, R.L., Olfactory memory formation in *Drosophila*: from molecular to systems neuroscience. Annu Rev Neurosci, 2005. 28: 275-302.

[79] de Kloet, E.R., M. Joels, and F. Holsboer, Stress and the brain: from adaptation to disease. Nat Rev Neurosci, 2005. 6(6): 463-75.

[80] de Kloet, E.R., M.S. Oitzl, and M. Joels, Stress and cognition: are corticosteroids good or bad guys? Trends Neurosci, 1999. 22(10): 422-6.

[81] Deng, R., W. Li, Z. Guan, J.M. Zhou, Y. Wang, Y.P. Mei, M.T. Li, G.K. Feng, W. Huang, Z.C. Liu, Y. Han, Y.X. Zeng, and X.F. Zhu, Acetylcholinesterase expression mediated by c-Jun-NH2-terminal kinase pathway during anticancer drug-induced apoptosis. Oncogene, 2006. 25(53): 7070-7.

[82] Derijard, B., M. Hibi, I.H. Wu, T. Barrett, B. Su, T. Deng, M. Karin, and R.J. Davis, JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell, 1994. 76(6): 1025-37.

[83] Desneux, N., A. Decourtye, and J.M. Delpuech, The sublethal effects of pesticides on beneficial arthropods. Annu Rev Entomol, 2007. 52: 81-106.

[84] Dhabhar, F.S., Stress-induced enhancement of cell-mediated immunity. Ann N Y Acad Sci, 1998. 840: 359-72.

[85] Dong, C., R.J. Davis, and R.A. Flavell, MAP kinases in the immune response. Annu Rev Immunol, 2002. 20: 55-72.

[86] Dostert, C., E. Jouanguy, P. Irving, L. Troxler, D. Galiana-Arnoux, C. Hetru, J.A. Hoffmann, and J.L. Imler, The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of drosophila. Nat Immunol, 2005. 6(9): 946-53.

[87] El Hassani, A.K., M. Dacher, V. Gary, M. Lambin, M. Gauthier, and C. Armengaud, Effects of sublethal doses of acetamiprid and thiamethoxam on the behavior of the honeybee (*Apis mellifera*). Arch Environ Contam Toxicol, 2008. 54(4): 653-61.

[88] English, J.D. and J.D. Sweatt, Activation of p42 mitogen-activated protein kinase in hippocampal long term potentiation. J Biol Chem, 1996. 271(40): 24329-32.

[89] Engström, Y., Induction and regulation of antimicrobial peptides in *Drosophila*. Dev and Comp Immunol, 1999. 23: 354-358.

[90] Erber, J., P. Kloppenburg, and A. Scheidler, Neuromodulation by serotonin and octopamine in the honeybee: behaviour, neuroanatomy and electrophysiology. Experientia, 1993. 49: 1073-1083.

[91] Etter, P.D., R. Narayanan, Z. Navratilova, C. Patel, D. Bohmann, H. Jasper, and M. Ramaswami, Synaptic and genomic responses to JNK and AP-1 signaling in *Drosophila* neurons. BMC Neurosci, 2005. 6: 39.

[92] Evans, J.D., K. Aronstein, Y.P. Chen, C. Hetru, J.L. Imler, H. Jiang, M. Kanost, G.J. Thompson, Z. Zou, and D. Hultmark, Immune pathways and defence mechanisms in honey bees *Apis mellifera*. Insect Mol Biol, 2006. 15(5): 645-56.

[93] Evans, P.D., Biogenic amines in the insect nervous system. Adv. Insect Physiol., 1980. 15: 317-473.

[94] Farooqui, T., Iron-induced oxidative stress modulates olfactory learning and memory in honeybees. Behav Neurosci, 2008. 122(2): 433-47.

[95] Feld, M., B. Dimant, A. Delorenzi, O. Coso, and A. Romano, Phosphorylation of extra-nuclear ERK/MAPK is required for long-term memory consolidation in the crab *Chasmagnathus*. Behav Brain Res, 2005. 158(2): 251-61.

[96] Fiala, A. and T. Spall, In vivo calcium imaging of brain activity in *Drosophila* by transgenic cameleon expression. Sci STKE, 2003. 2003(174): PL6.

[97] Fievet, J., D. Tentcheva, L. Gauthier, J. de Miranda, F. Cousserans, M.E. Colin, and M. Bergoin, Localization of deformed wing virus infection in queen and drone *Apis mellifera* L. Virol J, 2006. 3: 16.

[98] Fischer, T.W., G. Scholz, B. Knoll, U.C. Hipler, and P. Elsner, Melatonin reduces UV-induced reactive oxygen species in a dose-dependent manner in IL-3-stimulated leukocytes. J Pineal Res, 2001. 31(1): 39-45.

[99] Fleshner, M. and M.L. Laudenslager, Psychoneuroimmunology: then and now. Behav Cogn Neurosci Rev, 2004. 3(2): 114-30.

[100] Frank, M.G., N.P. Issa, and M.P. Stryker, Sleep enhances plasticity in the developing visual cortex. Neuron, 2001. 30(1): 275-87.

[101] Free, J.B., Insect pollination of crops. 1993: London: Academic Press. 768.

[102] Friedrich, A., U. Thomas, and U. Muller, Learning at different satiation levels reveals parallel functions for the cAMP-protein kinase A cascade in formation of long-term memory. J Neurosci, 2004. 24(18): 4460-8.

[103] Fukui, K., K. Onodera, T. Shinkai, S. Suzuki, and S. Urano, Impairment of learning and memory in rats caused by oxidative stress and aging, and changes in antioxidative defense systems. Ann N Y Acad Sci. , 2001. 928: 168-175.

[104] Gangadhar, N.M., S.J. Firestein, and B.R. Stockwell, A novel role for jun N-terminal kinase signaling in olfactory sensory neuronal death. Mol Cell Neurosci, 2008. 38(4): 518-25.

[105] Gao, Q., Y.J. Liu, and Z.Z. Guan, Oxidative stress might be a mechanism connected with the decreased alpha 7 nicotinic receptor influenced by high-concentration of fluoride in SH-SY5Y neuroblastoma cells. Toxicol In Vitro, 2008. 22(4): 837-43.

[106] Gauthier, M., M. Dacher, S.H. Thany, C. Niggebrugge, P. Deglise, P. Kljucevic, C. Armengaud, and B. Grunewald, Involvement of alpha-bungarotoxin-sensitive nicotinic receptors in long-term memory formation in the honeybee (*Apis mellifera*). Neurobiol Learn Mem, 2006. 86(2): 164-74.

[107] Gegear, R.J., M.C. Otterstatter, and J.D. Thomson, Bumble-bee foragers infected by a gut parasite have an impaired ability to utilize floral information. Proc Biol Sci, 2006. 273(1590): 1073-8.

[108] Gelderblom, M., S. Eminel, T. Herdegen, and V. Waetzig, c-Jun N-terminal kinases (JNKs) and the cytoskeleton--functions beyond neurodegeneration. Int J Dev Neurosci, 2004. 22(7): 559-64.

[109] Genersch, E., Development of a rapid and sensitive RT-PCR method for the detection of deformed wing virus, a pathogen of the honeybee (*Apis mellifera*). Vet J, 2005. 169(1): 121-3.

[110] Genersch, E., C. Yue, I. Fries, and J.R. de Miranda, Detection of Deformed wing virus, a honey bee viral pathogen, in bumble bees (*Bombus terrestris* and *Bombus pascuorum*) with wing deformities. J Invertebr Pathol, 2006. 91(1): 61-3.

[111] Glaser, R. and J.K. Kiecolt-Glaser, Stress-induced immune dysfunction: implications for health. Nat Rev Immunol, 2005. 5(3): 243-51.

[112] Gliński, Z. and J. Jarosz, Infection and immunity in the honey bee *Apis mellifera*. Apiacta, 2001. 36(1): 12-24.

[113] Goldberg, F., B. Grunewald, H. Rosenboom, and R. Menzel, Nicotinic acetylcholine currents of cultured Kenyon cells from the mushroom bodies of the honey bee *Apis mellifera*. J Physiol, 1999. 514 (Pt 3): 759-68.

[114] Grabensteiner, E., W. Ritter, M.J. Carter, S. Davison, H. Pechhacker, J. Kolodziejek, O. Boecking, I. Derakhshifar, R. Moosbeckhofer, E. Licek, and N. Nowotny, Sacbrood virus of the honeybee (*Apis mellifera*): rapid identification and phylogenetic analysis using reverse transcription-PCR. Clin Diagn Lab Immunol, 2001. 8(1): 93-104.

[115] Graves, L., A. Pack, and T. Abel, Sleep and memory: a molecular perspective. Trends Neurosci, 2001. 24(4): 237-43.

[116] Gregory, P.G., J.D. Evans, T. Rinderer, and L. de Guzman, Conditional immune-gene suppression of honeybees parasitized by *Varroa* mites. J Insect Sci, 2005. 5: 7.

[117] Gronli, J., R. Murison, E. Fiske, B. Bjorvatn, E. Sorensen, C.M. Portas, and R. Ursin, Effects of chronic mild stress on sexual behavior, locomotor activity and consumption of sucrose and saccharine solutions. Physiol Behav, 2005. 84(4): 571-7.

[118] Grünbaum, L. and U. Müller, Induction of a specific olfactory memory leads to a long-lasting activation of Protein Kinase C in the antennal lobe of the honeybee. The Journal of Neuroscience, 1998. 18(11): 4384–4392.

[119] Guan, Z.Z., Cross-talk between oxidative stress and modifications of cholinergic and glutaminergic receptors in the pathogenesis of Alzheimer's disease. Acta Pharmacol Sin, 2008. 29(7): 773-80.

[120] Guan, Z.Z., X. Zhang, M. Mousavi, J.Y. Tian, C. Unger, and A. Nordberg, Reduced expression of neuronal nicotinic acetylcholine receptors during the early stages of damage by oxidative stress in PC12 cells. J Neurosci Res, 2001. 66(4): 551-8.

[121] Guo, A., L. Li, S.Z. Xia, C.H. Feng, R. Wolf, and M. Heisenberg, Conditioned visual flight orientation in *Drosophila*: dependence on age, practice, and diet. Learn Mem, 1996. 3(1): 49-59.

[122] Halliwell, B., Reactive oxygen species and the central nervous system. J Neurochem, 1992. 59(5): 1609-23.

[123] Hammer, M., An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. Nature, 1993. 366: 59-63.

[124] Hammer, M. and R. Menzel, Learning and memory in the honeybee. J Neurosci, 1995. 15(3 Pt 1): 1617-30.

[125] Hammer, M. and R. Menzel, Multiple sites of associative odor learning as revealed by local brain microinjections of octopamine in honeybees. Learn Mem, 1998. 5(1-2): 146-56.

[126] Harris, J.W. and J. Woodring, Effects of stress, age, season, and source colony on levels of octopamine, dopamine and serotonin in the honeybee *(Apis mellifera)* brain. J Insect Physiol, 1992. 38: 29-35.

[127] Harris, J.W., J. Woodring, and J.R. Harbo, Effects of carbon dioxide on levels of biogenic amines in the brains of queenless worker and virgin queen honeybees *(Apis mellifera).* Journal of Apicultural Research, 1996. 35: 69–78.

[128] Hayes, E.B., N. Komar, R.S. Nasci, S.P. Montgomery, D.R. O'Leary, and G.L. Campbell, Epidemiology and transmission dynamics of West Nile virus disease. Emerg Infect Dis, 2005. 11(8): 1167-73.

[129] Hazel, M.H., R.J. Christensen, and M.J. O'Donnell, Inhibition of the SAPK/JNK pathway blocks the stimulatory effects of glutamine on fluid secretion by the Malpighian tubules of *Rhodnius prolixus.* J Insect Physiol, 2003. 49(10): 897-906.

[130] Hedges, L.M. and K.N. Johnson, Induction of host defence responses by *Drosophila* C virus. J Gen Virol, 2008. 89(Pt 6): 1497-501.

[131] Heisenberg, M., A. Borst, S. Wagner, and D. Byers, *Drosophila* mushroom body mutants are deficient in olfactory learning. J Neurogenet, 1985. 2(1): 1-30.

[132] Hendricks, J.C., J.A. Williams, K. Panckeri, D. Kirk, M. Tello, J.C. Yin, and A. Sehgal, A non-circadian role for cAMP signaling and CREB activity in *Drosophila* rest homeostasis. Nat Neurosci, 2001. 4(11): 1108-15.

[133] Hetru, C., L. Troxler, and J.A. Hoffmann, *Drosophila melanogaster* antimicrobial defense. J Infect Dis, 2003. 187 Suppl 2: S327-34.

[134] Heyman, J., Quantification of Activated Signal Transduction Proteins Using Fast Activated Cell-based ELISAs (FACE™) Nature Methods; Application Notes (18 April :doi:10.1038/an1562), 2006.

[135] Hibi, M., A. Lin, T. Smeal, A. Minden, and M. Karin, Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev, 1993. 7(11): 2135-48.

[136] Hirashima, A. and E. Morifuza, Chemical stress induced changes in the biogenic amine levels of *Periplaneta americana* L. Biochem Physiol, 1993. 46: 131-141.

[137] Hirashima, A., M. Sukhanova, and I. Rauschenbach, Biogenic amines in *Drosophila virilis* under stress conditions. Biosci Biotechnol Biochem, 2000. 64(12): 2625-30.

[138] Hoffmann, J.A., The immune response of *Drosophila*. Nature, 2003. 426(6962): 33-8.

[139] Ip, Y.T., *Drosophila* innate immunity goes viral. Nat Immunol, 2005. 6(9): 863-4.

[140] Iqbal, J. and U. Mueller, Virus infection causes specific learning deficits in honeybee foragers. Proc Biol Sci, 2007. 274(1617): 1517-21.

[141] Ishioka, S.-i., Y. Ezaka, K. Umemura, T. Hayashi, T. Endo, and T. Saito, Proteomic analysis of mechanisms of hypoxia-induced apoptosis in trophoblastic cells. Int. J. Med. Sci., 2007. 4(1): 36-44.

[142] Izquierdo, I., L.R. Bevilaqua, J.I. Rossato, J.S. Bonini, J.H. Medina, and M. Cammarota, Different molecular cascades in different sites of the brain control memory consolidation. Trends Neurosci, 2006. 29(9): 496-505.

[143] Jhoo, J.H., H.-C. Kimb, T. Nabeshima, K. Yamadad, E.-J. Shin, W.-K. Jhoo, W. Kimb, K.-S. Kange, S.A. Jo, and J.I. Woo, β-Amyloid (1–42)-induced learning and memory deficits in mice:involvement of oxidative burdens in the hippocampus and cerebral cortex. Behavioural Brain Research, 2004. 155: 185-196.

[144] Ji, R.R., R.W.t. Gereau, M. Malcangio, and G.R. Strichartz, MAP kinase and pain. Brain Res Rev, 2008.

[145] Joels, M., Z. Pu, O. Wiegert, M.S. Oitzl, and H.J. Krugers, Learning under stress: how does it work? Trends Cogn Sci, 2006. 10(4): 152-8.

[146] Johnson, G.L. and R. Lapadat, Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science, 2002. 298(5600): 1911-2.

[147] Johnson, G.L. and K. Nakamura, The c-jun kinase/stress-activated pathway: regulation, function and role in human disease. Biochim Biophys Acta, 2007. 1773(8): 1341-8.

[148] Johnson, O., J. Becnel, and C.D. Nichols, Serotonin 5-HT(2) and 5-HT(1A)-like receptors differentially modulate aggressive behaviors in *Drosophila melanogaster*. Neuroscience, 2008.

[149] Johnson, T.R., J. Trojan, S.D. Rudin, B.K. Blossey, J. Ilan, and J. Ilan, Effects of actinomycin D and cycloheximide on transcript levels of IGF-I, actin, and albumin in hepatocyte primary cultures treated with growth hormone and insulin. Mol Reprod Dev, 1991. 30(2): 95-9.

[150] Joiner, W.J., A. Crocker, B.H. White, and A. Sehgal, Sleep in *Drosophila* is regulated by adult mushroom bodies. Nature, 2006. 441(7094): 757-60.

[151] Jones, A.K., L.A. Brown, and D.B. Sattelle, Insect nicotinic acetylcholine receptor gene families: from genetic model organism to vector, pest and beneficial species. Invert Neurosci, 2007. 7(1): 67-73.

[152] Jordan, J.D., E.M. Landau, and R. Iyengar, Signaling networks: the origins of cellular multitasking. Cell, 2000. 103(2): 193-200.

[153] Josselyn, S.A., C. Shi, W.A. Carlezon, Jr., R.L. Neve, E.J. Nestler, and M. Davis, Long-term memory is facilitated by cAMP response element-binding protein overexpression in the amygdala. J Neurosci, 2001. 21(7): 2404-12.

[154] Kaiser, W. and J. Steiner-Kaiser, Neuronal correlates of sleep, wakefulness and arousal in a diurnal insect. Nature, 1983. 301(5902): 707-9.

[155] Kamita, S.G., K. Nagasaka, J.W. Chua, T. Shimada, K. Mita, M. Kobayashi, S. Maeda, and B.D. Hammock, A baculovirus-encoded protein tyrosine phosphatase gene induces enhanced locomotory activity in a lepidopteran host. Proc Natl Acad Sci U S A, 2005. 102(7): 2584-9.

[156] Kamitani, W., E. Ono, S. Yoshino, T. Kobayashi, S. Taharaguchi, B.J. Lee, M. Yamashita, T. Kobayashi, M. Okamoto, H. Taniyama, K. Tomonaga, and K. Ikuta, Glial expression of Borna disease virus phosphoprotein induces behavioral and neurological abnormalities in transgenic mice. Proc Natl Acad Sci U S A, 2003. 100(15): 8969-74.

[157] Kandel, E.R., The molecular biology of memory storage: a dialog between genes and synapses. Biosci Rep, 2001. 21(5): 565-611.

[158] Kandel, E.R., The molecular biology of memory storage: a dialogue between genes and synapses. Science, 2001. 294(5544): 1030-8.

[159] Kant, G.J., G.R. Meininger, K.R. Maughan, W.L. Wright, T.N. Robinson, 3rd, and T.M. Neely, Effects of the serotonin receptor agonists 8-OH-DPAT and TFMPP on learning as assessed using a novel water maze. Pharmacol Biochem Behav, 1996. 53(2): 385-90.

[160] Kelleher, R.J., 3rd, A. Govindarajan, H.Y. Jung, H. Kang, and S. Tonegawa, Translational control by MAPK signaling in long-term synaptic plasticity and memory. Cell, 2004. 116(3): 467-79.

[161] Kelly, A., A. Lynch, E. Vereker, Y. Nolan, P. Queenan, E. Whittaker, L.A. O'Neill, and M.A. Lynch, The anti-inflammatory cytokine, interleukin (IL)-10, blocks the inhibitory effect of IL-1 beta on long term potentiation. A role for JNK. J Biol Chem, 2001. 276(49): 45564-72.

[162] Keshishian, H., K. Broadie, A. Chiba, and M. Bate, The *Drosophila* neuromuscular junction: a model system for studying synaptic development and function. Annu Rev Neurosci, 1996. 19: 545-75.

[163] Kiyokawa, Y., T. Kikusui, Y. Takeuchi, and Y. Mori, Alarm pheromone that aggravates stress-induced hyperthermia is soluble in water. Chem Senses, 2005. 30(6): 513-9.

[164] Kiyokawa, Y., M. Shimozuru, T. Kikusui, Y. Takeuchi, and Y. Mori, Alarm pheromone increases defensive and risk assessment behaviors in male rats. Physiol Behav, 2006. 87(2): 383-7.

[165] Klein, M. and E.R. Kandel, Mechanism of calcium current modulation underlying presynaptic facilitation and behavioral sensitization in *Aplysia*. Proc. Nati. Acad. Sci. USA, 1980. 77(11): 6912-6916.

[166] Kralj, J., A. Brockmann, S. Fuchs, and J. Tautz, The parasitic mite *Varroa destructor* affects non-associative learning in honey bee foragers, *Apis mellifera* L. J Comp Physiol A Neuroethol Sens Neural Behav Physiol, 2007. 193(3): 363-70.

[167] Kujime, K., S. Hashimoto, Y. Gon, K. Shimizu, and T. Horie, p38 mitogen-activated protein kinase and c-jun-NH2-terminal kinase regulate RANTES production by influenza virus-infected human bronchial epithelial cells. J Immunol, 2000. 164(6): 3222-8.

[168] Lacoste, A., F. Jalabert, S.K. Malham, A. Cueff, and S.A. Poulet, Stress and stress-induced neuroendocrine changes increase the susceptibility of juvenile oysters (Crassostrea gigas) to Vibrio splendidus. Appl Environ Microbiol, 2001. 67(5): 2304-9.

[169] Laeger, T., Der Einfluss des Sättigungszustandes auf appetitive sensorische Prozessierung, Lernen und Gedächtnisbildung: Die Rolle der 5'-Adenosinmonophosphat-aktivierten Proteinkinase. Saarland University, Saarbruecken, Germany, 2007. PhD: 105.

[170] Lanzi, G., J.R. de Miranda, M.B. Boniotti, C.E. Cameron, A. Lavazza, L. Capucci, S.M. Camazine, and C. Rossi, Molecular and biological characterization of deformed wing virus of honeybees (*Apis mellifera* L.). J Virol, 2006. 80(10): 4998-5009.

[171] Lee, J.K., J. Park, Y.D. Lee, S.H. Lee, and P.L. Han, Distinct localization of SAPK isoforms in neurons of adult mouse brain implies multiple signaling modes of SAPK pathway. Brain Res Mol Brain Res, 1999. 70(1): 116-24.

[172] Leppa, S. and D. Bohmann, Diverse functions of JNK signaling and c-Jun in stress response and apoptosis. Oncogene, 1999. 18(45): 6158-62.

[173] Liu, Y., E.G. Shepherd, and L.D. Nelin, MAPK phosphatases--regulating the immune response. Nat Rev Immunol, 2007. 7(3): 202-12.

[174] Loesch, M. and G. Chen, The p38 MAPK stress pathway as a tumor suppressor or more? Front Biosci, 2008. 13: 3581-93.

[175] Lupien, S.J., F. Maheu, M. Tu, A. Fiocco, and T.E. Schramek, The effects of stress and stress hormones on human cognition: Implications for the field of brain and cognition. Brain Cogn, 2007. 65(3): 209-37.

[176] Mackiewicz, M., N. Naidoo, J.E. Zimmerman, and A.I. Pack, Molecular mechanisms of sleep and wakefulness. Ann N Y Acad Sci, 2008. 1129: 335-49.

[177] Maier, S.F., L.R. Watkins, and M. Fleshner, Psychoneuroimmunology. The interface between behavior, brain, and immunity. Am Psychol, 1994. 49(12): 1004-17.

[178] Mallon, E.B., A. Brockmann, and P. Schmid-Hempel, Immune response inhibits associative learning in insects. Proc Biol Sci, 2003. 270(1532): 2471-3.

[179] Maquet, P., The role of sleep in learning and memory. Science, 2001. 294(5544): 1048-52.

[180] Martin, S.J., The role of *Varroa* and viral pathogens in the collapse of honeybee colonies: a modelling approach. Ecology, 2001. 38: 1082-1093.

[181] Masamune, A., K. Kikuta, N. Suzuki, M. Satoh, K. Satoh, and T. Shimosegawa, A c-Jun NH2-terminal kinase inhibitor SP600125 (anthra[1,9-cd]pyrazole-6 (2H)-one) blocks activation of pancreatic stellate cells. J Pharmacol Exp Ther, 2004. 310(2): 520-7.

[182] Masterman, R., R. Ross, K. Mesce, and M. Spivak, Olfactory and behavioral response thresholds to odors of diseased blood differ between hygienic and non-hygienic honey bees (*Apis mellifera* L.). J Comp Physiol [A], 2001. 187(6): 441-52.

[183] Masterman, R., B.H. Smith, and M. Spivak, Brood odor discrimination abilities in honey bees (*Apis mellifera* L.) using proboscis extension reflex conditioning. J. Insect Behav, 2000. 13: 87–101.

[184] Mattila, H.R. and B.H. Smith, Learning and memory in workers reared by nutritionally stressed honey bee (*Apis mellifera* L.) colonies. Physiol Behav, 2008. 95(5): 609-16.

[185] Mayack, C. and D. Naug, Energetic stress in the honeybee *Apis mellifera* from Nosema ceranae infection. J Invertebr Pathol, 2009. (Article in press).

[186] McDermott, C.M., G.J. LaHoste, C. Chen, A. Musto, N.G. Bazan, and J.C. Magee, Sleep deprivation causes behavioral, synaptic, and membrane excitability alterations in hippocampal neurons. J Neurosci, 2003. 23(29): 9687-95.

[187] McEwen, B.S., Central effects of stress hormones in health and disease: Understanding the protective and damaging effects of stress and stress mediators. Eur J Pharmacol, 2008. 583(2-3): 174-85.

[188] McEwen, B.S. and A.M. Magarinos, Stress effects on morphology and function of the hippocampus. Ann N Y Acad Sci, 1997. 821: 271-84.

[189] McKay, S.E., A.L. Purcell, and T.J. Carew, Regulation of synaptic function by neurotrophic factors in vertebrates and invertebrates: implications for development and learning. Learn Mem, 1999. 6(3): 193-215.

[190] Meller, E., 5-HT1A receptor-mediated apoptosis: death by JNK? Biochim Biophys Acta, 2007. 1773(6): 691-3.

[191] Mendez, F. and R. Penner, Near-visible ultraviolet light induces a novel ubiquitous calcium-permeable cation current in mammalian cell lines. J Physiol, 1998. 507 (Pt 2): 365-77.

[192] Meneses, A., 5-HT system and cognition. Neurosci Biobehav Rev, 1999. 23(8): 1111-25.

[193] Menzel, R., Learning, memory and cognition in honey bees, In: Neurobiology of Comparative Cognition. 1990, R.P. Kesner and D.S. Olten. 237-292.

[194] Menzel, R., J. Erber, and T. Masuhr, Learning and memory in the honeybee, In: Experimental analysis of insect behaviour, L.B. Browne, Editor. 1974, Springer, Berlin Heidelberg New York. 195–217.

[195] Menzel, R., A. Heyne, C. Kinzel, B. Gerber, and A. Fiala, Pharmacological dissociation between the reinforcing, sensitizing, and response-releasing functions of reward in honeybee classical conditioning. Behav Neurosci, 1999. 113(4): 744-54.

[196] Menzel, R. and U. Müller, Learning and memory in honeybees: from behavior to neural substrates. Annu. Rev. Neurosci, 1996. 19: 379-404.

[197] Mercer, A.R. and J. Erber, The effects of amines on evoked potentials recorded in the mushroom bodies of the bee brain J Comp Physiol, 1983. 151: 469-476.

[198] Mercer, A.R. and R. Menzel, The effects of biogenic amines on conditioned and unconditioned responses to olfactory stimuli in the honeybee *Apis mellifera.* J. Comp. Physiol, 1982. 145: 363–368.

[199] Mielke, K., A. Damm, D.D. Yang, and T. Herdegen, Selective expression of JNK isoforms and stress-specific JNK activity in different neural cell lines. Brain Res Mol Brain Res, 2000. 75(1): 128-37.

[200] Miles, C.I. and R. Booker, Octopamine mimics the effects of parasitism on the foregut of the tobacco hornworm *Manduca sexta.* J Exp Biol, 2000. 203(Pt 11): 1689-700.

[201] Minden, A., A. Lin, T. Smeal, B. Derijard, M. Cobb, R. Davis, and M. Karin, c-Jun N-terminal phosphorylation correlates with activation of the JNK subgroup but not the ERK subgroup of mitogen-activated protein kinases. Mol Cell Biol, 1994. 14(10): 6683-8.

[202] Miyamoto, E., Molecular mechanism of neuronal plasticity: induction and maintenance of long-term potentiation in the hippocampus. J Pharmacol Sci, 2006. 100(5): 433-42.

[203] Mizutani, T., M. Kobayashi, Y. Eshita, O. Inanami, T. Yamamori, A. Goto, Y. Ako, H. Miyoshi, H. Miyamoto, H. Kariwa, M. Kuwabara, and I. Takashima, Characterization of JNK-like protein derived from a mosquito cell line, C6/36. Insect Mol Biol, 2003. 12(1): 61-6.

[204] Moore, J., Parasites and the behavior of animals. 2002: Oxford University Press USA.

[205] Mori, I., F. Goshima, T. Koshizuka, N. Koide, T. Sugiyama, T. Yoshida, T. Yokochi, Y. Nishiyama, and Y. Kimura, Differential activation of the c-Jun N-terminal kinase/stress-activated protein kinase and p38 mitogen-activated protein kinase signal

transduction pathways in the mouse brain upon infection with neurovirulent influenza A virus. J Gen Virol, 2003. 84(Pt 9): 2401-8.

[206] Moyano, S., J. Del Rio, and D. Frechilla, Role of hippocampal CaMKII in serotonin 5-HT(1A) receptor-mediated learning deficit in rats. Neuropsychopharmacology, 2004. 29(12): 2216-24.

[207] Mueller, U., Memory: Cellular and molecular networks. Cell. Mol. Life Sci., 2006. 63: 961-962.

[208] Mullenixa, P.J., P.K. Denbesten, A. Schunior, and W.J. Kernan, Neurotoxicity of sodium fluoride in rats. Neurotoxicol Teratol, 1995. 17(2): 169-177.

[209] Müller, U., Second messenger pathways in the honeybee brain: Immunohistochemistry of protein kinase A and protein kinase C. Microsc Res Tech, 1999. 45: 165-173.

[210] Müller, U., Prolonged Activation of cAMP-Dependent Protein Kinase during Conditioning Induces Long-Term Memory in Honeybees. Neuron, 2000. 27: 159-168.

[211] Müller, U., Learning in honeybees: from molecules to behaviour. Zoology, 2002. 105: 313-320.

[212] Müller, U. and H. Hildebrandt, Nitric oxide/cGMP mediated protein kinase A activation in the antennal lobes plays an important role in appetitive reflex habituation in the honeybee. J. Neurosci, 2002. 22: 8739-8747.

[213] Navajas, M., A. Migeon, C. Alaux, M. Martin-Magniette, G. Robinson, J. Evans, S. Cros-Arteil, D. Crauser, and Y. Le Conte, Differential gene expression of the honey bee *Apis mellifera* associated with Varroa destructor infection. BMC Genomics, 2008. 9: 301.

[214] Neckameyer, W.S. and H. Matsuo, Distinct neural circuits reflect sex, sexual maturity, and reproductive status in response to stress in *Drosophila melanogaster*. Neuroscience, 2008. 156(4): 841-56.

[215] Neckameyer, W.S. and J.S. Weinstein, Stress affects dopaminergic signaling pathways in *Drosophila melanogaster*. Stress, 2005. 8(2): 117-131.

[216] Nishio, H., H. Kuwabara, H. Mori, and K. Suzuki, Repeated fasting stress causes activation of mitogen-activated protein kinases (ERK/JNK) in rat liver. Hepatology, 2002. 36(1): 72-80.

[217] Noselli, S., JNK signaling and morphogenesis in *Drosophila*. Trends Genet, 1998. 14(1): 33-8.

[218] Oitzl, M.S. and E.R. de Kloet, Selective corticosteroid antagonists modulate specific aspects of spatial orientation learning. Behav Neurosci, 1992. 106(1): 62-71.

[219] Ono, M., T. Igarashi, E. Ohno, and M. Sasaki, Unusual thermal defence by a honeybee against mass attack by hornets Nature, 1995. 377: 334-336.

[220] Padgett, D.A. and R. Glaser, How stress influences the immune response. Trends Immunol, 2003. 24(8): 444-8.

[221] Park, C.R., M. Adam, Campbell, and D.M. Diamond, Chronic Psychosocial Stress Impairs Learning and Memory and Increases Sensitivity to Yohimbine in Adult Rats. BIOL PSYCHIATRY, 2001. 50: 994-1004.

[222] Pavlov, I.P., Conditioned Reflexes (G. V. Anrep, Trans.). 1927: London: Oxford University Press (Original work published in 1926).

[223] Pearson, G., F. Robinson, T. Beers Gibson, B.E. Xu, M. Karandikar, K. Berman, and M.H. Cobb, Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev, 2001. 22(2): 153-83.

[224] Perić-Mataruga, V., V. Nenadović, and J. Ivanović, Neurohormones in insect stress: A review. Archives of Biological Sciences 2006. 58(1): 1-12.

[225] Platt, K.B., K.J. Linthicum, K.S. Myint, B.L. Innis, K. Lerdthusnee, and D.W. Vaughn, Impact of dengue virus infection on feeding behavior of *Aedes aegypti*. Am J Trop Med Hyg, 1997. 57(2): 119-25.

[226] Polatnick, J. and R.B. Arlinghaus, Foot-and-mouth disease virus-induced ribonucleic acid polymerase in baby hamster kidney cells. Virology, 1967. 31(4): 601-8.

[227] Pugliese, A.M., M.B. Passani, and R. Corradetti, Effect of the selective 5-HT1A receptor antagonist WAY 100635 on the inhibition of e.p.s.ps produced by 5-HT in the CA1 region of rat hippocampal slices. Br J Pharmacol, 1998. 124(1): 93-100.

[228] Qi, X.L., J. Xiu, K.R. Shan, Y. Xiao, R. Gu, R.Y. Liu, and Z.Z. Guan, Oxidative stress induced by beta-amyloid peptide(1-42) is involved in the altered composition of cellular membrane lipids and the decreased expression of nicotinic receptors in human SH-SY5Y neuroblastoma cells. Neurochem Int, 2005. 46(8): 613-21.

[229] Rahmsdorf, H.J., Modulation of Fos and Jun in response to adverse environmental agents, In: The Fos and Jun Families of Transcription Factors P.E. Angel and P. Herrlich, Editors. 1994, CRC Press.

[230] Ratcliffe, N.A., A.F. Rowley, S.W. Fitzgerald, and C.P. Rhodes, Invertebrate immunity: basic concepts and recent advances. Int. Rev. Cytol, 1985. 97: 183-350.

[231] Reich, E. and I.H. Goldberg, Actinomycin and nucleic acid function. Prog Nucleic Acid Res Mol Biol, 1964. 3: 183-234.

[232] Ribere, M., J.-P. Faucon, and M. Pepin, Detection of chronic honey bee (*Apis mellifera* L.) paralysis virus infection: application to a field survey. Apidologie, 2000. 31: 567–577.

[233] Riddell, C.E. and E.B. Mallon, Insect psychoneuroimmunology: immune response reduces learning in protein starved bumblebees (*Bombus terrestris*). Brain Behav Immun, 2006. 20(2): 135-8.

[234] Rios-Barrera, D., A. Vega-Segura, V. Thibert, J.S. Rodriguez-Zavala, and M.E. Torres-Marquez, p38 MAPK as a signal transduction component of heavy metals stress in *Euglena gracilis.* Arch Microbiol, 2009. 191(1): 47-54.

[235] Robb, T. and M.R. Forbes, On understanding seasonal increases in damselfly defence and resistance against ectoparasitic mites. Ecological Entomology, 2005. 30: 334-341.

[236] Rohrbough, J. and K. Broadie, Electrophysiological analysis of synaptic transmission in central neurons of *Drosophila* larvae. J Neurophysiol, 2002. 88(2): 847-60.

[237] Roozendaal, B., A. Barsegyan, and S. Lee, Adrenal stress hormones, amygdala activation, and memory for emotionally arousing experiences. Prog Brain Res, 2008. 167: 79-97.

[238] Rosette, C. and M. Karin, Ultraviolet Light and Osmotic Stress: Activation of the JNK Cascade Through Multiple Growth Factor and Cytokine Receptors Science 1996. 274: 1194-1197.

[239] Rossato, J.I., L.R. Bevilaqua, R.H. Lima, J.H. Medina, I. Izquierdo, and M. Cammarota, On the participation of hippocampal p38 mitogen-activated protein kinase in extinction and reacquisition of inhibitory avoidance memory. Neuroscience, 2006. 143(1): 15-23.

[240] Rothenbuhler, W.C., Behaviour genetics of nest cleaning in honey bees. IV. Responses of F1 and backcross generations to disease-killed brood. Am Zool, 1964. 4: 111–123.

[241] Roxstrom-Lindquist, K., O. Terenius, and I. Faye, Parasite-specific immune response in adult *Drosophila melanogaster*: a genomic study. EMBO reports, 2004. 5(2): 207-212.

[242] Ruskin, D.N., C. Liu, K.E. Dunn, N.G. Bazan, and G.J. LaHoste, Sleep deprivation impairs hippocampus-mediated contextual learning but not amygdala-mediated cued learning in rats. Eur J Neurosci, 2004. 19(11): 3121-4.

[243] Sambrook, J. and D.W. Russell, Molecular Cloning: A Laboratory Manual. 3rd ed. Vol. 2. 2001, New York: Cold Spring Harbor Laboratory.

[244] Sandi, C., Stress, cognitive impairment and cell adhesion molecules. Nat Rev Neurosci, 2004. 5(12): 917-30.

[245] Sandi, C. and M.T. Pinelo-Nava, Stress and memory: behavioral effects and neurobiological mechanisms. Neural Plast, 2007. 2007: 78970.

[246] Sanyal, S., D.J. Sandstrom, C.A. Hoeffer, and M. Ramaswami, AP-1 functions upstream of CREB to control synaptic plasticity in *Drosophila.* Nature, 2002. 416(6883): 870-4.

[247] Saudou, F., U. Boschert, N. Amlaiky, J.L. Plassat, and R. Hen, A family of *Drosophila* serotonin receptors with distinct intracellular signalling properties and expression patterns. Embo J, 1992. 11(1): 7-17.

[248] Sauer, S., E. Herrmann, and W. Kaiser, Covariation of behavioural sleep signs and resting respiration in honey bees. J. Sleep Res, 1998. 7(Suppl. 2): S. 240.

[249] Sauer, S., E. Herrmann, and W. Kaiser, Sleep deprivation in honey bees. J Sleep Res, 2004. 13(2): 145-52.

[250] Schaeffer, H.J. and M.J. Weber, Mitogen-activated protein kinases: specific messages from ubiquitous messengers. Mol Cell Biol, 1999. 19(4): 2435-44.

[251] Schaffer, F.L. and M. Gordon, Differential inhibitory effects of actinomycin D among strains of poliovirus. J Bacteriol, 1966. 91(6): 2309-16.

[252] Scharlaken, B., D.C. de Graaf, K. Goossens, L.J. Peelman, and F.J. Jacobs, Differential gene expression in the honeybee head after a bacterial challenge. Dev Comp Immunol, 2008. 32(8): 883-9.

[253] Scheiner, R., Responsiveness to sucrose and habituation of the proboscis extension response in honey bees. J Comp Physiol A Neuroethol Sens Neural Behav Physiol, 2004. 190(9): 727-33.

[254] Scheiner, R., R.E. Page, Jr., and J. Erber, The effects of genotype, foraging role, and sucrose responsiveness on the tactile learning performance of honey bees (*Apis mellifera* L.). Neurobiol Learn Mem, 2001. 76(2): 138-50.

[255] Scheiner, R., S. Pluckhahn, B. Oney, W. Blenau, and J. Erber, Behavioural pharmacology of octopamine, tyramine and dopamine in honey bees. Behav Brain Res, 2002. 136(2): 545-53.

[256] Schiapparelli, L., A.M. Simon, J. Del Rio, and D. Frechilla, Opposing effects of AMPA and 5-HT1A receptor blockade on passive avoidance and object recognition performance: correlation with AMPA receptor subunit expression in rat hippocampus. Neuropharmacology, 2006. 50(7): 897-907.

[257] Schulz, D.J., A.B. Barron, and G.E. Robinson, A role for octopamine in honey bee division of labor. Brain Behav Evol, 2002. 60(6): 350-9.

[258] Sharma, S.K. and T.J. Carew, The roles of MAPK cascades in synaptic plasticity and memory in *Aplysia*: facilitatory effects and inhibitory constraints. Learn Mem, 2004. 11(4): 373-8.

[259] Shi, L., S.H. Fatemi, R.W. Sidwell, and P.H. Patterson, Maternal influenza infection causes marked behavioral and pharmacological changes in the offspring. J Neurosci, 2003. 23(1): 297-302.

[260] Shimanuki, H., N.W. Calderone, and D.A. Knox, Parasitic mite syndrome: the symptoms. Am bee J, 1994. 134(12): 827-828.

[261] Shors, T.J., Learning During Stressful Times. Learn Mem, 2004. 11: 137-144.

[262] Siegel, J.M., Do all animals sleep? Trends Neurosci, 2008. 31(4): 208-13.

[263] Sigg, D., C.M. Thompson, and A.R. Mercer, Activity-dependent changes to the brain and behavior of the honey bee, *Apis mellifera* (L.). J Neurosci, 1997. 17(18): 7148-56.

[264] Silva, A.J., J.H. Kogan, P.W. Frankland, and S. Kida, CREB and memory. Annu Rev Neurosci, 1998. 21: 127-48.

[265] Silverman, N., R. Zhou, R.L. Erlich, M. Hunter, E. Bernstein, D. Schneider, and T. Maniatis, Immune activation of NF-kappaB and JNK requires *Drosophila* TAK1. J Biol Chem, 2003. 278(49): 48928-34.

[266] Sluss, H.K., Z. Han, T. Barrett, D.C. Goberdhan, C. Wilson, R.J. Davis, and Y.T. Ip, A JNK signal transduction pathway that mediates morphogenesis and an immune response in *Drosophila*. Genes Dev, 1996. 10(21): 2745-58.

[267] Song, I. and R.L. Huganir, Regulation of AMPA receptors during synaptic plasticity. Trends Neurosci, 2002. 25(11): 578-88.

[268] Song, J.J. and Y.J. Lee, Differential activation of the JNK signal pathway by UV irradiation and glucose deprivation. Cell Signal, 2007. 19(3): 563-72.

[269] Soreq, H. and S. Seidman, Acetylcholinesterase--new roles for an old actor. Nat Rev Neurosci, 2001. 2(4): 294-302.

[270] Sparkman, N.L., R.A. Kohman, A.K. Garcia, and G.W. Boehm, Peripheral lipopolysaccharide administration impairs two-way active avoidance conditioning in C57BL/6J mice. Physiol Behav, 2005. 85(3): 278-88.

[271] Spittle, B., Fluoride and intelligence. Fluoride, 2000. 33(2): 49-52.

[272] Spivak, M., Honey bee hygienic behavior and defense against *Varroa jacobsoni*. Apidologie 1996(27): 245–260.

[273] Spivak, M. and M. Gilliam, Hygienic behaviour of honey bees and its application for control of brood diseases and *Varroa* mites. Part I: hygienic behaviour and resistance to American foulbrood. Part II: studies on hygienic behaviour since the Rothenbuhler era. Bee World, 1998. 79: 124–34, 165–82.

[274] Stankus, T., A review and bibliography of the literature of honey bee colony collapse disorder: A poorly understood dpidemic that clearly threatens the successful pollination of billions of dollars of crops in America. Journal of Agricultural & Food Information, 2008. 9(2): 115-143.

[275] Starks, P.T., C.A. Blackie, and T.D. Seeley, Fever in honeybee colonies. Naturwissenschaften, 2000. 87(5): 229-31.

[276] Stefanelli, C.C., J.G. Castilho, M.V. Botelho, R.E. Linhares, and C.M. Nozawa, Effect of actinomycin D on simian rotavirus (SA11) replication in cell culture. Braz J Med Biol Res, 2002. 35(4): 445-9.

[277] Stein, B., P. Angel, H. van Dam, H. Ponta, P. Herrlich, A. van der Eb, and H.J. Rahmsdorf, Ultraviolet-radiation induced c-jun gene transcription: two AP-1 like binding sites mediate the response. Photochem Photobiol, 1992. 55(3): 409-15.

[278] Stoltz, D., X.-R. Shen, C. Boggis, and G. Sisson, Molecular diagnosis of Kashmir bee virus infection. J Apic Res, 1995. 34: 153–160.

[279] Stronach, B., Dissecting JNK signaling, one KKKinase at a time. Dev Dyn, 2005. 232(3): 575-84.

[280] Stussi, T. and M.L. Harmelin, Research on the ontogenesis of the circadian rhythm of energy expenditure in bees. C R Acad Sci Hebd Seances Acad Sci D, 1966. 262(19): 2066-9.

[281] Sweatt, J.D., Mitogen-activated protein kinases in synaptic plasticity and memory. Curr Opin Neurobiol, 2004. 14(3): 311-7.

[282] Tentcheva, D., L. Gauthier, N. Zappulla, B. Dainat, F. Cousserans, M.E. Colin, and M. Bergoin, Prevalence and seasonal variations of six bee viruses in *Apis mellifera* L. and *Varroa destructor* mite populations in France. Appl Environ Microbiol, 2004. 70(12): 7185-91.

[283] Thany, S.H., G. Lenaers, M. Crozatier, C. Armengaud, and M. Gauthier, Identification and localization of the nicotinic acetylcholine receptor alpha3 mRNA in the brain of the honeybee, *Apis mellifera*. Insect Mol Biol, 2003. 12(3): 255-62.

[284] Thomas, G.M. and R.L. Huganir, MAPK cascade signalling and synaptic plasticity. Nat Rev Neurosci, 2004. 5(3): 173-83.

[285] Thomas, G.M., D.T. Lin, M. Nuriya, and R.L. Huganir, Rapid and bi-directional regulation of AMPA receptor phosphorylation and trafficking by JNK. Embo J, 2008. 27(2): 361-72.

[286] Thompson, P.M., R.A. Dutton, K.M. Hayashi, A.W. Toga, O.L. Lopez, H.J. Aizenstein, and J.T. Becker, Thinning of the cerebral cortex visualized in HIV/AIDS reflects CD4+ T lymphocyte decline. Proc Natl Acad Sci U S A, 2005. 102(43): 15647-52.

[287] Tierney, A.J., Structure and function of invertebrate 5-HT receptors: a review. Comp Biochem Physiol A Mol Integr Physiol, 2001. 128(4): 791-804.

[288] Tobler, I., R. Murison, R. Ursin, H. Ursin, and A.A. Borbely, The effect of sleep deprivation and recovery sleep on plasma corticosterone in the rat. Neurosci Lett, 1983. 35(3): 297-300.

[289] Toma, D.P., G. Bloch, D. Moore, and G.E. Robinson, Changes in period mRNA levels in the brain and division of labor in honey bee colonies. Proc Natl Acad Sci U S A, 2000. 97(12): 6914-9.

[290] Tomonaga, K., Virus-induced neurobehavioral disorders: mechanisms and implications. Trends Mol Med, 2004. 10(2): 71-7.

[291] Tournier, C., P. Hess, D.D. Yang, J. Xu, T.K. Turner, A. Nimnual, D. Bar-Sagi, S.N. Jones, R.A. Flavell, and R.J. Davis, Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. Science, 2000. 288(5467): 870-4.

[292] Turek, F.W. and P.C. Zee, Regulation of Sleep and Circadian Rhythms. 1999: Dekker, Inc, New York.

[293] Turner, J.H., M.N. Garnovskaya, and J.R. Raymond, Serotonin 5-HT1A receptor stimulates c-Jun N-terminal kinase and induces apoptosis in Chinese hamster ovary fibroblasts. Biochim Biophys Acta, 2007. 1773(3): 391-9.

[294] Tyler, E.R., S. Adams, and E.B. Mallon, An immune response in the bumblebee, Bombus terrestris leads to increased food consumption. BMC Physiol, 2006. 6: 6.

[295] Uni-California-Irvine, Short-term stress can affect learning and memory, in ScienceDaily. http://www.sciencedaily.com/releases/2008/03/080311182434.htm. 2008, March 13.

[296] Urlacher, E., B. Francés, M. Giurfa, and J.M. Devaud. Stress and learning in honeybees: effects of exposure to an alarm pheromone component on associative conditioning and memory. In: Göttingen Meeting of the German Neuroscience Society. 2007.

[297] van Engelsdorp, D., J. Hayes, Jr., R.M. Underwood, and J. Pettis, A survey of honey bee colony losses in the U.S., fall 2007 to spring 2008. PLoS ONE, 2008. 3(12): e4071.

[298] Vasconcelos, S.D., R.S. Hails, M.R. Speight, and J.S. Cory, Differential crop damage by healthy and nucleopolyhedrovirus-infected *Mamestra brassicae* L. (Lepidoptera: Noctuidae) larvae: a field examination. J Invertebr Pathol, 2005. 88(2): 177-9.

[299] Vereker, E., E. O'Donnell, and M.A. Lynch, The inhibitory effect of interleukin-1beta on long-term potentiation is coupled with increased activity of stress-activated protein kinases. J Neurosci, 2000. 20(18): 6811-9.

[300] Versteeg, H.H., E. Nijhuis, G.R. van den Brink, M. Evertzen, G.N. Pynaert, S.J. van Deventer, P.J. Coffer, and M.P. Peppelenbosch, A new phosphospecific cell-based ELISA for p42/p44 mitogen-activated protein kinase (MAPK), p38 MAPK, protein kinase B and cAMP-response-element-binding protein. Biochem J, 2000. 350 717-22.

[301] Wada, T., K. Nakagawa, T. Watanabe, G. Nishitai, J. Seo, H. Kishimoto, D. Kitagawa, T. Sasaki, J.M. Penninger, H. Nishina, and T. Katada, Impaired synergistic activation of stress-activated protein kinase SAPK/JNK in mouse embryonic stem cells lacking

SEK1/MKK4: different contribution of SEK2/MKK7 isoforms to the synergistic activation. J Biol Chem, 2001. 276(33): 30892-7.

[302] Waetzig, V. and T. Herdegen, Context-specific inhibition of JNKs: overcoming the dilemma of protection and damage. Trends Pharmacol Sci, 2005. 26(9): 455-61.

[303] Walker, M.P. and R. Stickgold, Sleep-dependent learning and memory consolidation. Neuron, 2004. 44(1): 121-33.

[304] Wang, L., D. Xu, W. Dai, and L. Lu, An ultraviolet-activated K+ channel mediates apoptosis of myeloblastic leukemia cells. J Biol Chem, 1999. 274(6): 3678-85.

[305] Wang, Q., D.M. Walsh, M.J. Rowan, D.J. Selkoe, and R. Anwyl, Block of long-term potentiation by naturally secreted and synthetic amyloid beta-peptide in hippocampal slices is mediated via activation of the kinases c-Jun N-terminal kinase, cyclin-dependent kinase 5, and p38 mitogen-activated protein kinase as well as metabotropic glutamate receptor type 5. J Neurosci, 2004. 24(13): 3370-8.

[306] Wang, W., L. Shi, Y. Xie, C. Ma, W. Li, X. Su, S. Huang, R. Chen, Z. Zhu, Z. Mao, Y. Han, and M. Li, SP600125, a new JNK inhibitor, protects dopaminergic neurons in the MPTP model of Parkinson's disease. Neurosci Res, 2004. 48(2): 195-202.

[307] Wang, Y., X. Zhang, M. Lebwohl, V. DeLeo, and H. Wei, Inhibition of ultraviolet B (UVB)-induced c-fos and c-jun expression in vivo by a tyrosine kinase inhibitor genistein. Carcinogenesis, 1998. 19(4): 649-54.

[308] Weed, M.R. and L.H. Gold, Paradigms for behavioral assessment of viral pathogenesis. Adv Virus Res, 2001. 56: 583-626.

[309] Wellmer, A., C. Noeske, J. Gerber, U. Munzel, and R. Nau, Spatial memory and learning deficits after experimental *Pneumococcal meningitis* in mice. Neurosci Lett, 2000. 296(2-3): 137-40.

[310] Whitfield, C.W., A.M. Cziko, and G.E. Robinson, Gene expression profiles in the brain predict behavior in individual honey bees. Science, 2003. 302(5643): 296-9.

[311] Widmann, C., S. Gibson, M.B. Jarpe, and G.L. Johnson, Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev, 1999. 79(1): 143-80.

[312] Willner, P., Animal models as simulations of depression. Trends Pharmacol Sci, 1991. 12(4): 131-6.

[313] Wilson-Rich, N., M. Spivak, N.H. Fefferman, and P.T. Starks, Genetic, Individual, and Group Facilitation of Disease Resistance in Insect Societies. Annu Rev Entomol, 2009. 54: 405-423.

[314] Wisdom, R., AP-1: one switch for many signals. Exp Cell Res, 1999. 253(1): 180-5.

[315] Wojda, I., P. Kowalski, and T. Jakubowicz, JNK MAP kinase is involved in the humoral immune response of the greater wax moth larvae *Galleria mellonella*. Arch Insect Biochem Physiol, 2004. 56(4): 143-54.

[316] Wolf, O.T., The influence of stress hormones on emotional memory: relevance for psychopathology. Acta Psychol (Amst), 2008. 127(3): 513-31.

[317] Woodrow, A.W. and E.C. Holst, The mechanism of colony resistance to American foulbrood. J Econ Entomol, 1942. 35: 327–330.

[318] Wüstenberg, D., B. Gerber, and R. Menzel, Short communication: long- but not medium-term retention of olfactory memories in honeybees is impaired by actinomycin D and anisomycin. Eur J Neurosci, 1998. 10: 2742--2745.

[319] Wüstenberg, D.G. and B. Grünewald, Pharmacology of the neuronal nicotinic acetylcholine receptor of cultured Kenyon cells of the honeybee, *Apis mellifera*. J Comp Physiol A, 2004. 190: 807-821.

[320] Xia, S.Z., L. Liu, C.H. Feng, and A.K. Guo, Nutritional effects on operant visual learning in *Drosophila melanogaster*. Physiol Behav, 1997. 62(2): 263-71.

[321] Yang, X. and D.L. Cox-Foster, Impact of an ectoparasite on the immunity and pathology of an invertebrate: evidence for host immunosuppression and viral amplification. Proc Natl Acad Sci U S A, 2005. 102(21): 7470-5.

[322] Yasuno, F., T. Suhara, T. Nakayama, T. Ichimiya, Y. Okubo, A. Takano, T. Ando, M. Inoue, J. Maeda, and K. Suzuki, Inhibitory effect of hippocampal 5-HT1A receptors on human explicit memory. Am J Psychiatry, 2003. 160(2): 334-40.

[323] Yin, J.C. and T. Tully, CREB and the formation of long-term memory. Curr Opin Neurobiol, 1996. 6(2): 264-8.

[324] Yoo, S.S., P.T. Hu, N. Gujar, F.A. Jolesz, and M.P. Walker, A deficit in the ability to form new human memories without sleep. Nat Neurosci, 2007. 10(3): 385-92.

[325] Yue, C., M. Schroder, K. Bienefeld, and E. Genersch, Detection of viral sequences in semen of honeybees (*Apis mellifera*): evidence for vertical transmission of viruses through drones. J Invertebr Pathol, 2006. 92(2): 105-8.

[326] Zhang, J.Y., H. Jiang, W. Gao, J. Wu, K. Peng, Y.F. Shi, and X.J. Zhang, The JNK/AP1/ATF2 pathway is involved in H2O2-induced acetylcholinesterase expression during apoptosis. Cell Mol Life Sci, 2008. 65(9): 1435-45.

[327] Zhang, S., S. Schwarz, M. Pahl, H. Zhu, and J. Tautz, Honeybee memory: A honeybee knows what to do and when. J Exp Biol, 2006. 209(Pt 22): 4420-8.

[328] Zhang, X., B.S. Rosenstein, Y. Wang, M. Lebwohl, and H. Wei, Identification of possible reactive oxygen species involved in ultraviolet radiation-induced oxidative DNA damage. Free Radic Biol Med, 1997. 23(7): 980-5.

[329] Zhen, X., W. Du, A.G. Romano, E. Friedman, and J.A. Harvey, The p38 mitogen-activated protein kinase is involved in associative learning in rabbits. J Neurosci, 2001. 21(15): 5513-9.

[330] Zhou, L. and H. Steller, Distinct pathways mediate UV-induced apoptosis in *Drosophila* embryos. Dev Cell, 2003. 4(4): 599-605.

[331] Zhu, Y., D. Pak, Y. Qin, S.G. McCormack, M.J. Kim, J.P. Baumgart, V. Velamoor, Y.P. Auberson, P. Osten, L. van Aelst, M. Sheng, and J.J. Zhu, Rap2-JNK removes synaptic AMPA receptors during depotentiation. Neuron, 2005. 46(6): 905-16.

[332] Zimmerman, J.E., N. Naidoo, D.M. Raizen, and A.I. Pack, Conservation of sleep: insights from non-mammalian model systems. Trends Neurosci, 2008. 31(7): 371-6.

[333] Zufall, F., Calcium imaging in the olfactory system: new tools for visualizing odor recognition Neuroscientist, 1999. 5(1): 4-7.

6 ABBREIVIATIONS

+ssRNA	Positive single stranded ribonucleic acid
5HT	5-hydroxytryptamine
5-HT_{1A} receptor	5-hydroxytryptamine (serotonin) receptor 1A
ABPV	Acute bee paralysis virus
AC	Adenylyl cyclase
ACh	Acetylcholine
AChE	Acetylcholinesterase
Act-D	Actinomycin-D
AMP	Adenosine monophosphate
AMPA-R	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AP1	Activator protein-1 (transcription factor)
ASK1	Apoptosis signal-regulating kinase
ATF2	Activating transcription factor 2
ATP	Adenosine triphosphate
Aβ	Beta- amyloid peptide
bp	Base pair
BQCV	Black queen cell virus
BSA	Bovine serum albumin
CA1	Cornu ammonis 1
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
CCD	Colony collapse disorder
c-Fos	c-Fos (transcription factor)
c-Jun	c-Jun (transcription factor)
CNS	Central nervous system
CO_2	Carbon dioxide
CR	Conditioned response
CREB	cAMP response element binding protein
CS	Conditioned stimulus
DCV	*Drosophila* C virus
DMSO	Dimethyl sulfoxide
DWV	Deformed wing virus

ECFP	Enhanced cyan fluorescent protein
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELK1	ELK1 (transcription factor)
ERK	Extra cellular regulated kinase
EtOH	Ethanol
EYFP	Enhanced yellow fluorescent protein
FAC	Ferrous ammonium citrate
FRET	Fluorescence resonance energy transfer
GPCR	G protein-coupled receptors
GR	Glucocorticoid receptors
GRE	Glucocorticoid response elements
IAA	Isoamyl acetate
IgG	Immunglobulin G
IL-1β	Interleukin 1 beta
INFs	Interferons
IPA	Isopentyl acetate
JAK-STAT	Janus kinase-signal transducers and activators of transcription
JNK	Jun-N-terminal kinase
KBV	Kashmir bee virus
kDa	Kilodalton
LPS	Lipopolysaccaride
LTM	Long-term memory
LTP	Long-term potentiation
M	Molar
MAPK	Mitogen activated protein kinase
MBs	Mushroom bodies
mM	Millimolar
MR	Mineralocorticoid receptors
MT	Malphigian tubules
MTM	Mid-term memory
nAChRs	Nicotinic acetylcholine receptors
NCBI	National Centre for Biotechnology Information
NF-kB	Nuclear factor-kappa B

OA	Octopamine
p38	p38 mitogen activated protein kinase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PER	Proboscis extension response
PKA	Cyclic AMP-dependent protein kinase
ROS	Reactive oxygen species
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SAPK	Stress activated protein kinase
SBV	Sacbrood bee virus
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SL	Shaking with light
STM	Short-term memory
TAK	Transforming growth factor
Thr	Threonine
TR	Threshold ratio
Tyr	Tyrosine
UCR	Unconditioned response
US	Unconditioned stimulus
UV	Ultraviolet
VUMmx1	Ventral unpaired median neuron maxillare 1
λ	Wavelength
μM	Micromolar

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10 SUMMARY

My work demonstrates a central role of JNK (Jun-N-terminal kinase), well known as central component in mammalian immune and stress response, in sensory processing and associative learning in honeybees. I describe the implication of JNK in mediating changes in behaviour induced by deformed wing virus (DWV) infection, and two other stress stimuli (UV light; UV and shaking with light; SL). DWV infected honeybees (3 days after infection) show changes in sensory processing, an impaired associative learning and memory formation, but intact non-associative learning. DWV infection activates JNK in a complex temporal pattern in the mushroom bodies (MBs) and acts via transcription to induce changes in behaviour. Interestingly, short-term UV exposure (≥15 min) and long-term SL treatment (overnight) induce similar behavioural changes as virus infection and lead to JNK activation in the brain. Inhibition of JNK fully restores stress induced biochemical as well as behavioural changes. UV induced behavioural changes require transcription while SL induced behavioural changes does not require transcription. Experiments on isolated *Drosophila* Kenyon cells show that UV light has an inhibitory effect on neuronal transmission and points to the MBs as the cellular location of stress induced physiological changes. Taken together, my data provide first evidence that JNK acts as central mediator between stress and behaviour of honeybee.

11 ZUSAMMENFASSUNG

In meiner Dissertation habe ich die Rolle von JNK (Jun-N-terminale Kinase), einer zentralen Komponente bei Immun- und Stressreaktionen, bei sensorischen Prozessen und assoziativen Lernen bei der Biene untersucht. Die Befunde zeigen, dass JNK bei der Vermittlung von Verhaltensänderungen die durch den deformed wing virus (DWV) und andere Stressfaktoren (UV-licht; UV und Schütteln mit Licht; SL) induziert werden, eine wichtige Rolle spielt. DWV infizierte Bienen zeigen eine veränderte sensorische Prozessierung und eine Beeinträchtigung des assoziativen Lernens und der Gedächtnisbildung, jedoch keine Änderungen bei nicht-assoziativen Lernformen. DWV-Infektion führt zu einem komplexen zeitlichen Aktivierungsmuster der JNK in den Pilzkörpern und benötigt Transkriptionsprozesse um die Verhaltensänderungen zu induzieren. Die kurzzeitige Exposition mit UV-Licht (≥15 min) und die langzeitige SL-Behandlung (über Nacht) führt zu ähnlichen Verhaltensänderungen wie die Virusinfektion und aktiviert die JNK im Bienengehirn. Diese stressinduzierten biochemischen und Verhaltensänderungen können durch die Inhibition von JNK verhindert werden. Während UV-induzierten Verhaltensänderungen transkriptionsabhängig sind, benötigen die durch SL-Behandlung induzierten Veränderungen keine Transkription. Experimente an isolierten *Drosophila* Kenyonzellen zeigen einen UV-Licht induzierten und transkriptionsabhängigen Effekt auf die neuronale Kommunikation und deuten auf eine wichtige Rolle der Pilzkörper bei der Prozessierung von Stresssignalen. Zusammengefasst zeigen die Befunde erstmals, dass JNK als zentraler Vermittler zwischen Stress und Verhalten agiert.

12 PUBLICATIONS

Parts of this work have been published in a peer reveiwed journal and also presented in different conferences as oral and poster presentations.

Iqbal, J. and U. Mueller, Stress activated protein kinase in learning and memory of honeybee: Implications for a role in sleep. 8th Göttingen Meeting of the German Neuroscience Society, T25-14A, 2009, (Poster).
https://www.nwg-goettingen.de/2009/upload/file/Proceedings_Goettingen2009.pdf

Iqbal, J. and U. Mueller, Involvement of stress activated protein kinase in learning and memory of honeybee. 6th FENS forum of European Neuroscience Geneva, Switzerland, FENS Abstr., vol.4, 193.18, 2008, (Poster).
http://fens2008.neurosciences.asso.fr/abstracts/Rpdf6/A193_18.pdf

Iqbal, J and U. Mueller, Stress activated protein kinase: Role in learning and memory of honeybee. 19th Neurobiologischer Doktoranden Workshop, University of Saarland, Germany, Abstr., 5: 19, 2008 (Poster).

Iqbal, J. and U. Mueller, Virus infection causes specific learning deficits in honeybee foragers. Proc Biol Sci, 2007. 274 (1617): 1517-21.
http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2176156

Iqbal, J and U. Mueller, Virus infection and ultraviolet light induced stress causes deficits in learning and memory of honeybee. 18th Neurobiologischer Doktoranden Workshop, University of Konstanz, Germany, Abstr.,: 34, 2007 (Talk).

Iqbal, J and U. Mueller, Impact of viral infection on learning, memory and the immune system of honeybee. 7th Göttingen Meeting of the German Neuroscience Society, T29-5B, 2007, (Poster).
http://www.neuro.uni-goettingen.de/archiv/2007/pdf/Proceedings-Goettingen2007.pdf

Iqbal, J and U. Mueller, Molecular diagnosis of viral infections and its impact on learning and memory in honeybees. 17th Neurobiologischer Doktoranden Workshop, Freie Universität Berlin, Germany, Abstr.,: 20, 2006 (Talk).
http://www.powerwissenschaft.de/abstracts.html#iqbal

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