LEARNING AND MEMORY IN THE AMERICAN COCKROACH, *PERIPLANETA AMERICANA*: NEW BEHAVIORAL PARADIGMS FOR ASSOCIATIVE LEARNING

By

Hyung-Wook Kwon

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF ENTOMOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2002

UMI Number: 3060983

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

THE UNIVERSITY OF ARIZONA ®
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Hyung-Wook Kwon

entitled Learning and Memory in the American Cockroach, *Periplaneta americana*: New Behavioral Paradigms for Associative Learning

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

Nicholas J. Strausfeld	7-17-02 Date
David N. Byrne	7-17-02 Date
Wulfila Gronenberg	7/17/02 Date
	Date
	Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director Nicholas J. Strausfeld — Date 7-17-02

STATEMENT BY AUTHOR

This thesis has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by copyright holder.

SIGNED: 

ACKNOWLEDGMENTS

For guidance during my progress as a scientist as well as for the encouragement to pursue my ideas I thank my Ph.D. advisor, Dr. Nicholas Strausfeld, who enabled me, as a scientist to be on the right track. I also appreciate the efforts of Dr. David Byrne, my co-advisor, who contributed much to my studies and the development of me as a scientist. The encouragement of both advisors allowed me many opportunities to mature as a scientist, such as attending a summer program at Woods Hole, MA, which was an invaluable experience. I am grateful to my committee members, Dr. Molly Hunter and Dr. Dan Papaj for their criticisms and advice on my thesis work. Particularly, I appreciate the help of my committee member Dr. Wulfila Gronenberg, for assistance in understanding scientific concepts and experimental design. I would also like to thank Dr. Liz Bernays and Dr. Reg Chapman for their tremendous amount of assistance in the understanding of insect learning as well as hands-on teaching of extracellular recordings; my lab rotation with them was crucial towards the achievement of my Ph.D. I am also very grateful to Dr. Leslie Tolbert, chair of neuroscience program; I thank her for comments and advice that helped me become a better neuroscientist with my background in Entomology. I am indebted to Mr. David Lent for help with these experiments, such as digitization of many cockroach videotapes and critical comments, as well as helping me with my English. Also, I thank current and previous members of the Strausfeld lab; I appreciate Dr. John Douglass for his interest in my experiment and discussion on neurophysiological problems. I also thank Dr. Holly Campbell for talking about her graduate experiences as well as her experiments, which was invaluable. I also thank Drs.

Sarah Farris, Irina Sinakevitch, Rudi Loesel for their critical comments on scientific approach and help with my presentation. I am grateful to Dr. Piotr Jablonski for help with statistics. I would also like to thank Dr. Marianna Pinter for her interest in my work and the sharing of her academic experiences. I would really like to thank Dr. Camilla Strausfeld for encouragement during my Ph.D. study as well as her unforgettable warm parties.

I also appreciate the guidance of friends, especially the Korean Biological Student Association at the University of Arizona, for their active discussion on biological issues and criticism. To my parents in Korea, Mahn-Ji Cho and Young-Chul Kwon, I appreciate their endless devotion and support as well as love. Also, my sister's and brother's family, I thank for their interest and love. For my mother-in-low, Choon-Yun Lee, I don't know how I can show enough appreciation for her assistance in taking care of my family here in the US. Without her care, I would have never finished my Ph.D. degree. And to my life companion, I am greatly thankful to my wife, Soo-Hee Lim, for her love, patience, solace, encouragement, and teaching me how to behave in society during my study here in the US. Finally, for my little kids, Jamie and Juliane, their presence during my Ph.D. course, being with their smile as well as playing with them was a mainspring of my current work.

DEDICATION

This work is dedicated to my parents.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

ABSTRACT

Although there is much information about insect associative learning, less is known about the underlying neural mechanisms. This is partly due to the lack of behavioral paradigms that provide a suitable model for studying learning mechanisms at the level of individual neurons. Honey bees and the fruitfly *Drosophila* have been used for molecular mechanisms underlying learning and memory but are generally unsuitable for intracellular studies. In contrast, the American cockroach, *Periplaneta americana*, possesses a relatively large brain which has been shown to be suitable for intracellular recordings. This taxon shows behavioral repertories, such as foraging and homing behaviors that can be used for learning and memory studies, so providing the entrée for underlying neural mechanisms.

This thesis describes the background to, and the demonstration of, two associative learning paradigms: visual associative learning and spatial learning. Both have been developed on the restrained cockroach so that later these methods can be employed in conjunction with electrophysiology. By projecting their antennae intermittently towards a position of potential food sources, cockroaches sample salient information. Here, this antennal behavior, called an "antennal projection response (APR)," is used to demonstrate long-term memory where an APR is elicited by a conditioning stimulus (the CS: green light) paired with a spatially coincident odor (unconditioned stimulus: the US). The acquired APR to the green light cue persists for up to 72 hours.

Spatial learning is also a vitally important behavior in most animals that must remember locations of food and landmarks and that must navigate. Spatial learning

abilities were here tested by observing APRs towards a cue, where the cockroach learns the position of a visual cue (CS) associated with a food odor (US), relative to the position of another visual stimulus in the contralateral visual field (the contralateral visual reference stimulus: ConRS). Memory of positional information, tested by altering the relative positions of the CS and ConRS, was investigated. Cockroaches showed significant APRs to visual cues not only when a position of the visual cue and spatial reference cue were exactly matched during training trials, but also during tests when the relative angles between the visual cue and spatial reference cue were matched but rotated around the head's vertical axis. When these angles were not the same as the angle used for training, the CS was not recognized. These results suggest that cockroaches employ two different mechanisms to find a food source: retinotopic matching and recognition of angular relationships between a source and landmark. The application of these paradigms to studies that could investigate possible neural mechanisms of these behaviors is discussed.

CHAPTER I

INTRODUCTION

1-1. Overview and significance of experiments

A comprehensive neuroethological understanding of animal behavior requires that the behaviors themselves can be accurately defined and that correlates can be achieved at various levels of neural complexity. These levels are: brain regions, pathways, identified neurons, and, finally circuits, their transmitters and their modulators. Ideally, molecular mechanisms of neuronal plasticity of the nervous system would add significantly to our understanding. However, from invertebrates to vertebrates such a comprehensive analysis has been partly achieved only once; namely, for the nematode *Caenorhabditis elegans* (review: Rose and Rankin, 2001).

Although it is generally understood that studies on learning and memory have focused on mammals, because of their importance for understanding the basis of psychiatric disorders in human (Kandel, 2001), such a view is misleading. In 1973, two Nobel Prize's for medicine were given to two extraordinary pioneers of animal behavior; Niko Tinbergen and Karl von Frisch. Tinbergen's work on animal behaviors which included: observations of place memory in the solitary wasp, *Philanthus*, (Tinbergen and Kruyt, 1938; Tinbergen, 1958). Studies on the mating behaviors of fish (Tinbergen, 1951) and ritualization (Tinbergen, 1951) set the stage for much of modern neuroethology. Likewise, von Frisch's lifetime's research on honey bee behaviors (von Frisch, 1967) has led to modern studies on honey bee learning and memory (Bitterman et al., 1983, Collett

and Cartwright, 1983; Gould, 1993), including insights into visual memory, generalization, and multiple memory retention (Srinivasan et al., 1998; Zhang et al., 1999; Srinivasan et al., 2000; Smith, 1993; Erber et al., 1980).

There are many advantages of working with insect brains. One is that the brains of most vertebrates, particularly "higher" mammals, are of such complexity that there is little likelihood of elucidating a defined memory circuit. Instead, arthropods appear more tractable. Fundamental similarities between arthropods and vertebrate central nervous systems were already recognized by Retzius (Retzius, 1890) in the late 19th Century, and by Zawarzin (Zawarzin, 1925) and Cajal (Cajal, 1915) in the early 20th Century (see Strausfeld, 1998 for references). Over the last two decades, it appears that cellular and molecular mechanisms of learning and memory are very similar across the animal kingdom. Invertebrates such as *Aplysia* (Kandel, 2001) and *Drosophila* (Waddell and Quinn, 2001; Roman and Davis, 2001) have fewer neurons comprising more defined neural networks than in mammals. Also, small ganglia that drive motor actions have proven to be unusually revealing regarding the plasticity of neural circuits in response to the action of neuropeptides. The best example is the stomatogastric nervous system in the lobster that controls the outputs of rhythmic contractions in the gastric mill and pylorus during digestion (Maynard, 1972; Selverston, 1977; Heinzel and Selverston, 1988). This neural network comprises 30 neurons that can be identified individually. Hence, intra- and extracellular recordings can be used to characterize a given neuron while rhythmic contraction is generated. Neuromodulators, such as FRMFamide, protolin, octopamine, and serotonin, modify the conductance of the different subsets of neurons in networks,

thus leading to changes of stomatogastric rhythms (Harris-Warrick et al., 1991; Elson and Selverston, 1992; Johnson and Hooper, 1992)

Such examples allow one to approach questions about the neural basis of behavior using integrated strategies: quantitative behavior; fictive behavior, which occur when certain nerves are activated even in the absence of the rest of the musculature and structure is removed, coupled with electrophysiology and pharmacology; molecular and circuit analysis of neuronal systems mechanisms. All these approaches can be used to investigate defined behaviors, and combinations of these strategies have to be employed in order to investigate the conceptually far more difficult phenomena of neural plasticity, learning, and memory. Like many vertebrate taxa, invertebrate animals show similarly rich behavioral repertories and exquisite adaptation to their ecological niches. Insects are ideal subjects for studying the relevance of brain structure and function in relation to an animal's specific demands from its environment (Eisenstein, 1997)

Using combined genetic, neurophysiological, and anatomical approaches, there is currently a consensus that specific neuropils in the insect brain, such as the antennal lobes (AL) and mushroom bodies (MBs), play crucial roles in olfactory learning (Erber et al., 1980; Hensenberg et al., 1985; Davis, 1993; Mauelshagen, 1993). Such notions are, however, not new. Dujardin in 1850 who discovered the mushroom bodies in hymenopteran insects in which size differences between casts in social insect species were suggested to relate to task differences. Although Dujardin did not claim their roles in learning and memory, he suggested that "intelligent" roles of the mushroom bodies over instinct by the fact that stereotyped motor pattern of locomotion was severely

impaired in decapitated animals with large mushroom bodies in comparison with those patterns in decapitated ones with small mushroom bodies (Dujadin, 1850, review: Stausfeld et al., 1998).

There is evidence that the MBs are also involved in place memory (Mizunami et al., 1998a), visual associative learning (Liu et al., 1999), and multimodal integration (Li and Strausfeld, 1997, 1999). However, the same centers may be involved in more basic functions such as odor coding in short-term memory of locusts (Stopfer and Laurent, 1999), associative olfactory learning in *Drosophila* (Heisenberg et al., 1985; de Belle and Heisenberg, 1994; Zars et al., 2000; Pascual and Preat, 2001), olfactory learning associated with food rewards in honey bees (Erber et al., 1980; Mauelshagen, 1993; Hammer and Menzel, 1995), cockroaches (Mizunami et al., 1998a), and the moth *Manduca sexta* (Daly and Smith, 2000; Vickers et al., 2001).

However, apart from two recent studies that ascribe specific parts of the mushroom bodies to short and to long term memory (Zars et al. 2000; Pascual and Preat, 2001) and one study on the role of a neuromodulatory neuron in *Drosophila* (Han et al., 1998), there is still little evidence about which neurons might be involved in memory formation. This might be due to the fact that too little attention has been given to developing behavioral paradigms of learning and memory that are robust enough, and which can be performed by a robust enough taxon, that would allow electrophysiological studies of defined brain regions during behavioral experiments. This thesis addresses this problem and describes experiments that will subsequently allow physiological and molecular correlates of learning and memory at the level of identified circuits.

Work described here focuses on the cockroach, *Periplaneta americana*, which was selected for the following reasons.

1. It is unusually robust and can tolerate long-term behavioral experiments and manipulations.

2. It tolerates surgical procedures as well as long-term, extra- and intracellular recordings (Eisenstein, 1997).

3. It's brain anatomy is well understood at the level of single neurons, neuronal architectures, neuromodulator distributions, and neural development (Ott and Elphick, 2002; Sinakevitch et al. 2001; Farris and Strausfeld, 2001).

4. It is the focus of robotics research, and thus provides important data about principles underlying motor control (Katz, 1996; Beer et al., 1998; Schmitt and Holmes, 2000; Webb, 2002).

5. There is information about higher centers that are thought to be central to memory formation, particular with respect to the relationships between the MBs and other defined parts of the brain (Mizunami et al., 1998a).

One rationale that drives the present study is the need to demonstrate the usefulness of the cockroach for neuroethological studies of learning and memory at all levels of analysis. In addition, the present study rectifies what has been a lack of quantifiable behavioral paradigms that can be used to investigate possible functions of higher centers of the brain.

My research has been devoted to developing, in highly restricted conditions, new behavioral paradigms that demonstrate associative learning between olfactory and visual

cues and place memory, using *P. americana*. By using the innate behavior of the antennal motor system, particularly the movement of an antenna towards an odor cue, "antennal projection responses (APRs), or flicking response (Mellon, 1997), cockroaches can inform us, the observer, that they are learning to associate visual cues with food odor cues. In other words, a quantifiable behavior can reveal the dynamic progress of memory acquisition. Results presented here demonstrate that restrained cockroaches not only can show antennal projection responses towards visual cues after learning, but also show that they can recognize a odor position relative to a spatial cue. Thus, learning protocols developed for this research can be readily employed to test physiological changes in the brain as well as pharmacological studies that aim to understand where, how, and when multimodal associative learning occurs. By characterizing changes in the brain that are incurred during memory acquisition, essential components of learning and memory circuits will be accessed. The results of behavioral experiments in the current study will provide new insight into neural correlates underlying learning and memory.

1-2. Behavioral studies on insect learning

Evidence of learning is found throughout Insecta. Foraging- and nutrition-related learning behaviors have been widely studied in honey bees (Gould, 1993; Menzel et al., 1993), ants (Schatz et al., 1999), flies (Prokopy et al., 1993; Campbell and Strausfeld, 2001), parasitoid wasps (Lewis and Takasu, 1990; Wäckers and Lewis, 1999), cockroaches (Balderrama, 1980; Durier and Rivault, 1999; Gadd and Raubenheimer, 2000), grasshopper and locusts (Bernays, 1993; Dukas and Bernays, 2000; Raubenheimer and

Tucker, 1997), and butterflies (Papaj, 1986). These studies demonstrate the capability of an insect of being able to associate between stimuli as well as being able to use spatial cues for learning and navigation. The employment of different types of learning such as non-associative learning, associative learning, and instrumental learning, all of which have been extensively used for psychological studies of humans, result in behavioral modifications in insects and suggest common underlying mechanisms (Bitterman et al., 1983; Smith, 1993; Gould, 1993; reviews for *Drosophila*: Heisenberg, 1997; Roman and Davis, 2001; Wadell and Quinn, 2001; review for honey bees: Menzel and Müller, 1996).

Non-associative learning is the waning (habituation) or waxing (sensitization) responses to repeated presentation of a stimulus without conditioning procedures. Cockroaches showed habituation towards repeated air puff to their cerci. When presented with mechanical stimuli to their legs, cockroaches also showed increase in leg movements to other stimuli (Zilber-Gachelin and Paupardin, 1974). Depending on the number and duration of sucrose presentation to an antenna, the proboscis extension reflexes (PER, see below) of the honey bee demonstrate the phenomenon of sensitization (Menzel et al., 1991). Stimulation with low concentrations of sucrose to the antennae of honey bees induces rapid habituation as demonstrated by PER (Braun and Bicker, 1992).

Associative learning plays a crucial role in foraging and homing in insects, allowing them to recognize the relationships between salient information and neutral information. Honey bees learn odor, color, and shape during foraging and they are able to memorize the distance as well as the direction of a food source (von Frisch, 1967; Gould, 1993; Cartwright and Collett, 1982; Srinivasan et al., 2000). Cartwright and Collett

(1982) demonstrated experimentally that during visits to a foraging area honey bees store panoramic snapshots of the landmarks from a odor position and they always find a place where images between their previous snapshots, also called eidetic images (Collet and Certwright, 1983), and retinal image of the landmarks is perfectly matched, regardless of the distance of the landmarks. Srinivasan et al. (2000) demonstrated that the amount of visual flow during navigation towards a food source is a crucial parameter for honey bees to calculate the distance. In this experiment, bees experiencing more visual flow while approaching a feeder inside a narrow tunnel, compared to bees flying the same distance but experiencing less visual flow, showed a higher probability to show waggle dance, thus communicating a longer distance.

Pipevine swallowtail butterflies, *Battus philenor* L., also learn leaf shapes that are associated with host plant extracts (Papaj, 1986; Allard and Papaj, 1996). In a predicted condition environmental scenario, in which locations and colors of nutrition-balanced diets remained unchanged, American grasshoppers, *Schistocerca americana* Drury, learned to associate these cues with the quality of foods (Dukas and Bernays, 2000). In addition, this polyphagous insect showed aversion learning to foods that were associated with toxic chemicals (Lee and Bernays, 1990; Bernays, 1993). Both protein- and carbohydrate-deprived locusts *Locusta migratoria* L. demonstrated learning because they were able to associate yellow or green color with diets containing the lacking nutrient they needed. After training, locusts learned to more frequently enter a cylinder decorated with a training color (Raubenheimer and Tucker, 1997). Depending on its oviposition experience of the host larvae presented against visual targets made of colors, shapes, or

patterns, parasitoid wasps, *Microplitis croceipes* Cresson, could learn to discriminate a rewarded target in choice tests (Wäckers and Lewis, 1999). This insect also learned to discriminate experienced odorants on the basis of behavioral needs, such as oviposition and food sources (Lewis and Takasu, 1990). Desert ants (*Cataglyphis cursor*), which use path integration for navigation, learn specific visual cues in association with internal home vectors (Schatz et al., 1999).

Under restrained laboratory conditions olfactory learning paradigms using PER in honey bees (Menzel et al., 1993), and using electric shock in *Drosophila* (Quinn et al., 1974), have been commonly used for studying learning mechanisms. The former have provided what is now accepted as a typical learning performance. The PER paradigm was originally developed by Kuwabara (1957) in which a harnessed honey bee reflexively extended its proboscis towards an odor stimulus (conditioned stimulus, CS) after its association of the odor with a sucrose reward (unconditioned stimulus, US) (see Takeda, 1961). The single pairing of CS and US in forward conditioning revealed a significant increase the probability of PER responses (Bitterman et al., 1983; Menzel and Müller, 1996). Moreover, this type of conditioning has shown that interstimulus intervals between CS and US are extremely important factors in learning performance (Menzel and Bitterman, 1983). In addition, in honey bees intertrial intervals during training have a significant effect on learning and memory of the PER (Menzel et al., 2001). The PER conditioning paradigm has been used to study more complicated learning abilities such as blocking in which, for example, animals are rewarded with sucrose on stimulus 1-hexanol (A+) in the first phase and in the second phase mixtures of 1-hexanol and geraniol are

paired with sucrose (AB+). After these training phases, the PER probability towards geraniol is tested to examine if experience with 1-hexanol with sucrose during the first phase of training (A+) fails to increase the response to geraniol (B) after training with AB+ (Smith and Cobey, 1994; Hosler and Smith, 2000). Generalization, which is an animal's tendency to respond to stimuli similar with a conditioned stimulus has been demonstrated by Smith (1993) and Daly et al. (2001) using the moth *Manduca sexta.* Overshadowing as also been demonstrated, in which animals rewarded first on AB mixture (AB+) fail to response to B alone, as has second-order conditioning, in which a reinforced stimulus is capable of serving as unconditioned stimulus (US) with another stimulus (Bitterman et al., 1983). Sensory preconditioning, which is an animal's learned ability to connect a reinforced stimulus (A+) with a stimulus (B) presented with an unreinforced mixture of A and B (AB-) during the pretraining phase, has been shown by Müller et al. (2000). The latter has also been demonstrated in free flying honey bees (Funayama et al., 1995; Couvillon et al., 1997).

Olfactory aversive learning paradigms in *Drosophila* (Quinn et al., 1974; Tully and Quinn, 1985) have been used to study neural mechanisms of learning and memory, combined with molecular biological techniques (Tully, 1996; Dubnau et al., 2001; McGuire et al., 2001; see 1-5). In this paradigm, association between attractive odorants (CS) and electric shocks (US) induces a pattern of classical conditioning in which *Drosophila* avoid the odor associated with electric shock after learning.

Operant learning in animals refers to learning a task or solving a problem to receive rewards or to avoid negative stimuli. Headless cockroaches have been used to

study learning mechanisms at the level of isolated ganglia in which cockroaches had to learn the leg position to avoid electric shock (Horridge, 1962). Tethered *Drosophila* in a flight simulator can learn to control their flight orientation towards an unusual position of the visual panorama when trained to do so using heat shock punishment. This operant conditioning is though to involve adjustments of the yaw torque circuits (Wolf and Heisenberg, 1991).

Recently, Giurfa et al. (2001) have demonstrated that freely flying honey bees are capable of forming 'sameness' and 'difference' concepts that were thought to be a cognitive capacity only of vertebrates. A honey bee is only rewarded with sucrose when it chooses the same stimulus matched with that at the entrance of the Y-maze. In a transfer test in which a different modality of stimuli decorated on the entrance of the Y-maze, the bee trained to choose the same grating patterns, for example, chooses a same pattern of the color compared to that at the entrance of the maze to obtain rewards, thus exhibiting association between different modalities to recall a salient event during foraging (Srinivasan et al., 1998). Honey bees can also choose a different pattern of stimuli to receive sucrose rewards, implying that honey bees have an ability to judge sameness and difference associated with foods in multimodal situation during foraging (Zhang et al., 1999; Giurfa et al., 2001).

1-3. Cockroach learning and memory

Cockroaches have been a model system used to study neuropeptides in the regulation of development, as well as metabolic and behavioral processes, certain of which are

mediated by "uniquely identifiable neurons" in the central nervous systems (Adams and O'Shea, 1983; Stay et al., 1992; Nässel et al., 2000).

Cockroaches have also been used to study learning and memory. Operant conditioning paradigms to avoid electric shock in headless insects have been employed to demonstrate neurophysiological mechanisms at the level of the single ganglion (Horridge, 1962; Eisenstein and Cohen, 1965). However, Chen et al. (1970) reported that there are important differences between headless and intact animals in which headless insects did not learn mild stimuli whereas intact animals show learning performance as well as long-term retention. Balderrama (1980) employed positive reinforcement rather than electric shocks to train freely moving cockroaches to the aversive odor, menthol. A dark avoidance learning paradigm employing choice behavior in a T-maze also revealed good learning performance, and has been used to demonstrate that protein synthesis is involved in learning and memory (Barraco et al., 1981). In this experiment, when the antibiotics cycloheximide and puromycin, which inhibit protein synthesis, were injected into the abdomen of a cockroach before training there was an inhibition of memory formation for 5 h after training. However, animals were able to form short-term memory, 1 h after training. The German cockroach, *Blatella germanica* L., has been used to demonstrate homing abilities, which employs path integration mechanisms (Durier and Rivault, 1999). All of these paradigms, however, have limitations; such as difficulties of handling during experiment, and time-consuming observations.

Mizunami et al. (1998a) have demonstrated the spatial learning capability of cockroaches using a modified Morris water maze (Morris et al., 1982). In this study,

intact *P. americana* discriminated visual patterns that decorated the wall of an arena. Using these distant visual cues, the cockroaches could learn to recognize an invisible cold area on the arena's surface. Despite the numerous cases of cockroach learning and memory, how cockroaches memorize cues and use these learned behaviors are as poorly understood. Again, there is a lack of quantifiable paradigms that could be used to elucidate neural correlates of these behaviors by recording neural activity during the acquisition of the behavior itself. In this regard, the present studies on associative and place learning, using antennal projection responses (APRs) towards assumed odor position during and after training, provides paradigms that can be used during recording from identifiable neurons (see Chapter IV).

Compared with honey bees and *Drosophila*, cockroaches have a large robust brain. Cockroaches also represent a basal group of neopteran insects and thus might be able to provide clues about how neural processing and the representation of sensory stimuli might have evolved.

1-4. The structure and central representation of the antenna

The antenna of insects is segmented, consisting of the scape, the pedicel, and the flagellum (Snodgrass, 1935; Chapman, 1998). The scape, consists of a single segment, is attached to membranous region of the head capsule in which a single marginal point, called the antennifer, attaches to the scape. This allows the scape to move in all directions. The second segment of the antenna, the pedicel, is usually the shortest. The third segment is called the flagellum, which consists of between one (in *Drosophila* for example) and

over 170 annuli, as in the American cockroach (Snodgrass, 1935; Seelinger and Tobin, 1981).

The antenna is moved by two basic sets of muscles, levators and depressors that allow the antenna to move all directions (Deshpande, 1984). One set originates from skeletal structures from the inside of head cuticle (the tentorium) and inserts into the base of the scape. The number of muscles is different among species. In ants, there are four muscles, in cockroaches, five muscles are attached to the scape. Extrinsic antennal muscles are located outside of the antenna whereas intrinsic antennal muscles arising in the scape insert into the base of the pedicel. These latter are the flexor and extensor muscles, which allow the antenna to move in a single plane (Richards and Davies, 1977). The flagellum contains no muscles. Antennal motorneurons controlling these muscles are provided from the dorsal part of the deutocerebrum, called the dorsal lobes (see below).

The main function of the insect antenna is to receive sensory information. The pedicel contain many different forms of proprioceptors, such as a chordotonal organ and Johnston's organ, which provides information about a position of the flagellum relative to the pedicel. Also, most sensilla on the scape, such as hair plates (Okada and Toh, 2000), act as proprioceptors that provide information about a position of these basal segments of antennae relative to the head. The flagellum contains olfactory and mechanoreceptors as well as hydro- and thermoreceptors (Seelinger and Tobin, 1981). Whereas the distribution of these sensilla is similar among each segment of the flagellum, specific receptors, such as contact chemoreceptors, are concentrated on the terminal segments.

Olfactory information from the flagellum is conveyed by sensory neuron axons to the antennal lobes, which are located in the brain and which are characterized by their distinct ball-like structures, called glomeruli, surrounded by glial cells (Tolbert and Hildebrand, 1981). Glomeruli receive the terminals of olfactory sensory neurons, the neurites of local interneurons (LNs) and centrifugal feedback neurons, and the dendrites of antennal projection neurons (PNs), which are the output neurons of the antennal lobes (Tolbert and Hildebrand, 1981; Kent et al., 1987; Homberg et al., 1989). The number of glomeruli varies depending on the insect species (Rospars and Hildebrand, 1992; Boeckh et al., 1990). For instance, *P. americana* and *Manduca sexta* have 124 and 64 glomeruli excluding male specific macroglomeruli complex (Boeckh, 1984; Rospars and Hildebrand 1992).

In addition to olfactory signal processing, the deutocerebrum is involved in the processing of mechanosensory inputs from antennae and visual inputs from the protocerebrum. Behavioral evidence from honey bees has indicated that antennal reflexes are elicited in response to moving gratings (black and white stripes), as well as olfactory or mechanical stimuli (Erber et al., 1993; Erber and Pribbenow, 1997). Anatomical findings from cockroaches and honey bees have indicated that mechanosensory inputs from the antennae, as well as visual inputs from the optic lobes arborize in the dorsal lobes of the deutocerebrum, a region of the brain that provides motor neurons controlling antennal movements (Ernst and Boeckh, 1983; Maronde, 1991; Kloppenburg, 1995). In several insect species, the number of the antennal motorneurons is 18~20 (Rospars, 1998; Honegger et al., 1990).

In this regard, in my present work, olfactory signal from the flagellum and directional information from the scape and the pedicel of the antenna are assumed to be integrated in the central nervous system. Moreover, learning a visual signal associated with a food odor should affect central representation of a odor position. However, little is known about how sensory integration occurs and how antennal movements to learned position are controlled by the dorsal lobes.

1-5. The structure and central representation of the compound eye

This brief introduction to the visual system of insects is important for understanding how neural circuits involved in visual associative learning work and how two different modalities (visual and olfactory signal) are integrated in the insect central nervous system.

Insects, including cockroaches, perceive light by their compound eyes, which are located laterally on the head. The compound eye is composed of many similar structures, called ommatidia, which are units that gather light. The number of ommatidia is variable. For example, adult *P. americana* has about 2000 ommatidia per compound eye (Seelinger and Tobin, 1981), while dragonflies and honeybees have about 10,000 and 5,500 ommatidia per compound eye, respectively (Chapman, 1998).

Ommatidium can be functionally divided into two parts: a light-collecting part and a sensory part. The light collecting and focusing parts consist of a corneal lens, which is produced by two primary pigment cells. Beneath the corneal lens is located a crystalline cone produced by four cone cells. The cone is hard and clear. Sensory elements consist of retinula cells. Generally, cockroaches (Butler, 1973) and other insects,

such as flies, contain eight retinular cells in each ommatidium. Each retinular cell has a rhabdomere in which visual pigment-contained structure, called microvilli, are located. The microvilli of each retinular cell are arranged towards the middle of the ommatidium, at right angle to the long axis of their parent cell. In cockroaches these rhabdoms are fused (Seelinger and Tobin, 1981) and share the same optical axis. But in flies, including *Drosophila*, the rhabdom is open and each retinular cell has a different optical alignment (Wolken et al., 1957).

The compound eye can be categorized by methods of image formation. In apposition eyes, retinal cells are located just beneath the lens and each lens forms an inverted image onto their tips. In superposition eyes, a clear zone exists between the lenses and retinal cells so that light rays from many adjacent lenses are collected at a single point of the tips of the rhabdoms. The arrangement of rhabdomeres in the apposition eyes of flies is "open." Seven adjacent ommatidia, which share the same visual field, send axons to same cartridge in the lamina (Franceschini, 1975). Hence, it is called the neural superposition eye.

In cockroaches, each of six "short" retinula cells sends an axon to the first-order optic neuropil, the lamina. There is a retinotopic mapping of neural elements onto the lamina in which each ommatidium relates top a specific column of cells (an optic cartridges) (Ribi, 1977). Long visual fibers (LVF), which are sensitive to ultraviolet (UV), pass through the lamina without making synapse to end in the second optic neuropil, the medulla. In flies, pathways from the six short retinular cells convey information to a third order neuropil, called the lobular plate, which is sensitive to motion detection (Laughlin,

1981; Strausfeld and Lee, 1991). Long visual fibers convey information to the lobular through transmedullary neurons, which are second order relay neurons of the medulla. Hence, there are two different visual pathways in insects, one that is involved in motion detection, the other in object recognition.

Visual projection neurons from optic lobes to higher center of the brain are quite well documented, especially from flies. Both small-field neurons from the lobula (Gilbert and Strausfeld, 1992) and wide-field neurons from the lobula plate (Gronenberg and Strausfeld, 1992) connect with descending neurons in premotor center in protocerebrum and deutocerebrum. Visual projection neurons from the lobula of the honey bee send axons to several neuropil areas in the brain, such as posterior-median protocerebrum and dorsal lobes of the deutocerebrum, where sensory fibers of the antennae overlap with visual projection neurons (Maronde, 1991). These regions are thought to be important centers for multimodal information processing between sensory, motor, and descending neurons in insects.

1-6. Neural mechanisms of insect learning and memory

Insects generally show a considerable range of adaptations to their ecological niches and sensory environments. For example, courtship and mating behaviors can undergo as many discrete stages of ritualized behaviors in insects as in mammals (fruit flies: Spieth, 1974; Hirsch and Tompkins, 1994). In this regard, insects can be studied as a means for elucidating experience-dependent modification of behaviors.

Using aforementioned learning behavioral paradigms in insects, putative neural substrates of learning and memory in the insect brain have been extensively studied. Many studies have demonstrated that the MBs and the AL might have independent roles in olfactory learning and memory in honey bees. Erber et al. (1980) demonstrated neural mechanisms of the honey bee brain on olfactory learning, where cooling of vertical lobes of the MBs blocks the short-term memory formation after one-trial olfactory conditioning. As a neural correlate of sucrose reinforcement in PER conditioning, the VUMmx1 neuron, whose unpaired cell body is situated ventrally in maxillary neuromere, sends arborizations to the antennal lobes, the calyces of the MBs, and the lateral protocerebrum, all of which are involved in olfactory processing (Hammer, 1993). This neuron is immunoreactive to octopamine (OA). Hammer and Menzel (1998) have demonstrated that injection of OA into the antennal lobes or calyces of the MBs paired with odor stimulation can replace the function of the unconditioned stimulus in olfactory conditioning. Moreover, this study has depicted that during memory acquisition OA injection into the AL enhances odor learning performance of the honey bee, while OA injection into the calyx leads to no enhancement of PER during test trials 30 minutes after training. Although diffusion of OA may remain an open question in this study, (where Lucifer Yellow is co-injected but this molecule is much larger compound than OA; thus, permeability of OA is unclear), this study at least indicates that AL and calyx of the MBs show different functions in memory formation. Injection into the ALs of double-stranded RNA (dsRNA) of OA receptors, which mediates interference of protein expression by combining with mRNA (Hunter, 1999; Clemens, 2000), impairs learning performance in

very early stages of learning, indicating that OA receptors might be up-regulated in ALs during the PER learning paradigm in honey bees (Farooqui et al., 2001).

Although functional differences of olfactory memory formation in ALs and MBs might contribute to the quick formation of olfactory memory in the ALs, little is known about how these two sites interplay in memory formation. It is known that the MBs play an important role in context-dependent memory formation of multimodal information, such as olfactory, visual, and mechanosensory signals (Liu et al., 1999), suggesting that memory formation in the MBs requires time to develop (Menzel, 2001).

Recordings from the PE-1 neuron, which is an efferent neuron of the α lobe of the honey bee's MBs and sends its axon into the posterior lateral protocerebrum, shows that its filtering properties and electrophysiological responses are modified by one trial and multiple conditioning procedures using associative memory paradigms. In this account, the PE-1 neuron shows different frequency modulation to the conditioned odor stimulus in which frequency reduction of the PE-1 neuron to the conditioned odor is found in early test trials. However, as trial numbers are increased (e.g. 4^{th} training trial) in multiple training, a spike frequency increase is detected. This indicates that one training trial of conditioning is sufficient to represent learned odor in a single efferent neuron and different computation mechanisms might be involved in olfactory learning as insects receive stimuli repeatedly (Mauelshagen, 1993). So far, however, it has been very hard to observe memory trace formation at the level of single neurons because intracellular recording from behaving animals is difficult to conduct in honey bees. In fruit flies, which are small and fragile, no such experiments have been successfully performed.

Genetic approaches have been used to study learning and memory in fruit flies. *Drosophila* can be conditioned to associate odors with an electric shock (Quinn, 1974) or heat (Wustmann and Heisenberg, 1997). Visual learning, associated with aversive odors (Guo and Götz, 1997) or heat (Liu et al., 1999), has also been demonstrated. Recent developments in molecular genetics, such as GAL4 enhancer-trap techniques by which tissue-specific transcriptional controls of transgenes are accessible (Fischer et al., 1988; Brand and Perrimon, 1993) and MB mutation in biochemical pathway (Heisenberg et al., 1985), as well as chemical ablation techniques with hydroxyurea (de Belle and Heisenberg, 1994), have revealed that the MBs are essential sites involved in these learning behaviors. For instance, adult flies whose MB neuroblasts in newly hatched larvae were selectively ablated by hydroxyurea (HU) show striking deficits in olfactory learning (de Belle and Heisenberg, 1994). In this account, while HU also reduces the AL volumes of flies, the ability of these flies to avoid tested odorants is not affected, indicating that MBs play an important role in the integration of olfactory signals. These studies, however, did not reveal functional roles of the MBs in memory formation.

By screening learning and memory mutants using olfactory learning paradigms (Quinn, 1974; Heisenberg et al., 1985; review: Waddell and Quinn, 2001), *Drosophila* mutants with learning and memory deficits, such as *dunce* (*dnc*), *rutabaga* (*rut*), and *amnesiac* (*amn*), provide biochemical mechanisms underlying memory formation. These mutants have deficits in components of the cAMP cascade, which is thought to play a crucial role in protein-dependent long-term memory (LTM) formation (Yin et al., 1994; Silva et al., 1998; Alberini, 1999). Cloned genes of the fly mutants, *dnc*, *rut*, and *amn*,

show that these mutants have deficits in cAMP-specific phosphorylase (Byers et al., 1981; Chen et al., 1986), Ca2+/calmodulin-dependent adenylyl cyclase (Livingstone et al., 1984; Levin et al., 1992), and a neuropeptide homologous to mammalian adenylate cyclase activating peptide (PACAP) increasing cAMP concentration (Feany and Quinn, 1995). cAMP is involved in the regulation of protein expression during memory acquisition and the phosphorylation of protein in synapses such as those found in *Aplysia* (Casadio et al, 1999). The target of a cAMP in the cell is cAMP-dependent protein kinase A (PKA), which phosphorylates aAMP-response-element-binding protein (CREB) by which the function of CREB can be activated to initiate gene expression (Silva et al., 1998; Alberini, 1999). Yin et al. (1994, 1995) have found that *Drosophila* with the inducible transgene of CREB shows LTM, indicating that CREB plays a crucial role in LTM formation. These protein products except *amn* products are highly expressed in the MB intrinsic cells (*dnc*: Nighorn et al., 1991; *rut*:Han et al., 1992). *amn* gene expression occurs in the dorsal paired medial (DPM) neurons, which are located outside the MBs, send axons to *Drosophila* MB lobes (Waddell et al., 2000). Using the restricted expression technique of *shibire* by GAL4 lines, this study has demonstrated that DPM neurons are important for long-term memory formation. Thus, although the MBs are thought to be a crucial center for LTM formation in many accounts, modulation by neuropeptide neurons outside the MBs are also crucial in memory formation. It is also still debated in that because the MBs are highly packed with small cells compared to other brain regions, high resolution of memory-related gene expression in the MBs may lead to misinterpretation (Ito et al., 1998).

Inhibition of gene expression (Heisenberg et al., 1985; McGuire et al., 2001; Dubnau et al., 2001) in the MBs in *Drosophila* can perturb or abolish learning and memory in olfactory and courtship conditionings. However, the localization of the memory trace is still elusive. McGuire et al. (2001) and Dubnau et al. (2001) have demonstrated location and temporal stages of memory formation by controlling expression of a temperature sensitive allele of the *shibire* that encodes dymanin guanosine triphosphatase, which is important in synaptic vesicle recycling, in the MBs of *Drosophila* GAL4 lines. These experiments can control neural activities spatiotemporally during learning. Transgenic GAL4 line flies with mutated *shibire* can be expressed at restricted temperature at 30-32°C but are not expressed at the permissive temperature at 20-25°C. These accounts indicate that synaptic transmission in the MBs of *Drosophila* is essential for retrieval but not for the acquisition or consolidation phase of memory in which flies are trained at permissive temperature and tested at restricted temperature. Although the results of these accounts are consistent, it still remains unknown where olfactory memory is stored.

Zars et al. (2000) demonstrated that one subcompartment of the MBs, called the γ lobes, may play an essential role in short-term memory formation. In this study, the rescue of wild-type *rut* gene by GAL4 enhancer trap elements demonstrated that rescued flies showed no difference from wild-type flies in short-term memory. Rescued flies among GAL4 lines showed that expression of the rescued gene occurred commonly in γ lobes of the MBs. Pascual and Preat (2001) have shown that the alpha-lobes-absent (*ala*) mutant *Drosophila* that receives spaced training in the olfactory learning paradigm shows

long-term memory deficit but not short-term memory. Taken together, these findings suggest that there could be functional differences within the lobes structure of the MBs in memory formation.

To this date, although studies on insect MBs at the neurophysiological and molecular biological levels have shown much progress, the site of memory formation is still uncertain and the mode in which input and output neurons are associated with memory formation and retrieval is still largely unknown.

1-7. Comparison with learning and memory of mammalian systems

As mentioned in the previous section, there is some evidence that various regions of the insect brain are involved in the formation of spatial memory and olfactory memory. Moreover, different subcompartments of the *Drosophila* MBs appear to differently function in memory formation in the olfactory associative learning paradigm (Zars et al., 2000; Pascual and Preat, 2001; Dubnau et al., 2001; McGuire et al., 2001).

The idea of distributed memory systems in the brain is not new in mammalian systems. Apart from the ethological schools of animal behaviors concerned with evolutionary mechanisms of innate behaviors (Tinbergen, 1951; von Frisch, 1967), psychological schools during the early 20th Century, such as Pavlov (1927), were concerned with species-independent behaviors, such as learning, memory, and perception as well as interaction between stimuli and behaviors. Combined with behavioral approaches, cognitive psychological schools are also interested in the mechanisms of information processing in the brain leading to these behaviors. During the mid 20th

Century, surgical treatments of medial temporal lobe regions to relieve severe psychiatric disorders in human, such as epileptic seizure, gave accidentally rise to deficits of recent memory of facts and events, called declarative memory which is involved in conscious recollection, in the absence of remote memory impairment (Milner and Penfield, 1955; Penfield and Milner; 1958; Penfield and Mathieson, 1974). The lesion of any identified components of the medial temporal lobe, which comprises the perirhinal cortex, the parahippocampal cortex, and the hippocampal formation composed of the dendate gyrus, CA3, CA1, the subuculum, and the entorhinal cortex, results in a deficit of the declarative memory (Squire and Zola-Morgan, 1991).

However, these patients showed no deficits in motor learning (non-declarative memory; see below), such as the mirror-drawing task (see Milner, 1965). These accounts have suggested that there are more than one centers involved in different kinds of memory formation. Distinct from declarative memory, learning abilities for non-declarative memory, such as changes in motor skills or the ability to respond to stimuli by previous experiences, have been tested in patients and experimental animals that had damage in various brain regions. Priming, which is a behavioral change in the ability to identify an object that is recently encountered, did not result in learning deficits in amnesic patients in whom temporal lobe areas of the brain were removed (Squire et al., 1993; Nielsen-Bohlman, 1997). In these accounts, the word-stem priming test was used to assess memory. Full-length of the words were first presented, such as "MOTEL", and later three-letter words, such as "MOT", were presented and a test patient was asked to complete a word with the first word in mind. These studies have indicated that deficits in

medial temporal lobe areas are not related with priming but a posterior neocortex region, which is outside the temporal lobes, is involved in this non-declarative memory formation (Squire et al., 1993; Nielsen-Bohlman, 1997). Other non-declarative learning, such as emotional learning by fear conditioning in rats, in which rats learn to associate a neutral stimulus, such as a tone, with an electric shock, have indicated that the amygdala plays a crucial role in fear conditioning (LeDoux, 1995; Davis et al., 1997). Also, there are other simple associative learning paradigms such as the eye-blink response of rabbits in which an animal learns to associate a neutral stimulus (tone) with an air puff to the eye. Lesion experiments have indicated that the substrate for this simple associative memory is located formed in the cerebellum (Thompson and Krupa, 1994). Hence, at the systems level of memory formation, different kinds of memory are formed in different places in the mammalian brain.

Another fundamental question of how memory is formed in the brain has been examined in mammalian systems, such as rats and mice, by using spatial learning paradigms, which involve declarative memory.

Since the effects of hippocampus lesion on learning and memory have been found in humans in which bilateral lesions of the CA1 area of the hippocampus (one components of the medial temporal lobe) induced deficits in memory formation (Zola-Morgan et al., 1986; Rempel-Clower et al., 1996), numerous research has focused on neural mechanisms underlying acquisition and consolidation of new information in rats as well as nonhuman primates. Hippocampal-lesioned rats showed learning deficits in place learning using two different paradigms: a radial arm maze in which rats were tested

to choose correct arms associated with distant spatial cues (Olton, 1977), and a Morris water maze in which rats have to swim to the location of a hidden platform (Morris et al., 1982). In these accounts, test rats whose hippocampus was damaged showed no spatial learning. However, other types of non-spatial learning such as cued learning were unaffected. Also, 'place cells', pyramidal cells in CA1 regions of hippocampus encoding a position in space, showed selective firing patterns in a specific area of an environment. Place cells not only fire when animals were confronted with novel environments, but they showed similar firing patterns in a dark condition of the same environment, implying that these cells might play a crucial role in spatial mapping (Muller et al., 1987; Best et al., 2001).

Also, the CA1 region is important for long-term memory formation. NMDA receptors, which are located in postsynaptic cell membranes, increase synaptic strength in which depolarization of postsynaptic cells as well as glutamate biding results in long-term potentiation by allowing Ca^{2+} to enter the inside of the postsynaptic cells, after which Ca^{2+} calmodulin kinase (CaMKII) and protein kinase C (PKC) is consistently active (Kauer et al., 1988). Blocking of NMDA receptor impairs spatial learning, where the NMDA antagonist, APV (aminophosphonovalerate), is used during spatial training (Morris et al., 1986). In addition to these functions, the hippocampus plays a role in context learning (Penick and Solomon, 1991) and consolidation during sleeping in which place cells show increased trends of the firing patterns after subsequent sleeping of animals (Wilson and McNaughton, 1994; Sutherland and McNaughton, 2000). Like *Drosophila* in which neurotransmission was blocked using GAL4 lines, CaMKII-

knockout mice in CA1 neurons of hippocampus showed severe impairment on spatial learning (Bach et al., 1995). Thus, recent studies on molecular mechanisms underlying memory formation in mammals indicate mechanisms similar to those in insects and other invertebrates, such as *Aplysia* (reviews: Silva et al., 1998; Kandel, 2001), indicating that common cellular pathways are involved in memory formation.

CHAPTER II

ANTENNAL MOVEMENTS REVEAL ASSOCIATIVE LEARNING IN THE AMERICAN COCKROACH, *PERIPLANETA AMERICANA*

Abstract

A new visual associative learning paradigm has been developed to investigate visual and olfactory associative memory using antennal movements as an indicator of learning and retention. Experiments were performed on the restrained cockroach, *Periplaneta americana*, which normally moves its antennae towards a localized odor source. In the present study, such "antennal projection responses" (APRs) are used to demonstrate long-term memory where an APR is elicited by a conditioning stimulus (CS: green light point source) paired with a spatially coincident odor (the unconditioned stimulus: US). Association of the CS with the US is established after 5 trials. The number of training trials required to establish APRs to the CS alone is significantly less using delayed conditioning compared with trace or backward conditioning, the latter two showing no significant differences. Before training, a visual cue alone does not elicit an APR, showing that this behavior is elicited by a visual cue only after pairing it with an odor stimulus. The acquired APR to the green light cue can persist for up to 72 hours, indicative of long-term memory. This paradigm is thus suitable for future studies of neural correlates of learning and memory on restrained animals. Implications of the present study for neural mechanisms underlying learning are discussed.

2-1. Rationale

In a continuously changing environment, animals have to identify stimuli, assess their relevance, and predict salient phenomena in order to successfully forage, find mates, and avoid predators. While it is self evident that learned associations between novel cues and biologically meaningful information are essential to survival, neural mechanisms underlying such associations, storage, and retrieval are not. While the focus on behavioral and genetic aspects of learning and memory in *Drosophila* provides insights into modulatory and second messenger systems involved in memory and retrieval (Tully and Quinn, 1985; Dubnau and Tully, 1998), there are comparatively few studies at a systems level on what kind of changes occur in identified neurons.

Studies on associative learning in insects have mainly focused on social taxa due to their elaborate foraging and navigation behaviors (von Frisch, 1967; Collett, 1996). Honey bees have been a particularly good model system for studying olfactory learning associated with food rewards. But while such investigations might suggest neural mechanisms underlying learning and memory (Smith and Cobey, 1994; Bitterman, 1996; Menzel and Müller, 1996) only one study has attempted to demonstrate specific changes that occur in neurons as a consequence of learning (Mauelshagen, 1983). In part, this deficit is because honey bees are quite fragile and are technically difficult. In contrast, cockroaches are by reputation experimentally resilient, a feature borne out by several recent studies that have recorded successfully from their higher brain centers and have shown specific examples of neuronal plasticity with respect to multimodal integration (Mizunami et al., 1998; Li and Strausfeld, 1995; Strausfeld and Li, 1998a). In addition,

behavioral studies on cockroaches have demonstrated their suitability for learning and memory studies (Balderrama, 1980; Gadd and Raubenheimer, 2000), including place memory (Mizunami et al., 1993, 1998) using a paradigm that first demonstrated this phenomenon in mammals (Morris et al, 1982).

A prerequisite for demonstrating change at the level of single identified neurons as a consequence of learning requires that a robust learning paradigm can be important to the electrophysiological station, requiring that associative learning by a freely moving animal must also be demonstrable under tethered conditions. A precedent is the proboscis extension reflex in honey bees, which can be conditioned by pairing a sucrose reward (the US) with an odor stimulus (CS). This paradigm was used by Mauelshagen (1983) for her intracellular study and is the mainstay of all biochemical studies on learning and memory in that species. Here we describe experiments that demonstrate a comparably plastic behavior that can be driven in immobilized cockroaches. The behavior, which is expressed by antennal movements (antennal projection responses: APRs) towards a stimulus source, can be further used for studies of spatial context in learning and memory thus providing a "place memory" paradigm for an animal that cannot move. This is described in the succeeding paper. APRs are reminiscent of sniffing behaviors in mammals (Gray and Skinner, 1988) as well as antennular flicking behaviors in crustaceans, such as crayfishes and spiny lobsters (Mellon, 1997; Derby, 2000). All of these intermittent movements allow the assessment of a continuously changing olfactory milieu, and provide the brain with data for locating a distant olfaction source. In lobsters, the frequency and directional control of antennular flicking behaviors are increased as

mixtures of odor components were increased (Mellon, 1997). Likewise, other modalities such as visual and tactile stimuli also triggered the same olfactory behaviors. For example, in honey bees, antennal scanning can be elicited by visual, olfactory, and mechanical cues (Erber et al., 1993). Antennal contact to a silver plate can be operantly conditioned with rewards in honey bees (Kisch and Erber, 1999). Crickets can also track antennae towards moving objects (Honegger, 1981), further showing that control of olfactory appendages can be influenced by a variety of different modalities.

This study describes the conditioning of cockroach APRs towards an odor source (the US) by presentation of a neutral stimulus, a green light (the CS). The study explores whether an APR is indicative of recognition by the visual system of a stimulus location. Although the paradigm used here is a simple form of association between visual and olfactory information without any rewards, the present study shows that antennal projection responses to odor cues can be used to test learning performance in immobilized cockroaches and that the cockroach indeed learns to associate food odors with visual cues.

2-2. Materials and Methods

2-2-1. Animal

Experiments were performed using male American cockroaches, *Periplaneta americana*, raised in a laboratory colony maintained on water and cat food (IAMS, Dayton, Ohio). The cockroaches were kept at 25±1°C on a 12:12 hour light-dark cycle. Only intact cockroaches were used in the experiments. Animals with any external damage (e.g.

missing antennal segments) were discarded. Each test cockroach was isolated from its colony and kept in a small plastic cage in which it was starved for 24 hours before behavioral experiments began. The cockroach was then cooled to 4°C for 6 min and then restrained in a small plastic tube holding the head in place but allowing the antenna to move freely. The head was immobilized using modeling wax, and 1:1 mixture of bee's wax, and pine resin. The tube holding the restrained cockroach was positioned horizontally in the middle of an arena, supported by modeling wax. This allowed the cockroach to move its antennae freely but without contact to the arena (Fig. 2-1A). The attitude held by the body was the same as that during walking on a flat surface. After restraining, the cockroach usually required 10 to 20 min until it began to show spontaneous antennal movements and its body movements subsided. Individuals showing no antennal movements to odor stimulation during training trials were discarded.

2-2-2. Arena and stimuli

All behavioral experiments were conducted inside a (1.5×1×1m) chamber surrounded uniformally by black curtains. An infrared heat lamp (Supreme Co., Mullins, SC) was positioned above the behavioral chamber to provide warmth and red light illumination for video-recording. The 30cm diameter arena was made of polyethylene with walls 10cm high walls. Green LEDs (peak wavelength, 565 nm diameter, 3 mm) (E166, Gilway Technical Lamp Co., Woburn, MA) were positioned at regular intervals on the wall of the arena, to the right of the cockroach's midline. These provided stationary light flashes (duration 2 sec). Green light was presented during the pretraining, training (conditioning)

trials, and during testing trials. A single red light diode (625 nm, E100, Gilway Technical Lamp Co., Woburn, MA) was positioned alongside the green diode used for conditioning and was used in a control test (see below) to determine if sounds from the light switches were being detected.

Food odors (peanut butter; Skippy; Bestfoods Co., Eaglewood Cliffs, NJ) were presented through an odor delivery system consisting of a syringe needle and a polyethylene tube (1 mm inner diameter) that were connected to odor sources. Pure air puffs (charcoal filtered; air pressure 10 Pascal; stimulus duration 1 sec) were blown through a cartridge containing the odor and controlled by a solenoid valve (General Valve Co. Fairfield, NJ). An exhaust fan system was placed above and behind the arena in order to remove odors from the inside of the arena between trials. Peanut butter odor was used for the unconditioned stimulus (US). Odor was delivered from immediately above the green LED used for conditioning trials.

Stimuli and their sequences were controlled by a Grass S88 stimulator (Grass instrument Co., Quincy, MA). Light and odor cues used for training trials were 15 cm distant the cockroach and at an angle of 30° with respect to the midline of the cockroach's head (Fig. 2-1A).

2-2-3. Training

Cockroaches were first trained to project their right antenna toward a green light as a conditioned stimulus (CS) coupled with a food odor as an unconditioned stimulus (US). Procedures tested were delayed, trace, and backward conditioning as shown in Fig. 2-1B.

In all three, the duration of visual and odor stimuli was 2 and 1 sec, respectively. In delayed conditioning, the CS and US were switched, the CS appearing first and overlapping the subsequent US for 1 sec (Fig. 2-1B-a). In trace conditioning, the CS was presented for 2 sec, followed by a 2 sec interval after which the US was presented (Fig. 2-1B-b). In backward conditioning, the US was presented first for 1 sec, followed by a 2 sec interval after which the CS was presented (Fig. 2-1B-c). Experimental procedures consisted of 3 pretraining trials, in which only the CS was presented, followed by 5 training trials in which the CS and US were presented, and 3 test trials 5 min after a last training trial in which the CS was presented again. Initial experiments showed that delayed conditioning was most effective (Fig. 2-4). Thereafter, further experiments used delayed conditioning only. To establish the optimal number of training trials, initial experiments were performed using different numbers (1, 3, 5, 7, 10 and 20) of training trials (Fig. 2-5). The inter-trial intervals of all trials were 1 min.

2-2-4. Control tests

1. Innate antennal response to the food odor alone and pure air puffs. Antennal responses to odor cues alone were tested to evaluate unconditioned responses (UR) of cockroaches. Without any other stimuli, odor stimulus (duration 1 sec) was presented 3 times with a 1 min interval. Pure air was used to determine whether cockroaches show antennal projection responses to pure air (Fig. 2-3). Five pure air puffs (duration 1 sec) were presented to each cockroach five times with 1 min interval. Responses were scored, as explained below.

2. Responses to acoustic switching. To control whether the sound of light switches rather than the lights themselves were providing the conditioned stimulus, a red light was presented with the same duration as green diode one minute after the last test trial (Fig. 2-1C).

3. Are visual cues associated with mechanical cues? Visual cues were coupled with pure air puffs without the food odor. During 5 training trials, clean air was paired with the green light (delayed paradigm; Fig. 2-1B-a). Five minutes after training, the learning performance of the test cockroach to a green light alone was examined (Fig. 2-6B).

2-2-5. Measurement of memory retention

Learned APRs to visual cues were measured at 5, 10, 20, and 30 min after training. Long-term memory retention was examined for up to 72 hours after training trials, in which APRs to visual cues were tested at 1, 3, 6, 12, 24, 48, and 72 hours after training trials. Throughout these tests, cockroaches remained restrained in their plastic tubes for 72 hours and were provided with water once a day or before tests to prevent dehydration.

2-2-6. Monitoring and video recording of antenna movements

Antennal movements were video-recorded with either 8mm Camcorder (Sony) or a digital video camera (Panasonic). Video sequences of 17 out of 21 test cockroaches were digitized every 167msec for 20 sec using Motus software (Peak Performance Technologies Inc. Englewood, CO), which produced about 105-120 images per trial. From the digitized images, antennal angles were measured from the tip and base of the

right antenna and the green light position (θ in Fig. 2-1A). Measured antennal angles from digitized images during pretraining, training, testing, and control testing were each averaged to compare differences. Timing of initial APRs to stimuli was also measured from video-recorded images of each test insect.

2-2-7. Scoring APRs and statistics

APRs were scored '1' if a cockroach projects its right antenna toward the cue position after stimulation or '0' if there was no response for 20 sec after stimulation. Percentages of APRs were calculated by summation of all scores during a given trial, as assessed by video observation. A learning index (LI) of each test cockroach was calculated by the percentage of APR to cues during testing minus the percentage of APR to cues during the pretraining for 20 sec periods after stimulation. Hence, 0 of the LI value indicates no learning, whereas 1 represents the maximum learning performance after training.

The Friedman test was used to compare APRs and LIs within subjects. Once a significant difference was established, the Wilcoxon signed-rank test was applied to compare each value of the repeated measurements. The Kruskal-Wallis test was performed to compare differences in LIs among groups. The Mann-Whitney U test was used to test differences between two groups. ANOVA with repeated measures was used for analyzing antennal angles during conditioning procedures. If significance was found, the Tukey HSD test was performed in parallel to compare two measurements of within groups. Values shown here depict means±standard errors (SE) and significance levels for all analysis were $P<0.05$.

2-3. Results

2-3-1. Patterns of antennal movements to stimuli

Figure 2-2 exemplifies antennal movements to stimuli by a single cockroach. The sequence of antennal angles (θ in Fig. 2-1A) measured from digitized images illustrates the actual antennal movement patterns for the 20 seconds after stimulation. Spontaneous antennal movements occurred with respect to the position of the green light cue (duration 2 sec) before training (A1-A3 in Fig. 2-2). During the 20 second observation period, the right antenna of a test insect showed no APR to the cue position. During a conditioning phase (B1-B5 in Fig. 2-2) with food odor and visual cues, antennal movements showed strong APR to the cue position, except in B4, in which no projection responses were shown. Antennal movements during the testing phase (C1-C3 in Fig. 2-2) to visual cues alone were significantly different compared to those during pretraining (A1-A3 in Fig. 2-2). Control tests to red light stimuli did not elicit APRs (D1-D3 in Fig. 2-2).

2-3-2. Responses to odor cues and pure air puffs

Innate responses (or unconditioned responses, UR) of APRs to food odor cues (US) were tested. Cockroaches showed 85% of APRs to food odors presented alone (N=21). On the other hand, APRs to pure air puffs (N=11) showed fewer responses (11%). Thus, APRs to odor cues showed significant difference from APRs to pure air puffs (Fig. 2-3; Mann-Whitney U test, $U=436.5$, $P<0.00001$).

2-3-3. Learning performance during different learning conditioning sequences

As shown in Fig. 2-4A, APRs elicited by three different conditioning procedures showed significant differences during pretraining, training, and testing (Friedman test, $p<0.003$). APRs to green LEDs were below 20% in the pretrainings, during which cockroaches responded spontaneously, if at all, to the light cues. During the five training trials, animals showed clear evidence of learning to associate light cues with food odors. APRs were significantly higher in delayed conditioning than those in trace and backward conditioning (Mann-Whitney U test, $P<0.001$). Five minutes after training, APRs of cockroaches to green LEDS were significantly increased compared to pretraining levels. APRs were retained after training using delayed conditioning, indicating that the number of training trials required for APRs to light alone was significantly less using delayed conditioning than using trace or backward conditioning. The LI of delayed conditioning, which was 0.65, was twice that of trace and backward conditionings (Fig. 2-4B; Mann-Whitney U test, $N=36$, $p<0.005$). LI's of trace conditioning showed no difference from L1's of backward conditioning (Mann-Whitney U test, $P>0.6$).

2-3-4. Optimal number of training trials

To determine the optimal number of training trials, the number of paired training trials was varied. Learning performance after different numbers of training was significantly different (Fig. 2-5; Kruskal-Wallis test, $H=37.25$, $df=5$, $P<0.0001$). Although cockroaches responded well during training regardless of the numbers of training trials, insects receiving a single ($N=10$) or triplet of trials ($N=8$) showed significantly lower learning performances compared to insects that received 5 ($N=9$), 7 ($N=8$), 10 ($N=10$), or

20 (N=11) training trials. LIs of insects given 5 or more training trials showed maximal performance with the asymptote lying between 5 and 7 training trials. This indicates that cockroaches learn to associate a neutral stimulus with food odors after about 5 repeated experiences. As training numbers increased to 10 and 20, their learning performance was diminished and was significantly different from that of groups given 5 or 7 training trials ($P<0.001$). As a result of this experiment, 5 training trials was determined to be the optimal number of trials for obtaining a maximized learning performance.

2-3-5. Learned antennal projection responses after training

To determine memory retention, APRs were tested to green LEDs presented 5, 10 20, and 30 min after training trials. APRs to the visual cue were retained for at least 30 min after training (Fig. 2-6A). After testing, a red LED was presented with the same duration as that of the green LED used beforehand. Cockroaches are insensitive to red light (Seelinger and Tobin, 1981), so that control tests with the LED should reveal whether or not the insect has learned to associate the odor with sensory modalities other than the green LED, such as the noise of the solenoids or light switches. However, only spontaneous antennal movements were observed in response to the red LED (Fig. 2-6A and Fig. 2-2 D1-D3) indicating that cockroaches learned to associate only visual cues with the odor but not other concurrent sensory stimuli. Coupling pure air puffs with green light cues during training trials also showed no significant increase in APRs (Fig, 2-6B, Wilcoxon signed-rank test, $N=12$, $P>0.5$).

2-3-6. Extinction of the learned response over time

Compared to control experiments that showed no learning performance (LI≤0), the learned responses to visual cues persisted for a long period of time, suggesting the establishment of long-term memory. The memory extinction curve showed in Fig. 2-7 indicates a gradual decrease over 72 hours. For the set of animals tested there was no significant difference of retention up to 72 hours (Friedman test, n=9, P>0.7). However, individual patterns of memory decay shown in Fig. 2-8 are surprisingly variable and are reminiscent of the kind of individual variation reported for place memory performance (Mizunami et al., 1998). Cockroaches A and B showed rather a similar pattern in that they exhibited stable memories for 24 hours but memory conspicuously decreased between 24 and 48 hours. LIs of 48 and 72 hours were 0 in cockroach A but were 0.33 in cockroach B and C. In contrast, cockroaches D, E, and F showed sustained memory in that LIs exceeded a score of 0.67 for up to 72 hours. Cockroach G and H showed a gradual increase in LI values 6 hours after training (LI≥ 0.67). Cockroach I showed low learning performance after training (LI≥ 0.67).

2-3-7. Average antennal angle during learning performance

Analysis of recorded antennal movements during pretraining, training, testing, and control testing in Fig. 2-9 showed a decrease in average antennal angles about the mean direction in training and in testing; indicating that the cockroach points its antenna accurately toward the position of a food source during the presentation of US with the CS coupling and afterwards tested with the CS alone (Fig. 2-9; ANOVA, N=17, P<0.005).

No significant differences in average antennal angles were found between training and testing. Average antennal angles for 20 sec in pretraining and control also showed no significant differences whereas average antennal angles during training and testing were significantly different from those during pretraining and control test (Fig. 2-9; Tukey HSD test, $P<0.001$).

Differences in the average antennal angles during pretraining, testing, and control testing were tested with ANOVA to analyze changes in APR patterns. Table 2-1 shows that significant differences were found among pretraining, testing, and control testing, supporting that antennal movement patterns were changed after training (ANOVA, $N=17$, $df=2$, $P<0.0001$). However, no difference was found with respect to trial numbers of each test (N; $P>0.7$), demonstrating that no upward or downward trends of antennal average angles were found within each test. No significant interaction was found between test trials (T) and trial number (N) (T×N, $P>0.8$). Average antennal angles among five training trials were analyzed; antennal movement patterns during training trials showed no significant difference (ANOVA, $N=17$, $df=4$, $P>0.4$). Thus, no trends of antennal movement patterns to visual cues were found (Table 2-2).

2-3-8. Delayed response to visual cues in test

A delay occurs between the presntation of an odor and an APR. However, after coupling the odor with light the delay of the APR to light alone significantly increased. (Fig. 2-10). The average response times of APRs to odor alone and during training, in which odor and visual cues were presented together, was 3.9 and 3.2 sec, respectively, whereas the

average response time in testing was 7.1 sec. The delays of APRs during testing was significantly different from the delays of APRs to odor alone or during training with the US and CS (Fig. 2-10; Tukey HSD test, $P<0.02$).

2-4. Discussion

2-4-1. Learned antennal responses to visual cues

Motor learning of antennal movements has been well described in honey bees (Erber et al., 1997). After scanning an object within the range of the antennae without rewards, honey bees showed the same motor behaviors of antennal movements for several minutes after the object was moved out of the range of antennae. This suggests that honey bees briefly remember the object and its position. In this experiment, the addition of a sucrose reward to antennal motor learning showed faster learning performance. Directed antennal movements, such as antennal scanning towards specific cues, have been previously demonstrated in honey bees where antennal extension can be elicited by olfactory, tactile, or visual cues such as moving stripes or small metal plates (Erber et al. 1993). Such antennal movements can be conditioned by simple association between a US and a sucrose reward or even with non-rewarding conditions. The present results likewise show in the cockroach that pointing or scanning movements by the antennae are directed towards the position of a relevant cue. The present study shows that such antennal responses can be exploited to analyze visual associative learning even without a reward and demonstrates that restrained cockroaches learn to associate two different modalities by projecting their antennae towards the conditioning stimulus after training to associate

it with the unconditioned stimulus, here peanut butter odor. That the acquisition of a response to the CS is due to the odor stimulus is shown by the lack of response elicited by pure air puffs coupled with visual cues.

2-4-2. Effects of stimulation intervals on learning performance

The interstimulus interval (ISI) and sequence of the unconditioned and conditioning stimuli strongly influence retention. In honey bees, olfactory learning, as assayed from the proboscis extension reflex, demonstrated that an optimal learning performance was achieved when the ISI was between 0 to 5 seconds between the presentation of the CS (odor) and the US (sucrose). In comparison, backward conditioning, in which the ISI of the US and CS exceeded 1 sec, showed a dramatic decrease in learning performance (Menzel et al., 1993) suggesting that contiguity between the CS and US is critical in reward-based learning performance. Furthermore, backward conditioning in honey bees showed that an ISI of 15 seconds between the US and CS induced maximum inhibition of learning performance (Hellstern et al., 1998). Similarly, the acquisition of a gill-withdrawal reflex after using electric shock as a negative reinforcer in *Aplysia* showed that an ISI of 0.5 sec between the backward pairing of the CS and US induced no learning (Hawkins et al., 1986).

In the present study, ISIs between the CS and US were 2 sec in trace and backward conditioning. Under these conditions, animals showed a weak learning performance compared to their performance using delayed conditioning, indicating that animals can still learn to associate temporal association of the two different stimuli (Fig.

2-4). Interestingly, short ISIs between the odor and visual cues in backward conditioning elicited moderate learning performance, indicating that cockroaches can associate the visual cue with a food source even when the stimuli are not concurrent. In nature, animals detect salient cues during foraging prior to the rewards, implying that the ISI is a critical factor in reinforcement-based conditioning. On the other hand, visual learning with food odors used here suggests that cockroaches can learn to associate visual cues with food odors regardless of the sequence of the two stimuli within a 2-sec ISI window.

2-4-3. Effect of inter-trial interval on learning

Intervals between training trials play have an important influence on learning and memory retention. Working with honey bees and using sucrose rewards in olfactory learning, Menzel et al. (2001) examined the effect of different ITIs, comparing massed training, with intervals of 30 sec between each training trial, with spaced training where the ITIs were 3 and 10 min.. These authors demonstrated that proboscis extension reflexes evoked during training did not different between the massed and spaced training trials. However the two procedures clearly demonstrated differences in long-term memory (>2 days) but not in mid-term memory (24 hours), Long term memory being significantly impaired by massed training, suggesting that different mechanisms might be involved in different forms of memory. At the level of gene expression spaced training of *Aplysia* results in the expression of new protein synthesis, which is essential for long-term memory formation, whereas massed training did not (Alberini, 1999).

In this study, inter-trial intervals (ITI) were 1 min between training trials. Cockroaches showed a significant learning performance after 5 training trials (Fig. 2-4 and Fig. 2-6A). Although learning behaviors to repeated odor presentation were not tested, we provide clues that an ITI as short as 1 min with repeated presentation of mutimodal information in the absence of rewards can be sufficient to elicit long-term memory and represent a "spaced training" protocol, although this still remains open to further experimental investigation.

2-4-4. Timing delay of APRs after stimulation

After training, there is a characteristic delay in the APR response to the CS (Fig. 2-10). There was an approximately 7.1 sec delay of an APR to the visual cue alone during testing compared to the unconditioned response to the odor alone or the response to both the odor and visual cue during training. These differences suggest that the strength of the association between an odor and visual cue might be weak compared to what might be achieved by reward-based learning. Possibly, visual learning takes longer than olfactory learning if the visual pathways to higher centers involve more delays than do the olfactory pathways. For example, Li and Strausfeld (1997) showed delayed activity in efferent neurons of mushroom bodies to visual stimulation, the neural activity of these neurons being delayed by as much as 1.5 sec after stimulation, in contrast to efferent neuron responses to tactile or olfactory cues. In honey bees, olfactory learning can be achieved just after one trial of conditioning, whereas visual learning requires 5 to 7 trials to obtain an asymptote of learning performance (see Gould, 1993). Cockroaches also

showed that one trial conditioning with odor associated with a sucrose reward is enough to elicit strong learning and memory retention (Balderrama, 1980). However, learning to locate a target using visual cues takes more than 5 training trials (Mizunami et al, 1998).

Fig. 2-1. The visual associative learning paradigm employed to train restrained cockroaches. **A.** Experimental set-up. Restrained cockroaches were positioned at the center of the arena. The distance from the head to the position of visual and olfactory cues was 15cm. Green and red LEDs (I.D. 3mm) as visual cues were positioned in parallel with an odor cue about 30° from the midline of the head. **B.** The best learning performance was determined from classical conditioning procedures. These are delayed conditioning, backward conditioning, and trace conditioning. The duration of the conditioning stimulus (CS: light cue) and unconditionjed stimulus (US: odor cue) were 2 and 1 sec, respectively. The time between the onset of the CS and the US indicates inter-stimulus intervals (ISI). The ISI in delayed conditioning was 1 sec. The CS and US overlap and cease simultaneously. In trace conditioning, the CS is given in its entirety and after a 2 second ISI is followed by the US. In backward conditioning, an ISI of 2 sec separates the US from the following by 2 second long CS. There is overlap between the CS and US in trace and backward conditioning. **C.** Basic training regimens consisted of 3 pretraining trials, 5 training trials, 3 testing trials, and 3 control testing trials. A 2 second exposure to a red diode (650nm) was used to control for other incidental stimuli (see text).

FIGURE 2-1

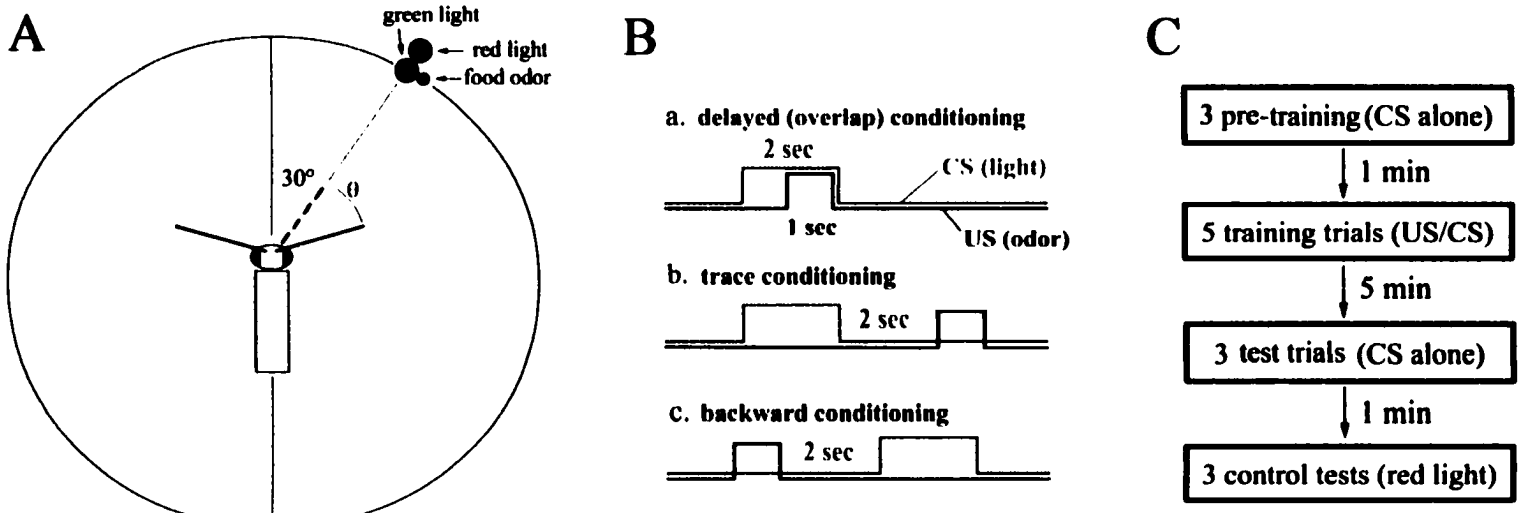
A
green light
red light
food odor
30°
θ
B
a. delayed (overlap) conditioning
2 sec
CS (light)
1 sec
US (odor)
b. trace conditioning
2 sec
c. backward conditioning
2 sec
C
3 pre-training (CS alone)
1 min
5 training trials (US/CS)
5 min
3 test trials (CS alone)
1 min
3 control tests (red light)

Fig. 2-2. Patterns of movements of antenna were analyzed with digitized images after video-recording during the delayed conditioning procedure. APRs are the pointing behaviors of the right antenna to the cue position after a 2 second stimulation during a 20 second observation period. The horizontal blues line at the stimulus position at 0^{o} on the X axes represents the 20 duration of the observation period. The thickened green lines represent the on timer of the green LED. The thickened red lines indicate on-time of the control red LED. Black traces are continuous changes of antennal angles throughout the 2 second light-on stimulus and the following 18 second observation period. During the pretraining (A1-A3), there are spontaneous antennal movements but no APRs to the LED position. During training (B1-B5), antenna movements after LED onset shows strong projection toward the cue position followed by gradual diminution although training trial 5 (B5) showed no APRs in this particular animal. During testing C1-C3, APRs were induced by the LED but they occur with a longer delay than in B1-B4. This animal showed no APR during third trial of the test (C3). Control tests (D1-D3) did not result in APRs to red LED stimulation.

FIGURE 2-2

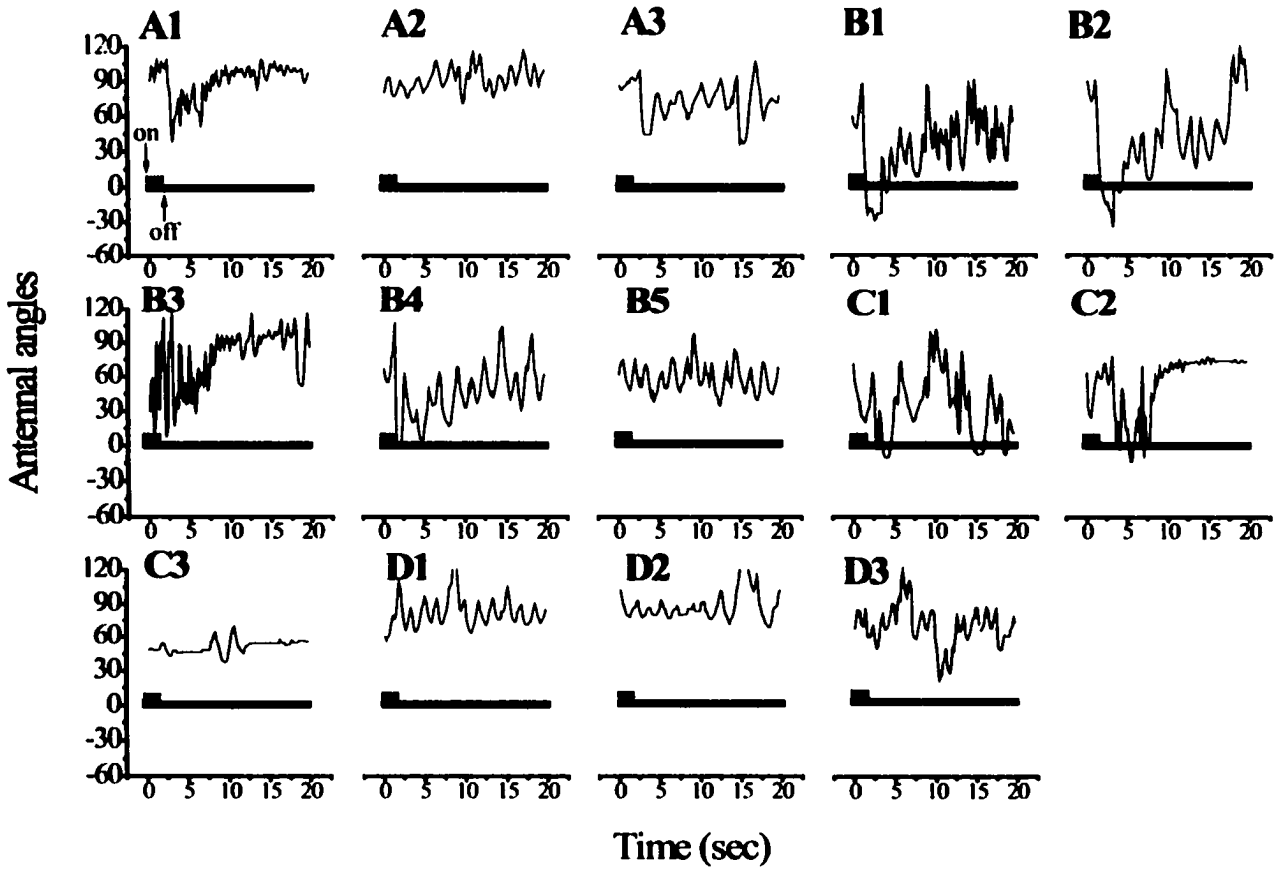
A1
A2
A3
B1
B2
B3
B4
B5
C1
C2
C3
D1
D2
D3
on
off
120
90
60
30
0
-30
-60
0 5 10 15 20
Antennal angles
Time (sec)

Fig. 2-3. Percentage of antennal projection responses (APRs) to puffs of food odor alone (85%: N=21) and to pure air puffs (11%: N=11). APRs to odor alone differed significantly from those to pure air puffs (Mann-Whitney U test, P<00001). Values depict mean±standard error (SE).

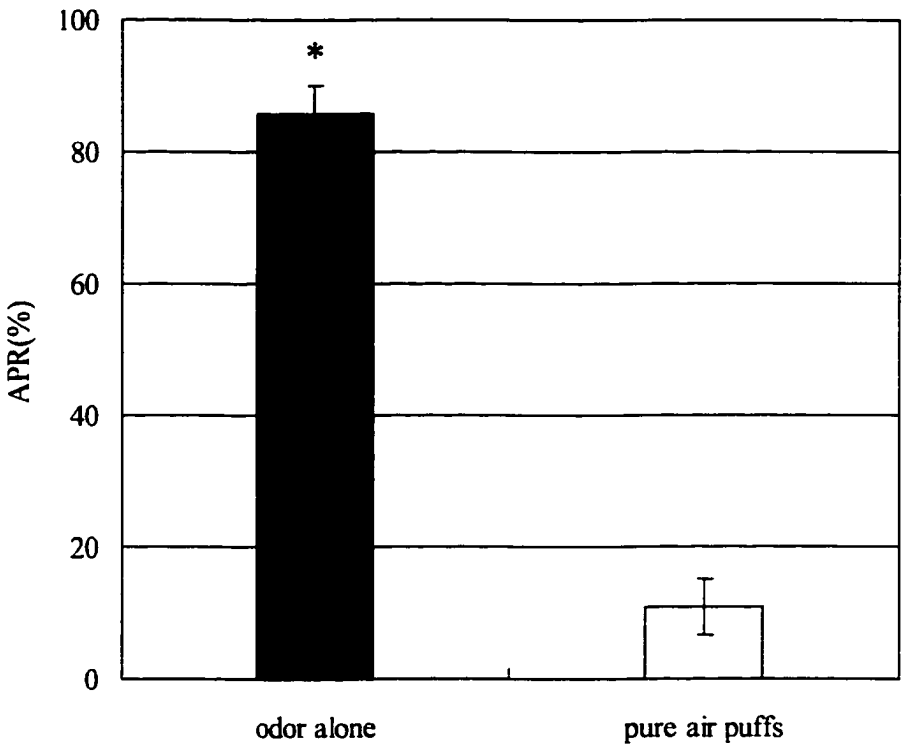

FIGURE 2-3

Fig. 2-4. Antennal projection responses (APR) and learning performance of restrained cockroaches during delayed (N=21), trace (N=13), and backward (N=11) conditioning procedures. **A.** APRs during training and testing trials were increased significantly in all three conditioning procedures compared to pretraining trials (Friedman test, $p<0.003$). APRs during training and testing in each conditioning procedure showed no difference (Wilcoxon signed-rank test, $P>0.19$). APRs during delayed conditioning training were significantly different from those of other procedures (Mann-Whitney U test, $P<0.01$). APRs during training showed no difference between trace and backward conditioning (Mann-Whitney U test, $P>0.5$). APRs during testing after delayed conditioning differed from those after trace and backward conditionings (Mann-Whitney U test, $P<0.001$). There was no significant difference in APRs during testing in trace and backward conditionings ($P>0.2$). **B.** The learning index (LI) was calculated by APRs (%) during testing minus APRs during pretraining. LI of delayed conditioning was significantly different from those of trace and backward conditionings (Mann-Whitney U test, $P<0.003$). LIs between trace and backward conditionings showed no difference (Mann-Whitney U test, $P>0.6$). Values are mean±SE.

FIGURE 2-4

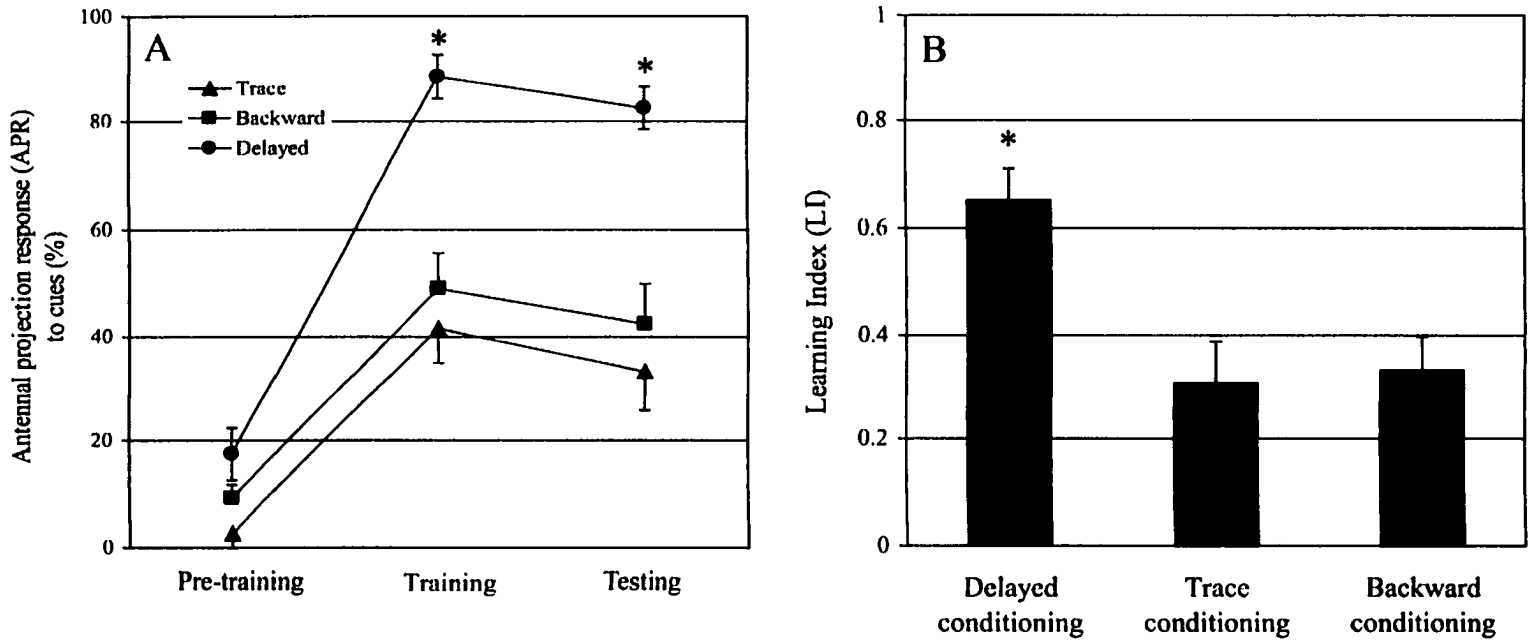
A
Trace
Backward
Delayed
Antennal projection response (APR) to cues (%)
100
80
60
40
20
0
*
*
Pre-training
Training
Testing
B
Learning Index (LI)
1
0.8
0.6
0.4
0.2
0
*
Delayed conditioning
Trace conditioning
Backward conditioning

Fig. 2-5. Comparision of learning performances of cockroaches receiving different number of training trials. In each group, cockroaches were trained with 1 (N=10), 3 (N=8), 5 (N=9), 7 (N=8), 10 (N=10), and 20 (N=11) training trials. Learning performances were significantly different among trials numbers (Kruskal-Wallis test, H=37.25, df=5, P<0.0001). The optimal numbers of training trials were obtained by asymptote of LI values; namely, between 5 and 7 training trial groups. LIs of cockroaches experiencing between 1 and 3 training trials were not significantly different from each other (Mann-Whitney U test, P>0.2) but showed significant differences from those experiencing 5, 7, 10, or 20 training trials (Mann-Whitney U test, P<0.05). LIs of cockroaches received 10 training trials decreased compared to those receiving 5 and 7 training trials (P<0.04). The group of cockroaches that received 20 training trials showed no difference from groups that received 5, 7, and 10 training trials (P>0.06). Cockroaches receiving 5 training trials showed a significant learning performance during testing. Same letters above each value indicate no significant difference between those values. Different symbols indicate significant difference at P<0.05 level. Values are mean±SE.

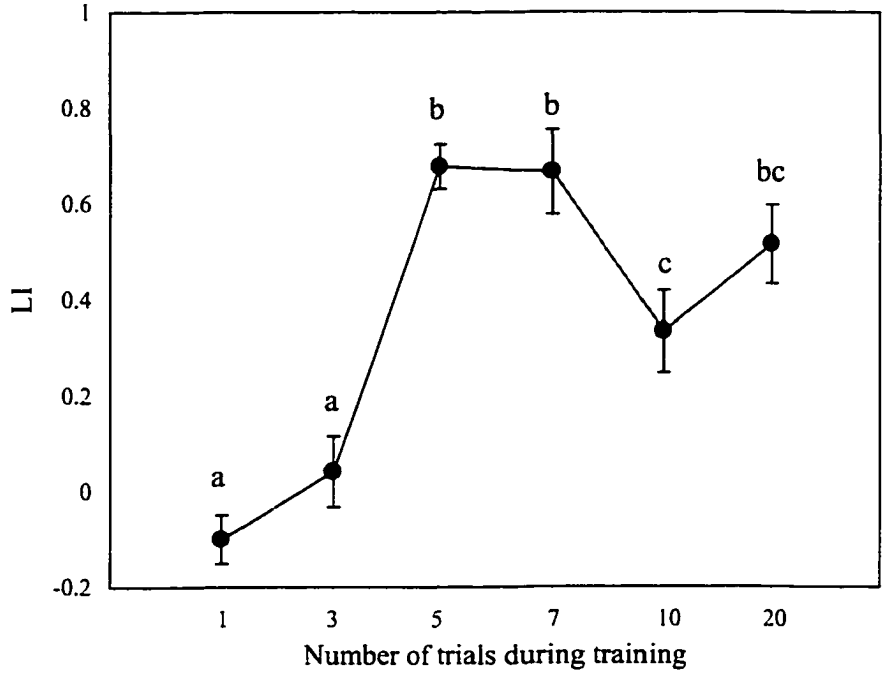
1
0.8
0.6
0.4
0.2
0
-0.2
LI
a
a
b
b
c
bc
1
3
5
7
10
20
Number of trials during training

FIGURE 2-5

Fig. 2-6. Percentage of APRs shortly after training (A) and learning performances after trained with a pure air puff (B). **A.** Learning performances (assessed by % of APRs) were tested for up to 30 min after 5 training trials with delayed conditioning. Antennal projection responses before and after training within-groups showed significant difference (Friedman test, N=18, P<0.0001). A high % of APRs to the visual cue were retained after 5, 10, 20 and 30 min after training and showed no difference in these intervals (Wilcoxon signed-rank test, P>0.3). The responses at these times were significantly different from pretraining and control test levels (N=18, P<0.0005). Pretraining and control test showed no significant difference (Wilconxon signed-rank test, P>0.09). The same symbols above each value indicate no significant difference. Different symbols indicate significant difference at P<0.05 level. **B.** APRs after trained with pure air puffs showed no significant (N.S.) learning performance during test trials (Wilcoxon signed-rank test, N=12, P>0.5). Values depict mean±SE.

FIGURE 2-6

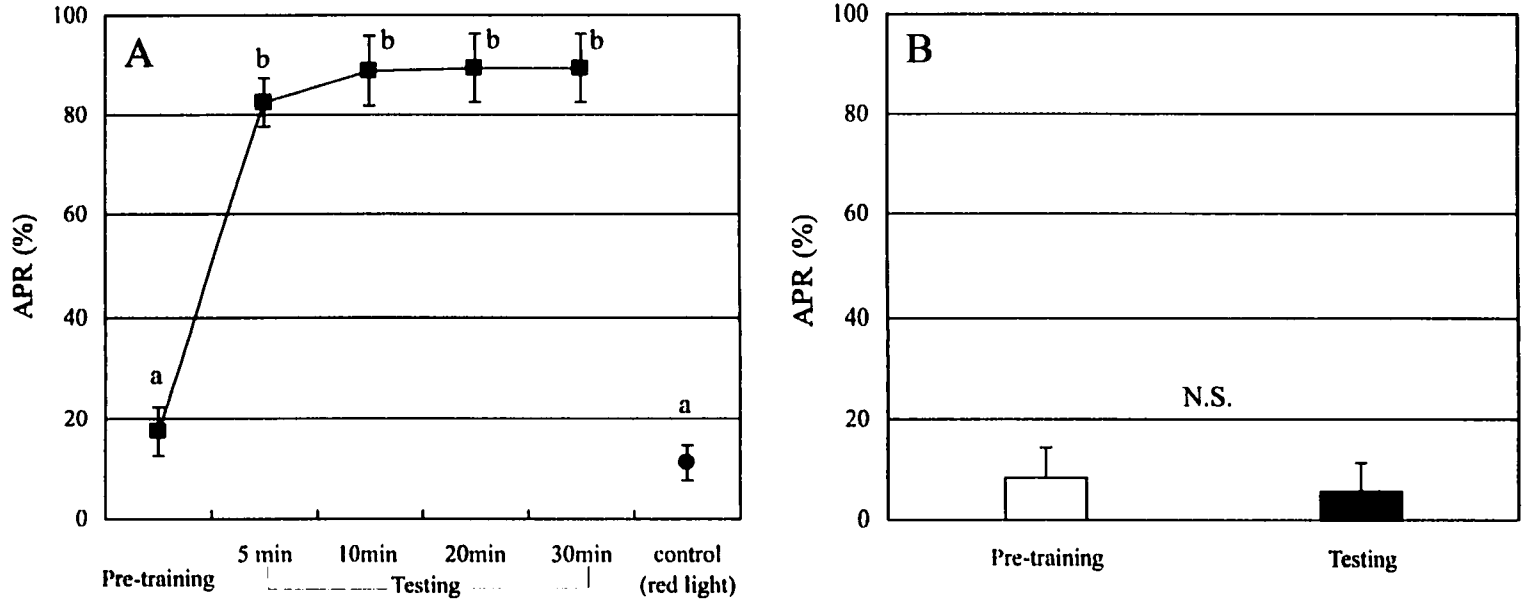
A
B
APR (%)
100
80
60
40
20
0
a
b
b
b
b
a
5 min
10min
20min
30min
control
(red light)
Pre-training
Testing
N.S.
Pre-training
Testing

Fig. 2-7. Tests for long-term memory of APRs. After training, APRs to the green light cue were tested for up to 72 hours. APRs showed no difference with time after training, meaning that cockroaches retained this learned behaviors for long periods (Friedman test, N=9, P>0.7). Control experiments, in which test cockroaches were trained with pure air puffs, showed no learning performance. Values depict mean±SE.

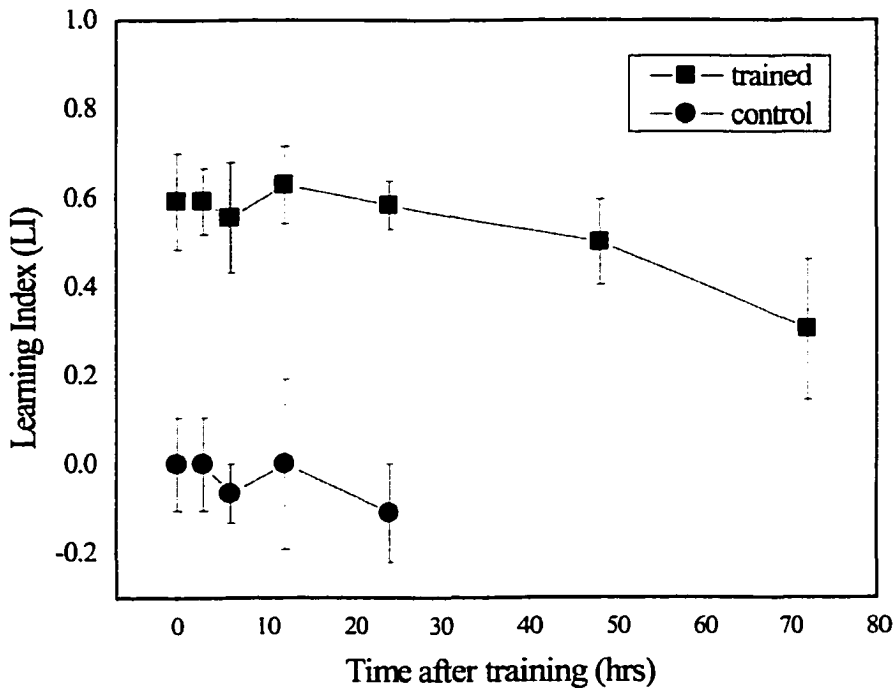
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
Learning Index (LI)
trained
control
0
10
20
30
40
50
60
70
80
Time after training (hrs)

FIGURE 2-7

Fig. 2-8. Individual patterns of memory retention and individual difference of memory decay. Cockroaches A, B, and C showed a similar pattern in that memory gradually decreased over 72 hours. Cockroaches D, E, and F, however, showed consistent memory retention over 72 hours after training. Cockroaches G and H showed a gradual increase of memory retention with time whereas cockroach I exhibited memory for 10 hours and then a negligible memory retention.

FIGURE 2-8

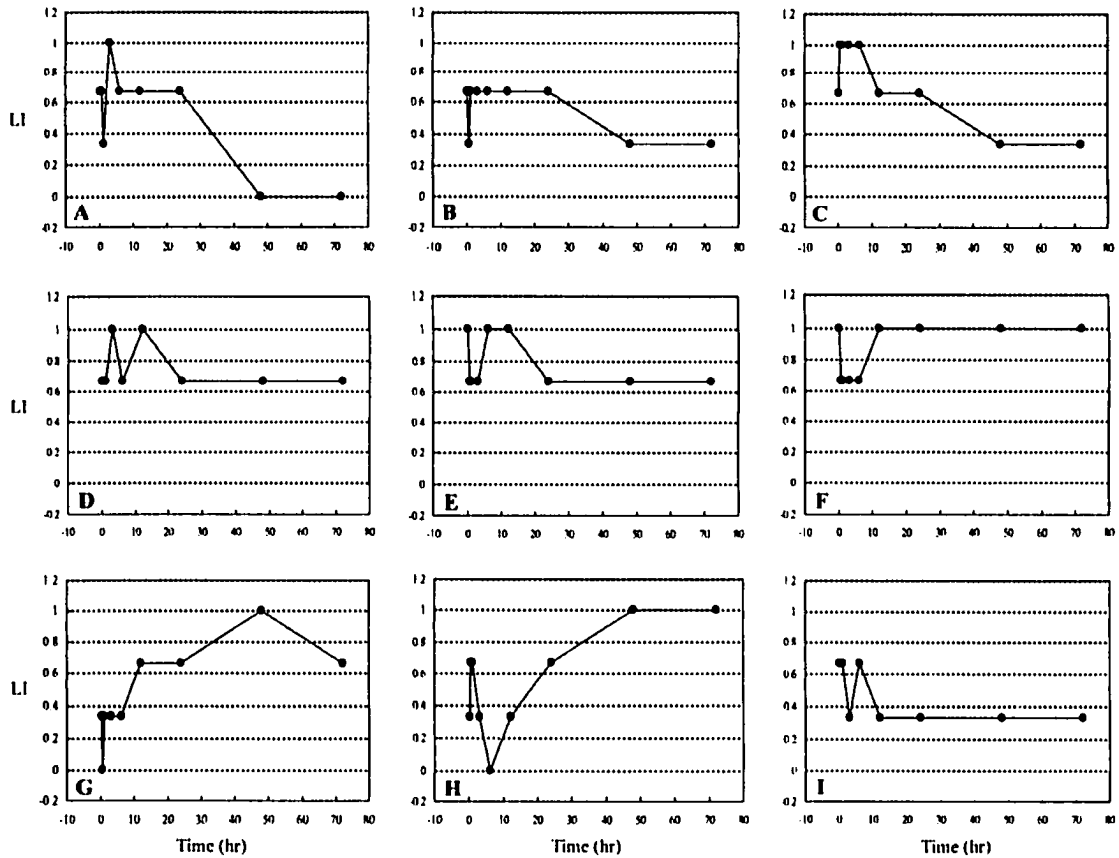

Fig. 2-9. Average antennal angles during delayed conditioning procedures. Average antennal angles showed significant difference by training procedures (ANOVA, df=3, N=17, P<0.0001). During pretraining and control test in which green light and red light were presented, respectively, average antennal angles were not different (Tukey HSD test, P>0.6). During training and testing, average antennal angles during training and testing were significantly smaller compared to those during pretraining and control test due to the antennal projection responses to visual cues after stimulation (Tukey HSD test, P<0.001). Average angles during pretraining and control test as well as training and testing for 20 sec observation period after stimulation, however, showed no significant difference. Same symbols above each value indicate no significant difference. Different symbols indicate significant difference at P<0.05 level. Values depict mean±SE.

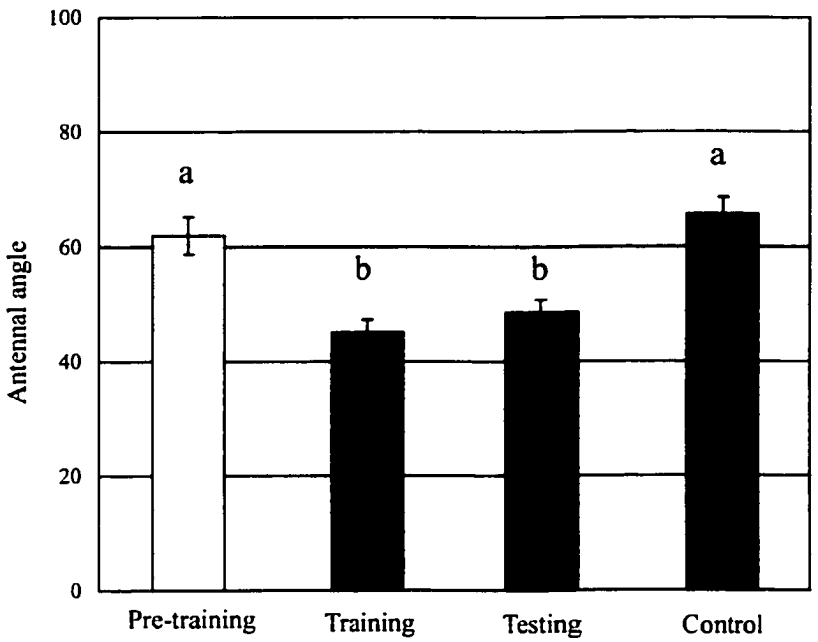
100
80
60
40
20
0
Antennal angle
a
b
b
a
Pre-training
Training
Testing
Control

FIGURE 2-9

Fig. 2-10. Timing of antennal projection responses after stimulation. Timing of initial APRs differed depending on the type of stimuli (AVONA, N=16 for US alone, N=23 for training, N=17 for testing: CS alone, df=2, F=7,57, P<0.002). Timings of antennal projection responses during US alone and training trials (US/CS pairing) were not significantly different (Tukey HSD test, P>0.7). Timing of testing (CS alone) was delayed about 3 sec compared to those of US alone and training (US/CS), showing significant difference from those of US alone and training trials (Tukey test, P<0.02). Values indicate mean±SE.

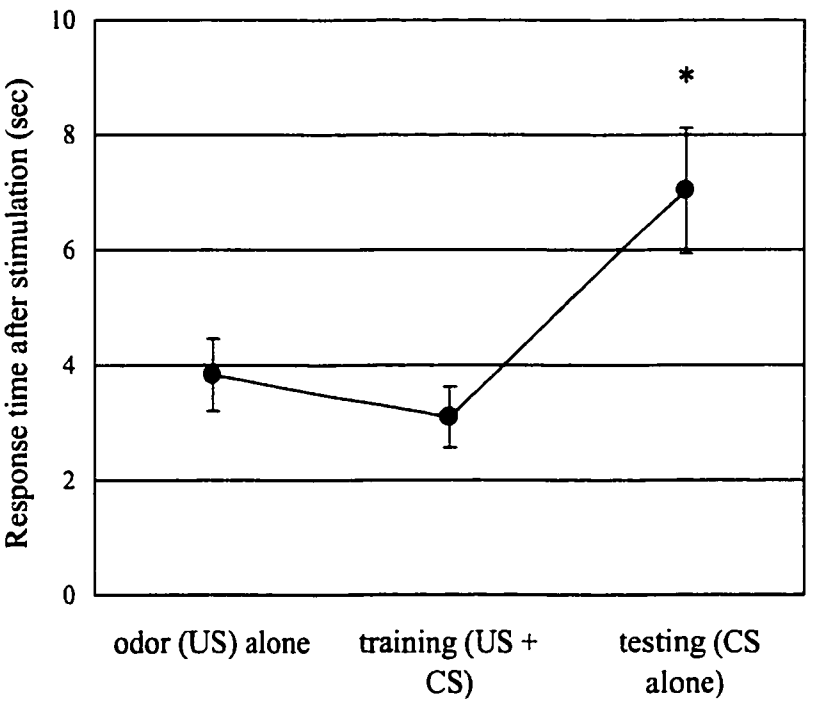
Response time after stimulation (sec)
10
8
6
4
2
0
*
odor (US) alone
training (US + CS)
testing (CS alone)

FIGURE 2-10

Table 2-1. Analysis of patterns of average antennal angles during pretraining, testing and control test (3 trials in each test). Significant differences were found among pretraining, testing and control testing (ANOVA, N=17, df=2, F=16.2, P<0.0001). No differences were found with respect to trials numbers (P>0.7); thus, there was no specific trend of antennal movements as test cockroaches received continuous stimulation in conditioning procedures. Also, no significant interaction between test trials and trial number was found (P>0.8).

	df	*F*	*P*
Test Trials (T)	2	16.2168	<0.0001
Trial Number (N)	2	0.2419	0.7865
T × N	4	0.2728	0.8944

TABLE 2-1

Table 2-2. The analysis of average antennal angles during training trials. Antennal movement patterns during training trials showed no significant difference (ANOVA, N=17, P>0.4). Thus, there was no increase or decrease in antennal projection responses during training.

	df	*F*	*P*
Training trial number (N)	4	0.8755	0.4836

TABLE 2-2

CHPATER III

SPATIAL LEARNING IN THE RESTRAINED AMERICAN COCKROACH, *PERIPLANETA AMERICANA*

Abstract

Spatial learning abilities were tested in restrained cockroaches by observing antennal projection responses towards the positions of a learned visual cue perceived monocularly by one eye in the context of a second stimulus provided to the contralateral eye. Memory of the position of the conditioning stimulus relative to the contralateral reference stimulus was tested by altering the relative positions of the two stimuli. Memory of the conditioning stimulus is retained if the angle between the conditioning stimulus and the contralateral reference stimulus is maintained. The results suggest that during learning the insect recognizes spatial relationships between the conditioning stimulus and the contralateral reference stimulus. Possible mechanisms, such as retinotopic matching versus angular matching, are discussed.

3-1. Rationale

Finding directions, places, and objects that convey meaningful information requires strategies for memorizing landmarks (Collett, 1996). In diurnal foragers, such as ants and bees, spatial orientation relies on the perception of the pattern of polarized light, the position of the sun, as well as salient landmarks of the visual world (Collett et al., 1999). The use of landmarks for navigation has been demonstrated to employ retinotopic matching, in which a series of snapshots of previously memorized images are retinotopically matched with current scenes (Cartwright and Collett, 1982; Dill et al., 1993; Judd and Collett, 1998). Although not demonstrated for insects, mammals are able to form experiential or cognitive maps, as evidenced by the activity of hippocampal neurons that fire only when the animal is at a specific position in its learned environment (Barnes et al., 1997; Poe et al., 2000). Nevertheless, there is evidence that cockroaches might have comparable abilities because they are able learn to relate distant visual cues with hidden targets (Mizunami et al., 1998a) in a manner that is similar to place memory behavior of mammals (Morris, 1984). However, little is known about the underlying neural mechanisms and brain regions that support such spatial learning abilities in insects. This is in part due to the fact that few behavioral paradigms are available for studying spatial learning in an immobilized animal that might then be subjected to intracellular study. This account rectifies this deficit.

In contrast to many behavioral paradigms that rely on movement of the whole animal through space, the antennal motor system of insects can be used to develop novel behavioral paradigms for studying associative memory in the previous chapter and, by

extension, place memory. Antennal motor actions can be elicited by different modalities, including olfactory, tactile, and visual stimuli (Erber et al., 1993). Antennal movements demonstrate active exploration as revealed by restrained honey bees that move their antennae towards the direction of a moving grating. Visual inputs have also been shown to control antennal movements in crickets (Honegger, 1981). Such behaviors have led to experiments that operantly conditioned antennae to touch a target in order to receive a reward (Erber et al., 1993; Kisch and Erber, 1999). In nature, antennal movements towards a defined target, called antennal projection responses (APRs), are used for locating the direction of a stimulus, such as an odor, mate, or predator (Bell, 1981). As shown by the preceding chapter, APRs can be conditioned to point to a visual cue after it's learned association with a food odor.

The present account describes a novel visual association paradigm to demonstrate spatial learning on restrained cockroaches, gain exploiting antennal movements as indicators of learning. The present results answer the question whether restrained insects can learn to recognize spatial relationships between distant cues. The results provide a crucial step towards developing a spatial learning protocol that can be used for intracellular studies of learning and memory.

3-2. Materials and Methods

3-2-1. Animal

Male American cockroaches (*Periplaneta americana*) raised in a laboratory colony maintained on water and IAMS cat food (IAMS, Dayton, Ohio) were used in the

behavioral experiments. Cockroaches were kept at 25±1°C on a 12:12 hour light-dark cycle. Test animals were isolated from colonies. They were maintained individually in small plastic cages and starved for 24 hours before behavioral experiments began. Cockroaches were restrained in plastic tubes, as described in the previous chpater. The test insect was positioned in the center of an arena the walls of which were decorated with a series of green and white LEDs, as schematized in Fig. 3-1A. Experiments were run after spontaneous antennal movements began to occur 10-30 minutes after being placed in the arena, and after body struggling movements abated. Individuals showing no antennal movements to odor stimulation during training trials were rejected.

3-2-2. Arena and stimuli

As described in the previous accounts, experiments were conducted in an arena enclosed within a visually uniform chamber illuminated with an infrared lamp (Fig. 3-1A). A restrained cockroach was positioned in the middle of arena and aligned with respect to five green LEDs that were positioned on the arena wall at 15° intervals to the right of the insect (Fig. 3-1A). The distance from the insect's head to the position of these cues was 15cm. Each diode was given a number, 1- 5. Four white LEDs (E1000, Gilway Technical Lamp Co., Woburn, MA) were positioned on the wall of the arena to the left of the insect. These contralateral reference stimuli (ConRS) were also spaced at 15° intervals with respect to the long axis of the cockroach and named A – D.

Food odors controlled by a solenoid valve were presented through an odor delivery system positioned at green diode 1, as described in the previous chapter. The

duration of the odor stimulation was 1 sec. An exhalation system was placed above the arena to remove odor after each trial (see Chapter II for details). Because cockroaches are insensitive to red light (Seelinger and Tobin, 1981), a red LED placed above the green LED 1 was used to control whether antennal responses were elicited by the sound of the light switches rather than illumination itself.

3-2-3. Training procedures

During the pretraining and training trials, the white diode at position A was switched on. One pretraining trial was followed by 5 training trials. These were succeeded by between 3 to 8 test trials, depending on the experiment. Between the last training trial, and between each succeeding test trial, the animal was covered by a black box while the position of the contralateral white LED was changed from position A to one of the positions A-D. The box was then removed, and the CS (green ipsilateral LED at one of the positions 1-5) was presented for 2 seconds. These protocols are summarized in Fig. 3-1B.

During the training trials, peanut butter odor was emitted under solenoid control at a position coincident with the green LED 1 to provide the unconditioned stimulus (US). This elicited antennal projection responses (APRs). The green LED at position 1 served as the conditioned stimulus (CS). A Grass S88 stimulator controlled the sequence of the US and CS. As described in the previous chapter, the US was given 1 sec after CS onset to provide delayed conditioning. In all experiments, except experiment 1 (see below), pretraining consisted of a 2 second presentation of the green LED at position 1, without

an odor cue, during continuous illumination by the white diode (ConRS) at contralateral position A (referred to as A+1). In the training trials, the green LED at position 1 was coupled with the food odor. The CS and US were presented in the context of continuous contralateral reference illumination by the white LED at position A (A+1+odor). Post training tests (tests) began 5 min after the last training trial and each lasted 1 minute with a 3-minute interval between each test. For the duration of each test, one of the white contralateral LEDs at one of the positions A-D was illuminated. Then one of the green LEDs at one of the positions 1- 5 was presented for 2 seconds. Between each test, the animal was covered with a black box (15×15×20cm) while a white LED was switched on at a new position (A-D) after which the box was removed. After 40 seconds the animal's APR was tested by illuminating the ipsilateral eye with a green LED at a new position 1-5 for 2 seconds. Because of the time required to change the positions of the contralateral visual cues (Fig. 3-1B) three minute ITIs were maintained during test trials.

3-2-4. Experiment 1

Because antennal projection responses (APRs) towards the unchanged position of a visual cue are significantly increased when the visual cue is coupled with a food odor, the questions arises whether the insect would project its antenna towards the green LED if its position was changed. This experiment tested if cockroaches project their right antenna towards the CS when its original location changed. During the test trials, positions of the green LED were changed to 2, 3, or 4. This experiment is the only experiment to omit a contralateral reference stimulus.

3-2-5. Experiment 2

This experiment establishes that a cockroach can project its right antenna towards the unchanged position of the green light cues when the position of contralateral reference stimulus (ConRS) was maintained at the A position. The positions of the ConRS and the CS at position 1 (A+1) were thus maintained throughout this experiment. APRs to the CS at position 1 were scored.

3-2-6. Experiment 3

In this experiment, the position of the CS was changed from position 1 after training, whereas the position of the ConRS was maintained at position A throughout the experiment. During the pretraining, APRs to the green LED at position 1 were tested in the absence of odor cues but in the presence of the ConRS at position A. After training of APRs to the CS+US in the presence of the ConRS associated, insects were then tested with altered position of the CS while maintaining the unaltered ConRS. Thus, insects were tested with A+1, A+2, A+3, and A+4. In the last trial of the test, APRs were tested with A+1, named "A+1 again".

3-2-7. Experiment 4

After training, ConRS positions were changed to from A, to B, C, or D, and then again returned to A, whereas the position of the CS was maintained at position 1. Thus APRs were tested with A+1, B+1, C+1, D+1, and "A+1 again". The sequence of changed positions of the ConRS was randomized, but the first and last trials were always A+1.

3-2-8. Experiment 5

APRs towards changed positions of the CS were tested in conjunction with changes in the positions of the ConRS such that the same as well as different angular relationships between the CS and ConRS were compared with the original angular relationship of the ConRS at position A and the CS at position 1 (see Fig. 3-1A). In the tests, the original angular relationships were preserved when A was shown with 1 (A+1), B with 2 (B+2), C with 3 (C+3), and D with 4 (D+4). Random sequences were tested, after which APRs were tested using the different angular relationships, such as B+3, C+4, and D+5. The final test was APRs towards "A+1 again".

3-2-9. Monitoring and video recording of antenna movements

Antennal movements were recorded with either a 8mm Camcorder (Sony) or a digital video camera (Panasonic) and recorded with a video recorder. The duration of an APR and the frequencies of APRs towards the positions of the green LED were discriminated from spontaneous antennal movements. Digitized images provided the numbers of antennal movements towards the position of a green LED in each trial. Antennal movements were video-recorded for 20 sec after stimulation and were digitized by the Motus program (Peak Performance Technologies Inc. Englewood, CO), which captured images every 167 msec, producing about 105-120 images per trial. From these digitized images, the tip and base of the right antenna of each test cockroach, and the position of the green light cue, was recorded to obtain angle data with respect to the midline of the head. This provided antennal angles and thus quantified antennal movements with respect

different positions of the green LED. Antennal movements in response to the green LED (±2.5°) were counted, allowing the discrimination of active antennal movements from antennal tremor or spontaneous movements.

3-2-10. Scoring antennal projection responses and statistics

Antennal projection responses (APRs) towards green LED positions were scored as '1' if cockroaches project their right antenna towards a green LED cue for 20 sec after stimulation. A score of '0' was if there was no response. The percentages of APRs were calculated by summing all scores during a given trial, divided by the number of trials multiplied by 100. Numbers of antennal movements to green LED illumination were derived from video analyses, as described above.

Non-parametric analytical tests were performed to compare APRs during pretraining and testing. The Friedman test was used to compare APRs within subjects. Once a significant difference was shown, a Wilcoxon signed-rank test (Z statistic) was performed in parallel to compare each value of every trial. The Kruskal-Wallis test (H statistic) was performed to compare the antennal responses between groups. Mann-Whitney U tests (U statistic) were used to test the differences between two groups. The ANOVA test with repeated measures was applied to analyze the significance in numbers of antennal movements to cue positions. In parallel with ANOVA, Tukey HSD tests were performed to compare two measurements of within-groups. To analyze the numbers of antennal movements to green light positions in the same and different angular relationships, paired t-tests were applied due to inadequate sample sizes for ANOVA

tests. Values shown here depict means±standard error (SE). The significance level for all analysis was $P<0.05$.

3-3. Results

3-3-1. Experiment 1

APRs towards the changed position of the green LEDs, 1, 2, 3, 4, and "position 1 again", showed significant differences during the tests (Friedman test, $\chi^2=17.8$, df=5, $P<0.004$). The APR to the green LED at position 1 during the pretraining was below 20 percent. After training, APRs to the green LED at position 1 were significantly increased, showing a significant difference from the pretraining (Wilcoxon signed-rank test, N=15, $Z=2.80$, $P<0.01$). APRs towards the green LED at position 2 during the tests, which was displaced 15° from position 1, were also significantly increased relative to the pretraining ($Z=2.07$, $P<0.04$). APRs to green LEDs at positions 3 and 4, which displaced 30° and 45° from position 1, showed no difference from those in pretraining but the Wilcoxon test also showed that they were not significantly different ($P>0.09$) from the CS at the first two trials (positions 1 and 2). APRs toward the green position 1 at the end of the test showed a significant increase compared to pretraining APRs ($Z=2.67$, $P<0.008$).

The numbers of antennal movements towards each green light position (±2.5°), calculated from digitized images, showed that antennal movements to cue positions could increase significantly during tests compared with pretraining (ANOVA, $F= 7.51$, df=5, $P<0.0001$). For example, antennal movements towards the green LED positions 1 and 2 after training were significantly increased compared to antennal movements during

pretraining (Fig. 3-2; Tukey HSD test, $P<0.05$). However, the numbers of antennal movements to the green LED positions 3 and 4 were not significantly different from the numbers of antennal movements recorded during the pretraining (Fig. 3-2). These results show interesting ambiguities with respect to the animal's recognition of the CS when it is moved from its original trained position. These ambiguities are abolished with the presence of a contralateral reference stimulus, as shown in the next results. These differences are returned to again in the Discussion.

3-3-2. Experiment 2

Using an established learning protocol in the preceding chapter, we tested whether or not cockroaches can learn the position of a food source by its association with a visual cue accompanied by a ConRS in the contralateral visual field (Fig. 3-1A). In this experiment, we tested if cockroaches project their right antenna towards a green light position in the presence of ConRS in which positions of both visual cues, A and 1 (A+1), were fixed throughout the experiment. In this test, APRs of the right antenna towards the green light position after training were significantly increased (solid bars in Fig. 3-3; Wilcoxon signed-rank test, $N=7$, $P<0.02$). Also, the number of antennal movements towards the green light position (±2.5°), counted from image analyses, showed a significant increase during tests compared to the pretraining (hatched bars in Fig. 3-3; t-test, $N=5$, $P<0.02$). This indicates that overall antennal movements towards the odor position after training were significantly increased compared to those during the pretraining.

3-3-3. Experiment 3

In this experiment, the position of the green light was changed while the position of the white ConRS was fixed throughout tests. This regimen tested whether cockroaches could determine the odor position when the ConRS position was fixed at the A position and green light positions were changed during tests. Throughout tests, APRs to different green light positions showed significant differences (solid bars in Fig. 3-4; Friedman test, N=18, χ^2=33.82, df=5, P<0.0001). The first test in response to A+1 (the ConRS A shown during green LED at position 1), revealed a significant increase in APRs compared to the pretraining (Fig. 3-4; Wilcoxon signed-rank test, N=18, Z=2.95, P<0.005). APRs to A+2, A+3, and A+4 were no different from that of pre-test, however (Wilcoxon test, N=18, P>0.1). APRs to "A+1 position again" in the tests showed a significant difference in comparison to tests using A+2, A+3, and A+4 (Wilcoxon signed-rank test, N=18, P<0.05).

The numbers of antennal movements towards the green LED (±2.5°) also showed similar results. ANOVA with repeated measures indicated a significant difference in the numbers of antennal movements to green light positions in this experiments (hatched bars in Fig. 3-4; ANOVA, N=9, F= 9.01, df=5, P<0.0001). Antennal movements towards green light position 1 coupled with ConRS A, A+1, were significantly increased compared to those during the pretraining, or tests using the combinations of A+3 and A+4 (Tukey HSD test, N=9, P<0.01). The numbers of antennal movements to green light positions with other combinations between the positions of ConRS and the green light significantly decreased compared to those to the green LED at position 1 (Tukey HSD

test, N=9, P<0.03), except that the numbers of antennal movements to A+2 was not significantly different from others (Tukey HSD test, N=9, P>0.1). The numbers of antennal movements towards "A+1 again" was significantly increased compared to the pretraining (Tukey HSD test, N=9, P<0.002).

3-3-4. Experiment 4

In this experiment, cockroaches were tested to demonstrate their ability to project an antenna towards the CS at position 1 when the original ConRS position was changed from A to B, to C, and then to D and returned to A again. APRs to the CS at position 1 coupled with the altered positions of the ConRS were significantly different throughout the tests (solid bars in Fig. 5A; Friedman test, N=17, χ^2=38.2, df=5, P<0.0001). APRs towards A+1 and "A+1 again" were significantly different from those of APRs in pretraining (Wilcoxon signed-rank test, N=17, P<0.01). However, APRs elicited by B+1, C+1, and D+1 were not significantly different from APRs in pretraining (Wilcoxon signed-rank test, N=17, P>0.5).

The numbers of antennal movements to the position of the green LED (±2.5°) also showed significant effects throughout this test (ANOVA, N=11, df=5, P<0.0001). Antennal movements towards A+1 and "A+1 again" were significantly increased compared to those during pretraining (Tukey HSD test, N=11, P<0.005). The numbers of antennal movements towards B+1, C+1, and D+1 were not different from those during pretraining (Tukey HSD test, N=11, P>0.3).

Do cockroaches project their antenna to the assumed position of the odor source because its original position was associated with the contralateral reference stimulus rather than the position of the green LED in the presence of the ConRS? To determine this, we analyzed the number of antennal movements towards the fictive odor source at position 2 in B+1, at 3 in C+1, and at 4 in D+1. The angles between the fictive odor and ConRS positions, B+2, C+3, and D+4, were the same as between the green LED and ConRS at position A+1 (see Fig. 1A). The numbers of antennal movements towards the fictive odor position 2 in B+1, 3 in C+1, and 4 in D+1 showed no significant difference from the number of antennal movements at pretraining (hatched bars in Fig. 5B; Tukey HSD test, N=8, P>0.9). Antennal movements towards the green LED at position 1 in A+1 were significantly different from those towards the fictive odor position 2 in B+1, 3 in C+1 and 4 in D+1 (Fig 5B; Tukey HSD test, N=8, P<0.001). Thus, it is unlikely that the contralateral reference stimulus is serving as the CS during training.

3-3-5. Experiment 5

This experiment investigated the ability of cockroaches to project their ipsilateral antenna towards a changed position of the green light source, accompanied by a change of the position of the ConRS. APRs towards A+1, B+2, C+3, and D+4, all which had the same angular relationships showed significant differences from the pretraining (Fig. 3-6A; Wilcoxon signed-ranked test, N=17, P<0.04). However, APRs towards B+3 (N=14), C+4 (N=16), and D+5 (N=14), all of which had angular relationships that were different from the combination of the training stimulus A+1, were not significantly different from APRs

of the pretraining (Fig. 3-6A; Wilcoxon signed-rank test, P=0.14, 0.59, and 0.36, respectively). Within APRs to the same angular relationships, responses to D+4 were significantly different from those towards A+1 and B+2 (Wilcoxon signed-rank test, N=17, P<0.03) but not C+3 (Wilcoxon signed-rank test, N=17, P=0.14). APRs towards B+3 (angle mismatch) showed no difference from C+3 (Wilcoxon signed-rank test, N=14, P=0.067) and D+4 (Wilcoxon signed-rank test, N=14, P=0.46). APRs towards "A+1 again" increased significantly in the last trial of the tests, and were significantly different from APRs of the pretraining (Wilcoxon signed-rank test, N=7, P<0.03).

The numbers of antennal movements to the green light positions when the angle between ConRS and a green light position was maintained differed significantly from the numbers observed in the pretraining (paired t-test, N=14, P<0.05). However, antennal movements to D+4 were not different from those in the pretraining (P=0.36). Antennal movements to green light when there was an angle mismatch such as B+3 (N=12) and D+5 (N=13) were not different from the numbers of antennal movements observed in the pretraining (paired t-test, P>0.01). In addition, antennal movements to C+4 were significantly lower compared to those during the pretraining (paired t-test, N=13, P<0.005).

Based on these results, APRs and numbers of antennal movements towards the same and discrepant angular relationships of the green light and ConRS were pooled to compare overall behaviors in this experiment. Fig. 3-6B elaborates on the patterns of APRs and the numbers of antennal movements towards the same and discrepant angular relationships compared with the pretraining. In this experiment, APRs to the green light

showed a significant influence by the ConRS, being generally maintained when the angles between the green light and ConRS were maintained (Kruskal-Wallis test, N=53, 68, and 44 for pretraining, same, and different angular relationships, respectively, H=40.95, P<0.0001). The average APRs to green light positions when angular relationships between the ConRS and green light positions were maintained were about 70% (Fig. 3-6B). This was significantly different from APRs in response to the green light positions when the angle between these and ConRS were altered (Mann-Whitney U test, U=746, P<0.0001). Also, compared to APRs towards A+1 in the tests (N=21) in which positions of visual cues were not changed throughout tests shown in Experiment 2 (Fig. 3-3), APRs towards green light positions in the same angular relationships in this experiment (N=68) showed no significant difference (Mann-Whitney U test, U=640, P=0.47). APRs during pretraining and towards green light positions in different angular relationships compared to A+1 showed no difference (Mann-Whitney U test, U=1162.5, P>0.9). In accordance with results from the APRs, the numbers of antennal movements to green light positions in same angular relationships with ConRS significantly increased in comparison with those in the pretraining and to green light positions in different angular relationships (t-test, P<0.04).

3-4. Discussion

3-4-1. Retinotopic matching versus spatial relationship matching

Experiment 1 shows interesting ambiguities. The animal can remember the original position of the CS at position 1, and shows strong APRs to the CS when it is displaced

forward by 15° to position 2. When the CS is rotated further forwards, there are fewer antennal movements and APRs diminish. This suggests that the position of the CS can be shifted across the retina by about 30° after which its associative relevance, namely for food odor, becomes ambiguous. This suggests that retinal matching might operate with regard to recognition of the CS as a putative odor source. However, the subsequent experiments 2-4 appear to demonstrate that in the presence of spatial cues to the contralateral eye, cockroaches not only associate visual cues with food odors presented at the same location, but a shift of the CS across the retina ceases to be ambiguous when the ConRS is held at the same location, or vice versa. Thus, when a reference cue is stabilized on the contralateral retina, APRs towards a CS shifted by more than 15° across the ipsilateral retina (Experiment 3; A+2 in Fig. 3-4) showed no difference from pretraining. This indicates that the spatial reference stimuli in the contralateral visual fields play a crucial role in the recall of the original position of the CS and that shifts in this position render the CS meaningless.

Does such angular matching suggest that the recognition of the learned CS is a function of retinotopic matching; namely, a point-to-point matching of the visual cues present during training on the contralateral and ipsilateral compound eyes? Evidence speaking against this comes from experiments in which the position of the CS is changed in conjunction with a changed position of the ConRS. When the arc distances (angle) between the two stimuli are kept the same the animal can project its antenna to the new location of the CS (Fig. 3-6A). However cockroaches did not respond to the changed positions of the CS when the arc distances were different from that during training (e.g.

B+3, C+4, and D+5 in Fig. 3-6A). This suggests that recognition of the CS in the context of a second visual cue relies on the recognition of angular matching rather than retinotopic matching.

Does this occur in nature? Rust et al. (1976) showed that cockroaches turn their heads towards a pheromone source in order to facilitate antennal scanning towards the directions of the odor plume. This behavior indicates that head movements follow antennal movements and that by realigning their antennae insects achieve greater precision of information about an odor source (Murlis, 1992). However, as far as we are aware, the role of visual cues in such olfactory-driven behaviors has not been investigated.

3-4-2. Behavioral and neural correlates of spatial learning

Up to date, little is known about neural mechanisms underlying spatial learning, even though behavioral evidence suggests that spatial learning is crucial to survival (von Frisch, 1967; Collett, 1996). But where and how are memory templates of visual scenes learned, stored, and compared with current visual images? One requirement for investigating underlying mechanisms, using electrophysiological methods, is to have behavioral paradigms that mimic spatial learning on restrained animals that can in some manner indicate recognition of direction and spatial context even when subject to intracellular recordings.

In this regard, the current study is the first to establish spatial learning abilities in a restrained insect by using antennal movement patterns that provide reliable behavioral indicators. Evidence from lesion studies suggests that it is the mushroom bodies that play

a crucial role in visual associative and spatial learning (Mizunami et al., 1998a). Hopefully, when adapted for intracellular recordings, the present behavioral paradigm should provide new insight into mechanisms of spatial learning in insects.

Fig. 3-1. The spatial learning paradigm. **A.** The restrained cockroach is placed in the center of the arena. White LEDs (A-D) and green LEDs (1-5) subtend its left and right visual fields, respectively. In any trial (except in experiment 1, see text) one of the white LEDs serves as the contralateral reference stimulus (ConRS) and is illuminated throughout that trial. LEDs are positioned at 15° intervals with respect to the middle of the cockroach's head. Each white LED is labeled A-D. Emission of the food odor is at the green LED position 1. **B. C.** Experimental procedures. Pretraining consisted of one trial in which the positions of the ConRS and green light were fixed at A and 1, respectively (referred to in the text as A+1). The ConRS was on for the whole trial, during which the green LED was illuminated for 2 seconds. No odorants were presented in these initial tests. Training trials consisted of five presentations during which the ConRS A was illuminated throughout and the green LED (CS) illuminated for 2 second, with the odor present during the second half of the CS. During tests only visual cues were provided. The positions of the ConRSs and green LEDs could be variously changed. The CS was present for 1-5 seconds during continuous illumination by the ConRS. Projection responses of the ipsilateral antenna to illuminated green LEDs were scored in each trial.

FIGURE 3-1

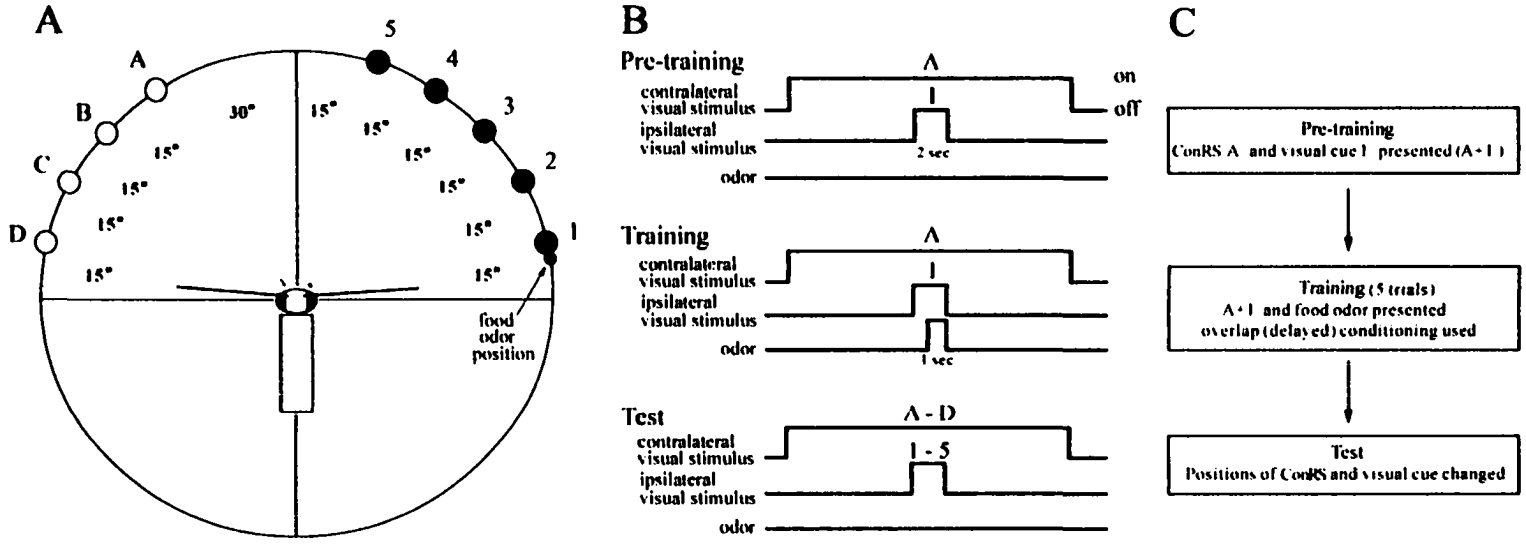
A
5
4
3
2
1
A
B
C
D
30°
15°
15°
15°
15°
15°
15°
15°
15°
15°
15°
food
odor
position
B
Pre-training
A
1
on
off
contralateral
visual stimulus
ipsilateral
visual stimulus
2 sec
odor
Training
A
1
contralateral
visual stimulus
ipsilateral
visual stimulus
odor
1 sec
Test
A - D
1 - 5
contralateral
visual stimulus
ipsilateral
visual stimulus
odor
C
Pre-training
ConRS A and visual cue 1 presented (A+1)
Training (5 trials)
A+1 and food odor presented
overlap (delayed) conditioning used
Test
Positions of ConRS and visual cue changed

Fig. 3-2. Antennal projection responses (APRs) and numbers of antennal projections in response to changed positions of green LEDs without a contralateral reference. APRs to LED positions 1 and 2 were significantly different from pretraining (Wilcoxon signed-rank test, N=15, $P<0.04$). APRs to LED position 3 and 4 were not different from pretraining ($P>0.1$). APRs to green LEDs at position 1 again (the last posttraining trial showed significantly different response to APRs during pretraining ($P<0.01$). Common letters above the solid bars represent no significant differences. Different symbols represent differences at a significance level of 0.05. The numbers of antennal movements (hatched bars) to green light positions 1, 2, and "green light 1 again" were significantly different from that of pretraining (Tukey HSD test, N=9, $P<0.05$). The numbers of antennal movements to green light at positions 3 and 4 did not differ from pretraining ($P>0.9$). Asterisks above hatched bars indicate significant difference from pretraining. Values represent means±standard errors (SE). Each schematic diagram above graph is aligned to its relevant column and represents the relevant test. A positive responses I indicated where the right antenna points towards the position of the green LED.

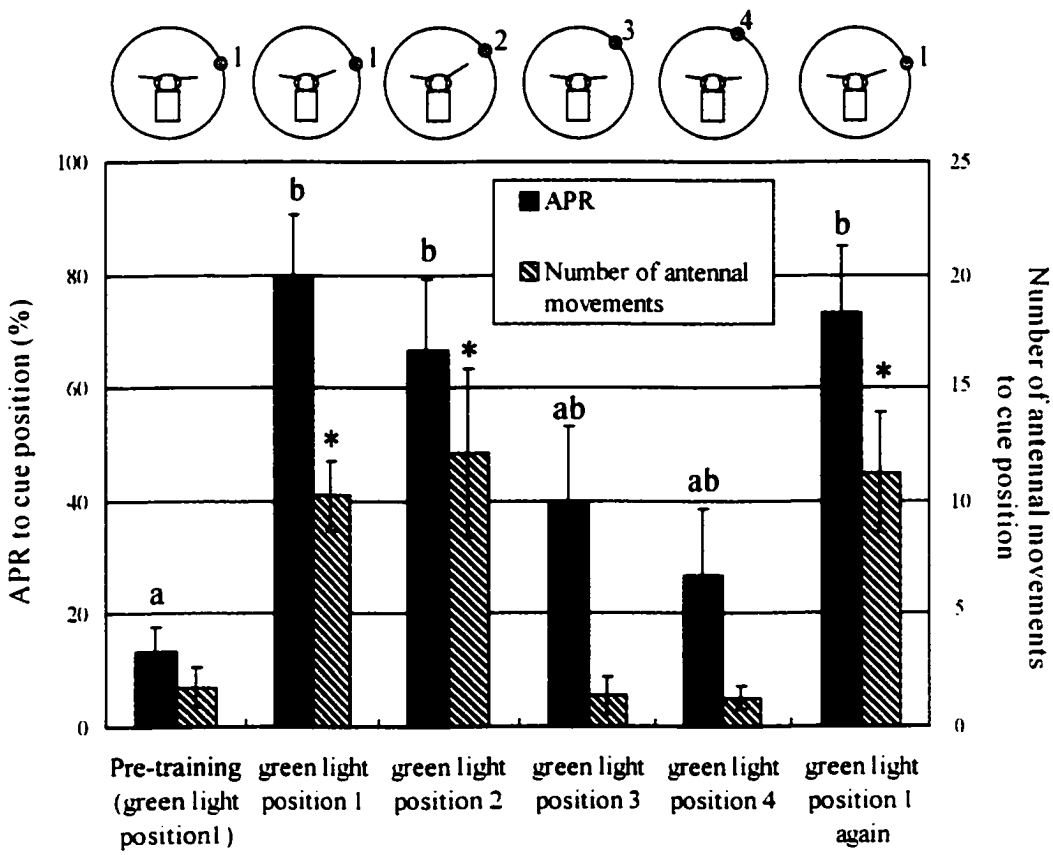
1
1
2
3
4
1
APR
Number of antennal movements
APR to cue position (%)
Number of antennal movements to cue position
100
80
60
40
20
0
25
20
15
10
5
0
a
b
b
ab
ab
b
*
*
*
Pre-training (green light position1)
green light position 1
green light position 2
green light position 3
green light position 4
green light position 1 again

FIGURE 3-2

Fig. 3-3. APRs and number of antennal movements to the green light position 1 in which the positions of ConRS and green light, A+1, were maintained throughout the experiment. APRs elicited by post-training tests (solid bars) were significantly different from those of pretraining (Wilcoxon signed-rank test, $N=7$, $P<0.02$). The number of antennal movements to the green LED (hatched bars) also showed significant increase during the post training tests compared to those during pretraining (t-test, $N=5$, $P<0.02$). Asterisks above hatched bars indicate significant difference from pretraining. Values depict means±SE. Each schematic diagram above graph is aligned with each test in x-column. Right antenna pointing towards the positions of green lights depicts positive responses.

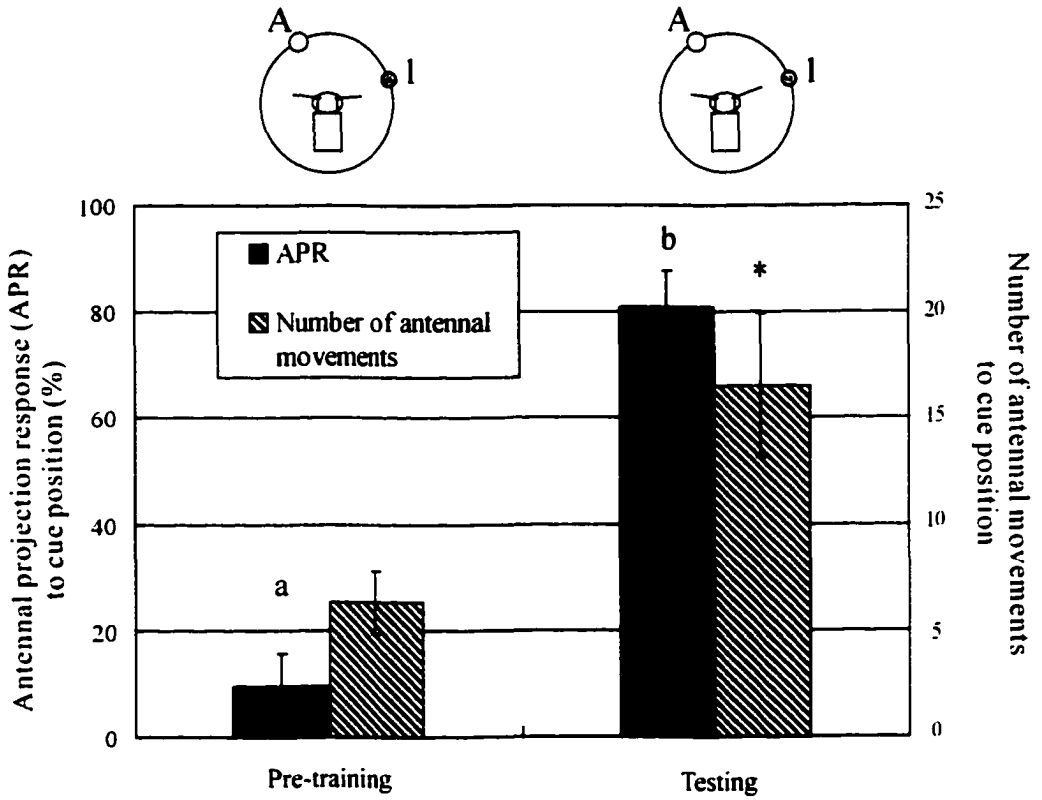
A
l
A
l
100
80
60
40
20
0
25
20
15
10
5
0
APR
Number of antennal movements
a
b
*
Antennal projection response (APR) to cue position (%)
Number of antennal movements to cue position
Pre-training
Testing

FIGURE 3-3

Fig. 3-4. APRs (solid bars) and number of antennal movements (hatched bars) to green light positions in which green light positions were changed after training trials and ConRS position was fixed. APRs to green light position 1 during the unchanged ConRS at position A, A+1 and "A+1 again", showed significant difference from each other (Wilcoxon signed-rank test, N=18, $P<0.05$). The numbers of antennal movements to the LED at position 1 was significantly different from pretraining (Tukey HSD test, N=9, $P<0.03$). The numbers of antennal movements to the LED at position 2, 3, and 4 were not different from that of pretraining ($P>0.1$). Values depict means±SE. Different symbols above the solid bars indicate difference with each other at a significance level of 0.05. Asterisks above hatched bars indicate significant difference from pretraining. The schematic diagram aligned above each column summarizes the behavioral responses. A right antenna pointing towards the positions of green lights indicates a positive response.

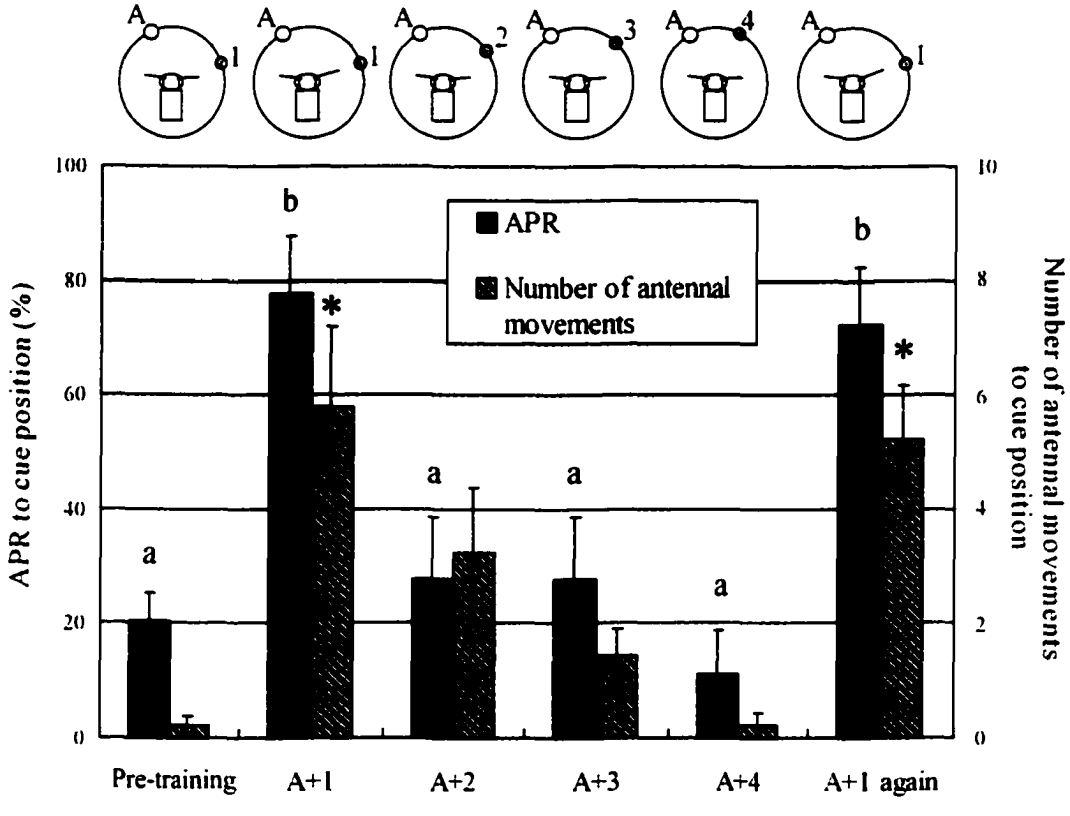
A
1
2
3
4
APR
Number of antennal movements
APR to cue position (%)
Number of antennal movements to cue position
100
80
60
40
20
0
10
8
6
4
2
0
a
b
a
a
a
b
*
*
Pre-training
A+1
A+2
A+3
A+4
A+1 again

FIGURE 3-4

Fig. 3-5. APRs and number of antennal movements to the LED at position 1 (A) and the number of antennal movements to fictive odor position 2 in B+1, 3 in C+1, and 4 in D+1 (B). **A.** APRs (solid bars) to the green LED at position 1 in conjunction with a ConRS at position A. APRs to position 1 at A+1 and to position 1 at "A+1 again" were significantly different from those in pretraining (Wilcoxon signed-rank test, N=17, $P<0.01$). APRs to the green LED at position 1, in conjunction with a ConRS at positions B, C, or D, respectively named B+1, C+1, and D+1, were not different from those in pretraining (Wilcoxon signed-rank test, N=17, $P>0.5$). The number of antennal movements to the green LED at position 1 (hatched bars) in A+1 and "A+1 again" were significantly different from those in pretraining (Tukey HSD test, N=11, $P<0.005$). The number of antennal movements towards the green LED 1 in B+1, C+1 and D+1 were not significantly different from those in pretraining (Tukey HSD test, N=11, $P>0.3$). **B.** The number of antennal movements towards the fictive odor source (that is. the fictive green LED) at positions 2, 3, and 4 during B+1, C+1, and D+1, respectively. The numbers of antennal movements to the fictive odor position 2 in B+1, 3 in C+1, and 4 in D+1 were not different from pretraining (Tukey HSD test, N=8, $P>0.9$), whereas these numbers differed from that of the actual odor at position 1 in A+1 (Tukey HSD test, N=8, $P<0.001$). Values depict mean±SE. Different symbols above the bars indicate significant difference betweeb each other at a significance level of 0.05. Asterisks above the hatched bars in A indicate significant difference from pretraining.

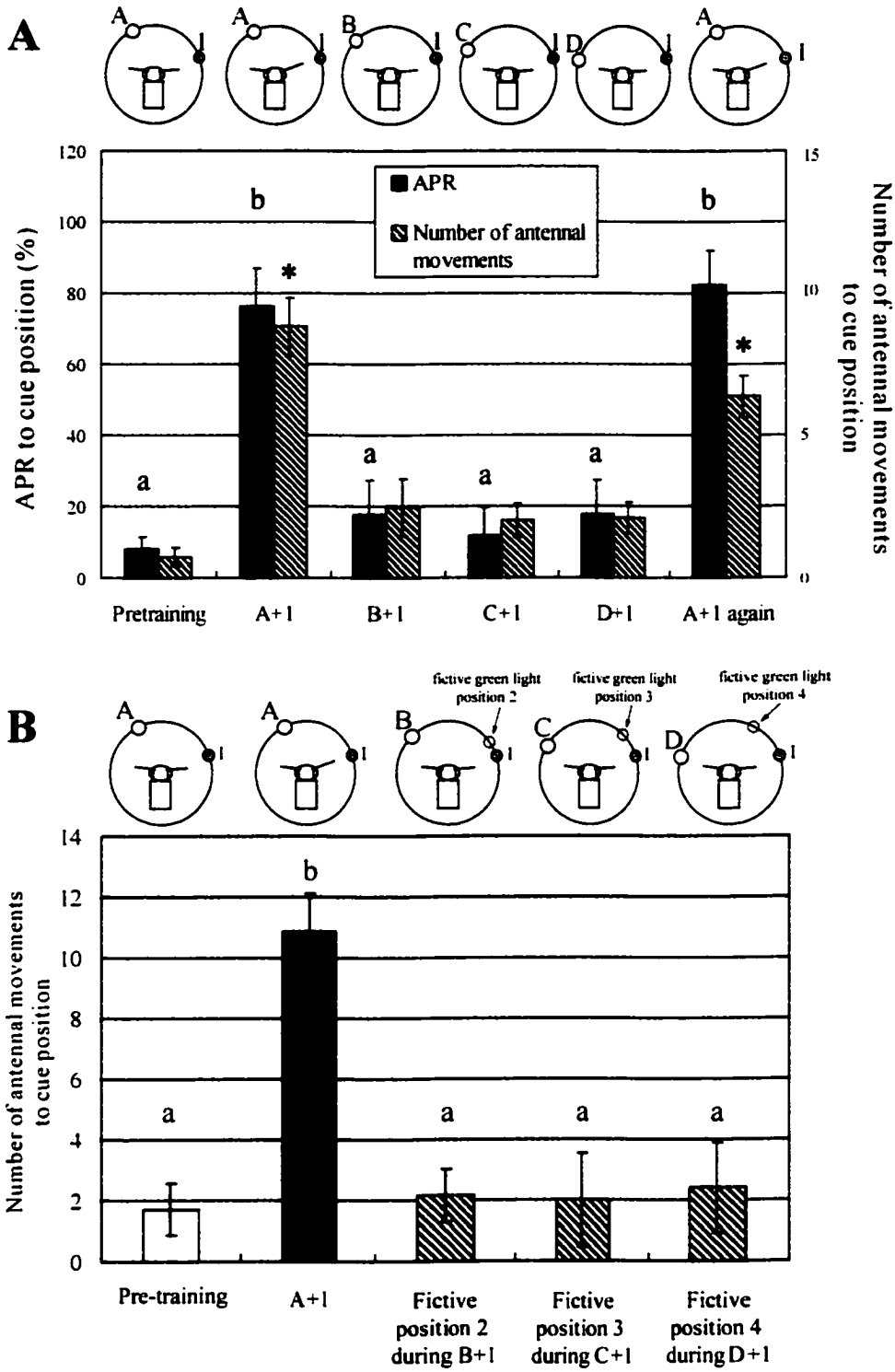
A
A
A
B
C
D
A
APR
Number of antennal movements
APR to cue position (%)
Number of antennal movements to cue position
120
100
80
60
40
20
0
15
10
5
0
a
b
a
a
a
b
*
*
Pretraining
A+1
B+1
C+1
D+1
A+1 again
B
fictive green light position 2
fictive green light position 3
fictive green light position 4
Number of antennal movements to cue position
14
12
10
8
6
4
2
0
a
b
a
a
a
Pre-training
A+1
Fictive position 2 during B+1
Fictive position 3 during C+1
Fictive position 4 during D+1

FIGURE 3-5

Fig. 3-6. The percentage of APRs (solid bars) and the number of antennal movements (hatched bars) to positions of the green LED in which the positions of the LEDs and ConRS were changed together to either maintain or to alter their angular relationships. Angles between B and 2, C and 3, and D and 4 were the same as those between A and 1, whereas B and 3, C and 4, and D and 5 were different (see Fig. 3-1A). **A.** APRs to the green LED positions having the same angular relationships showed a significant difference from pretraining (Wilcoxon signed-rank test, N=17, P<0.04), whereas APRs to green LED positions having different angular relationships with the ConRSs (i.e., B+3 [N=14], C+4 [N=16], and D+5 [N=14]) compared to A+1 showed no difference from APRs at pretraining (P>0.1). APRs to the green LED position 1 in the last trials ("A+1 again") were increased compared to pretraining (P<0.03). The numbers of antennal movements to green LED positions when these had the same angular relationships with the ConRS as between A and 1 were significantly increased compared to pretraining (paired t-test, N=14, P<0.05), with the exception of movements to D+4 which were not significantly different from pretraining (paired t-test, N=14, P>0.3). The numbers of antennal movements to green LED positions having different angular relationships with the ConRS as compared with that between A and I (namely, B+3 [N=12] and D+5 [N=13]), were not significantly different from pretraining (paired t-test, P>0.1). The number of antennal movements to C+4 (N=13) showed a significant decrease compared to pretraining (paired t-test, P<0.005). **B.** APRs and numbers of antennal movements shown pooled with respect to pretraining (N=53), same angular relationships (N=68), and different angular relationships (N=44). APRs to green LED positions having the same

angular relationships with their respective ConRS showed a significant increase compared to those of pretraining and green LED positions when these had a different angular relationship with their respective ConRS (Mann-Whitney U test, $P<0.0001$). Same symbols above the solid bars indicate no statistical difference of antennal responses. Asterisks above the hatched bars indicate significant differences from pretraining at a significance level of 0.05. Values are means±SE.

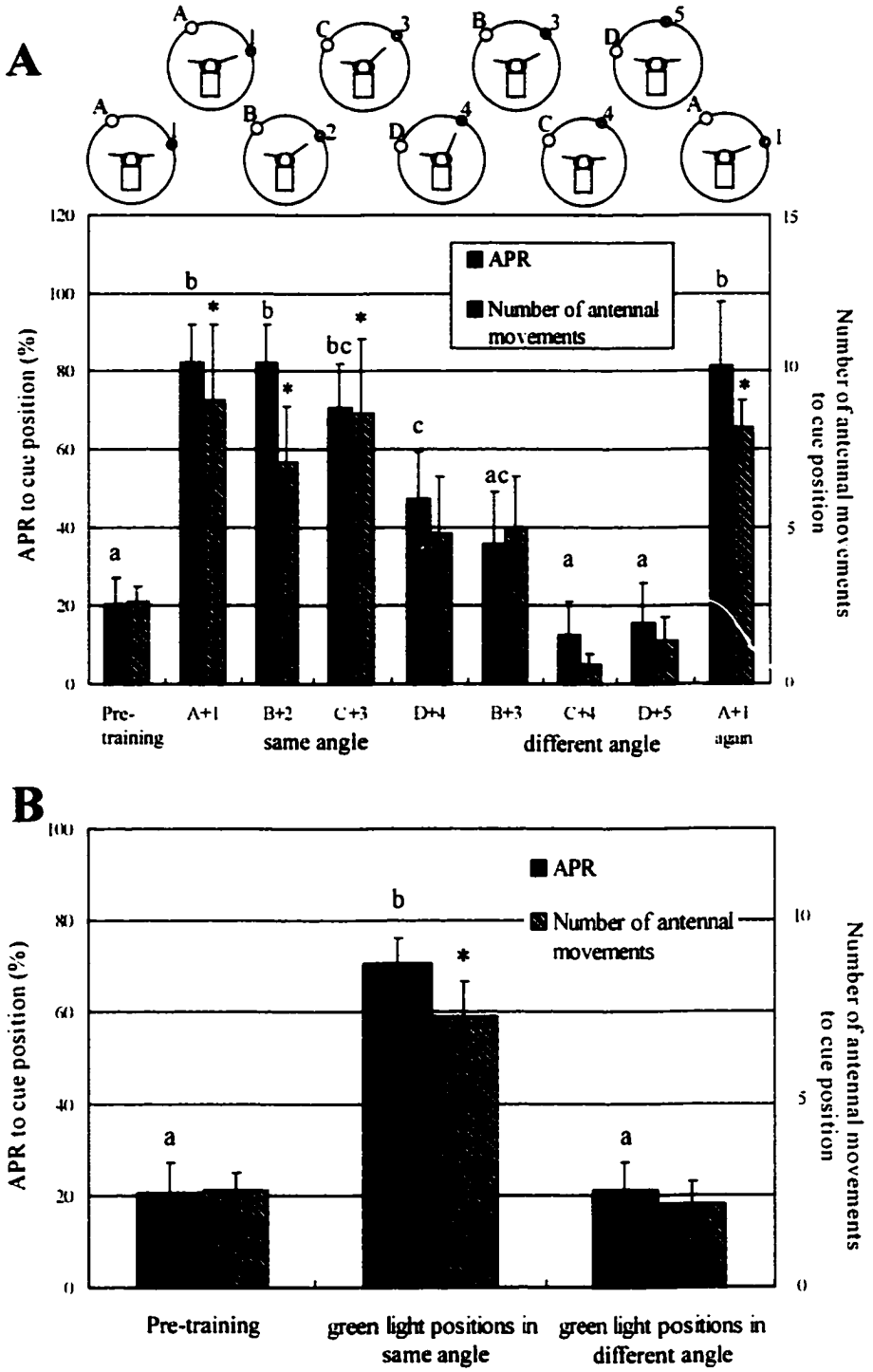
A
APR
Number of antennal movements
APR to cue position (%)
Number of antennal movements to cue position
Pre-training
A+1
B+2
C+3
D+4
B+3
C+4
D+5
A+1 again
same angle
different angle
B
APR
Number of antennal movements
APR to cue position (%)
Number of antennal movements to cue position
Pre-training
green light positions in same angle
green light positions in different angle

FIGURE 3-6

CHAPTER IV

SUMMARY AND FUTURE RESEARCH

4-1. Overview and Significance of the current research

Up to the present, cockroaches have been occasionally used to study learning ability in a maze in which cockroaches learn to avoid one side arm associated with electric shock (Longo, 1964; Barraco et al., 1981) as well as neural mechanisms underlying operant learning at the level of their thoracic ganglia (Horridge, 1962; Eisenstein and Cohen, 1965; Chen et al., 1970). However, little research has investigated classical conditioning in restrained conditions. Recent neuroanatomical evidence based on cockroach brain architectures (Mizunami et al., 1998b; Strausfeld and Li, 1999) and studies on mushroom body functions in place memory (Mizunami et al., 1998a), suggests that cockroaches provide a suitable model system to study learning mechanisms. Compared with honey bees and *Drosophila*, where neural mechanisms underlying learning and memory have been extensively studied, cockroaches possess a larger brain and, compared with bees, are behaviorally independent of a colony. In these aspects, the fact that the cockroach is experimentally robust, allows the study of systems and mechanisms underlying memory formation. With these merits as a model insect, new learning paradigms using behaving cockroaches will provide novel insights about the neural mechanisms underlying learning and memory in the brain.

Studies described in Chapters II and III demonstrate that cockroaches in the restrained condition show reliable patterns of learned behaviors as evidenced by their

antennal movements towards cues. In the visual associative learning paradigm, cockroaches showed significant changes in learned antennal projection responses to visual cues associated with food odors after multiple training trials. The learned behaviors persist up to 72 hours, indicating long-term memory. Also, cockroaches did not associate pure air puffs with visual cues, indicating that cockroaches recognize the visual cue as a food source not mechanosensory input itself. This learning paradigm can in the future be extended to test classical conditioning to tactile stimulation as well as learning experiments that are based on context dependent associations.

Spatial learning is also a vitally important behavior in most animals that must remember the locations of food sources relative to landmarks and must navigate from and back to their starting point, such as a burrow or nest. Spatial learning has been tested in freely moving cockroaches (Mizunami et al., 1998a), showing that after only a few trials cockroaches can learn to associate distant spatial cues (both olfactory and visual) with a hidden location that provides safety from an otherwise noxious (heated) surface environment. In the present study, antennal projection behaviors are strongly suggestive of retinotopic matching by binocular retinotopic angular matching in conditions where the test stimulus to the ipsilateral eye is associated with a white light reference stimulus given to the contralateral eye. This means that cockroaches showed significant antennal projection responses to visual cues not only when a position of the visual cue and spatial reference cue were exactly matched during training trials, but also when relative angles between visual cue and spatial reference cue were matched during training trials.

An important question is whether cockroaches employ types of computational tools to find food sources in nature. For example, behavioral experiments are needed to test whether cockroaches can recognize the position of a visual cue when the positions of the spatial reference cue and visual cues are reversed. In the present study, only one position of the visual cue was used during training. Various positions of visual cues associated with food odors during training can be also used to study if there are significant effects on learning performance.

Overall, restrained cockroaches provide reliable subjects for testing their learning abilities, such as cue association and place learning. Because all of the paradigms described and discussed so far have been used on restrained animals, these paradigms will be most useful for investigating neuronal plasticity at the level of the brain. In principle, they can be employed during intra and extracellular recordings on similarly restrained animals.

4-2. The quest for neural correlates of learning and memory

Most learning paradigms so far have focused on olfactory learning to study learning mechanisms of classical conditioning. In fact, learning paradigms in honey bees (Menzel, 2001) and *Drosophila* (Heisenberg et al., 1985; McGuire et al., 2001; Dubnau et al., 2001) have been providing clues about the functional roles of two specific neuropils, the antennal lobes and the mushroom bodies. However, the mushroom bodies, which many considered to be crucial center for olfactory learning and memory, have been shown to process many different sensory modalities, such as olfactory, visual, tactile, acoustic, and

mechanosensory signals. The mushroom bodies have been shown to play a crucial role in spatial learning (Mizunami et al., 1998a) as well as context learning (Liu et al., 1999) in which this neuropil must configure incoming information in some way with respect to salient phenomena. Modification of mushroom body neuron responses with respect to ambient sensory contexts has been directly shown in cockroaches: efferent neurons from the mushroom body lobes that respond, for example, to an acoustic signal do so differently depending upon whether the signal is preceded by olfactory or tactile stimulation.

In this respect, the current study on visual/olfactory associative learning in restrained cockroaches could lead to the first experiments that directly study neural correlates of associative learning in mutimodal situations. A working hypotheses that suggests the involvement of the mushroom bodies and antennal lobes in olfactory/visual associative learning could be initially approached using targeted and highly restricted ablation studies as by Mizunami et al. (1998a) to show the involvement of the pedunculus and medial lobes, but not the vertical lobe, in place memory. In *Drosophila*, chemical ablation has been used to delete neuroblasts that give rise to the mushroom bodies (de Belle and Heisenberg, 1994). Such experiments result in the loss of the ability to form olfactory associative memory, although the experiments do not prove the necessary and sufficient role of the mushroom bodies in such memory formation. Possibly, similar targeted ablation experiments, using hydroxyurea (HU), might allow specific regions of the brain, such as specific populations of mushroom body neurons, to be deleted at an early nymphal stage. Chemical ablation techniques using HU have been used to on larval

and pupal honey bees to delete parts of their mushroom bodies (Malun, 1998) and on early embryonic *Drosophila* (de Belle and Heisenberg, 1994), as discussed above.

Recent studies on *Drosophila*, cockroaches, and honey bees have shown that their lobes are divided into discrete subdivisions, each with its own afferent supply and efferent output. Do these different components support different learning and memory functions? If HU ablation techniques are to provide useful information, then these will have to be refined so as to delete specific subdivisions and not others. Also, unlike experiments by Malun (1998), the method has to be refined such that deletions are bilaterally symmetric and can be repeated from experiment to experiment.

Another challenge is to answer questions that address the relationships between the antennal lobes, and the optic lobes, with the mushroom bodies in the visual/olfactory associative learning. In locusts, repeated odor presentation induced synchronized oscillation in neurons of olfactory lobes and mushroom bodies, suggesting that this neuronal activity would be important for animals to identify or localize odor sources (Stopfer and Laurent, 1999). Hence, precise and simultaneous firing of subsets of projection neurons in antennal lobes could facilitate computation of the mushroom bodies in identifying learned information. Does this phenomenon also occur in cockroaches, and would similar repetitive stimulation of the visual system provide comparable oscillations? Such studies must, however, employ intracellular recording techniques.

Using paradigms described here in chapters II and III, questions about the functional relationships between the antennal lobes and the mushroom bodies in integrating both olfactory and non-olfactory information can be tested using

electrophysiological recordings from their respective neurons, and from neurons connecting these centers with each other. Focused injection of GABA antagonists (Christensen et al., 1998; MacLeod et al., 1998), such as pictrotoxin, into the antennal lobes and mushroom body areas during learning, might also provide information about the possible roles of inhibitory neurons, already identified by immunocytology in several insect species (moths: Hoskins et al., 1986; Hildebrand et al., 1992; cockroaches: Nishino and Mizunami, 1998; Strausfeld and Li, 1999a; honey bees: Bicker et al., 1985; Grunewald, 1999; locusts: Leitch and Laurent, 1996), in memory acquisition and consolidation. Little is, however, studied about functional roles of these neurons in learning and memory, even though the anatomical and physiological evidence of GABAergic neurons in antennal lobes and mushroom body areas provide a possible role in shaping Kenyon cell responses (Nishino and Mizunami, 1998; Strausfeld and Li, 1999a; Stopfer and Laurent, 1999).

One long-term goal will be to obtain intracellular recordings from mushroom body outputs (the efferent neurons), which previous studies have already shown respond to multimodal stimulation (Li and Strausfeld, 1997, 1999). Do these neurons alter their multimodal sensitivity during learning, as has been shown for the Pe1 neuron in the honey bee (Mauelshagen, 1993)? Because such efferent neurons alter their tuning properties depending on the background or salient modality, it is likely that they would show plastic responses if the mushroom bodies indeed support learning and memory. However, it should be cautioned that thus far evidence for this is still indirect and comes mainly from the fruitfly *Drosophila* (Zars et al., 2000; Pascual and Preat, 2001).

How can the learning paradigm described here be used to determine changes in identified neurons of the mushroom bodies? Preliminary studies suggest that antennal responses can be monitored not from antennal movements but from the activity of nerves and muscles that mediate such movements. In order to accomplish this, extracellular recording from antennal motor neurons or antennal muscles will be performed after cutting the main antennal muscle attachments so as to reduce indirect brain movements. Afterwards, extracellular hook electrodes can be used to record "fictive" antennal movements during learning paradigms described here, where the arena is replaced in the electrophysiological rig by a parabolic screen.

In addition to these planned experiments, a pilot study using antennal projection responses has shown that antennal behaviors can be used to monitor olfactory aversion learning. Furthermore, the antennal projection response can be used in animals in which the supraesophageal brain is split into two halves. This method is currently being used to investigate gene expression–that is, up- or downregulation–associated with learning and memory. One side of the brain serves as the experimental half and is trained while the other untrained brain half serves as the control. Eventually, proteins that are detected in association with memory acquisition may be used to provide appropriate antisera that could be used to reveal in what brain areas such proteins are upregulated. In conclusion, these ongoing and future studies on neural mechanisms of visual associative learning, all of which are or will use the learning paradigms described here, should provide new insights into neural mechanisms underlying visual/olfactory associative and possibly suggest which brain areas are involved.

REFERENCES

Adams, M. E. and O'Shea, M. (1983). Peptide cotransmitter at a neuromuscular junction. *Science* 221, 286-289.

Alberini, C. M. (1999). Genes to remember. *J. Exp. Biol.* 202, 2887-2891.

Allard, R. A. and R., P. D. (1996). Learning of leaf shape by pipevine swallowtail butterflies: A test using artificial leaf models. *J. Insect Behav.* 9, 961-967.

Bach, M. E., Hawkins, R. D., Osman, M., Kandel, E. R. and Mayford, M. (1995). Impairment of spatial but not contextual memory in CaMKII mutant mice with a selective loss of hippocampal LTP in the range of the theta frequency. *Cell* 81, 905-915.

Balderrama, N. (1980). One trial leanring in the American cockroach, *Periplaneta americana*. *J. Insect Physiol.* 26, 499-504.

Barnes, C. A., Suster, M. S., Shen, J. and McNaughton, B. L. (1997). Multistability of cognitive maps in the hippocampus of old rats. *Nature* 388, 272-275.

Barraco, D. A., Lovell, K. L. and Eisenstein, E. M. (1981). Effects of cycloheximide and puromycin on learning and retention in the cockroach, *P. americana*. *Pharmacol. Biochem. Behav.* 15, 489-494.

Beer, R. D., Chiel, H. J., Quinn, R. D. and Ritzmann, R. E. (1998). Biorobotic approaches to the study of motor systems. *Curr. Opin. Neurobiol.* 8, 777-782.

Bell, W. J. (1981). Pheromones and behaviour. In *The American cockroach* (ed. W. J. Bell and K. G. Adiyodi), pp. 371-397. London, New York: Chapman and Hall.

Bernays, E. A. (1993). Aversion learning and feeding. In *Insect learning: Ecological and evolutionary perspectives* (ed. P. D. R. and A. C. Lewis), pp. 1-17. New York, London: Chapman & Hall.

Best, P. J., White, A. M. and Minai, A. (2001). Spatial processing in the brain: the activity of hippocampal place cells. *Annu. Rev. Neurosci.* 24, 459-486.

Bicker, G., Schafer, S. and Kingan, T. G. (1985). Mushroom body feedback interneurones in the honeybee show GABA-like immunoreactivity. *Brain Res.* 360, 394-397.

Bitterman, M. E. (1996). Comparative analysis of learning in honeybees. *Anim. Learn. Behav.* 24, 123-141.

Bitterman, M. E., Menzel, R., Fietz, A. and Schafer, S. (1983). Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *J Comp Psychol* 97, 107-119.

Boeckh, J., Distler, P., Ernst, K. D., Hosl, M. and Malun, D. (1990). Olfactory bulb and antennal lobe. In *Chemosensory Information Processing* (ed. D. Schild), pp. 201-227. Berlin: Springer.

Boeckh, J., Ernst, K. D., Sass, H. and Waldow, U. (1984). Anatomical and physiological characteristics of individual neurons in the central anatennal pathways of insects. *J. Insect Physol.* 30, 15-26.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118, 401-415.

Braun, G. and Bicker, G. (1992). Habituation of an appetitive reflex in the honeybee. *J. Neurophysiol.* 67, 588-598.

Butler, R. and Horridge, G. A. (1973). The electrophysiolgy of the retina of *Periplaneta americana*. I. Changes in receptor acuity upon light/dark adaptation. *J. Comp. Physiol.* 83, 263-278.

Byers, D., Davis, R. L. and Kiger, J. A., Jr. (1981). Defect in cyclic AMP phosphodiesterase due to the dunce mutation of learning in Drosophila melanogaster. *Nature* 289, 79-81.

Campbell, H. R. and Strausfeld, N. J. (2001). Learned discrimination of pattern orientation in walking flies. *J. Exp. Biol.* 204, 1-14.

Cartwright, B. A. and Collett, T. S. (1982). How honey bees use landmarks to guide their return to a food source. *Nature* 295, 560-564.

Casadio, A., Martin, K. C., Giustetto, M., Zhu, H., Chen, M., Bartsch, D., Bailey, C. H. and Kandel, E. R. (1999). A transient, neuron-wide form of CREB-mediated long-term facilitation can be stabilized at specific synapses by local protein synthesis. *Cell* 99, 221-237.

Chapman, R. F. (1998). Vision. In *The insects: Structure and function* (ed. R. F. Chapman), pp. 587-609. Cambridge, UK: Cambridge University Press.

Chen, C. N., Denome, S. and Davis, R. L. (1986). Molecular analysis of cDNA clones and the corresponding genomic coding sequences of the Drosophila dunce+ gene, the structural gene for cAMP phosphodiesterase. *Proc. Natl. Acad. Sci.* 83, 9313-9317.

Chen, W. Y., Aranda, L. C. and Luco, J. V. (1970). Learning and long and short-term memory in cockroaches. *Anim. Behav.* 18, 725.

Christensen, T. A., Waldrop, B. R. and Hildebrand, J. G. (1998). Multitasking in the olfactory system: context-dependent responses to odors reveal dual GABA-regulated coding mechanisms in single olfactory projection neurons. *J. Neurosci.* 18, 5999-6008.

Clemens, J. C., Worby, C. A., Simonson-Leff, N., Muda, M., Maehama, T., Hemmings, B. A. and Dixon, J. E. (2000). Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. *Proc. Natl. Acad. Sci.* 97, 6499-6503.

Collett, M., Collett, T. S. and Wehner, R. (1999). Calibration of vector navigation in desert ants. *Curr. Biol.* 9, 1031-1034.

Collett, T. (1996). Insect navigation en route to the goal: multiple strategies for the use of landmarks. *J. Exp. Biol.* 199, 227-235.

Collett, T. S. and Cartwrigt, B. A. (1983). Eidetic images in insects: their role in navigation. *Trends Neurosci.* 6, 101-105.

Couvillon, P. A., Arakaki, L. and Bitterman, M. E. (1997). Intramodal blocking in honeyees. *Anim. Learn. Behav.* 25, 277-282.

Daly, K. C. and Smith, B. H. (2000). Associative olfactory learning in the moth *Manduca sexta*. *J. Exp. Biol.* 203, 2025-2038.

Davis, M., Walker, D. L. and Lee, Y. (1997). Amygdala and bed nucleus of the stria terminalis: differential roles in fear and anxiety measured with the acoustic startle reflex. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 352, 1675-1687.

Davis, R. L. (1993). Mushroom bodies and *Drosophila* learning. *Neuron* 11, 1-14.

de Belle, J. S. and Heisenberg, M. (1994). Associative odor learning in *Drosophila* abolished by chemical ablation of mushroom bodies. *Science* 263, 692-695.

Derby, C. D. (2000). Learning from spiny lobsters about chemosensory coding of mixtures. *Physiol. Behav.* 69, 203-209.

Deshpande, S. B. (1984). Studies on the morphological evolutionary trends of antennary muscular patterns in insects. *J. Anim. Morphol. Physiol.* 31, 53-66.

Dill, M., Wolf, R. and Heisenberg, M. (1993). Visual pattern recognition in *Drosophila* involves retinotopic matching. *Nature* 365, 751-753.

Dubnau, J., Grady, L., Kitamoto, T. and Tully, T. (2001). Disruption of neurotransmission in *Drosophila* mushroom body blocks retrieval but not acquisition of memory. *Nature* 411, 476-480.

Dubnau, J. and Tully, T. (1998). Gene discovery in *Drosophila*: new insights for learning and memory. *Annu. Rev. Neurosci.* 21, 407-444.

Dujadin, F. (1850). Mémoire sur le système nerveux des insects. *Ann. Sci. Nat. Zool.* 14, 195-206.

Dukas, R. and Bernays, E. A. (2000). Learning improves growth rate in grasshoppers. *Proc. Natl. Acad. Sci.* 97, 2637-2640.

Durier, V. and Rivault, C. (1999). Path integration in cockroach larvae, *Blatella germanica* (L.) (Insect: Dictyoptera): Direction and distance estimation. *Anim. Learn. Behav.* 27, 108-118.

Eisenstein, E. M. (1997). Selecting a model system for neurobiological studies of learning and memory. *Behav. Brain Res.* 82, 121-132.

Eisenstein, E. M. and Cohen, H. J. (1965). Learning in an isolated prothoracic insect ganglion. *Anim. Behav.* 13, 104.

Elson, R. C. and Selverston, A. I. (1992). Mechanisms of gastric rhythm generation in the isolated stomatogastric ganglion of spiny lobsters: bursting pacemaker potentials, synaptic interactions, and muscarinic modulation. *J. Neurophysiol.* 68, 890-907.

Erber, J., Masuhr, T. and Menzel, R. (1980). Localization of short-term memory in the brain of the bee, *Apis mellifera. Physiol. Emtomol.* 5, 343-358.

Erber, J. and Pribbenow, B. (1997). Tactile motor learning in the antennal system of the honeybee (*Apis mellifera* L.). *J. Comp. Physiol. A* 181, 355-365.

Erber, J., Pribbenow, B., Bauer, A. and Kloppenburg, P. (1993). Antennal reflexes in the honeybee: tools for studying the nervous system. *Apidologie* 24, 283-296.

Ernst, K. D. and Boeckh, J. (1983). A neuroanatomical study on the organization of the central antennal pathways in insects. III. Neuroanatomical characterization of physiologically defined response types of deutocerebral neurons in *Periplaneta americana. Cell Tissue Res.* 229, 1-22.

Farooqui, T., Vaessin, H. and Smith, B. H. (2001). Modulation of early olfactory processing by an octopaminergic reinforcement pathway in the honey bee. In *Society for Neuroscience 31st Annual Meeting*, pp. 622.19. San Diego, CA.

Farris, S. M. and Strausfeld, N. J. (2001). Development of laminar organization in the mushroom bodies of the cockroach: Kenyon cell proliferation, outgrowth, and maturation. *J. Comp. Neurol.* 439, 331-351.

Feany, M. B. and Quinn, W. G. (1995). A neuropeptide gene defined by the *Drosophila* memory mutant amnesiac. *Science* 268, 869-873.

Fischer, J. A., Giniger, E., Maniatis, T. and Ptashne, M. (1988). GAL4 activates transcription in *Drosophila*. *Nature* 332, 853-856.

Franceschini, N. (1975). Sampling of the visual environment by the compound eye of the fly: Fundamentals and application. In *Photoreceptor optics* (ed. S. A. W. and R. Menzel), pp. 97-125. Berlin: Springer.

Funayama, E. S., Couvillo, P. A. and Bitterman, M. E. (1995). Compound conditioning in honeybees: Blocking tests of the independence assumption. *Anim. Learn. Mem.* 23, 429-437.

Gadd, C. A. and Raubenheimer, D. (2000). Nutrient-specific learning in an omnivorous insect: The American cockroach *Periplaneta americana* L. learns to associate dietary protein with the odors citral and carvone. *J. Insect Behav.* 13, 851-864.

Gilbert, C. and Strausfeld, N. J. (1992). Small-field neurons associated with oculomotor and optomotor control in muscoid flies: functional organization. *J. Comp. Neurol.* 316, 72-86.

Giurfa, M., Zhang, S., Jenett, A., Menzel, R. and Srinivasan, M. V. (2001). The concepts of 'sameness' and 'difference' in an insect. *Nature* 410, 930-933.

Gould, J. L. (1993). Ethological and comparative perspectives on honey bee learning. In *Insect learning: Ecological and evolutionary perspectives* (ed. P. D. R. and A. C. Lewis), pp. pp. 18-50. New York, London: Chapman & Hall.

Gray, C. M. and Skinner, J. E. (1988). Centrifugal regulation of neuronal-activity on the olfactory-bulb of the walking rabbit as revealed by reversible cryogenic blockade. *Exp. Brain Res.* 69, 378-386.

Gronenberg, W. and Strausfeld, N. J. (1992). Premotor descending neurons responding selectively to local visual stimuli in flies. *J. Comp. Neurol.* 316, 87-103.

Grunewald, B. (1999). Morphology of feedback neurons in the mushroom body of the honeybee, *Apis mellifera*. *J. Comp. Neurol.* 404, 114-126.

Guo, A. and Gotz, K. G. (1997). Association of visual objects and olfactory cues in *Drosophila*. *Learn. Mem.* 4, 192-204.

Hammer, M. (1993). An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. *Nature* 366, 59-63.

Hammer, M. and Menzel, R. (1995). Learning and memory in the honeybee. *J. Neurosci.* 15, 1617-1630.

Hammer, M. and Menzel, R. (1998). Multiple sites of associative odor learning as revealed by local brain microinjections of octopamine in honeybees. *Learn. Mem.* 5, 146-156.

Han, K. A., Millar, N. S. and Davis, R. L. (1998). A novel octopamine receptor with preferential expression in *Drosophila* mushroom bodies. *J. Neurosci.* 18, 3650-3658.

Han, P. L., Levin, L. R., Reed, R. R. and Davis, R. L. (1992). Preferential expression of the *Drosophila* rutabaga gene in mushroom bodies, neural centers for learning in insects. *Neuron* 9, 619-627.

Harris-Warrick, R. M. and Marder, E. (1991). Modulation of neural networks for behavior. *Annu. Rev. Neurosci.* 14, 39-57.

Hawkins, R. D., Carew, T. J. and Kandel, E. R. (1986). Effects of interstimulus interval and contingency on classical conditioning of the *Aplysia* siphon withdrawal reflex. *J. Neurosci.* 6, 1695-1701.

Heinzel, H. G. and Selverston, A. I. (1988). Gastric mill activity in the lobster. III. Effects of proctolin on the isolated central pattern generator. *J. Neurophysiol.* 59, 566-585.

Heisenberg, M. (1997). Genetic approaches to neuroethology. *Bioessays* 19, 1065-1073.

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

Hellstern, F., Malaka, R. and Hammer, M. (1998). Backward inhibitory learning in honeybees: A behavioral analysis of reinforcement processing. *Learn. Mem.* 4, 429-444.

Hildebrand, J. G., Christensen, T. A., Harrow, I. D., Homberg, U., Matsumoto, S. G. and Waldrop, B. R. (1992). The roles of local interneurons in the processing of

olfactory information in the antennal lobes of the moth *Manduca sexta*. *Acta Biol. Hung.* 43, 167-174.

Hirsch, H. V. and Tompkins, L. (1994). The flexible fly: experience-dependent development of complex behaviors in *Drosophila melanogaster*. *J. Exp. Biol.* 195, 1-18.

Homberg, U., Christensen, T. A. and Hildebrand, J. G. (1989). Structure and function of the deutocerebrum in insects. *Annu. Rev. Entomol.* 34, 477-501.

Honegger, H. W. (1981). A preliminary note on a new optomotor response in cricket: Antennal tracking of moving targets. *J. Comp. Physiol.* 142, 419-421.

Honegger, H. W., Allgauer, C., Klepsch, U. and Welker, J. (1990). Morphology of antennal motoneurons in the brains of two crickets, *Gryllus bimaculatus* and *Gryllus campestris*. *J. Comp. Neurol.* 291, 256-268.

Horridge, G. A. (1962). Learning of leg position by the ventral nerve code in headless insects. *Proc. R. Soc. London Ser. B* 157, 33.

Hoskins, S. G., Homberg, U., Kingan, T. G., Christensen, T. A. and Hildebrand, J. G. (1986). Immunocytochemistry of GABA in the antennal lobes of the sphinx moth *Manduca sexta*. *Cell Tissue Res.* 244, 243-252.

Hosler, J. S. and Smith, B. H. (2000). Blocking and the detection of odor components in blends. *J. Exp. Biol.* 203, 2797-2806.

Hunter, C. P. (1999). Genetics: a touch of elegance with RNAi. *Curr. Biol.* 9, 440-442.

Ito, K., Suzuki, K., Estes, P., Ramaswami, M., Yamamoto, D. and Strausfeld, N. J. (1998). The organization of extrinsic neurons and their implications in the functional roles of the mushroom bodies in *Drosophila melanogaster* Meigen. *Learn. Mem.* 5, 52-77.

Johnson, B. R. and Hooper, S. L. (1992). Overview of the stomatogastric nervous system. In *Dynamic Biological Networks* (ed. R. M. Harris-Warrick , E. Marder, A. I. Selverston and M. Moulins), pp. 1-30. Cambridge, MA: MIT Press.

Judd, S. P. D. and Collett, T. S. (1998). Multiple stored views and landmark guidance in ants. *Nature* 392, 710-714.

Kandel, E. R. (2001). The molecular biology of memory storage: a dialogue between genes and synapses. *Science* 294, 1030-1038.

Katz, P. S. (1996). Neurons, networks, and motor behavior. *Neuron* 16, 245-253.

Kauer, J. A., Malenka, R. C. and Nicoll, R. A. (1988). NMDA application potentiates synaptic transmission in the hippocampus. *Nature* 334, 250-252.

Kent, K. S., Hoskins, S. G. and Hildebrand, J. G. (1987). A novel serotonin-immunoreactive neuron in the antennal lobe of the sphinx moth *Manduca sexta* persists throughout postembryonic life. *J. Neurobiol.* 18, 451-465.

Kisch, J. and Erber, J. (1999). Operant conditioning of antennal movements in the honey bee. *Behav. Brain. Res.* 99, 93-102.

Kloppenburg, P. (1995). Anatomy of the antennal motoneurons in the brain of the honeybee (*Apis mellifera*). *J. Comp. Neurol.* 363, 333-343.

Laughlin, S. (1981). A simple coding procedure enhances a neuron's information capacity. *Z. Naturforsch.* 36, 910-912.

LeDoux, J. E. (1995). Emotion: clues from the brain. *Annu. Rev. Psychol.* 46, 209-264.

Lee, J. C. and Bernays, E. A. (1990). Food tasts and toxic effect: associative learning by the polyphagous grasshopper *Shistocerca americana* (Drury) (Orthoptera: Acrididae). *Anim. Behav.* 39, 163-173.

Leitch, B. and Laurent, G. (1996). GABAergic synapses in the antennal lobe and mushroom body of the locust olfactory system. *J. Comp. Neurol.* 372, 487-514.

Levin, L. R., Han, P. L., Hwang, P. M., Feinstein, P. G., Davis, R. L. and Reed, R. R. (1992). The Drosophila learning and memory gene rutabaga encodes a Ca2+/Calmodulin-responsive adenylyl cyclase. *Cell* 68, 479-489.

Lewis, W. J. and Takasu, K. (1990). Use of learned odours by a parasitic wasp in accordance with host and food needs. *Nature* 348, 635-636.

Li, Y. and Strausfeld, N. J. (1997). Morphology and sensory modality of mushroom body extrinsic neurons in the brain of the cockroach, *Periplaneta americana*. *J. Comp. Neurol.* 387, 631-650.

Li, Y. and Strausfeld, N. J. (1999). Multimodal efferent and recurrent neurons in the medial lobes of cockroach mushroom bodies. *J. Comp. Neurol.* 409, 647-663.

Liu, L., Wolf, R., Ernst, R. and Heisenberg, M. (1999). Context generalization in *Drosophila* visual learning requires the mushroom bodies. *Nature* 400, 753-756.

Livingstone, M. S., Sziber, P. P. and Quinn, W. G. (1984). Loss of calcium/calmodulin responsiveness in adenylate cyclase of rutabaga, a *Drosophila* learning mutant. *Cell* 37, 205-215.

Longo, N. (1964). Probability-learning and habit-reversal in the cockroach. *Am. J. Psychol.* 77, 29.

MacLeod, K., Backer, A. and Laurent, G. (1998). Who reads temporal information contained across synchronized and oscillatory spike trains? *Nature* 395, 693-698.

Malun, D. (1998). Early development of mushroom bodies in the brain of the honeybee Apis mellifera as revealed by BrdU in corporation and ablation experiments. *Learn. Mem.* 5, 90-101.

Maronde, U. (1991). Common projection areas of antennal and visual pathways in the honeybee brain, *Apis mellifera. J. Comp. Neurol.* 309, 328-340.

Mauelshagen, J. (1993). Neural correlates of olfactory learning paradigms in an identified neuron in the honeybee brain. *J. Neurophysiol.* 69, 609-625.

Maynard, D. M. (1972). Simpler networks. *Ann. N. Y. Acad. Sci.* 193, 59-72.

McGuire, S. E., Le, P. T. and Davis, R. L. (2001). The role of *Drosophila* mushroom body signaling in olfactory memory. *Science* 293, 1330-1333.

Mellon, D. (1997). Physiological characterization of antennular flicking reflexes in the crayfish. *J. Comp. Physiol. A* 180, 553-565.

Menzel, R. (2001). Searching for the memory trace in a mini-brain, the honeybee. *Learn. Mem.* 8, 53-62.

Menzel, R. and Bitterman, M. E. (1983). Learning by honeybees in an unnatural situation. In *Neuroethology and Behavioral Physiology* (ed. F. Huber and H. Markl), pp. 206-215. Berlin: Springer.

Menzel, R., Greggers, U. and Hammer, M. (1993). Functional organization of appetitive learning and memory in a heneralist pollinator, the honey bee. In *Insect learning: Ecological and evolutionary perspectives* (ed. P. D. R. and A. C. Lewis), pp. pp. 79-125. New York, London: Chapman & Hall.

Menzel, R., Hammer, M., Braun, G. and Mauelshagen. (1991). Neurobiology of learning and memory in honeybees. In *The behaviour and physiology of bees* (ed. L. J. Goodman and R. C. Fisher), pp. 323-353. Willingford, UK: CAB Int.

Menzel, R., Manz, G., Menzel, R. and Greggers, U. (2001). Massed and spaced learning in honeybees: The role of CS, US, the internal interval, and the test interval. *Learn. Mem.* 8, 198-208.

Menzel, R. and Muller, U. (1996). Learning and memory in honeybees: From behavior to neural substrates. *Annu. Rev. Neurosci.* 19, 379-404.

Milner, B. (1965). Cognitive Processes and the Brain (ed. P. M. Milner and S. E. Glickman). Princeton, NJ: Van Nostrand.

Milner, B. and Penfield, W. (1955). The effect of hippocampal lesions on recent memory. *Trans. Am. Neurol. Assoc.* 80, 42-48.

Mizunami, M., Iwasaki, M., Okada, R. and Nishikawa, M. (1998b). Topography of modular subunits in the mushroom bodies of the cockroach. *J. Comp. Neurol.* 399, 153-161.

Mizunami, M., Weibrecht, J. M. and Strausfeld, N. J. (1998). Mushroom bodies of the cockroach: Their participation in place memory. *J. Comp. Neurol.* 402, 520-537.

Mizunami, M., Weibrecht, J. M. and Strausfeld, N. J. (1998a). Mushroom bodies of the cockroach: Their participation in place memory. *J. Comp. Neurol.* 402, 520-537.

Morris, R. (1984). Developments of a water-maze procedure for studying spatial learning in the rat. *J. Neurosci. Methods* 11, 47-60.

Morris, R. G., Anderson, E., Lynch, G. S. and Baudry, M. (1986). Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. *Nature* 319, 774-776.

Morris, R. G. M., Garrud, P., Raulins, J. N. P. and O'Keefe, J. (1982). Place navigation impaired in rats with hippocampal lesions. *Nature* 297, 681-683.

Muller, D., Gerber, B., Hellstern, F., Hammer, M. and Menzel, R. (2000). Sensory preconditioning in honeybees. *J. Exp. Biol.* 203, 1351-1364.

Muller, R. U., Kubie, J. L. and Ranck, J. B., Jr. (1987). Spatial firing patterns of hippocampal complex-spike cells in a fixed environment. *J. Neurosci.* 7, 1935-1950.

Murlis, J. (1992). Odor plumes and how insects use them. *Annu. Rev. Entomol.* 37, 505-532.

Nassel, D. R., Persson, M. G. and Muren, J. E. (2000). Baratin, a nonamidated neurostimulating neuropeptide, isolated from cockroach brain: distribution and actions in the cockroach and locust nervous systems. *J. Comp. Neurol.* 422, 267-286.

Nielsen-Bohlman, L., Ciranni, M., Shimamura, A. P. and Knight, R. T. (1997). Impaired word-stem priming in patients with temporal-occipital lesions. *Neuropsychologia* 35, 1087-1092.

Nighorn, A., Healy, M. J. and Davis, R. L. (1991). The cyclic AMP phosphodiesterase encoded by the *Drosophila* dunce gene is concentrated in the mushroom body neuropil. *Neuron* 6, 455-467.

Nishino, H. and Mizunami, M. (1998). Giant input neurons of the mushroom body: intracellular recording and staining in the cockroach. *Neurosci. Lett.* 246, 57-60.

Okada, J. and Toh, Y. (2000). The role of antennal hair plates in object-guided tactile orientation of the cockroach (*Periplaneta americana*). *J. Comp. Physiol. A* 186, 849-857.

Olton, D. S. (1977). Spatial learning. *Sci. Am.* 236, 82-98.

Ott, S. R. and Elphick, M. R. (2002). Nitric oxide synthase histochemistry in insect nervous systems: Methanol/formalin fixation reveals the neuroarchitecture of formaldehyde-sensitive NADPH diaphorase in the cockroach *Periplaneta americana*. *J. Comp. Neurol.* 448, 165-185.

Papaj, D. R. (1986). Conditining of leaf-shape discrimination by chemical cues in the butterfly, *Battus philenor*. *Anim. Behav.* 34, 1281-1288.

Pascual, A. and Preat, T. (2001). Localization of long-term memory within the *Drosophila* mushroom body. *Science* 294, 1115-1117.

Pavlov, I. P. (1927). Conditioned Reflexes. London: Oxford University Press.

Penfield, W. and Mathieson, G. (1974). Memory: autopsy findings and comments on the role of the hippocampus in experiential recall. *Arch. Neurol.* 31, 145-154.

Penfield, W. and Milner, B. (1958). Memory deficits induced by bilateral lesions in the hippocampal zone. *Am. Med. Assoc. Arch. Neurol. Psychiary* 79, 475-497.

Penick, S. and Solomon, P. R. (1991). Hippocamus, context, and conditioning. *Behav. Neurosci.* 105, 611-617.

Poe, G. R., Teed, R. G., Insel, N., White, R., McNaughton, B. L. and Barnes, C. A. (2000). Partial hippocampal inactivation: effects on spatial memory performance in aged and young rats. *Behav. Neurosci.* 114, 940-949.

Prokopy, R. J., Cooley, S. S. and Papaj, D. R. (1993). How well can relative specialist *Rhagoletis* flies learn to discriminate fruit for oviposition? *J. Insect Behav.* 7, 663-677.

Quinn, W. G., Harris, W. A. and Benzer, S. (1974). Conditioned behavior in *Drosophila melanogaster*. *Proc. Nat. Acad. Sci.* 71, 708-712.

Raubenheimer, D. and Tucker, D. (1997). Associative learning by locusts: pairing of visual with consumption of protein and carbohydrate. *Anim. Behav.* 54, 1449-1459.

Rempel-Clower, N. L., Zola, S. M., Squire, L. R. and Amaral, D. G. (1996). Three cases of enduring memory impairment after bilateral damage limited to the hippocampal formation. *J. Neurosci.* 16, 5233-5255.

Ribi, W. A. (1977). Fine structure of the first optic ganglion (lamina) of the cockroach, *Periplaneta americana*. *Tissue Cell* 9, 57-72.

Richards, O. W. and Davies, R. G. (1977). Imm's general textbook of entomology Volume I: Structure, physiology and development. London: Chapman and Hall.

Roman, G. and Davis, R. L. (2001). Molecular biology and anatomy of *Drosophila* olfactory associative learning. *Bioessays* 23, 571-581.

Rose, J. K. and Rankin, C. H. (2001). Analyses of habituation in *Caenorhabditis elegans*. *Learn. Mem.* 8, 63-69.

Rospars, J. P. (1988). Structure and development of the insect antennodeutocerebral system. *Int. J. Insect Morphol. Embryol.* 17, 243-249.

Rospars, J. P. and Hildebrand, J. G. (1992). Anatomical identification of glomeruli in the antennal lobes of the male sphinx moth *Manduca sexta*. *Cell Tissue Res.* 270, 205-227.

Rust, M. K., Burk, T. and Bell, W. J. (1976). Pheromone-stimulated locomotory and orientation responses in the American cockroach. *Anim. Behav.* 24, 52-67.

Schatz, B., Chameron, S., Beugnon, G. and Collett, T. S. (1999). The use of path integration to guide route learning in ants. *Nature* 399, 769-772.

Schmitt, J. and Holmes, P. (2000). Mechanical models for insect locomotion: dynamics and stability in the horizontal plane I. Theory. *Biol. Cybern.* 83, 501-515.

Seelinger, G. and Tobin, T. R. (1981). Sense organs. In *The American Cockroach* (ed. W. J. Bell and K. G. Adiyodi), pp. 217-222. London, New York: Chapman and Hall.

Selverston, A. I. (1977). Neural circuitry underlying oscillatory motor output. *J. Physiol.* 73, 463-470.

Silva, A. J., Kogan, J. H., Frankland, P. W. and Kida, S. (1998). CREB and memory. *Annu. Rev. Neurosci.* 21, 127-148.

Sinakevitch, I., Farris, S. M. and Strausfeld, N. J. (2001). Taurine-, aspartate- and glutamate-like immunoreactivity identifies chemically distinct subdivisions of Kenyon cells in the cockroach mushroom body. *J. Comp. Neurol.* 439, 352-367.

Smith, B. H. (1993). Merging mechanism and adaptation: An ethological approach to learning and generaization. In *Insect learning: Ecological and evolutionary perspectives* (ed. P. D. R. and A. C. Lewis), pp. 126-157. New York, London: Chapman & Hall.

Smith, B. H. and Cobey, S. (1994a). The olfactory memory of the honeybee *Apis mellifera*: II. Blockig between odorants in binary mixtures. *J. Exp. Biol.* 195, 91-108.

Smith, B. H. and Cobey, S. (1994b). The olfactory Memory of the honeybee *Apis mellifera*: II. Blocking between odorants in binary mixtures. *J. Exp. Biol.* 195, 91-108.

Snodgrass, R. E. (1935). Principles of Insect Morphology, pp. 130-156. New York; London: Mcgraw-Hill Book Company, Inc.

Spieth, H. T. (1974). Courtship behavior in Drosophila. *Annu. Rev. Entomol.* 19, 385-405.

Squire, L. R., Knowlton, B. and Musen, G. (1993). The structure and organization of memory. *Annu. Rev. Psychol.* 44, 453-495.

Squire, L. R. and Zola-Morgan, S. (1991). The medial temporal lobe memory system. *Science* 253, 1380-1386.

Srinivasan, M. V., Zhang, S., Altwein, M. and Tautz, J. (2000). Honeybee navigation: nature and calibration of the "odometer&quot. *Science* 287, 851-853.

Srinivasan, M. V., Zhang, S. W. and Zhu, H. (1998). Honeybees links sights to smells. *Nature* 396, 637-638.

Stay, B., Chan, K. K. and Woodhead, A. P. (1992). Allatostatin-immunoreactive neurons projecting to the corpora allata of adult *Diploptera punctata*. *Cell Tissue Res.* 270, 15-23.

Stopfer, M. and Laurent, G. (1999). Short-term memory in olfactory network dynamics. *Nature* 402, 664-668.

Strausfeld, N. J. (1998). Crustacean-insect relationships: the use of brain characters to derive phylogeny amongst segmented invertebrates. *Brain Behav. Evol.* 52, 186-206.

Strausfeld, N. J., Hansen, L., Li, Y., Gomez, R. S. and Ito, K. (1998). Evolution, discovery, and interpretations of arthropod mushroom bodies. *Learn. Mem.* 5, 11-37.

Strausfeld, N. J. and Lee, J. K. (1991). Neuronal basis for parallel visual processing in the fly. *Vis. Neurosci.* 7, 13-33.

Strausfeld, N. J. and Li, Y. (1999). Organization of olfactory and multimodal afferent neurons supplying the calyx and pedunculus of the cockroach mushroom bodies. *J. Comp. Neurol.* 409, 603-625.

Strausfeld, N. J. and Li, Y. (1999a). Organization of olfactory and multimodal afferent neurons supplying the calyx and pedunculus of the cockroach mushroom bodies. *J. Comp. Neurol.* 409, 603-625.

Strausfeld, N. J. and Li, Y. (1999b). Representation of the calyces in the medial and vertical lobes of cockroach mushroom bodies. *J. Comp. Neurol.* 409, 626-646.

Sutherland, G. R. and McNaughton, B. (2000). Memory trace reactivation in hippocampal and neocortical neuronal ensembles. *Curr. Opin. Neurobiol.* 10, 180-186.

Takeda, K. (1961). Classical conditioned response in the honey bee. *J. Insect Physiol.* 6, 168-179.

Thompson, R. F. and Krupa, D. J. (1994). Organization of memory traces in the mammalian brain. *Annu. Rev. Neurosci.* 17, 519-549.

Tinbergen, N. (1951). The Study of Instinct. London: Oxford University Press.

Tinbergen, N. (1958). Curious Naturalists. New York: Basic Books, Inc.

Tinbergen, N. and Kruyt, W. (1938). uber die Orientierung des Bienenwolfes (*Philanthus triangulum* Fabre). III. Die Bevorzugung bestimmter Wegmarken. *Z. Vgl. Physiol* 25, 292-334.

Tolbert, L. P. and Hildebrand, J. G. (1981). Organization and synaptic ultrastructure of glomeruli in the antennal lobes of the moth *Manduca sexta*: a study using thin sections and freeze-fracture. *Proc. R. Soc. Lond. B* 213, 279-301.

Tully, T. (1996). Discovery of genes involved with learning and memory: an experimental synthesis of Hirschian and Benzerian perspectives. *Proc. Natl. Acad. Sci.* 93, 13460-13467.

Tully, T. and Quinn, W. G. (1985). Classical conditioning and retention in normal and mutant Drosophila melanogaster. *J Comp Physiol [A]* 157, 263-77.

Vickers, N. J., Christensen, T. A., Baker, T. C. and Hildebrand, J. G. (2001). Odour-plume dynamics influence the brain's olfactory code. *Nature* 410, 466-470.

von Frisch, K. (1967). The dance language and orientation of bees. London: Oxford University Press.

Wäckers, F. L. and Lewis, W. J. (1999). A comparison of color-, shape- and pattern-learning by the hymenoptern parasitoid *Microplitis croceipes*. *J. Comp. Physiol. A* 184, 387-393.

Waddell, S., Armstrong, J. D., Kitamoto, T., Kaiser, K. and Quinn, W. G. (2000). The amnesiac gene product is expressed in two neurons in the *Drosophila* brain that are critical for memory. *Cell* 103, 805-813.

Waddell, S. and Quinn, W. G. (2001). Neurobiology. Learning how a fruit fly forgets. *Science* 293, 1271-1272.

Webb, B. (2002). Robots in invertebrate neuroscience. *Nature* 417, 359-363.

Wilson, M. A. and McNaughton, B. L. (1994). Reactivation of hippocampal ensemble memories during sleep. *Science* 265, 676-679.

Wolf, R. and Heisenberg, M. (1991). Basic organization of operant behavior as revealed in *Drosophila* flight orientation. *J. Comp. Physiol. A* 169, 699-705.

Wolken, J. J., Capenos, J. and Turano, A. (1957). Photoreceptor structures. III. *Drosophila melanogaster*. *J. Biophy. Biochem. Cytol.* 3, 441-447.

Wustmann, G. and Heisenberg, M. (1997). Behavioral manipulation of retrieval in a spatial memory task for *Drosophila melanogaster*. *Learn Mem* 4, 328-336.

Yin, J. C., Del Vecchio, M., Zhou, H. and Tully, T. (1995). CREB as a memory modulator: induced expression of a dCREB2 activator isoform enhances long-term memory in *Drosophila*. *Cell* 81, 107-115.

Yin, J. C., Wallach, J. S., Del Vecchio, M., Wilder, E. L., Zhou, H., Quinn, W. G. and Tully, T. (1994). Induction of a dominant negative CREB transgene specifically blocks long-term memory in *Drosophila*. *Cell* 79, 49-58.

Zars, T., Fischer, M., Schulz, R. and Heisenberg, M. (2000). Localization of a short-term memory in *Drosophila*. *Science* 288, 672-675.

Zhang, S. W., Lehrer, M. and Srinivasan, M. V. (1999). Honeybee memory: navigation by associative grouping and recall of visual stimuli. *Neurobiol. Learn. Mem.* 72, 180-201.

Zilber-Gachelin, N. F. and Paupardin, D. (1974). Sensitization and dishabituation in the cockroach. Main characteristics and localization of the changes in reactivity. *Comp. Biochem. Physiol. A* 49, 441-470.

Zola-Morgan, S., Squire, L. R. and Amaral, D. G. (1986). Human amnesia and the medial temporal region: enduring memory impairment following a bilateral lesion limited to field CA1 of the hippocampus. *J. Neurosci.* 6, 2950-2967.