5 General Discussion

The aim of this investigation was the localization of the NMDA glutamate receptor subunit R1 (AmNR1) mRNA and protein in the adult and at different developmental stages of the honeybee. In addition, *in situ* hybridization and immunohistochemistry were used for the detection of the expression of AmNR1 gene at different seasonal stages and hives and the co-localization of AmNR1 with protein discs-large (DLG). Localization of AmNR1 mRNA and protein in the adult bee is addressed in the Chapter 1. Localization of AmNR1 mRNA and protein at different developmental stages is addressed in the Chapter 2 and the seasonal basis expression of AmNR1 gene and the co-localization of AmNR1 with protein discs-large (DLG) is addressed in the Chapter 3.

5.1: Chapter 1

Learning induces multiple phases of memory through the changes in synaptic connection (synaptic plasticity) as the substrate of experience-dependent behavioral plasticity that is measured by changes in an animal's behavior some time after learning (Milner et al., 1998; for review see, Abel and Lattal 2001). In this proccess, a number of transcription factors are involved. One of these factors is CREB-1 (cAMP-response element binding protein-1) which switches on the genes needed for long-term memory. Several groups have reported involvement of the cAMP/PKA/CREB signaling pathway in the long-term potentiation (LTP) and learning and memory in mammals, honeybee, *Drosophila* and *Aplysia* (Kogan et al., 1997; Dubnau and Tully 1998; Fiala et al., 1999). In insects, involvement of NMDA receptor in long-term memory has been reported by several groups, for example, in *Drosophila* (Xia et al., 2005), and in the honeybee (Si et

al., 2004; Locatelli et al., 2005). In the present investigation, using *in situ* hybridization with DIG-labeled RNA probes revealed that the AmNR1 mRNA is expressed in the mushroom body and the antennal lobes, both of which are involved in associative learning. These results are consistent with two other glutamate receptors, AmGluRA and AmGluRB that are expressed in the mushroom body and the antennal lobes (Funada et al., 2004). Similarly, in *Drosophila*, NMDAR1 mRNA was also expressed in the adult fly head (Ultsch et al., 1993). Therefore, the expression of AmNR1 mRNA in the mushroom body and the antennal lobe that are consistent with the expression of two other glutamate receptors in the bee suggests that this receptor might be involved in the mechanism of learning and memory.

In the optic lobes, the high-level expression of AmNR1 mRNA was observed that are consistent with the expression of honeybee metabotropic glutamate receptors (Funada et al., 2004) and glutamate transporter gene (Kucharski et al., 2000). Similarly, a high-level of glutamate transporter was also identified in the visual system of other animals, such as the salamander and *Drosophila* (Eliasof et al., 1998; Besson et al., 1999). Moreover, the AmNR1 mRNA was also highly expressed in the dorsal lobes, ventral and lateral soma rind of the suboesophageal ganglion that contain sensory and motor neurons responsible for the movement of antennae and mouthparts (Rehder 1989). This widespread distribution of AmNR1 mRNA in the bee brain further suggests that it might participate in the processing of higher order sensory information including visual and olfactory information as well as learning and memory.

Immunoenzyme histochemical analysis showed that the AmNR1 immunoreactivity was strong in the optic lobes, the antennal lobes, the central body and the suboesophageal ganglion. While the mushroom body showed weak AmNR1 immunoreactivity. These results are consistent with expression of AmNR1 mRNA in the honeybee. Further, weak

AmNR1 immunoreactivity in the mushroom body is also consistent with the expression of glutamate-like immunoreactivity reported by Bicker et al., in 1988 and high-level AmNR1 immunoreactivity in the optic lobes is consistent with the expression of glutamate transporter gene in the honeybee (Kucharski et al., 2000). In *Drosophila*, high-levels of glutamate enzymatic activity were identified in the retina and the lamina (Chase and Kankel 1987). Therefore, high-level expression of AmNR1 mRNA and protein together with the presence of glutamate-like immunoreactivity and glutamate transporter in the optic lobes and ocellar system indicates functional involvement of glutamate as a meurotransmitter in the visual system of insects.

5.2: Chapter 2

The expression of AmNR1 transcript in the developmental stages and also in the adult bees are summarized in figure 5. The AmNR1 transcript was strongly detected in all brain neuropils and in most of the cells including Kenyon cell neuroblasts in all pre-adult stages that was decreased in young adult bees. Although, in the young adult bees, a reduction of the expression of AmNR1 was observed, but it was still higher than the foraging bees, with the exception of Kenyon cell somata of the mushroom body, which showed very low or no expression at all at this stage. In the pre-adult stages, the expression of AmNR1transcript is not comparable with the expression of AmNR1 protein because experimental results showed that very low or almost no AmNR1 immunoreactivity in the 0 day old bees. Two main reasons can be accounted here: First, the AmNR1 protein may not be expressed in the pre-adult stages. Second, the AmNR1 protein may be expressed in the pre adult stages but below the detectable level with the antibody. Further, the AmNR1 mRNA was highly detected in the young adult bees,

whereas AmNR1 protein was detected at low level at these stages. If we compare side-specific expression of AmNR1 transcript and protein in different brain neuropils, any correlation can be investigated. By example, high-levels of AmNR1 mRNA were detected in the optic lobe, antennal lobe, protocerebral lobe, and the central body, but not in the mushroom body that was correlated with the expression of AmNR1 protein in the young adult bees. In the honeybee, the AmNR1 transcript and protein was expressed differentially at different developmental stages that are consistent with the expression of honeybee glutamate transporter gene (Am-EAAT, excitatory amino acid transporters; Kucharski et al., 2000), transferring-gene (AmTRF; Adriana et al., 2004) and D2 type dopamine receptor (AmDop2 gene; Humphries et al., 2003). High-levels expression of AmNR1 transcript in the pre-adult stages suggests that it may be involved in developing the brain including the terminal differentiation and the synapse formation of the central nervous system. On the other hand, age related increase of AmNR1 protein may be correlated with behavioral maturation of the bee which underlies learning and memory.

In developmental stages, the expression of AmTRF is influenced greatly by ecdysteriods that was confirmed by the experimental treatment of spinning-stages larvae with 20-hydroxyecdysone and of fourth instar-larvae with juvenile hormone (JH). The strongest reduction of the expression of AmTRF in the first spinning-stage (S1-stage) was observed when the endogenous ecdysteroid titer was increased in consequence of the JH treatment in the fourth instar. In the cockroach (*Diploptera punctata*), the regulation of JH synthesis by NMDA receptors was reported by Chiang et al. in 2002.

In insects, JH is responsible for preventing premature metamorphosis and is necessary for maturation of the eggs that synthesize and release from the corpora allata. Without this hormone, females are sterile. Therefore, involvement of NMDA receptor to regulate JH synthesis and the strong expression of AmNR1 transcript in the developmental

stages suggest that might play same role in the honeybee like honeybee transferring-gene.

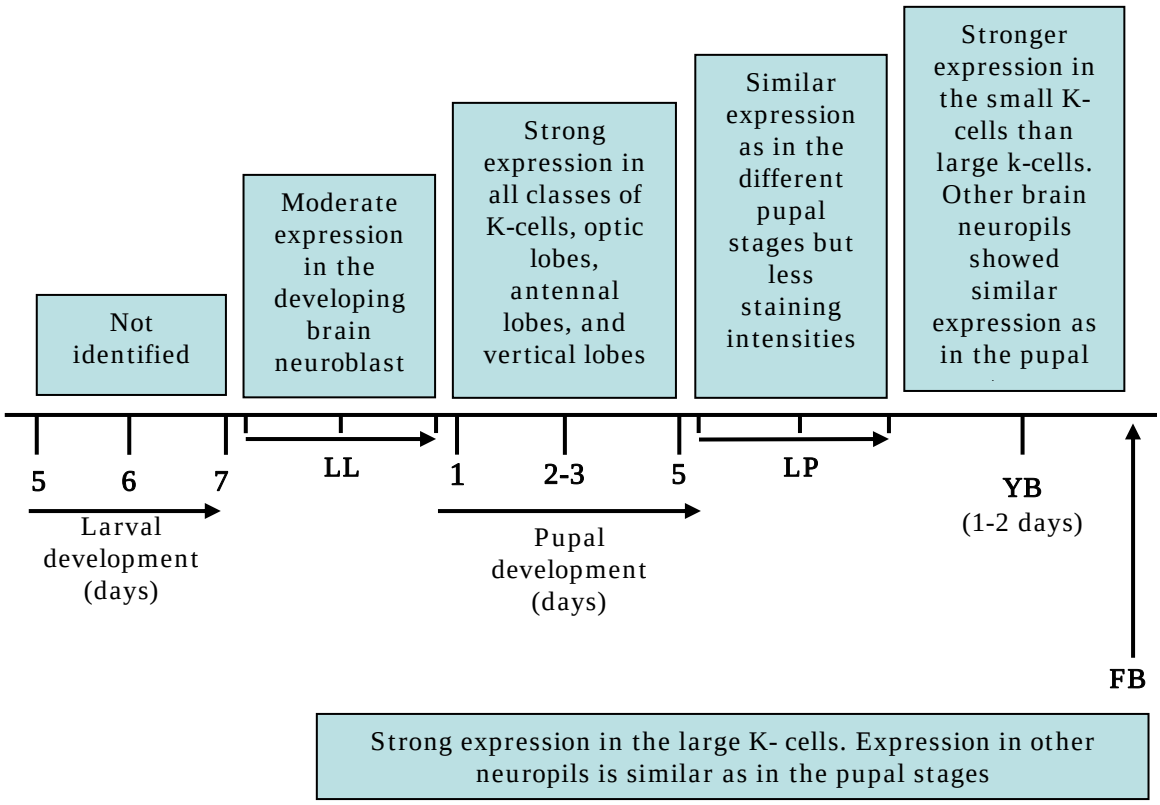

Figure 5.1: Diagram summarizing the expression profile of AmNR1 transcript at different developmental stages and also in the adult bee brain. Late larval stage (LL), late pupal stages (LP), young bee (YB) and foraging bee (FB).

5.3: Chapter 3

In this investigation, different probes (DNA and RNA) were used to investigate the functional properties and to detect differences between them. In both cases, a very similar expression profile of AmNR1 transcript was found in all brain neuropils, except for the Kenyon cells somata of the mushroom body. A strong hybridization signal was detected in a discrete population of Kenyon cells (large Kenyon cells and small class II Kenyon cells) with the DNA probe, whereas the RNA probe recognized transcript in almost all Kenyon cells with different staining intensities. There are at least two

possibilities. a) The RNA probe is much more sensitive than the DNA probe. Therefore, the RNA probe can detect even low-level expression of transcript. b) The position of two different probes within the sequence of the clone is not same. The DNA probe was 291 bp long and contained a specific region between 1906 and 2196 nucleotides of the AmNR1-1 variant. On the other hand, the RNA probe was 221 bp long and contained nucleotides between 2202 and 2423 of the AmNR1-1 variant. Recently, we have demonstrated that the AmNR1 gene possesses 17 exons and encodes at least 11 splice variants (termed AmNR1-1 to AmNR1-11) (Zannat et al., 2006). The sequence of the DNA probe is specific for 2 adjacent exons of the gene (from position 16417 in the exon 13 to the position 17352 in the exon 15). These two exons were found in the AmNR1-2, AmNR1-3 and AmNR1-8 variants. Therefore, the DNA probe can identify these three additional variants and might not be identified gene sequence. The sequence of the RNA probe probe is specific for 2 adjacent exons of the gene (from position 17358 in the exon 14 to the position 17660 in the exon 15) which were also found in several splice variants of the AmNR1 subunits (AmNR1-2, AmNR1-3, AmNR1-8 and AmNR1-9) (Fig. 5.2), which are most likely identified by the RNA probe. The AmNR1-1 mRNA and the probe sequences for *in situ* hybridization were compared to the honeybee genome with the BLASTn algorithm (default parameters) and showed that they matched to the nmdar1 gene sequence (Zannat et al., 2006). Therefore, we can assume unambiguously that the hybridization signal obtained with DNA and RNA probes are specific for AmNR1 transcript, and both probes are applicable for *in situ* hybridization.

DIG-labeled RNA probe was further used for *in situ* hybridization for the localization of AmNR1 transcript in the summer and winter bees and NR1-mab363 primary antibody was used for the localization of AmNR1 protein in the bees, which were collected from different seasons (winter, spring and summer) and hives to investigate

seasonal and colony type effect on the expression of AmNR1 gene. Experimental results showed no differences of the localization of AmNR1 transcript between summer and winter bees. Further, no difference was observed of the localization of AmNR1 protein in different seasonal stages and hives bees. These results are consistent with the expression of *Per* gene in the honeybee, which is not affected by light, flight experience, colony size, and colony demography (Bloch et al., 2004). In the adult canary, Singh et al. (2003) reported that NR2B is affected by season but not NR1 subunit. It suggests that the expression of NMDAR1 gene is not influenced by social and environmental factors.

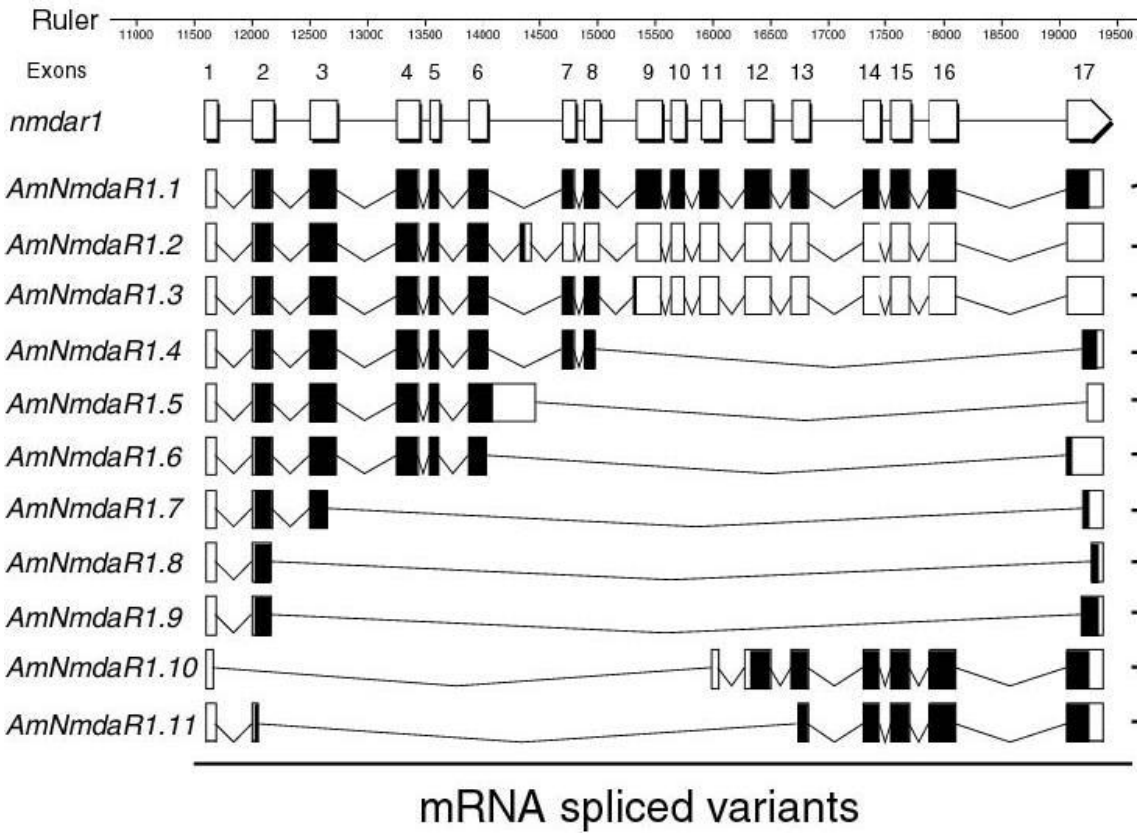

Figure 5.2: Identification of several AmNR1 mRNA variants. The mRNA splice variants (AmNmdaR1.1 to AmNmdaR1.11) were aligned with the nmdar1 gene. The 17 exons composing the gene are represented by boxes. The mRNAs variants are characterized by different open reading frames (black filled boxes). Ruler: nucleotide numbering of the genomic clone.

Double staining was performed for the co-localization of AmNR1 and protein discs-large (DLG) to investigate whether these two proteins are expressed in the same region of the bee brain. DLG is a tumor suppressor gene of *Drosophila* (Daniel et al., 1991; Woods and Bryant 1989) encodes a guanylate kinase homolog that abundantly expressed in the post-synaptic densities, septate junctions and in the CNS (Daniel et al., 1991). This protein is a member of the membrane associated guanylate kinase (MAGUK) and directly interacts with NMDA receptors for clustering in the post synaptic plasmamembrane (Kim et al., 1995, 1996; Kornau 1995; Niethammer et al., 1996). Present results showed that similar AmNR1 and DLG immunoreactivity in all brain neuropils with different staning intensities. Different staning intensities of DLG in different brain neuropils might indicate that it clusters with other membrane bound proteins. Therefore, we can conclude that the AmNR1 and DLG are co-expressed in the brain of honeybee.

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