4 Chapter 3

NMDA glutamate receptor subunit R1 gene is not affected by season, or colony type and this receptor protein is co-localized with protein discs-large

4.1: Abstract

The localization of the expression of AmNR1 transcript and protein was investigated at different seasonal stages and hives bees by *in situ* hybridization and immunohistochemistry. No difference was identified in the expression of AmNR1 transcript and protein in the bees, which were collected from different seasons and hives. It suggests that the expression of AmNR1 gene is not influenced by season or colony type. A comparative immunoreactivity between AmNR1 and DLG and their potential co-localization were investigated by immunohistochemistry and immunofluorescent on brain cryosections and vibratome sections. Immunohistochemical and immunofluorescent singnals revealed very similar immunoreactivity of AmNR1 and DLG throughout the entire bee brain but appeared with different staining intensities and these two molecules are mostly co-localized in the same brain neuropils.

4.2: Introduction

Genes can influence behavior in different ways. For example, allelic variation causes alternative behavioral phenotypes, and changes in gene expression can influence the initiation of behavior at different ages (Ben-Shahar et al., 2002). The same gene or

orthologs of a gene can influence behavior in both ways. For example, in *Drosophila melanogaster*, allelic differences in *foraging (for)* gene (for^R and for^S) result in two different foraging behaviors (Osborne et al., 1997). In the honeybee, this gene is associated with the age at onset of foraging, foragers having higher levels of *for* transcript than nurses (Ben-Shahar et al., 2002).

Mattila and Seeley (2007) reported that genetic diversity in the honeybee colonies enhances productivity and fitnees. After swarming genetically diverse colonies founded new colonies faster than genetically uniform colonies. In the genetically diverse colonies, comb, foraging rates, food storage, and population growth were higher than colonies with genetically uniform populations. Genetic variation and the rate of behavioral development among workers influence the division of labor and the expression of colony defensive behavior in the honeybee (Giray et al., 2000; Page and Robinson 1991; Robinson 2002). Honeybee colonies which composed of workers with faster rates of adult behavioral development are more defensive than colonies composed of workers with slower behavioral development (Giray et al., 2000). Further, numerous investigations have shown that bees of some genotypes show a faster rate of maturation and start foraging at younger ages than workers of other genotypes (Guzman-Novoa et al., 1994; Robinson 2002)

Many experimental results suggest that gene expression is influenced by various social and environmental factors, such as light, flight experience, age demography, colony type, and size of the colony (Bloch et al., 2004; Jodo et al., 2005; Messager et al., 2000; Sumova et al., 2002; Robinson 1992, 2002). In the honeybee, *period* (*per*) mRNA levels are higher at night and in older bees (Bloch et al., 2004; Toma et al. 2000). This result is consistent with the expression of PER-like immunoreactivity, where in the bee brain it is higher at night, and in foragers (Block et al., 2003). Further, Toma et al. (2000) reported

differences of *per* expression in bees collected from small laboratory colonies and one typical field colony with free-flying bees. In single-cohort colonies, the absence of older bees causes some individuals to exhibit precocious foraging. Young workers showed high levels of forager-like *per* mRNA compared to bees in more typical colonies (Block et al., 2001; Huang and Robinson 1992; Toma et al., 2000).

In the adult canaries, song learning requires activation of NMDARs within the forebrain song region IMAN (Basham et al., 1996), and the expression of NMDARs is affected by season (Singh et al., 2002). Canary exhibits vocal plasticity throughout the adulthood regulated by seasonal changes in day length and testosteron (T) levels. Songs produced by both wild and domesticated adult canaries (*Serinus canaries*) are stable during the spring breeding season that changes mainly during the fall and winter time (Leitner et al., 2001). In the early spring, vocal stability is associated with high plasma T levels stimulated by increasing day length. From many experimental results, it is well documented that gene expression is affected by various social and environmental factors in different animals however no work has been done on NMDARs in the honeybee.

In this context, we have investigated the effect of season and colony type on the expression of AmNR1 transcript and protein in foraging bees by *in situ* hybridization and immunohistochemistry. In addition, double staining was performed for the co-localization of AmNR1 protein and protein discs-large (DLG). DLG is a *Drosophila* homolog of the PSD-95 MAGUK and expressed at the post synaptic sites to form a multimeric scaffold for the clustering of NMDA receptors and Shaker K+ channel subunits (Mori er al., 1998; Standley et al., 2000; Steigerwald et al., 2000). Thereby, we have investigated to know whether these two molecules are expressed at the same brain region or not.

4.3: Materials and methods

4.3.1: Animal's maintenance

Honeybee (*Apis mellifera carnica*) colonines were maintained in the flight room in winter and kept in natural conditions in the garden in summer, which was used throughout the experiments. Forager honeybees were collected in the morning or afternoon at the entrance of the hive depending on the type of experiments, weather and seasons. For seasonal basis experiments, foragers were collected from three different seasons: winter, spring, and summer. For colony type experiments, bees were collected from four different colonies. Two of them were maintained in natural condition in the garden and other two colonies were maintained in the flight room in winter.

4.3.2: Preparation of DIG-labeled dsDNA and RNA probe

A cDNA template was used to generate a 291 bp fragment of DIG-labeled dsDNA probe for *in situ* hybridization by using forward (5'- AACACTGACGGTACCGAAGAGGAT -3') and reverse (5'- ATGTGCCACGGTAAAAGGTTCTGCT -3') primer pairs. The PCR protocol consisted of 40 cycles of 30 s at 95°C, 1 min at 66 °C, 1 min at 72°C, and followed by a 5 min incubation at 72°C. For in detail, please see the appendix. DIG-labeled dsDNA probes were purified using probeQuant ^{TM} G-50 micro columns (Amersham Biosciences, Freiburg, Germany) and quantified by a spectrophotometer. DIG-labeled anti-sense and sense RNA probes were prepared as described in the materials and methods in Chapter 1.

4.3.3: Preparation of DIG-labeled CAT dsDNA probe

pBlueBac4.5/V5-His CAT plasmid was used as a template for polymerase chain reaction (PCR) to amplify the 300 bp fragments of DIG-labeled CAT dsDNA probe using forward (5'- GTATGGCAATGAAAGACGGTGAGC-3') and reverse (5'-TGAAAACGGGGGCGAAGAAGT-3') primers. The same PCR protocol was applied as described for DNA probe. DIG-labeled probe was purified and quantified as described above.

4.3.4: *In situ* hybridization

Bee brains were dissected and pre-fixed in 4 % paraformaldehyde for 2 hours on ice. Cryoprotected overnight at 4^{o}C with 30% sucrose in 1× PBS, 0.1% Triton X-100 and embedded in Jung medium. Brains were then sliced at 14 µm thickness and air-dried for 20 minutes at RT. Air-dried slides were post fixed in 4% paraformaldehyde for 10 minutes at RT and then washed 3 times with 1× PBS for 3 minutes each. Then the slides were acetylated with acetylating solution for 10 minutes. Subsequently, the slides were washed with 1× PBS 3 times for 5 minutes each at RT and under constant agitation. Prehybridization was performed with 500 µl of hybridization solution at 65^{o}C in a humidified box for 2 hours. 200 µl of hybridization solution and 65 ng of DIG-labeled dsDNA probe were mixed gently in a sterile microcentrifuge tube on ice and denatured at 95^{o}C for 5 minutes. After denaturization, the probe solutions were placed on ice immediately to cool down. 200 µl of denature probe solution was overlaid on each slide and covered with glass cover slip and hybridized at 65^{o}C for 18-22 hours. Following hybridization, the slides were washed with preheated 5× SSC (approximately 30 minutes before heated at 65^{o}C) for 5 minutes. Posthybridization washes were carried out with pre-heated 5× SSC for one time and with 0.2% SSC for 3× 30 minutes each at

65°C. Slides were then washed with 1× PBS-T for 10 minutes at RT and blocked with 2% blocking solution (2% BSA in 1× PBS-T) for 2 hours at RT. The slides were then incubated with anti-digoxigenin antibodycouple to alkaline phosphatase (diluted 1:500 in the blocking solution) overnight at 4°C. After antibody incubation, the slides were washed with 1× PBS-T 3 times for 30 minutes each and equilibrated with the detection buffer (0.1M Tris pH 9.5, 0.1M NaCl, 50 mM $MgCl_2$, 0.1% Tween-20) for 10 minutes at RT under agitation. Subsequently, slides were incubated with the color development solution (0.1M Tris pH 9.5, 0.1M NaCl, 50 mM $MgCl_2$, 0.1% Tween-20, 165 µg/ml BCIP, 330 µg/ml NBT) in a humidified box in dark conditions at RT for few minutes to several hours with a piece of parafilm. Thereafter, the slides were washed with 1× PBS-T for 5 minutes. Finally, the slides were rinsed with distilled water for 10 minutes. The slides were then air-dried and mounted in melted glycergel covered with a 24×60 mm glass cover slip and observed under the light microscope.

In situ hybridization was performed with DIG-labeled RNA probes as described in the materials and methods in Chapter 1. Briefly stated here, bee brains were fixed in 4% paraformaldehyde for 10 minutes on ice and incubated with 30% sucrose in 1× PBS-T overnight at 4°C. The brains were then embedded and sectioned at 14 µm thicknesses. Each slide was hybridized with overlay of preheated and chilled 200 µl of hybridization solution which contained 150 ng to 200 ng of DIG-labeled sense and/or anti-sense probes (sense or anti-sense probes were mixed with the 200 µl of hybridization solution and heated at 70°C for 10 minutes and immediately placed on ice to cool down) at 65°C for 16-24 hours. Subsequently, anti-digoxigining antibody incubation and color development were performed as described for DIG-labeled DNA probe.

4.3.5: Immunohistochemistry

Immunoenzyme histochemical analysis was performed as described in the section 2.3 in materials and methods in Chapter 1 and 2. Briefly stated here, the brains were disected and fixed in 4% paraformaldehyde for 3 hours on ice. Afterwards, the brains were cryoprotected with 30% sucrose solution at 4^{o}C for overnight and sectioned at 14 μm thicknesses. The slides were blocked with 3% blocking solution at RT for 2 hours and incubated with NR1-mab363 primary antibody overnight at 4^{o}C. The slides were then incubated with anti-mouse secondary antibody coupled to alkaline phosphatase and developed with NBT/BCIP solution.

4.3.6: Immunofluorescent and double staining of AmNR1 and DLG

Brain preparation and fixation were done according to section 2.3 in materials and methods in Chapter 1. The brains were then dehydrated and redydrated with ethanol (50, 70, 90, 99 and 100% for 2 times) and washed with 1× PBS for 3 times 15 minutes each. The brains were embedded in 7% low melting agarose at 65^{o}C and cool down on ice, and sectioned at 100 μm thicknesses using a vibratome machine and collected in an ELISA plate that contained 1× PBS solution. The sections were blocked in the blocking solution (1% BSA in 0.3% 1× PBS-T) for 24 hours at 4^{o}C. After blocking, sections were incubated with primary antibodies, NR1-mab363 or NR1-pan (diluted 1:200 in the blocking solution each) or AF3 anti-DLG (diluted 1:100 in the blocking solution, 300 μl/whole in the ELISA plate) for 24 hours.

For the co-localization of AmNR1 and DLG, sections were incubated simultaneously with NR1-pan (1:200) and AF3 anti-DLG (1:100) antibodies. Then the sections were washed with 0.3% 1× PBS-T for 3 times for 10 minutes each and one time for 24 hours (1 ml/whole in the ELISA plate) at 4^{o}C. The following day, sections were incubated

with secondary antibodies; anti-mouse CY3 or CY5 and anti-rabbit CY2 for 24 hours at 4^{o}C (diluted 1:500 in the blocking solution each) and washed with 0.3% 1× PBS-T 3 times for 10 minutes each and one time for 24 hours at 4^{o}C. Finally, sections were kept in 50% glycerol and the fluorescent signal was detected under the confocal microscope.

4.4: Results and discussion

Before doing the comparative study of the expression of AmNR1 transcript and protein in different seasonal stages and hive bees, a comparative study for the localization of AmNR1 transcript between DIG-labeled DNA and RNA probes were investigated by *in situ* hybridization to understand whether different probes show any different staining patterns of AmNR1 transcript. Some part of the results using DIG-labele sense and anti-sense RNA probes were previously published and are described in the Chapter 1 as a published manuscript (Zannat et al., 2006). Here, we are further considering these data shortly with additional unpublished data to compare with the results of DIG-labeled dsDNA probe. Table of experiments are presented in the appendix.

4.4.1: Synthesis of probes

A cDNA that encode a homologue of the NMDA glutamate receptor subunit R1 (AmNR1) used as a template to generate PCR. PCR products were then used as a template to generate DIG-labeled DNA and RNA probes. DNA probe was 291 bp long and contained a specific region between 1,906 and 2,196 nucleotides of the NMDAR1-1 variant. The RNA probe was 221 bp long and contained nucleotides between 2,202 and 2,423 of the NMDAR1-1 variant. Expected size of PCR products and both probes were confirmed by 1% agarose gel electrophoresis with ethidium bromide (Fig. 3.1 and Fig.

4.1). The yield of DIG-labeled incorporation of RNA probes was estimated by Dot blot on a nitrocellulose membrane that showed almost same staining intensity of DIG-labeling (Fig. 3.1E). DIG-labeled CAT dsDNA probe was used as a negative control for *in situ* hybridization using DIG-labeled AmNR1 DNA probe and DIG-labeled sense RNA probe was used as a negative control for *in situ* hybridization using DIG-labeled anti-sense RNA probe to clarify the specificity of the hybridization signal. In both cases, did not yield any signals. (Fig. 4.2D and Fig. 4.3D).

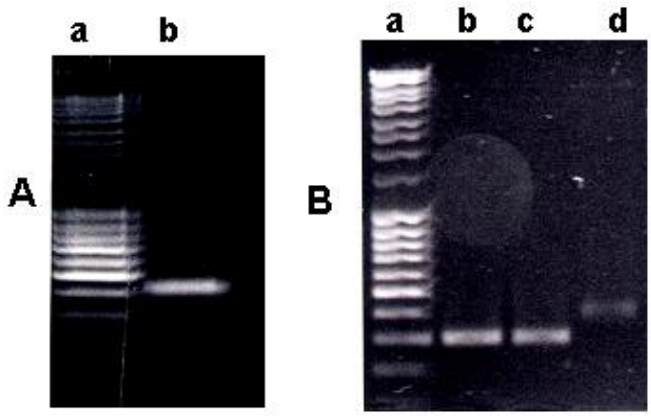

Figure 4.1: Analysis of PCR DIG-labeled probes by gel electrophoresis. A: Column 'a' indicates DNA ladder and column 'b' indicates DIG-labeled AmNR1 DNA probes. B: DIG unlabeled (column a, b) and labeled CAT DNA probes (column c).

4.4.2: Localization of AmNR1 transcript in the adult bee brain

Using DIG-labeled DNA probe, strong AmNR1 signal was detected in the antennal lobe, optic lobes, sub-oesophageal ganglion, and dorsal lobe (Fig. 4.2A-C, E and F). In the mushroom body, a heterogeneous AmNR1 signal was detected in the Kenyon cells, in which predominant staining was detected in the large Kenyon cells and in the clawed Kenyon cells, whereas moderate staining was detected in the small class I Kenyon cells (Fig. 4.4A-H). Difference of the expression of AmNR1 transcript was also detected in the class I Kenyon cells. In the most sections, strong AmNR1 signal was detected in the

somata of the small class I Kenyon cells located above the calyces (Fig. 4.4B, C and E). Sometimes, weak and/or no signal was detected in the class I Kenyon cells located within the calyces (Fig. 4.4A-H). These features were observed systematically in anterior site to posterior sites of cryosections through the bee brain. A similar expression profiles were also detected with anti-sense RNA probes (Fig. 4.3A-C and Fig. 4.41-N). In some sections, the signal was detected uniformly throughout the all types of Kenyon cells located within the calyces (Fig. 4.4I-K). In addition to that, a strong signal was detected in the large Kenyon cells and clawed Kenyon cells, whereas moderate signal was detected in the small class I Kenyon cells (Fig.L-N). These results are consistent with the expression of AmNR1 immunoreactivity and are also consistent with the expression of glutamate transporter gene in the honeybee (Kucharski et al., 2000; Zannat et al., 2006). Heterogeneous expression of AmNR1 transcript and protein suggests populations of different subset of Kenyon cells in the mushroom body of the honeybee. Kenyon cell populations were investigated in much detail in the honeybee by several groups using a variety of antibodies and modulatory peptides, such as, FMRF-amide and gastrin-cholecystokinin (Schörmann and Erber 1990, Straufeld et al., 2000); the amino acids taurine and glutamate (Bicker et al., 1988; Bicker 1991 and 1999); and honeybee PKA and PKC (Eisenhardt et al., 2001; Fiala et al., 1999; Müller 1997 and 1999). Heterogeneous immunoreactivity in the Kenyon cells has also been described for other insect species, for example, glutamate-like immunoreactivity in the cricket and cockroach (Shürmann et al., 2000; Sinakevitch et al., 2001); taurine-like immunoreactivity in the *Drosophila*, *Locusta* and cockroach (Bicker et al., 19991; Sinakevitch et al., 2001), and aspartate-like immunoreactivity in the cockroach (Sinakevitch et al., 2001). All these results indicate heterogeneity of the Kenyon cells population and heterogeneity of their function in insects including honeybee.

Heterogeneity of the Kenyon cells population can be identified based on the following criteria: i) cellular morphology, ii) modulatory peptide, and iii) neurotransmitters (Sinakevitch et al., 2001; Strausfeld et al., 2000).

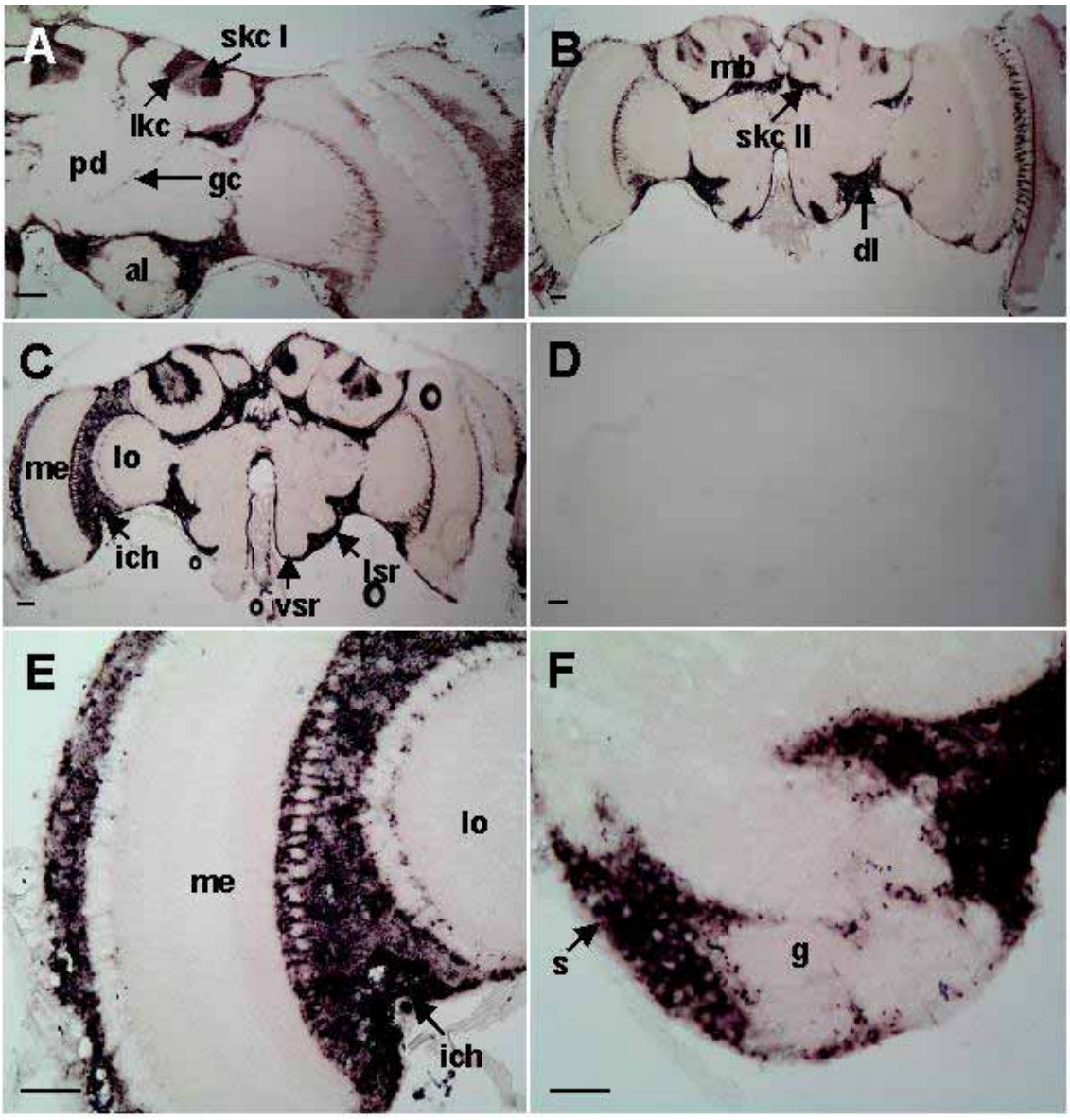

Figure 4.2: *In situ* hybridization to AmNR1 transcript hybridized with DIG-labeled AmNR1 DNA probes. A-C: Serial sections from same brain. D: Control experiment hybridized with DIG-labeled chloramphenicol acetyl transferase (CAT) gene probes. E and F: Higher magnification of the optic lobe and the antennal lobe. Mushroom body (mb), antennal lobe (al), small class I kenyon cells (skc I), small class II kenyon cells (skc II), large kenyon cells (lkc), dorsal lobe (dl), inner chiasma (ich), lateral and ventral soma rind of the suboesophageal ganglion (lsr, vsr), lobula (lo), medulla (me), peduncle (pd), cell somata of the antennal lobe (s), glial cells (gc), and glomeruli (g). Scale bar = 0.1 mm.

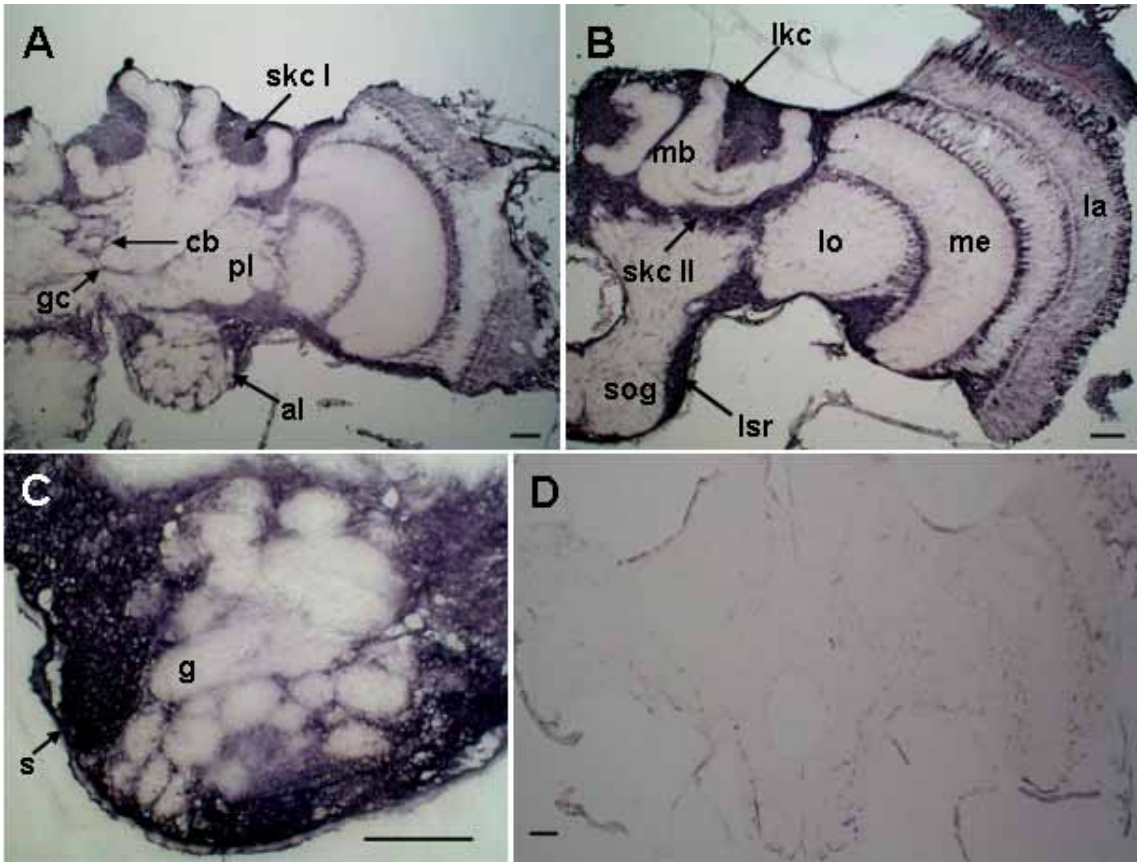

Figure 4.3: *In situ* hybridization to AmNR1 transcript hybridized with DIG-labeled AmNR1 anti-sense RNA probes. A and B: Sections from different bee brain. C : Higher magnification of the antennal lobe. D: Control experiment hybridized with DIG-labeled AmNR1 sense RNA probes. Mushroom body (mb), antennal lobe (al), small class I kenyon cells (skc I), small class II kenyon (skc II), large kenyon cells (lkc), lateral soma rind of the suboesophageal ganglion (lsr), lobula (lo), medulla (me), lamina (la), glial cells (gc), cell somata of the antennal lobe (s), glomeruli (g), suboesophageal ganglion (sog), and central brain (cb). Scale bar = 0.1 mm.

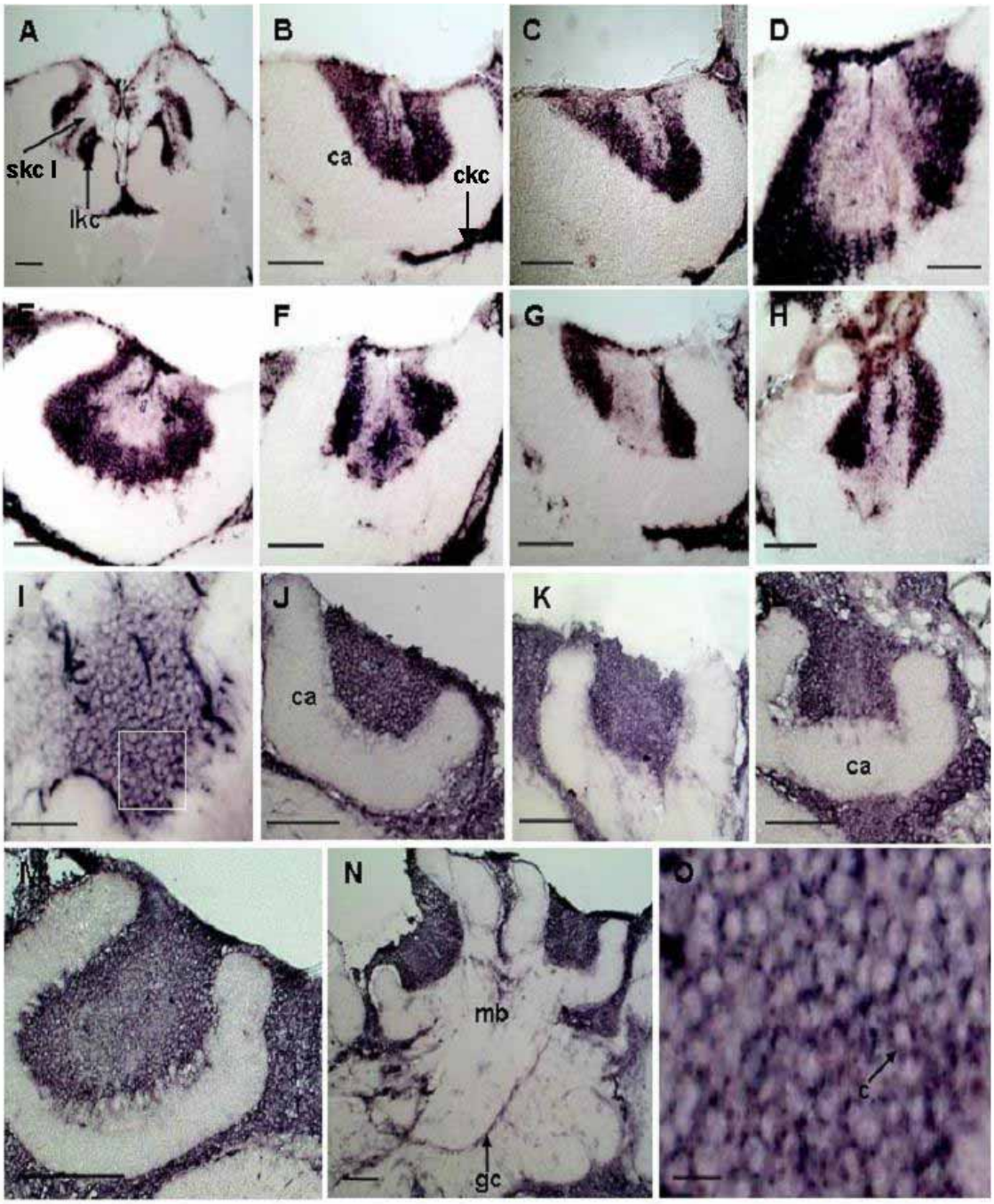

Figure 4.4: Expression of AmNR1 transcript in the mushroom body. A-H: Serial sections of the mushroom body from same specimen hybridized with digoxigenin-labeled AmNR1 DNA probes are shown preferential expression of AmNR1 transcripts. I-M: Preferential expression of AmNR1 transcripts in the mushroom body from different specimens hybridized with digoxigenin-labeled AmNR1 anti-sense RNA probes. N: Expression of AmNR1 transcripts in the whole mushroom body. O: Enlargement of the Kenyon cells that indicates by boxed area in I. Calyx (ca), large Kenyon cells (lkc), small Class I Kenyon cells (skc I), clawed Kenyon cells (ckc), mushroom body (mb), glial cells (gc), cytoplasm (c). Scale bar = A-N, 0.1 mm and O, 25 μm.

4.4.3: Localization of AmNR1 transcript in two different seasonal bees

A comparative analysis for the localization of AmNR1 transcript was studied in the summer and winter bees by *in situ* hybridization with DIG-labeled RNA probes to investigate the seasonal effects on the expression of AmNR1 gene. *In situ* hybridization signal for AmNR1 transcripts did not reveal any differences between the brains of summer and winter bees (Fig. 4.5A and C). In both cases, strong AmNR1 signal was detected in the optic lobe, in the antennal lobe, in the lateral and ventral soma rind of the suboesophageal ganglion, in the dorsal lobe, and in the protocerebral lobe compared to mushroom body.

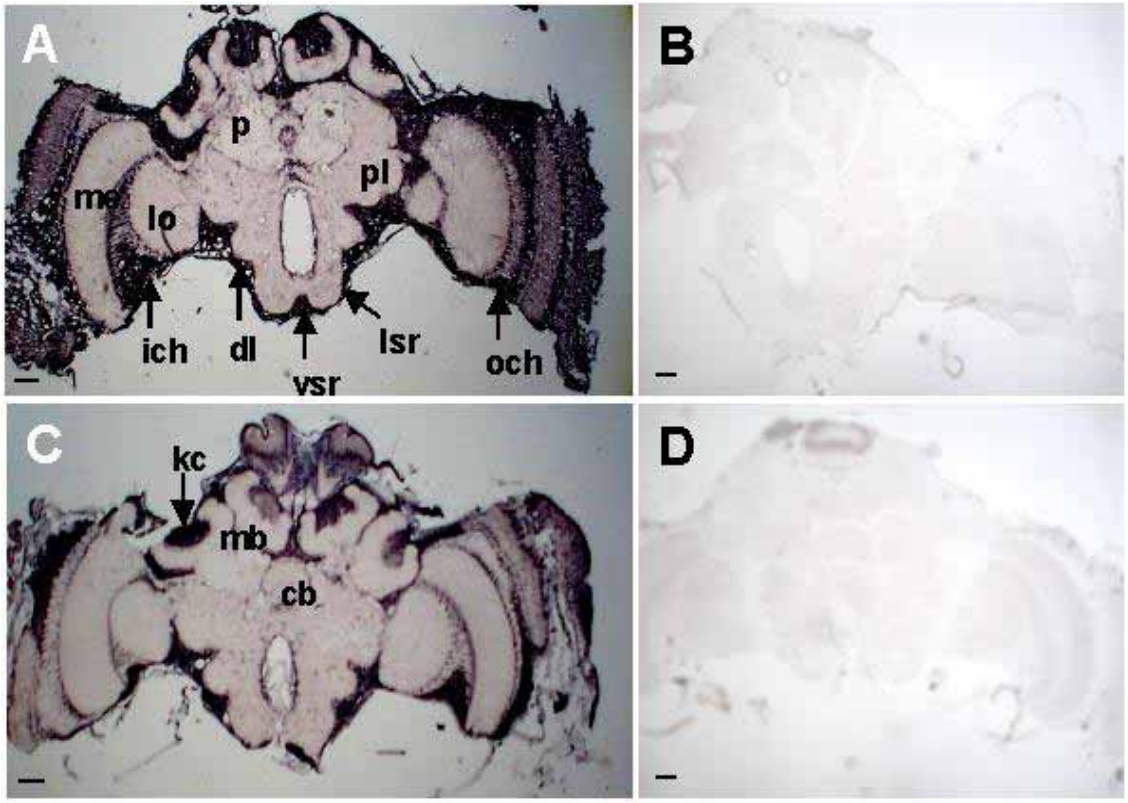

Figure 4.5: Seasonal basis expression of AmNR1 transcript in the worker bee brain revealed by *in situ* hybridization with DIG-labeled AmNR1 anti-sense RNA probes. A: AmNR1 transcripts expression in the summer bee. B: Control experiment for summer bee hybridized with DIG-labeled AmNR1 sense RNA probe. C: AmNR1 transcripts expression in the winter bee. D: Control experiment for winter bee hybridized with DIG-labeled AmNR1 sense RNA probe. Peduncle (p), protocerebral lobe (pl), lobula (lo), medulla (me), outer chiasma (och), inner chiasma (ich), lateral and ventral soma rind of suboesophageal ganglion (lsr and vsr), dorsal lobe (dl), mushroom body (mb), central brain (cb), Kenyon cells (kc). Scale bar = 0.2 mm.

In the mushroom body, a preferential AmNR1 signal was detected in which strong AmNR1 signal was detected in the large Kenyon cells and in the clawed Kenyon cells. Whereas moderate and/or weak AmNR1 signal was detected in the small class I Kenyon cells. No signal was detected in the negative control bee brains which were hybridized with DIG-labeled sense RNA probes (Fig. 4.5B and D). These results suggest that the expression of AmNR1 gene is not influenced by season.

4.4.4: Localization of AmNR1 protein in different seasonal stages and hives bees

Immunoenzyme histochemical analysis was performed for the localization of AmNR1 protein with NR1-mab363 primary antibody in three different seasonal stages (winter, spring and summer) and four different hives bee. No remarkable differences were identified of the expression of AmNR1 protein in different seasonal stages bee (Fig. 4.6A-C, Fig. 4.7A-C and Fig. 4.8A-D). In all cases, strong AmNR1 imuunoreactivity was detected in the antennal lobe, in the optic lobe, in the central brain, in the suboesopheal ganglion, and in the protocerebral lobe, while very weak AmNR1 imuunoreactivity was detected in the mushroom body. These results are consistent with the expression of AmNR1 transcript in the honeybee revealed by *in situ* hybridization (as described above) and with the expression of NR1 subunit in the adult canary (Singh et al., 2003). Further, no differences were also identified of the expression of AmNR1 protein in bees that were collected from the different colonies (Fig. 4.9A-C, 4.10A-C, 4.11A-C and 4.12A-C). These results are further consistent with the expression of *Per* gene in the honeybee (Bloch et al., 2004). Bloch et al. reported that the expresstion of *Per* gene is not influenced by light, flight experience and/or colony type. The specificity of AmNR1 protein was realized with negative control where the primary antibody was

omitted did not reveal any AmNR1 immunoreactivity (Fig. 4.6D, 4.7D, 4.8D, 4.9D, 4.10D, 4.11D and Fig. 4.12D). Together these results with the localization of AmNR1 transcript further suggest that the expression of AmNR1 gene is not influenced by season and colony type.

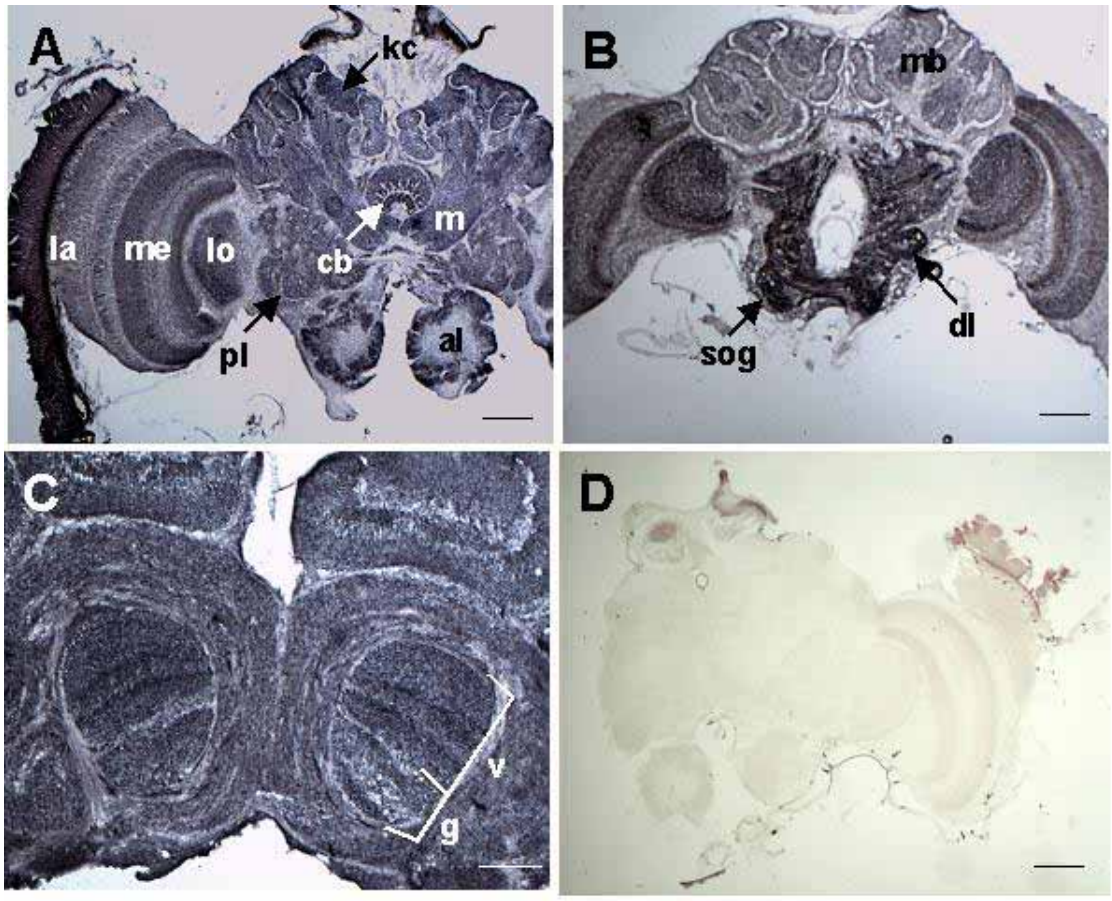

Figure 4.6: AmNR1 immunoreactivity in the winter bee realized with NR1-mab363 primary antibody. A and B: Frontal sections from different bee brain. Vertical (v) and gamma (g) lobes in C. D: Control experiment for AmNR1 immunoreactivity where primary antibody was omitted. Mushroom body (mb), protocerebral lobe (pl), lobula (lo), medulla (me), lamina (la), central brain (cb), antennal lobe (al), dorsal loba (dl), medial lobe 8m), and suboesophageal ganglion (sog). Scale bar = 0.2 mm in A, B, and D, and 0.1 mm in C.

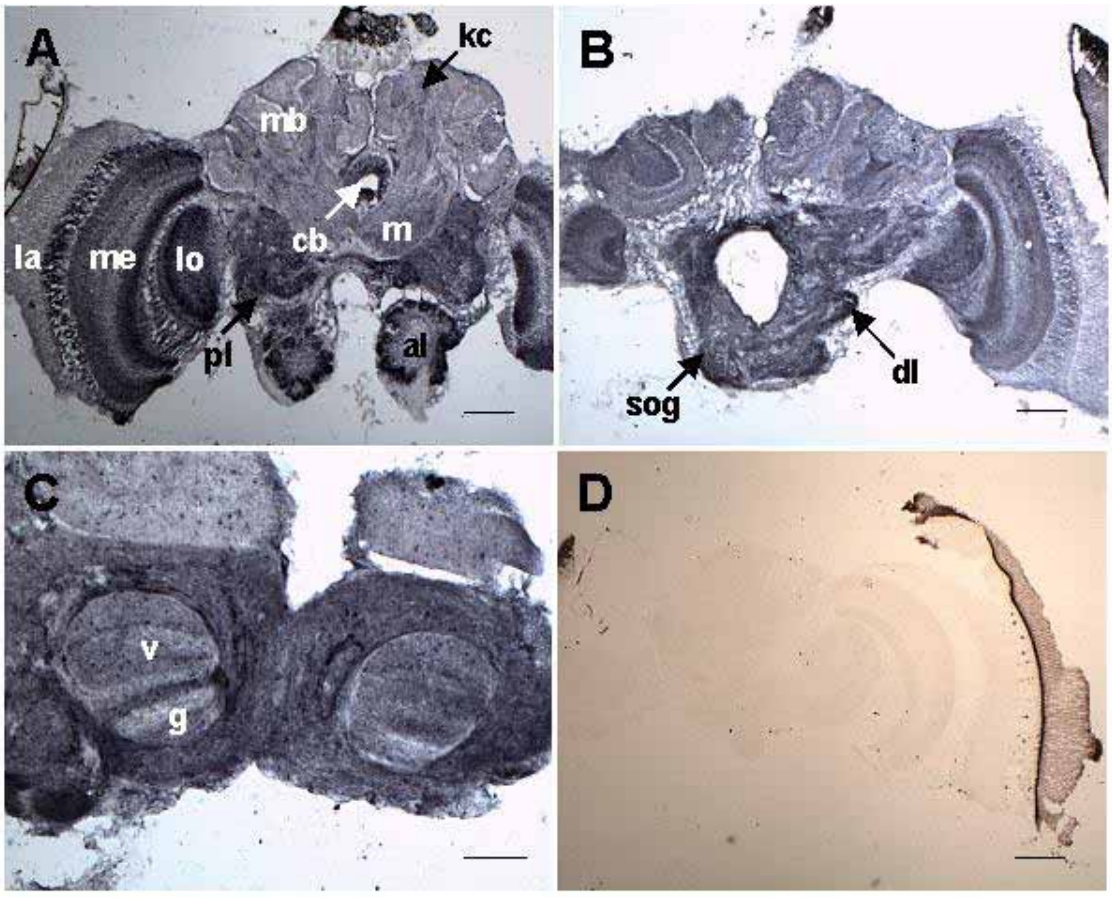

Figure 4.7: AmNR1 immunoreactivity in the spring bee realized with NR1-mab363 primary antibody. A and B: Frontal sections from different bee brain. Vertical (v) and gamma (g) lobes in C. D: Control experiment for AmNR1 immunoreactivity where primary antibody was omitted. Kenyon cells (kc), mushroom body (mb), protocerebral lobe (pl), lobula (lo), medulla (me), lamina (la), central brain (cb), antennal lobe (al), dorsal loba (dl), medial lobe (m), and suboesophageal ganglion (sog). Scale bar = 0.2 mm in A, B, and D, and 0.1 mm in C.

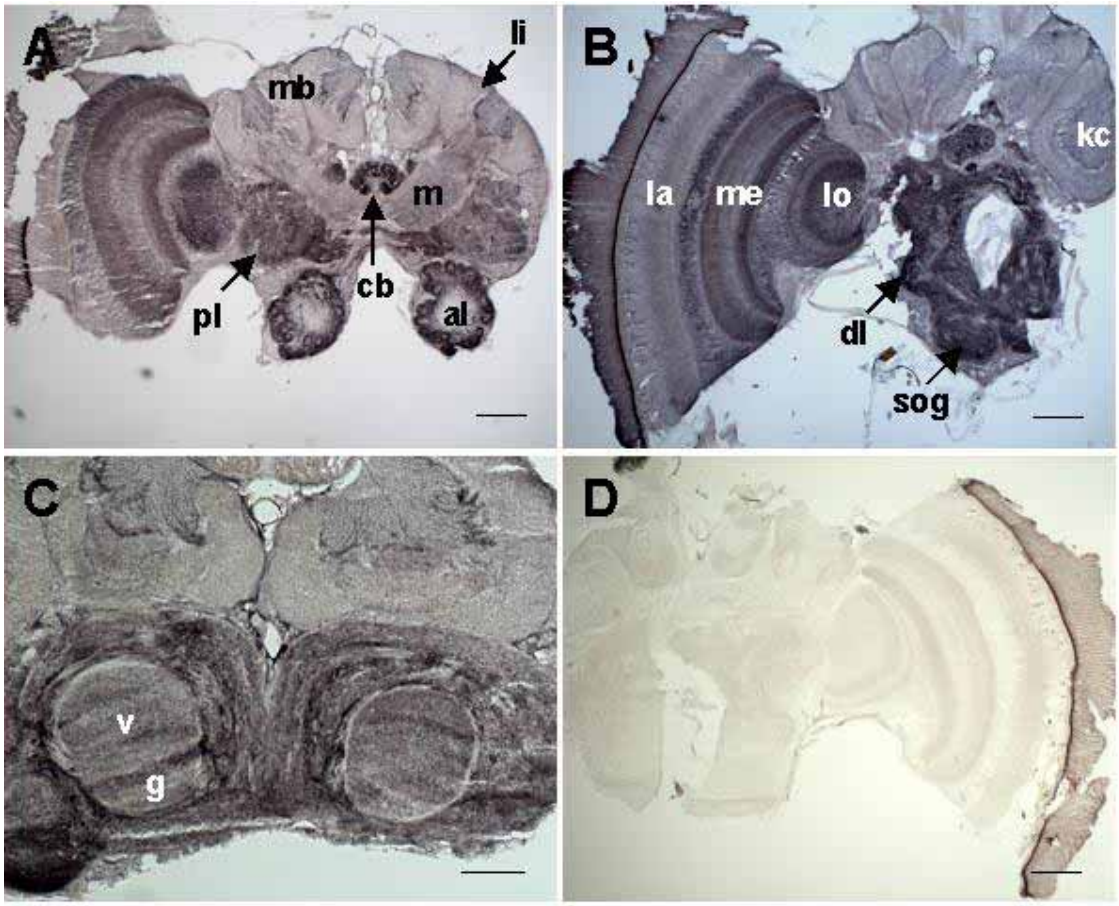

Figure 4.8: AmNR1 immunoreactivity in the summer bee realized with NR1-mab363 primary antibody. A and B: Frontal sections from different bee brain. Vertical (v) and gamma (g) lobes in C. D: Control experiment for AmNR1 immunoreactivity where primary antibody was omitted. Kenyon cells (kc), mushroom body (mb), lip (li), protocerebral lobe (pl), lobula (lo), medulla (me), lamina (la), central brain (cb), antennal lobe (al), dorsal loba (dl), medial lobe (m), and suboesophageal ganglion (sog). Scale bar = 0.2 mm in A, B, and D, and 0.1 mm in C.

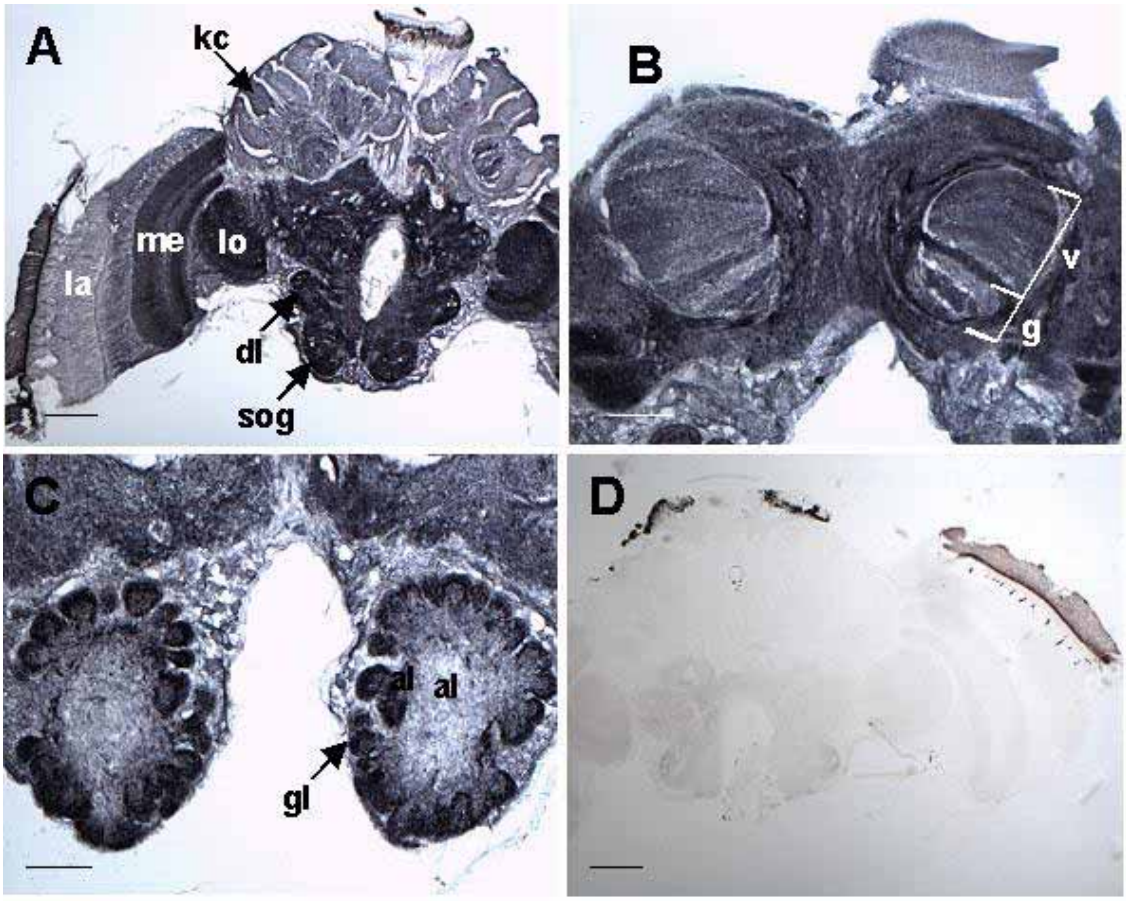

Figure 4.9: AmNR1 immunoreactivity in the garden bee collected from hive ´A` realized with NR1-mab363 primary antibody. A: Frontal section from the posterior part of the bee brain. Vertical (v) and gamma (g) lobes in B. Antennal lobe (al) and glomeruli (gl) in C. D: Control experiment for AmNR1 immunoreactivity. Kenyon cells (kc), lobula (lo), medulla (me), lamina (la), dorsal loba (dl), and suboesophageal ganglion (sog). Scale bar = 0.2 mm in A, and D, and 0.1 mm in B and C.

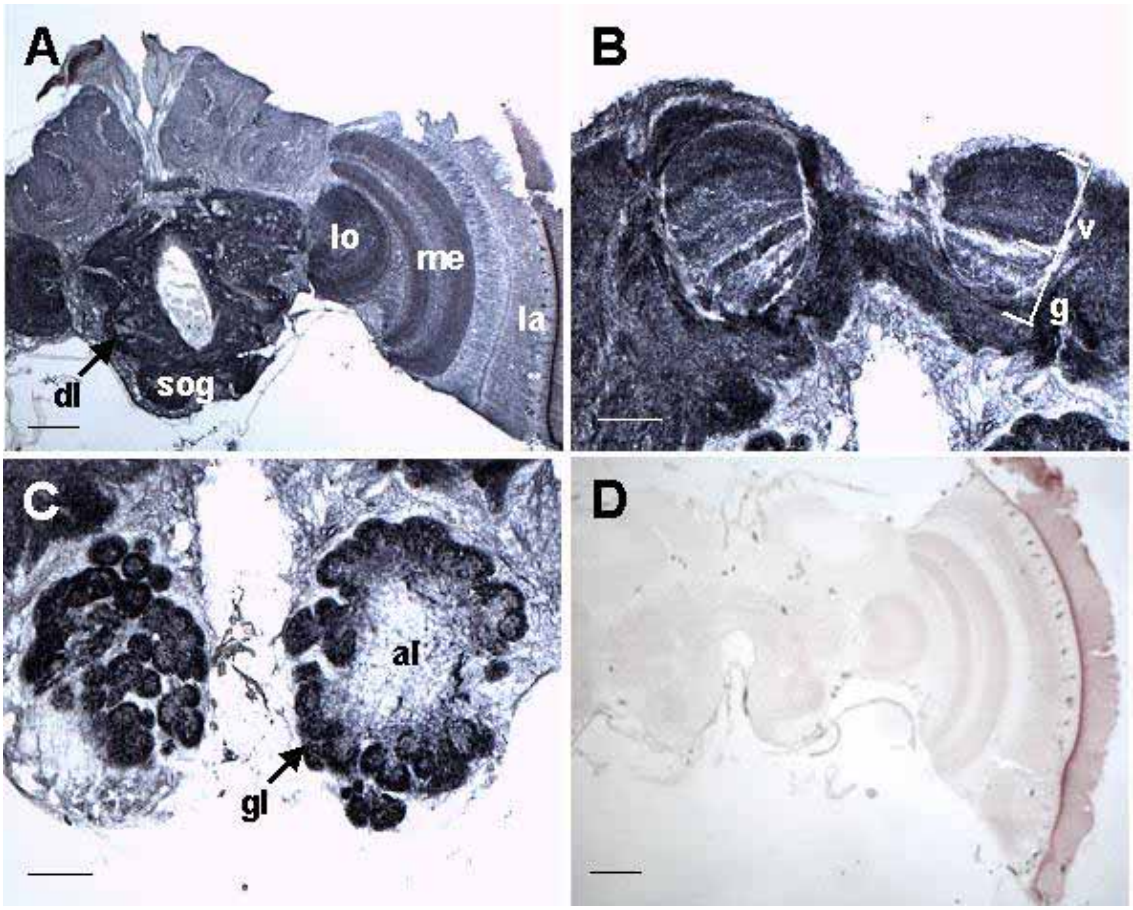

Figure 4.10: AmNR1 immunoreactivity in the garden bee (hive B) realized with NR1-mab363 primary antibody. A: Frontal section from the posterior part of the bee brain. Vertical (v) and gamma (g) lobes in B. Antennal lobe (al) and glomeruli (gl) in C. D: Control experiment for AmNR1 immunoreactivity. Lobula (lo), medulla (me), lamina (la), dorsal loba (dl), and suboesophageal ganglion (sog). Scale bar = 0.2 mm in A, and D, and 0.1 mm in B and C.

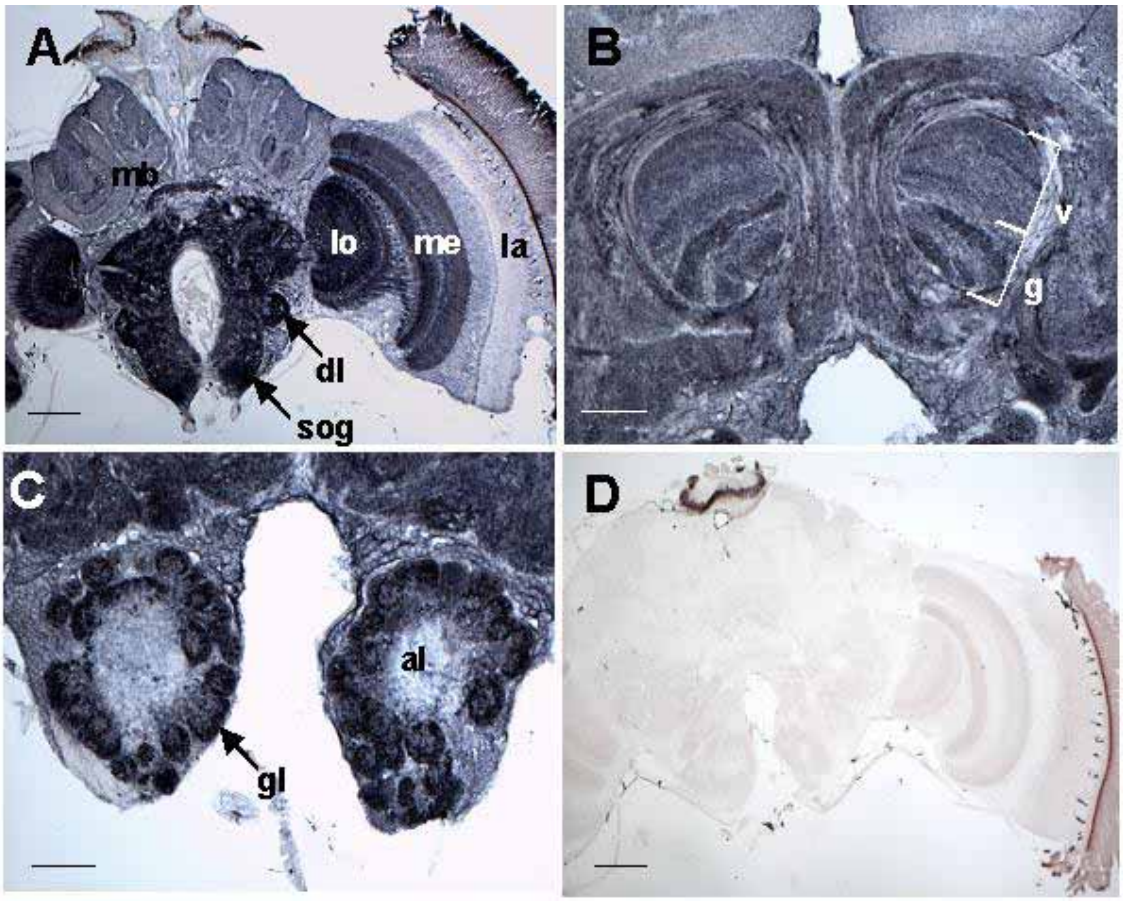

Figure 4.11: AmNR1 immunoreactivity in the flight room bee (hive A) realized with NR1-mab363 primary antibody. A: Frontal section from the middle part of the bee brain. Vertical (v) and gamma (g) lobes in B. Antennal lobe (al) and glomeruli (gl) in C. D: Control experiment. Mushroom body (mb), lobula (lo), medulla (me), lamina (la), dorsal loba (dl), and suboesophageal ganglion (sog). Scale bar = 0.2 mm in A, and D, and 0.1 mm in B and C.

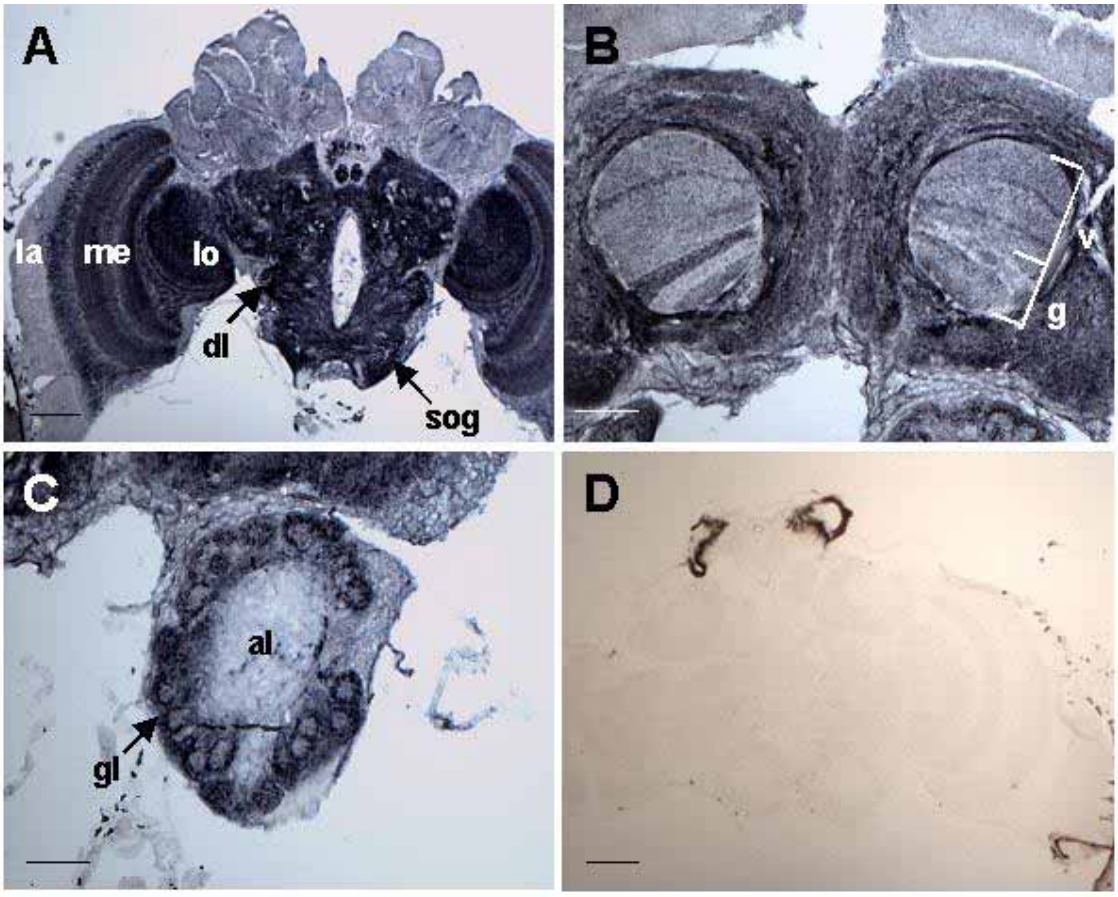

Figure 4.12: AmNR1 immunoreactivity in the flight room bee (hive B) realized with NR1-mab363 primary antibody. A: Frontal section from the middle part of the bee brain. Vertical (v) and gamma (g) lobes in B. Antennal lobe (al) and glomeruli (gl) in C. D: Control experiment. Lobula (lo), medulla (me), lamina (la), dorsal loba (dl), and suboesophageal ganglion (sog). Scale bar = 0.2 mm in A, and D, and 0.1 mm in B and C.

4.4.5: Comparisons and co-localization of AmNR1 immunoreactivity with DLG

A comparative immunoreactivity between AmNR1 and DLG and their potential co-localization were performed on brain cryosections and vibratome sections by immunohistochemistry, immunofluorescent, and double staining to know whether these two proteins are expressed in the same synaptic site. Immunoenzyme histochemical staining on brain cryosections revealed very similar immunoreactivity of AmNR1 and DLG throughout the entire bee brain but staining intensities were different. The AmNR1 immunoreactivity was prominent than the DLG in all brain neuropils, except central body (Fig. 4.13A-C). In vibratome sections, strong DLG immunofluorescence was detected in the calyces, while AmNR immunoreactivity was detected moderately in this neuropil. In the calyces, both AmNR1-1 and DLG immunoreactivity were identified differentially in different sub compartments of the collar and the basal ring (Fig. 4.14). This suggests that differential staining intensity in different brain neuropils might be a consequence of the sensitivity of the detection technique and/or sections were not from same level of the bee brain rather than a problem of the specificity of the antibody. Further, double labeling for AmNR1 and DLG on vibratome sections revealed that in general, the distribution of AmNR1 and DLG immunoreactivity was overlapped (Fig. 4.15). However, the staining intensity differed between these two molecules. Most prominent DLG immunoreactivity was detected in the mushroom bodies compared to other neuropils (Fig. 4.15B). In contrst, AmNR1 immunoreactivity is predominantly detected in the protocerebral lobes, central body, and in the somata of the Kenyon cells (Fig. 4.15A and C). Prominent DLG immunoreactivity in the mushroom body might indicate that it clusters with other membrane bound proteins, since it is a member of the membrane associated guanylate kinase super family.

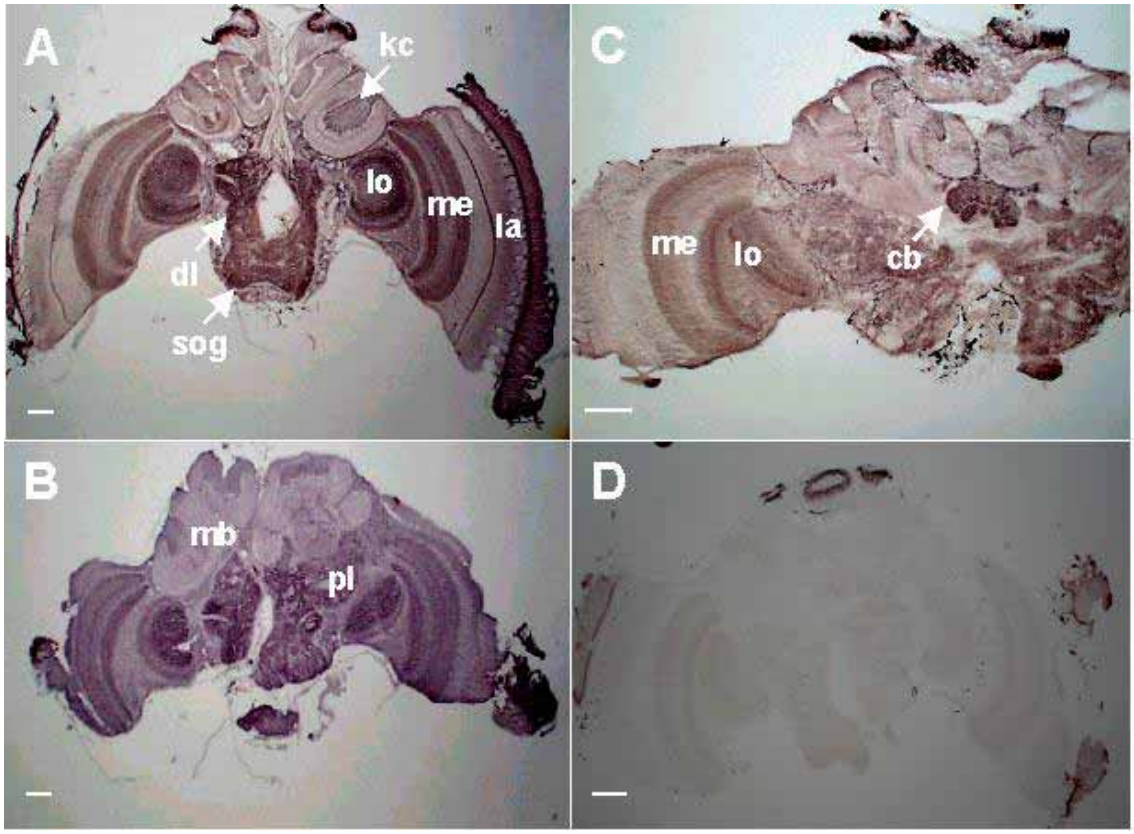

Figure 4.13: Immunostaining of AmNR1 and DLG in the worker bee brain. A: AmNR1 immunoreactivity with NR1-pan antibody. B: AmNR1 immunoreactivity with NR1-mab1586 antibody. C: DLG immunoreactivity with AF3 anti-DLG antibody. D: Control experiment where primary antibody was omitted. Mushroom body (mb), central brain (cb), optic lobe (ol), antennal lobe (al), protocerebral lobe (pl), Kenyon cells (kc), suboesophageal ganglion (sog), dorsal lobe (dl), lobula (lo), and medula (me). Scale bar = 0.1 mm.

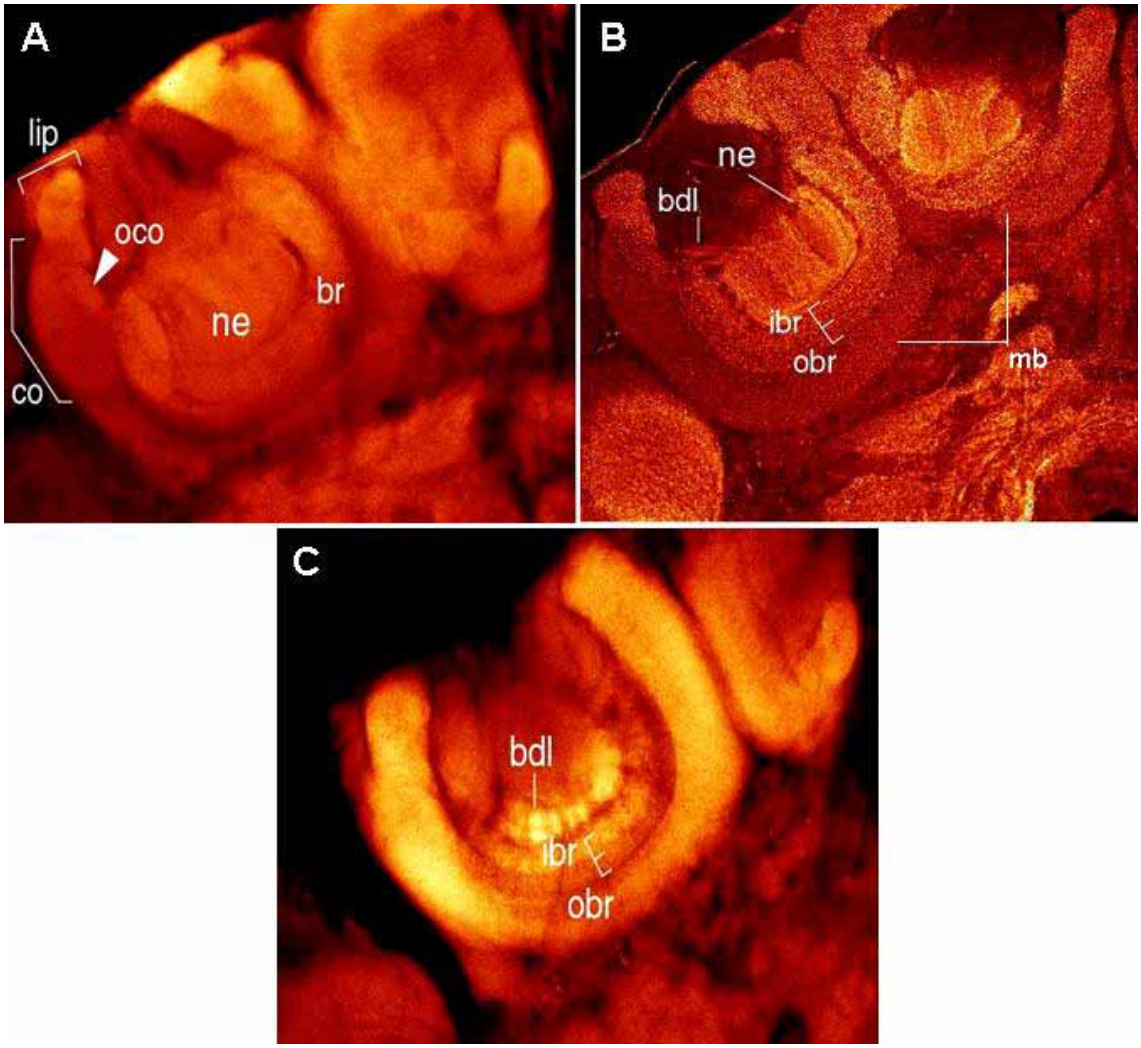

Figure 4.14: Comparative immunoreactivity of AmNR1 and DLG in the mushroom body on brain vibratome sections. A and B: AmNR1 immunostaining with NR1-mab363 and NR1-pan antibodies. C: DLG immunostaining with AF3 anti-DLG antibody. Mushlroom body (mb), ip (li), collar (co), outer collar (oco), basal ring (br), inner basal ring (ibr), outer basal ring (obr), neck of the peduncle (ne) and bundles of axons (bdl).

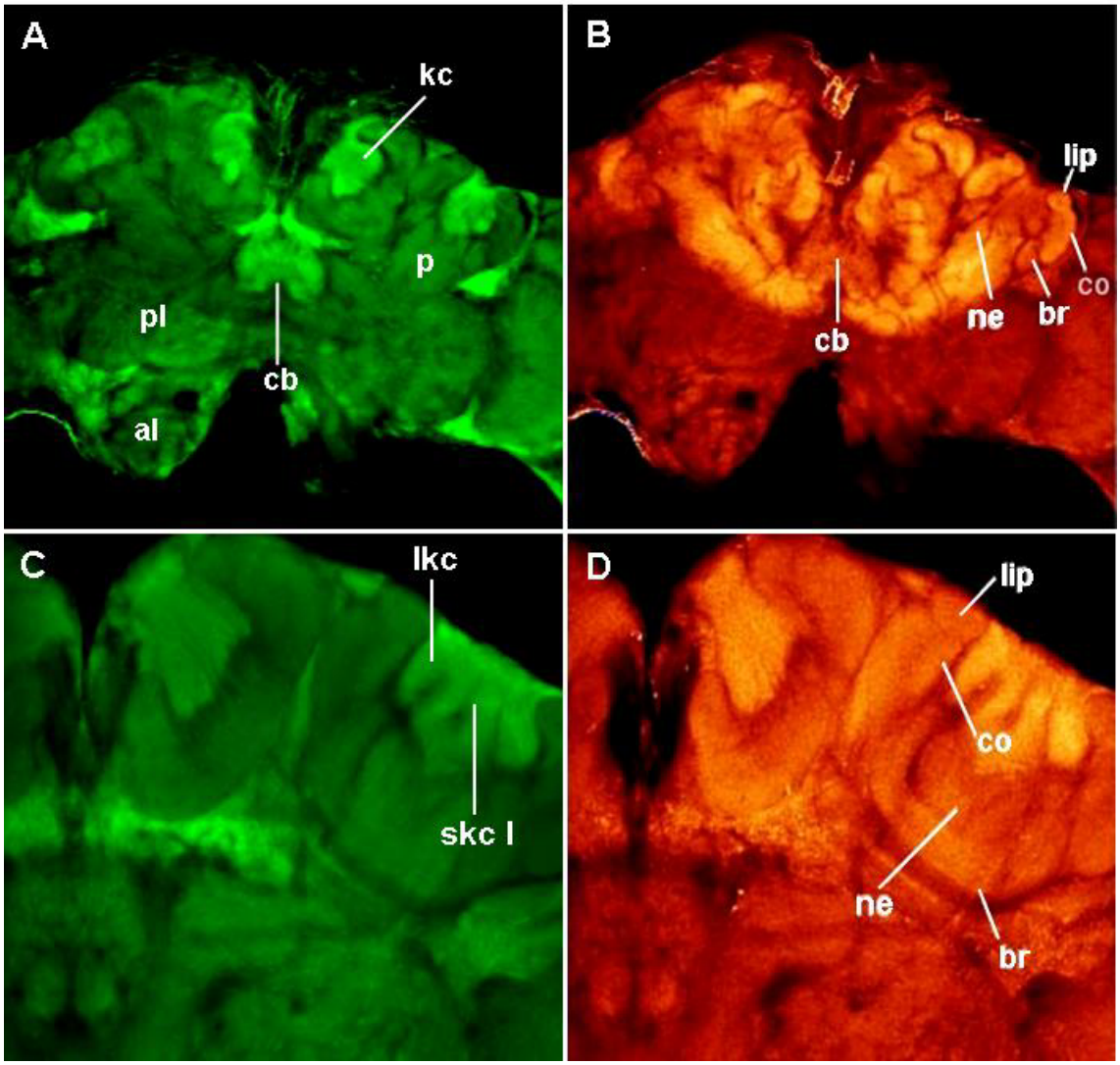

Figure 4.15: Co-localization of AmNR1 and DLG proteins in the bee brain. A and C: AmNR1 immunofluorescence staining with NR1-pan antibody. B and D: DLG immunofluorescence staining with AF3 anti-DLG antibody. C and D: Higher magnification of mushroom bodies. Peduncle (p), Kenyon cells (kc), large Kenyon cells (lkc), small Kenyon cells class I (skc I), lip (li), collar (co), basal ring (br), neck of the peduncle (ne), protocerebral lobe (pl), central brain (cb), and antennal lobe (al).

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