1 Introduction

1.1: Thesis outline

This thesis consists of 3 (three) Chapters

Chapter 1 presents the localization of the AmNR1 transcript and protein in the adult honeybee brain. The AmNR1 transcripts and protein is expressed abundantly throughout the entire brain, which will provide a great opportunity to study site-specific involvement of AmNR1 in the mechanism of learning and memory in the honeybee bee.

Chapter 2 presents the localization of the AmNR1 transcript and protein in the developmental stages of the honeybee. The AmNR1 transcript is highly expressed in the pre-adult stages compared to adult honeybee, and the AmNR1 protein is increased with age. Here, it is concluded that the AmNR1 gene is developmentally regulated and may be involved with the behavioral maturation and learning and memory.

Chapter 3 describes the expression of the AmNR1 gene at different seasonal stages and hives bees, and the co-localization of the AmNR1 protein with protein discs-large (DLG). No differences were identified of the localization of AmNR1 protein in the bees that were collected from different seasons and hives. The AmNR1 protein is co-localized with DLG. It suggests that the AmNR1 gene is not influenced by season or colony type and the AmNR1 protein is co-expressed with DLG in the honeybee.

1.2: General introduction

In the present investigation, honeybee used as a model organism, and studying area was the central nervous system of the honeybee *Apis mellifera*. In the following sections, honeybee in research and different brain neuropils are described to gain a better

understanding about their research importance and morphological structure of the bee brain that are involved in different brain activities and learning and memory.

1.2.1: Honeybee in research

The honeybee has provided a useful organism for studying a diverse range of disorders and diseases in humans such as mental illnesses, immunology, allergic reactions, antibiotic resistance, embryonic and nervous development, venom toxicology, infectious diseases and genetic (X chromosome) diseases (Amos and Harwood 1998; Harbo and Harris 2001; Insel and young 2001; Kolecki 1999; Ono 2000). Furthermore, researchers are interested in studying social behavior and complex cognitive tasks i.e., learning and memory on different behavioral and physiological levels, pharmacological and physiological properties of different receptors and their intracellular signaling pathways, and single neuron recording or optophysiological registrations of the brain activity (Blenau and Baumann 2001; Fiala et al., 1999; Hammer 1993; Menzel and Müller 1996; Menzel 2001). An additional advantage of these insects is that their brain contains 950,000 neurons (Witthöft 1967) that can be identified individually.

1.2.2: Brain Structures of the honeybee

The predominant parts of the bee brain are the mushroom bodies, the antennal lobes, the protocerebral lobes, the optic lobes and the central complex (Fig. 1.1). The mushroom bodies, the antennal lobes, lateral parts of the protocerebrum, and the suboesephageal ganglion are the main neuropils that are involved in the associative proboscis extension learning.

Mushroom bodies

The mushroom bodies (MB) are paired neuropils that are located in the dorsal part of the insect brain. In insect, mushroom bodies are involved in a diverse range of behavioral functions and are important structures for memory formation which receive multimodal sensory information from different sensory modalities (Heisenberg 1998; Menzel 2001; Menzel and Giurfa 2001; Rybak and Menzel 1993; Zars 2000). In the honeybee, each mushroom body consists of a pair of cup-shaped structures called calyces, connected by short necks to a common peduncle that is subdivided into two different lobes: the α- (also called vertical lobe) and the β-lobe (also called medial lobe) (Mobbs 1982; Strausfeld 2002) (Fig.1.2). In the worker bee, each mushroom body is composed of 170,000 densely packed intrinsic neurons called Kenyon cells (K cells: Witthöft 1967), first described by Dujardin (1850) and by Kenyon (1896). The calyx receives sensory input from both the antennal lobes and the optic lobes, whereas in the peduncle, the α- and β- lobes form the output region of the mushroom bodies (Mobbs 1982; Vowles's 1955). Each calyx of the mushroom body is subdivided into three major concentric neuropil zones: the lip, the collar, and the basal ring (Mobbs 1982) (Fig. 1.2A). These zones are further subdivided into smaller zones according to their sensory afferent and efferent supply, morphology of intrinsic neurons and immunohistological affinities. Strausfeld et al. (2000) first proposed that the lip neuropil is subdivided into two discrete zones, the collar is subdivided into five zones, and the basal ring is subdivided into four zones. Later, he (2002) described that the lip is subdivided into at least three zones and the collar is subdivided at least three zones: called the outer collar zone; the intermediate collar zone; and the inner collar zone. The basal ring is subdivided into two zones: the inner basal ring and the outer basal ring. The lip receives input from the antennal lobes and the collar receives input from the optic lobes. On the

other hand, the basal ring receives input from the antenna, compound eyes, and the suboesephageal ganglion (Mobbs 1982, 1984, 1985; Rybak and Menzel 1993).

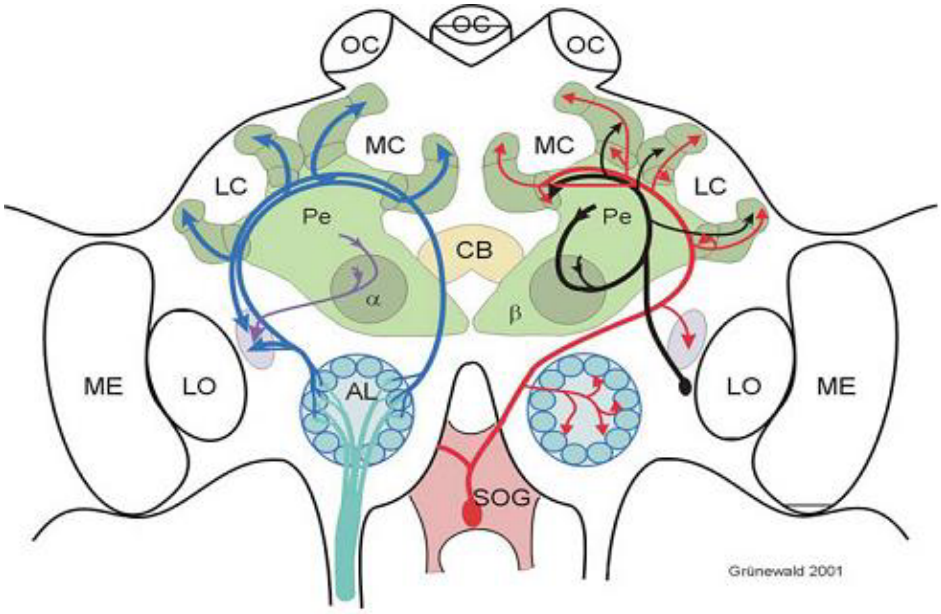

Figure 1.1: Schematic diagram of adult honeybee brain (frontal view). It has medial calyx (MC), lateral calyx (LC), peduncle (Pe), alpha lobe (α; vertical lobe), beta lobe (β; medial lobe), projection neurons (blue), VUMmx1 (red), inhibitory feedback loop (black), indirect input from the mushroom body to lateral protocerebrum (also called lateral horn, violet), lobula (LO), medulla (ME), suboesophageal ganglion (SOG), central brain (CB), antennal lobe (AL) and ocelli (OC). (Source: Dr. Grunwald Bern).

The cups of the calyces are filled with Kenyon cell bodies, whereas their dendritic arborizations are found throughout the entire calyces. Each Kenyon cell sends a long projection into the peduncle. Projections either split into two branches and enter the α- and β-lobes, or send a single projection to only one lobe (Strausfeld 2002). The Kenyon cell bodies are subdivided into two groups based on their diameters: the small and large Kenyon cells (4-5 μm and 6-7 μm in diameter, respectively). The somata of large Kenyon cells are located on the inside edges of the calyces. Small Kenyon cells are subdivided into two classes: the somata of class I Kenyon cells are located on the inner core of each calyx and the somata of class II Kenyon cells are located in the outer

surface of the calyx (Mobbs 1982; Strausfeld 2002) (Fig. 1.2B). The distribution of Kenyon cells within the mushroom body is depended on their birth date during mushroom body development (Farris et al., 1999, 2001; Farris and Strausfeld 2001; Kurusu et al., 2002; Malaterre et al., 2002). The class II small type of Kenyon cells (also called clawed Kenyon cell), which can be identified by their clawed dendritic specializations (Strausfeld 2002; Strausfeld et al., 2003) are born early in the center of the calyces and are gradually pushed outwards to more peripheral locations, whereas class I small type of Kenyon cells are born later and remain closer to the center of proliferation (Farris et al., 1999; Malaterre et al., 2002).

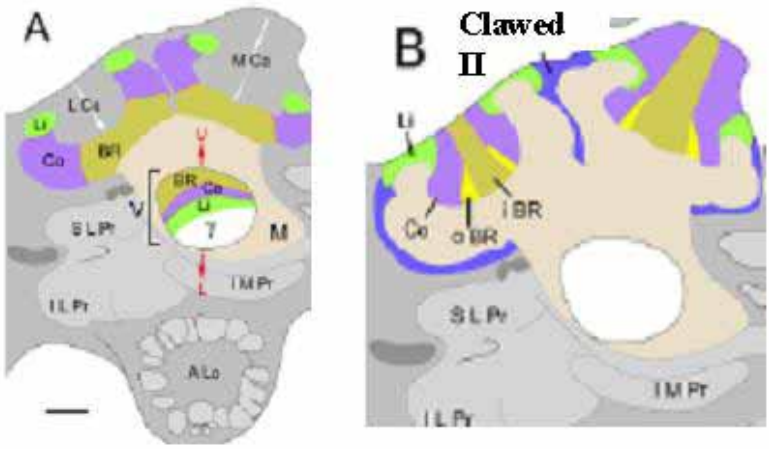

Figure 1.2: Organization of the mushroom body of honeybee. A: The median (M) and lateral calyces (L Ca) are each subdivided into three major regions, such as, lip (Li), collar (Co), and basal ring (BR) which are represented in the vertical lobe (V; also called α - lobe) by three subdivisions that lie above the adjoining gamma lobe (γ). The medial lobe (M; also referred as β - lobe). B: Distribution of class II (clawed) and class I Kenyon cell bodies in the calyces. Class I Kenyon cell bodies are indicated by the calycal regions: lip, collar, outer basal ring (oBR) and inner basal ring (iBR). Scale bars =100 μ m. Source: Strausfeld N. J., 2002 (publish with permission).

The lip receives dendrites projection from the somata of large K cells, which are surrounded inside edges of the calyces and also from the somata of small class II K cells. The collar receives dendrites from the somata of large K cell and the somata of small

class II K cells that are located underneath the collar region. The basal ring receives dendritic projections from the somata of small K cell class I located in the central parts of the cup and also from the somata of small K cell class II located on the underside of the calyces of the basal ring (Mobbs 1982). Kenyon cell axons that originate from the lip, the collar, and the basal ring are subdivided into three regions within the α- and β-lobes (Mobbs 1982). Kenyon cell axons from the lip are represented in the lower third of the vertical lobe, whereas Kenyon cell axons from the collar and the basal ring are represented in the middle and upper thirds of the vertical lobe, respectively. A recent study has revealed that Kenyon cell axons from the lip are not represented in the lower third of the vertical lobe. This part of the vertical lobe receives axon projection from the small class II Kenyon cells (Strausfeld 2002). Strausfeld proposed one additional zone, the gamma lobe in the vertical lobe that is anatomically and developmentally similar to the *Drosophila* gamma lobe (Strausfeld 2002) (Fig. 1.2A). More recently, histological and immunochemical investigation suggests that small class II Kenyon cells are further subdivided into four classes according to their axons projection into the peduncle and lobes (Farris et al., 2004).

Antennal lobe

The antennal lobes are another pair of structured neuropils located at the front of the bee brain. Each antennal lobe is compartmentalized into 166 glomeruli spherical neorupils called glomeruli that form functional units of the olfactory code (Arnold et al., 1985; Flanagan and Mercer 1989; Galizia et al., 1999). In total, both antennal lobes contain about 10,000 neurons that receive input from the chemosensory in the antennae and are responsible for the preprocessing of olfactory and chemosensory information. The two lobes are connected with each other by a bundle of nervous tissue called the supraeosophageal commissure and they are connected with the mushroom bodies by the

olfactorio-globularis tract.

Optic lobe and central complex

The optic lobes are located laterally at both sides of the brain, and are responsible for the processing visual information gathered by the compound eyes. Each optic lobe is subdivided into three major neuropile zones: lobula, medulla, and lamina. The central complex is located in the center of the honeybee brain. The real function of this part of the brain is not known well due to the complexity of neural circuits and it is less studied than other parts of the brain. The central complex has four major structures: the fan shaped body, the protocerebral-bridge, the ellipsoid body, and the noduli.

1.2.3: Honeybee and learning and memory

Honeybee has a rich behavioral repertory that is easily accessible by appetitive training. Bees are able to form an association between an olfactory stimuli and a sucrose reward by using a classical conditioning paradigm of the proboscis extension reflex. If one antennae of a hungry bee comes in contact with sucrose solution, the bee extends its proboscis towards the food source and licks it. This characteristic phenomenon does not appear when other odors or olfactory stimuli are presented to the antenna alone. However, they can form an association if an odor is presented immediately before presenting the sucrose solution such that after several learning trials the odor by itself can trigger proboscis extension response (PER) (Bitterman et al., 1983; Kuwabara 1957; Menzel 1990; Takeda 1961).

In the honeybee as in other animals, memory formation following a 3-trial classical conditioning is a dynamic and multiphase process in which different brain regions are involved and a sequence of events lead to the long-lasting memory passing through the multiple phases of transient memories (Menzel and Müller 1996; Menzel 1999; Menzel

2001; Menzel and Giurfa 2001). In this process, the antennal lobes are involved in the initial stages and mushroom bodies are involved in the later stages (Menzel 2001). Many experimental results suggest that different neurotransmitters and their receptors are involved in learning and memory processes, such as acetylcholine (Lozano et al., 2001; Shapira et al., 2001), octopamine (Hammer and Menzel 1998; Menzel 2001), and glutamate (Si et al., 2004; Maleszka et al., 2000). In this thesis, neurotransmitter receptor NMDA glutamate receptor subunit R1 which has a functional involvement in the mechanism of learning and memory in the bees is focused upon.

1.2.4: Glutamate receptors

Glutamate is an important excitatory neurotransmitter in the mammalian central nervous system (CNS) (Danbolt 2001¸Headley and Grillner 1990). The role of glutamate in the CNS has been studied on the basis of the receptor proteins that have been identified with molecular cloning (Dingledine et al., 1999; for review, see Danbolt 2001; Hollmann and Heinemann 1994; Nakanishi et al., 1998; Ozawa et al., 1998). There are two kinds of glutamate receptors, ionotropic glutamate receptors and metabotropic glutamate receptors. Ionotropic glutamate receptors are ligand-gated ion channels which are subdivided into three groups on the basis of their responses to specific agonists: NMDA (N-methyl-D-aspartate), AMPA (α-amino-3hydroxy-5-methylisoxazole propionic acid), and kainate (Nakanishi et al., 1998). Ionotropic receptors are expressed on the postsynaptic cell membrane and are responsible for many sequences of neuronal events. However, present evidence suggests that these receptors can also be found on the presynaptic terminals (Anastassios and Nicoll 2004).

Among the ionotropic receptors, the NMDA receptor is well characterized in the mammalian central nervous system and has been shown to be expressed both at the

presynaptic and the postsynaptic cell membrane (Anastassios and Nicoll 2004; Danbolt 2001). Postsynaptic NMDA receptors are responsible for the induction of long-term potentiation (LTP) that typically produces slower but longer-lasting responses than the AMPA/kainite receptors. Therefore, NMDA receptor containing synapses produce longer-lasting excitatory postsynaptic currents (EPSCs) than the synapses which containing only AMPA/kainate types of receptors (Hestrin et al., 1990; Nicoll et al., 1990). In fact, some evidences suggest that many glutamate synapses contain both NMDA and non-NMDA receptors and synaptic responses greatly depend on the properties of the receptor types (Forsythe and Westbrook 1988; Lester et al., 1990).

A characteristic NMDA receptor is the heterometric receptor complex comprised of a common NR1 subunit and one of four NR2 subunits (NR2A-NR2D) combined at an undefined ratio. The subunits are derived from seven different genes (NR1, NR2A-NR2D and NR3A-3B) (Ciabarra et al., 1995; Hollmann and Heinemann 1994; Monyer et al., 1992; Sucher et al., 1995). Until recently, it was believed that the NMDA receptor has at least five pharmacologically distinct sites: i) a glutamate binding site, ii) a glycine binding site, iii) a channel binding site that binds channel blockers such as phencyclidine and related compounds, iv) a voltage dependent Mg^{2+}binding site, and v) a Zn^{2+} binding site. Two unique properties are required for the activation of the NMDA receptors. First, activation of the NMDA receptor depends on the simultaneous binding of two agonists glutamate released from synaptic vesicles and glycine from the extrasynaptic fluids (Klecker and Dingledine 1988; Johnson and Ascher 1987). Second, the NMDA receptor is highly permeable to Ca^{2+} in a voltage-dependent manner that can be blocked by extracellular Mg^{2+} at resting potential. When depolarization following a high firing rate of the presynaptic neuron is sufficient, Mg^{2+} is expelled from the mouth of the NMDA receptor allowing Ca^{2+} to enter the postsynaptic cell through the NMDA

receptor channel, thereby initiating a sequence of steps that leads to the induction of LTP (MacDonald et al., 1982; Mayer et al., 1984). LTP is subdivided into two phases: early and late phases of LTP. A single train of stimulation is sufficient to produce the early phases of the LTP by the activation of NMDA receptors, whereas repeated trains are required for the late phases of LTP (Bliss and Collingridge 1993; Collingidge and bliss 1995; Ozawa et al., 1998).

In arthropods, glutamate uptake has been studied in neural and neuromuscular tissues (Duce 1988; Gardiner 2002). Furthermore, the L-glutamate olfactory receptor has also been identified in the Caribbean spiny lobster *Panulirus argus* (Burgess and Derby 1997). In the molluscs *Aplysia californica*, a cellular model of LTP has been described that depends on the activation of NMDA receptors (Murphy and Glanzman 1997; 1999). In insects, the role of glutamate has been intensively studied as a neurotransmitter in the central nervous system (CNS), skeleton muscle and at the neuromuscular junction (Delgado 1989; Johansen et al., 1989a and b; Petersen et al., 1997; Sigrist et al., 2002; Usherwood 1994). Glutamate transporter has been cloned from the cabbage looper *Trichoplusia ni* (Donly et al., 1997), the fruit fly *Drosophila melanogaster* (Besson et al., 1999; Seal et al., 1998), the honeybee *Apis mellifera* (Kucharski et al., 2000), and the cockroach *Diploptera punctata* (Donly et al., 2000). In addition, by immunohistological staining, a glutamate transporter has been identified at the neuromuscular junction of the skeleton muscle and in the ganglionic neuropile of the CNS of lepidopteran insects (the cabbage looper *Trichoplusia ni*; Gardiner et al., 2002). Glutamate-like immunoreactivity has also been described in the brain of honeybees (Bicker et al., 1988; Bicker 1999), crickets (Schürmann et al., 2000), and cockroaches (Sinakevitch et al., 2001).

The kainate and metabotropic glutamate receptors have been identified in the CNS of embryos of *Drosophila* (Parmentier et al., 1996; Ultsch et al., 1992). Two ionotropic

glutamate receptors have been identified in the CNS of *Drosophila* embryos by whole-mount of *situ* hybridization that also clarified by the immunofluorescence analysis on adult fly brain cryosections (Völkner et al., 2000). Further, NMDA receptor subunit R1 has also been detected in the different developmental stages in *Drosophila* heads by Northern blot analysis (Ultsch et al., 1993) and in the weakly electric fish *Apteronotus leptorhynchus* (Dunn et al., 1999). Recently, metabotropic glutamate receptors have been characterized in the honeybee *Apis mellifera* (Funada et al., 2004) and ionotropic glutamate receptors from the cockroach *Diploptera punctata* (Chiang et al., 2002).

The role of NMDA receptor in the mechanisms of learning and memory has been investigated by two noncompetitive NMDA receptor antagonists memantine and MK-801 in the honeybee and it was shown that receptor blocking impairs the formation of long-term (24h) memory but not short-term (1h) memory (Si et al., 2004). Neuhaus and Müller (2001) found that inhibition of glutamate-recycling before a single training trial improves short-term memory, without affecting long-term memory. The involvement of the NMDA receptors in an associative learning and memory has also been described in *Drosophila* (Xia et al., 2005) and *Aplysia* (Robert and Glanzman 2003). These results suggest that glutamate plays an important role in the mechanisms of learning and memory in invertebrates. However, most of the results of the olfactory learning and memory have been obtained by applying classical biochemical techniques and molecular basis of behavior and learning and memory in the bee is greatly unknown.

Further, in insect and other invertebrates, the role of glutamate in the specific brain function is poorly understood and controversial for the following reasons: a) specificity of drugs that have been developed in mammal on the basis of their physiological

effectiveness of the specific glutamate agonists and antagonists are not same in invertebrates due to the different pharmacological profiles and functionalities of the receptor channels, b) all drugs have physiological side effect that can interfere with other biochemical pathways. Therefore, to overcome these problems and to understand the precise role of the glutamatergic neurotransmission in the adult and developing central nervous system of the honeybee requires the characterization and localization of its receptors.

1.3: The aim of this thesis

The aim of this thesis was to the localization of the expression of NMDA glutamate receptor subunit R1 (AmNR1) transcript and protein in the adult and at different developmental stages of the honeybee, and the co-localization of this receptor protein with protein discs-large (DLG). The following questions were investigated in this thesis:

1. In which honeybee brain neuropils are the AmNR1 gene expressed?
2. Does the localization of the AmNR1 protein corelate with the localization of AmNR1 transcripts?
3. Is the expression of the AmNR1 gene developmentally regulated?
4. Is the expression of the AmNR1 gene influence by season or colony type?
5. Does the AmNR1 protein co-localize with DLG?

Question 1 and 2 are addressed in Chapter 1, question 3 is addressed in Chapter 2 and question 4 and 5 are addressed in Chapter 3.

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