3 Chapter 2

Developmental expression of the NMDA glutamate receptor subunit R1 gene in the central nervous system of the honeybee

3.1: Abstract

Using *in situ* hybridization and immunohistochemistry, the expression of the NMDA glutamate receptor subunit R1 (AmNR1) gene was investigated in the different developmental stages of the honeybee brain. The AmNR1 mRNA was detected as early as in 0 day old pupae which gradually increased until 2 day old bees and then slightly decreased in the foraging bees. Conversely, the AmNR1 protein was detected at low level in 0 day old bees (0 day old bees refers to those just before emerging) then increased in 2 day old, 7 day old, 15 day old, and in the foraging bees. These results suggest that the AmNR1 gene in the honeybee brain is developmentally regulated.

3.2: Introduction

Based on numerous investigations, it is well documented that fast forms of synaptic transmission are mediated by glutamate, which acts as a primary excitatory neurotransmitter in the central nervous system in both vertebrates and invertebrates (Bicker 1999; Danbolt 2001; Headley and Grillner 1990; Sinakevitch et al., 2001). Nevertheless, it is also identified as a neurotransmitter in insect skeleton muscle, and at the neuromuscular junction (Delgado 1989; Johansen at al., 1989a and b). In recent years, glutamate receptors have generated considerable interest due to their unique

pharmacological, physiological, pathophysiological and electrophysiological properties and they play an important role in synapse refinement, excitotoxicity, neuronal development, neuronal plasticity as well as learning and memory (Bliss and Collingride 1993; Bradley et al., 2006; Michaelis 1998; Nakanishi et al., 1998; Mohn et al., 1999).

In vertebrates, the role of glutamate and its receptors have been studied intensively in adults and developmental stages (Bliss and Collingridge 1993; Gore et al., 2002; Imataka et al., 2006; Lee-Rivera et al., 2003; Maric et al., 2000). In insects, functional involvement of glutamate and NMDA receptors in the mechanism of learning and memory has also been reported by several groups, for example, in the *Aplysia* (Robert and Glanzman 2003), *Drosophila* (Xia et al., 2005), and honeybee (Locatelli et al., 2005; Si et al., 2004). However, the role of glutamate receptors in the developing central nervous system in insects is greatly unknown. Therefore, to gain a better understanding about their functional involvements in the developing central nervous system requires identification of glutamate receptors in the developmental stages. Although some of the glutamate receptors have already been identified in *Drosophila* (Parmentier et al., 1996; Ultsch et al., 1992 and 1993; Völkner et al., 2000), honeybee *Apis mellifera* (Funada et al., 2004), and cockroach *Diploptera punctata* (Chiang et al., 2002), no attempt has been realized with ionotropic glutamate receptors, particularly with the NMDA glutamate receptor subunit R1 in the honeybee.

In the previous investigation, we identified the NR1 subunit homologue of the NMDA glutamate receptor in the honeybee, *Apis mellifera* refered to AmNR1-1 (also called AmNR1) and described the expression sites of its mRNA and protein level in the adult bee brain (Zannat et al., 2006). In the current context, the localization of the AmNR1 transcript and protein during honeybee central nervous system development was investigated by using *in situ* hybridization and immunohistochemistry to understand the

developmental stages at which neural cells begin to express this receptor.

3.3: Materials and methods

3.31: Bees

For *in situ* hybridization, larvae and pupae were collected from the brood comb of the hive. To collect the young bees (1 and 2 day old bees), a piece of comb containing honeybee larvae and pupae was taken out the hive and kept them in an incubator at a constant temperature of 34^{o}C and a relative humidity of 90% for several days. Emerging adult insects were separated and reared in a plastic case supplied with 30% sucrose solution for 1 to 2 days.

For immunohistochemistry, five different adult stages were examined: 0 day old, 2 day old, 7 day old, 15 day old bees and foragers. To collect the different age bees, a piece of comb with honeybee larvae and pupae placed in an incubator at 34°C and a relative humidity of 65% for several days. Emerging adult bees were separated and marked with opalithplate (Werner Seip, Butzbach-Ebersgöns). The following days, bees were reared in a mini hive (Werner Seip, Butzbach-Ebersgöns) in the presence of a queen in the flight room in the winter. During this time a piece of comb with honey was supplied as a food source. The queen was collected from Neichen (Imkermeister, Anerkannter Lehrbetrieb, Neichen). Foragers were collected from the entrance of the hive in the afternoon, when bees returning to their hive with loads of pollen in their pollen baskets, conspicuously located on the hind legs.

3.3.2: Preparation of DIG-labeled sense and anti-sense RNA probes

DIG-labeled RNA probes were prepared according to section 2.3 in the materials and methods in Chapter 1 that were previously published (Zannat et al., 2006). Briefly, to

generate DIG-labeled sense and anti-sense RNA probes, template DNA was obtained by polymerase chain reaction (PCR) using the forward (5′-CATGTATTTCCGTCGCCAAGTC-3′) and the reverse (5′-TTCTGTAAACCAATCCCATAGC-3′) primers that were modified by addition of T7 and T3 promoter sequence at the 5′ extremity. Purified PCR products were then used as a template for in vitro transcription. For in detail, please see the appendix

3.3.3: Brain preparation and *in situ* hybridization

Brain preparation and *in situ* hybridization were performed as described for DIG-labeled RNA probes in section 2.3 in Chapter 1. Briefly, pupal brains were prefixed in 4% paraformaldehyde for 30 minutes and adult brains were prefixed in 4% paraformaldehyde for 10 minutes. The brains were cryoprotected with 30% sucrose solution overnight at 4°C. The brains were embedded in the embedding medium and sectioned at 14 μm thickness. Each slide was hybridized with 200 μl of hybridization solution which contained 150 ng to 200 ng of DIG-labeled sense and /or anti-sense probes at 65°C for 16-24 hours. Finally, the slides were developed with NBT/BCIP.

3.3.4: Immunohistochemistry

Brains were dissected and immunohistochemical analysis was manipulated as described in section 2.3 in Chapter 1, with the exception of the following parameters. Brains were fixed in 4% paraformaldehyde for 3 hours and then dehydrated and rehydrated by a series of ethanol (50, 70, 90, 99 and 100%) for 10 minutes each. Sections were blocked with 3% blocking solution for 2 to 3 hours at room temperature and incubated with NR1-mab363 primary antibody overnight at 4°C (diluted 1:200 in the 3% blocking solution). Subsequently, sections were incubated with anti-mouse coupled to alkaline

phosphatase secondary antibody for 2 hours at RT and developed with BNT/BCIP solution.

3.4: Results

3.4.1: Expression of AmNR1 mRNA at different developmental stages

DIG-labeled RNA probes were used for *in situ* hybridization to detect the expression of AmNR1 gene at different developmental stages of the honeybee brain neuropils (Fig. 3.1). *In situ* hybridization revealed that the AmNR1 mRNA is expressed differentially depending on the different developmental stages. In 1 day old pupae, the AmNR1 mRNA signal was detected throughout the entire brain with stronger signals in the neuroblasts of the mushroom body (Fig. 3.2A, and B). In 2-3 day old pupae, an AmNR1 mRNA signal was detected in the same regions as in 1 day old pupae. At this developmental stage more structural features of the optic lobes become recognizable and the staining pattern in sub-structures of the optic lobe was very similar to the adult bee brain, where only weak AmNR1 mRNA signal was detected in the lamina (Fig. 3.2C, and D). Higher magnification revealed that the AmNR1 signal is more intense in the Kenyon cell neuroblasts, while some cells show no AmNR1 expression (Fig. 3.2E and F).

In 5 day old pupae, the AmNR1 mRNA signal was also detected everywhere in the brain, such as mushroom body, optic lobe, and antennal lobe (Fig. 3.3). At this stage, the complete structure of the bee brain was visible. In the mushroom body, all type of Kenyon cells showed a homogeneous staining pattern (Fig. 3.4A). In the optic lobe, the signals resembled those that were detected in adult inner chiasma, outer chiasma and the glial cells (Fig. 3.3C). In the vertical lobe, a strong AmNR1 mRNA signal was observed

within a restricted band/column that may indicate the glial cells (Fig. 3.4B, C, and D). In the late pupae, an AmNR1 mRNA signal was detected in the same regions as in 5 day old pupae but the staining was weaker overall (Fig. 3.4E).

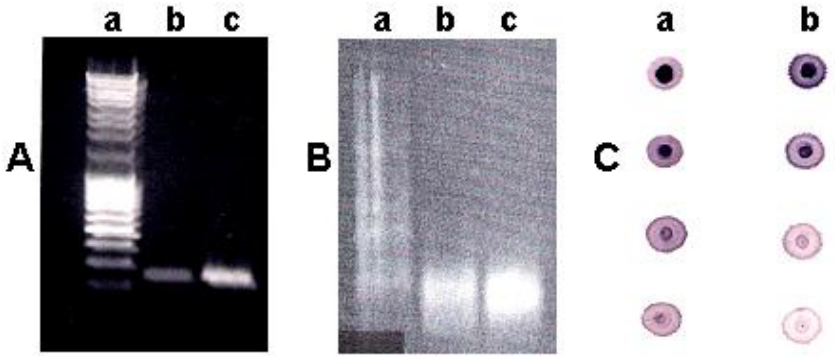

Figure 3.1: Gel electrophoresis and Dot blot for DIG-labeled probes. A: PCR amplification of 221 bp fragment from a cDNA that was generated from the AmNmdaR1 mRNA and used as a template to produce the sense and anti-sense probes. Column 'a' indicates DNA ladder, column 'b' and 'c' indicate anti-sense and sense PCR products. B: DIG-labeled anti-sense (column b) and sense (column c) RNA probes. C: Column 'a' indicates Dot blot for DIG-labeled anti-sense RNA probes and column 'b' for DIG-labeled sense RNA probes.

In the young adult bees (1-2 days old), the expression pattern closely resembles the one detected in adult worker bees but the general staining intensity was higher than the adult workers (Fig. 3.5). In all pre-adult stages, the neuroblasts showed more intense staining and the gene expressed by a majority of Kenyon cells was detected at very low level in the Kenyon cells of the young adult bees. The specificity of AmNR1 mRNA signals was compared with negative control where DIG-labeled sense RNA probes were used and did not yield any hybridization signals (Fig. 3.4F). *In situ* hybridization was performed at least five times.

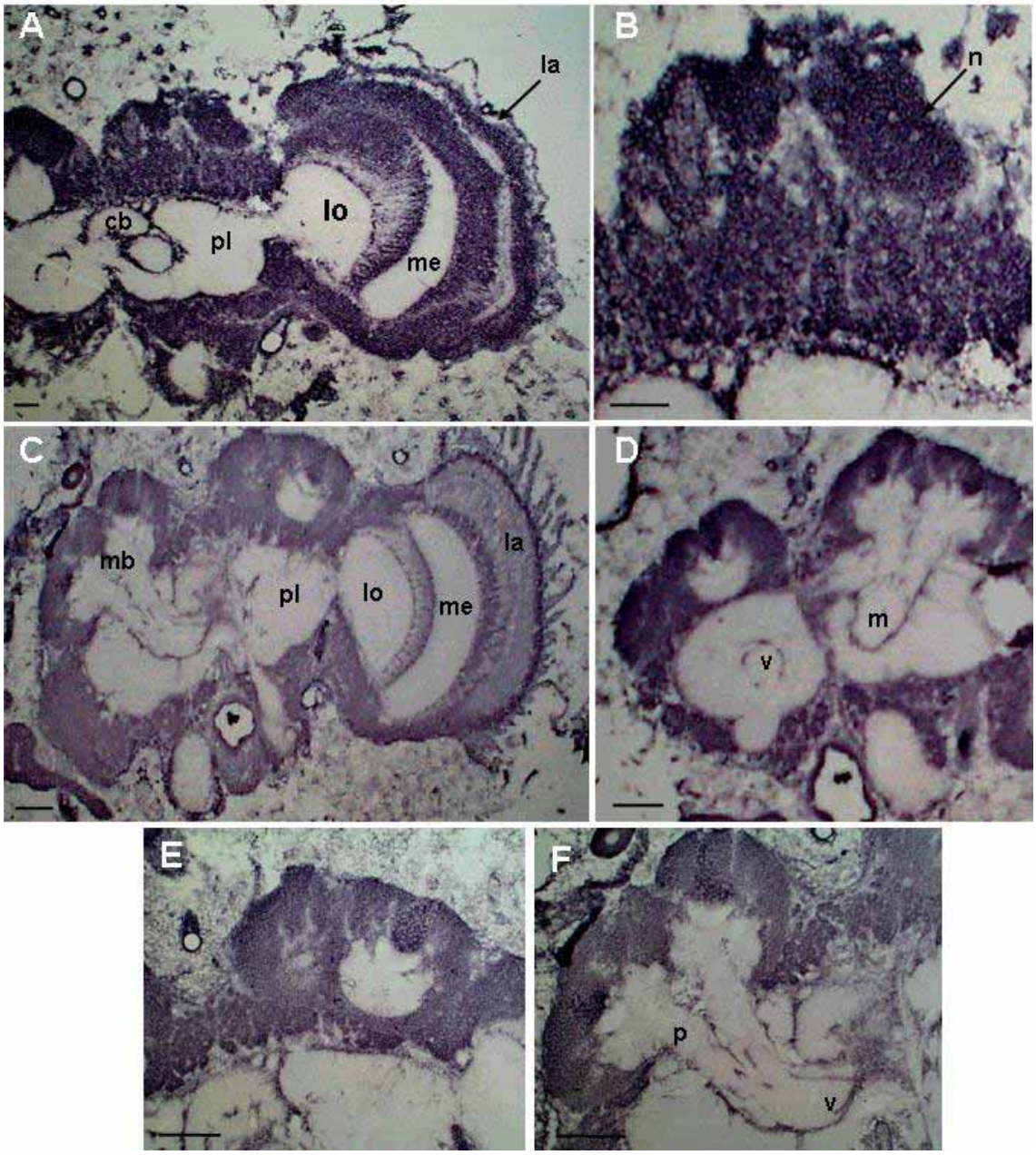

Figure 3.2: Expression of AmNR1 transcript in young pupae hybridized with DIG-labeled AmNR1 anti-sense RNA probes. A: Brain section from 1 day old pupae. B: Higher magnification of Kenyon cells somata of the mushroom body of 1 day old pupae. C and D: Brain sections from 2-3 day old pupae. E and F: Higher magnification of the mushroom body from upper and middle parts of the brain of 2-3 day old pupae. Mushroom body (mb), protocerebral lobe (pl), central brain (cb), lobula (lo), medulla (me), lamina (la), Kenyon cell neuroblasts (n), median lobe (m), and vertical lobe (v). Scale bar = 0.1 mm in A, C, D, E, and F and 25 μm in B.

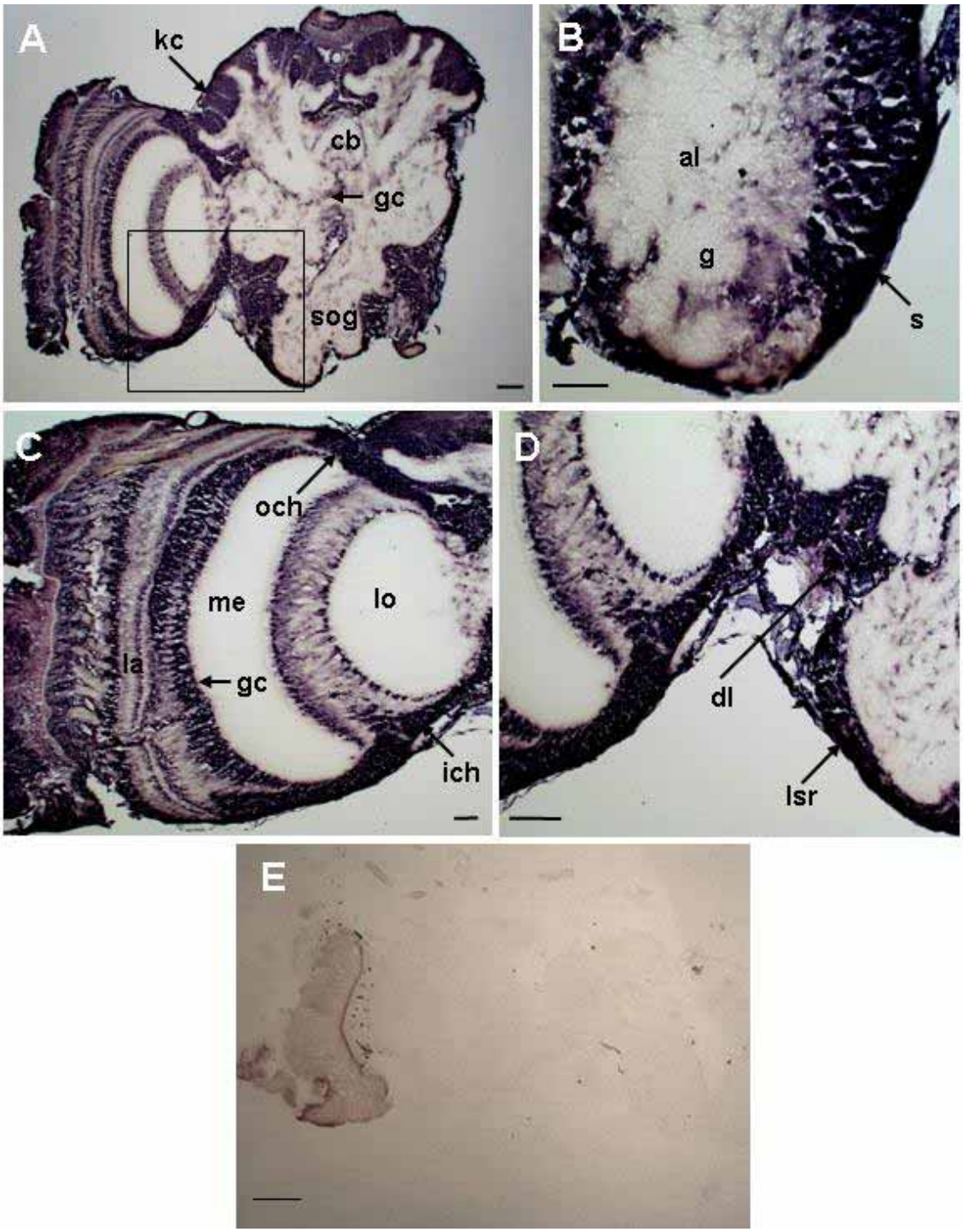

Figure 3.3: *In situ* hybridization to AmNR1 transcript in 5 day old pupae with DIG-labeled AmNR1 anti-sense RNA probes. A: Over all distribution of AmNR1 transcripts in the brain of 5 day old pupae. B and C: Higher magnification of antennal and optic lobes, respectively. D: Higher magnification of boxed area of A. Kenyon cells (kc), central brain (cb), suboesophageal ganglion (sog), antennal lobe (al), glomeruli (g), lobula (lo), medulla (me), lamina (la), inner chiasma (ich), outer chiasma (och), dorsal lobe (dl), and lateral soma rind of the suboesophageal ganglion (lsr). Scale bar = 0.2 mm in A and E; 0.1 mm in B, C and D.

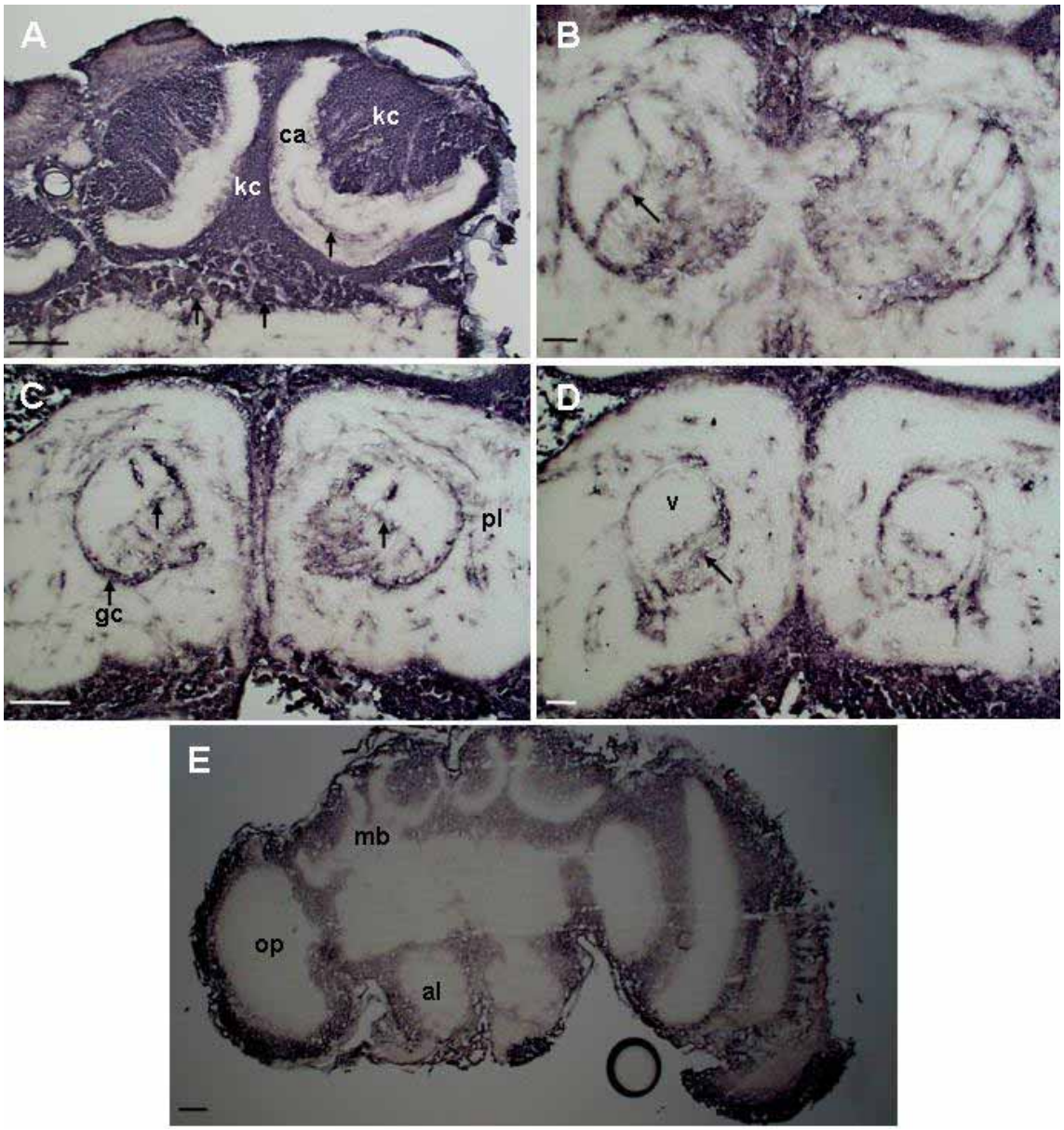

Figure 3.4: Expression of AmNR1 transcript in the mushroom body and the vertical lobe of 5 day old pupae, and in the late pupae with DIG-labeled AmNR1 anti-sense RNA probes. A: Mushroom body; similar staining intensity is detected in the Kenyon cells located inside and outer periphery of the calyces (kc). Single arrow indicates AmNR1 expression in the basal ring and double arrows indicate prominent AmNR1 expression in the large cells that located above the central brain and the protocerebral lobe. B-D: Band and/or finger like expression of AmNR1 transcripts in the vertical lobes indicated by arrows. E: Distribution of AmNR1 transcripts in late pupal brain. F: Control experiment hybridized with DIG-labeled AmNR1 sense RNA probe. Mushroom body (mb), optic lobe (op), antennal lobe (al), vertical lobe (v), protocerebral lobe (pl), calyx (ca), Kenyon cells (kc), and glial cells (gc). Scale bar = A-D, 0.1 mm and 0.2 mm in E and F.

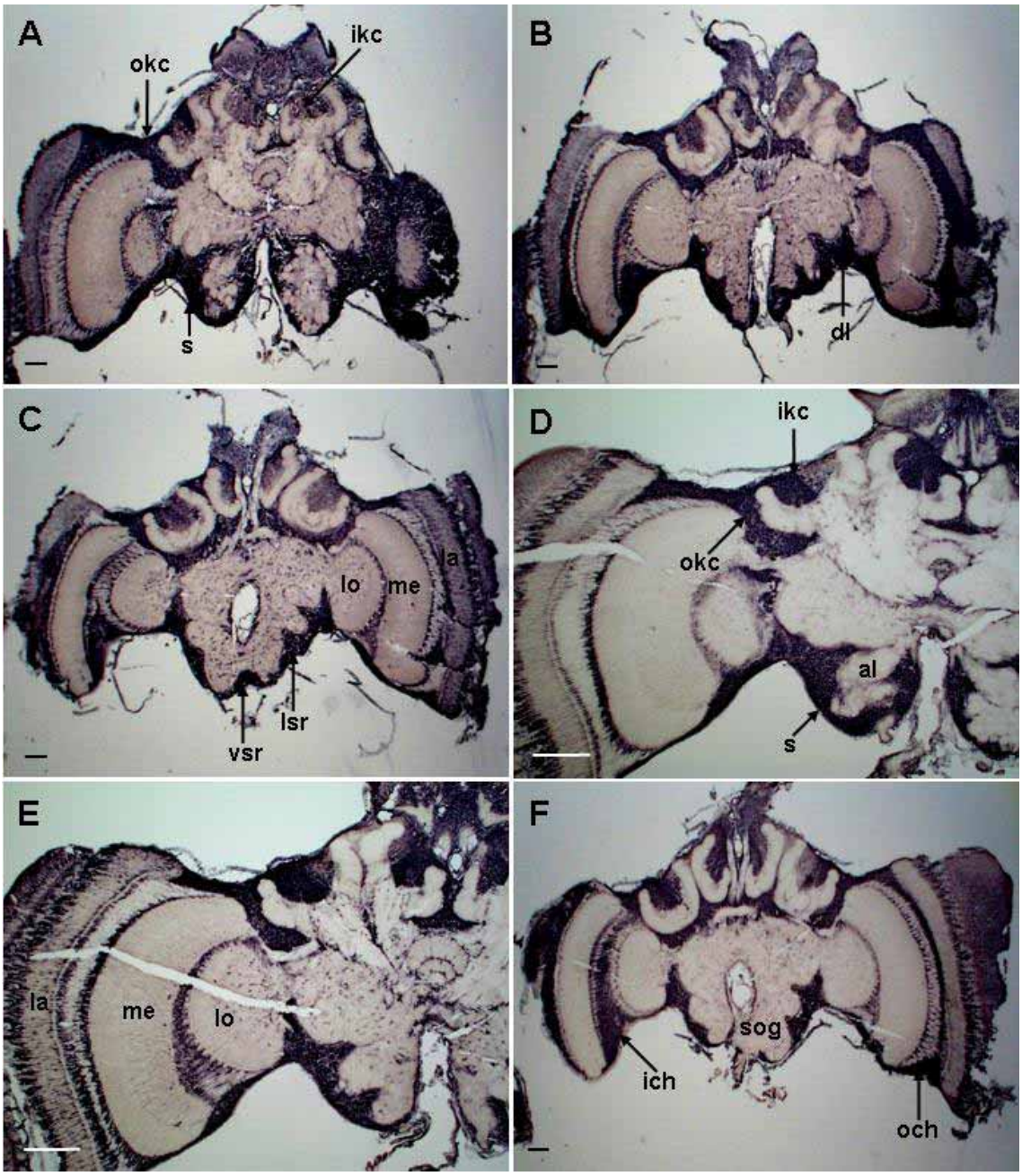

Figure 3.5: AmNR1 mRNA expression in the young bees revealed by *in situ* hybridization with DIG-labeled AmNR1 anti-sense RNA probes. A-C: Series of frontal sections from 1 day old bee and D-F from 2 day old bee. Inner Kenyon cells (ikc), outer Kenyon cells (okc), lobula (lo), medulla (me), lamina (la), cell somata of the antennal lobe (s), inner chiasma (ich), outer chiasma (och), suboesophageal ganglion (sog), antennal lobe (al), dorsal lobe (dl), lateral and ventral soma rind of the suboesophageal ganglion. Scale bar = 0.2 mm.

3.4.2: Expression of AmNR1 protein at different developmental stages

Immunoenzyme histochemical analysis was performed for the detection of AmNR1 protein at different developmental stages of the honeybee (0 day old bees refers to those just before emerging, along with 2 day old, 7 day old, and 15 day old bees) with NR1-mab363 primary antibody. In 0 day old bees, almost no AmNR1 immunoreactivity was detected in the mushroom body calyces and optic lobes, but sometimes very low level of AmNR1 immunoreactivity was detected in the peduncle of mushroom body and in the lobula of the optic lobe (Fig. 3.6A-D). Furthermore, weak AmNR1 immunoreactivity was detected in the protocerebral lobe, antennal lobe, vertical lobe, and suboesophageal ganglion. At this stage, the central brain showed stronger AmNR1 immunoreactivity than the other brain neuropils (Fig. 3.6A-D).

In 2 day old bees, the AmNR1 immunoreactivity was stronger when compared to 0 day old bees in all brain neuropils (Fig. 3.7A-D), which were strongest in the 7 day old bees (Fig. 3.8A-D). In the 15 day old bees, AmNR1 immunoreactivity was weaker than the 2 day and 7 day old bees (Fig. 3.9A-D). In the foraging bees, the AmNR1 immunoreactivity resembles to those were found in 7 day old bees (Fig. 3.10A-D). The specificity of the AmNR1 immunoreactivity was confirmed with negative control where primary antibody was omitted and no AmNR1 immunoreactivity was detected in the control bees (Fig. 3.6E, 3.7E, 3.8E, 3.9E and Fig. 3.10E). The age of the bees was strictly maintained, except foragers, and each experiment was done at least 6 times or more.

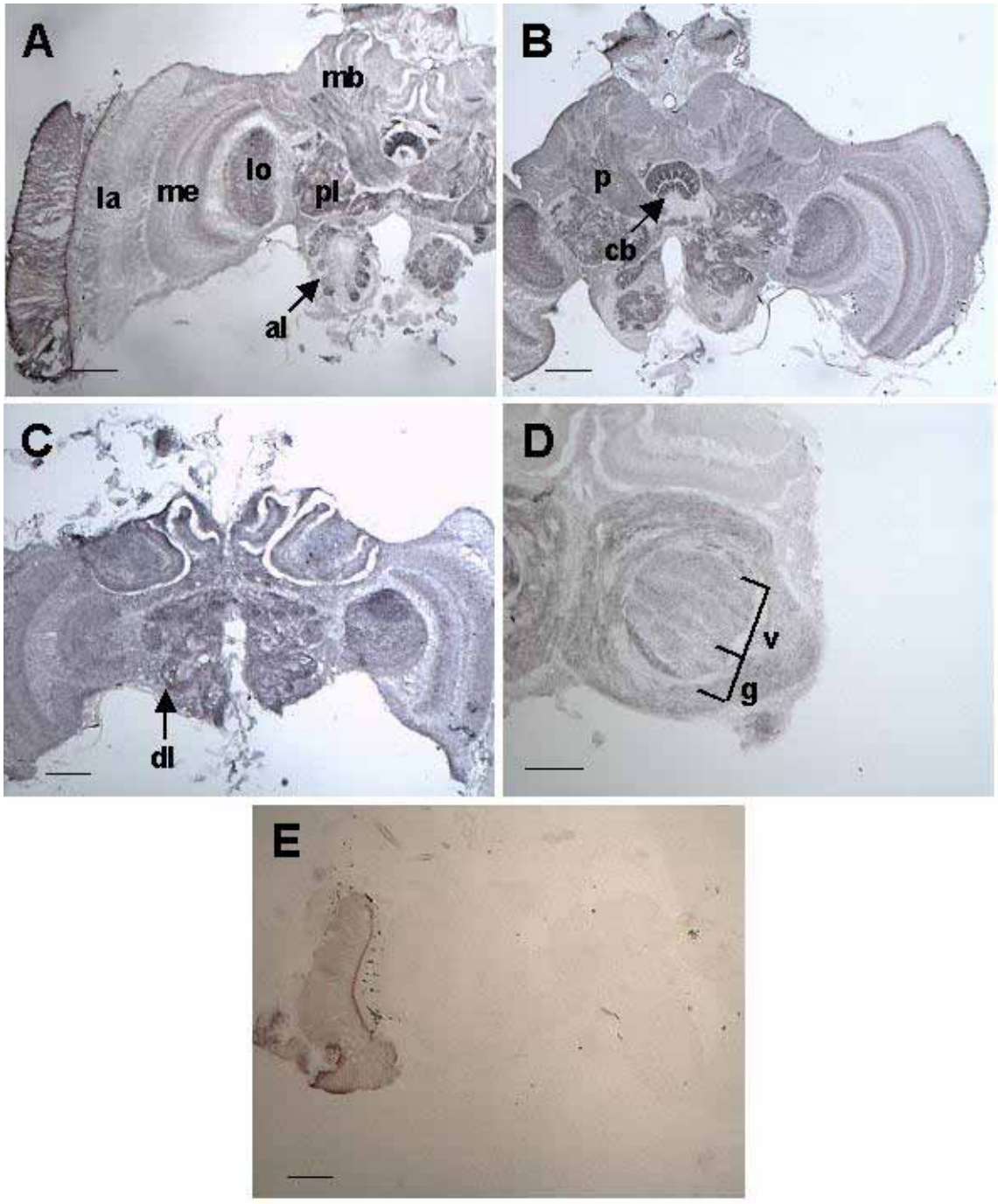

Figure 3.6: AmNR1 immunoreactivity in 0 day old bees realized with NR1-mab363 primary antibody on brain cryosections. A-C: Sections from different level of the bee brain. Vertical (v) and gamma (g) lobes in D. E: Control experiment for AmNR1 immunoreactivity where primary antibody was omitted. Mushroom body (mb), protocerebral lobe (pl), peduncle (p), lobula (lo), medulla (me), lamina (la), central brain (cb), dorsal lobe (dl), and antennal lobe (al). Scale bar = 0.2 mm in A-C, E and 0.1 mm in D.

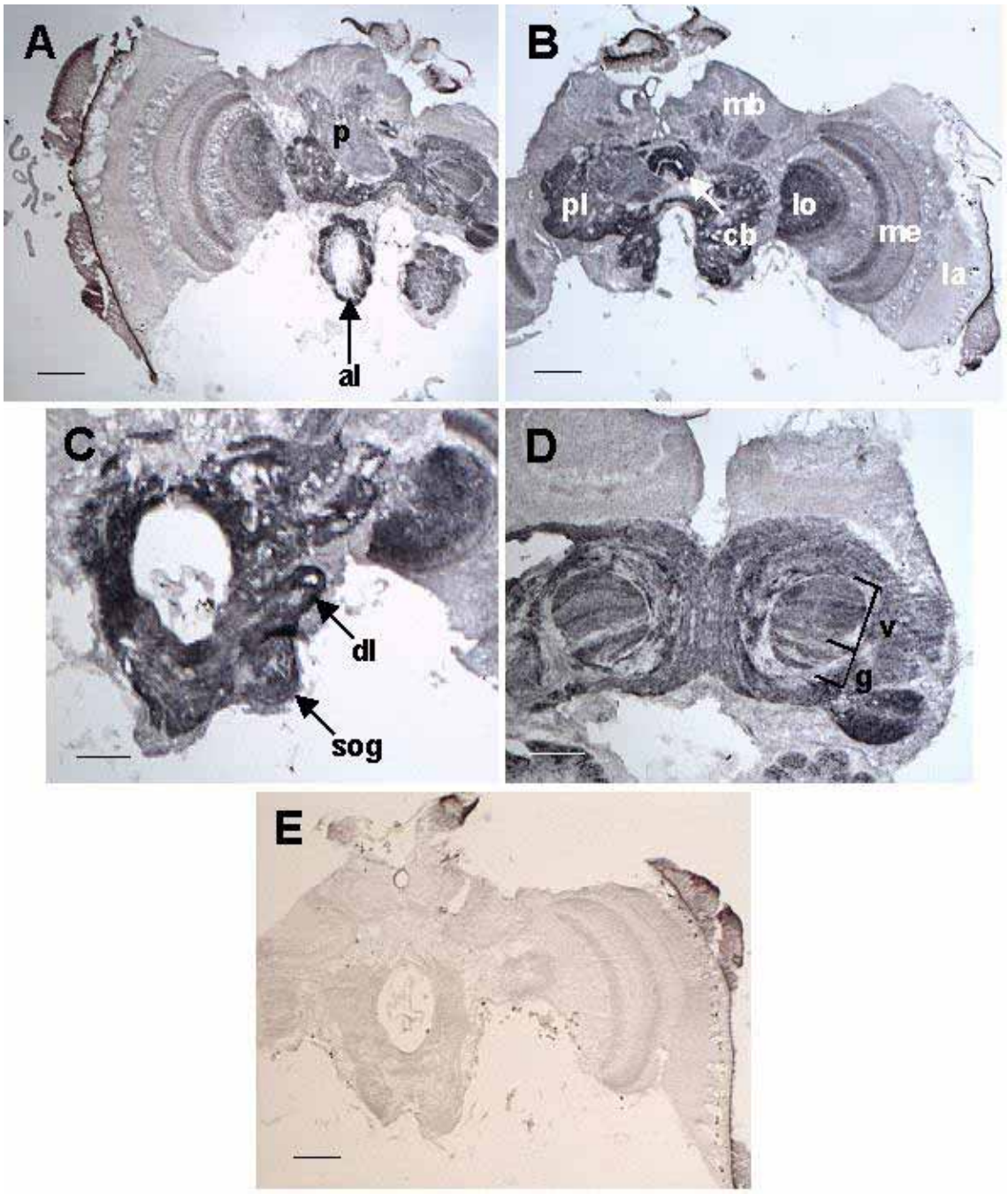

Figure 3.7: AmNR1 immunoreactivity in 2 day old bees realized with NR1-mab363 primary antibody on brain cryosections. A and B: Frontal sections of the bee brain. Dorsal lobe (dl) and suboesophageal ganglion in C. Vertical (v) and gamma (g) lobes in D. E: Control experiment for AmNR1 immunoreactivity where primary antibody was omitted. Mushroom body (mb), protocerebral lobe (pl), peduncle (p), lobula (lo), medulla (me), lamina (la), central brain (cb), and antennal lobe (al). Scale bar = 0.2 mm in A, B, and E, and 0.1 mm in C and D.

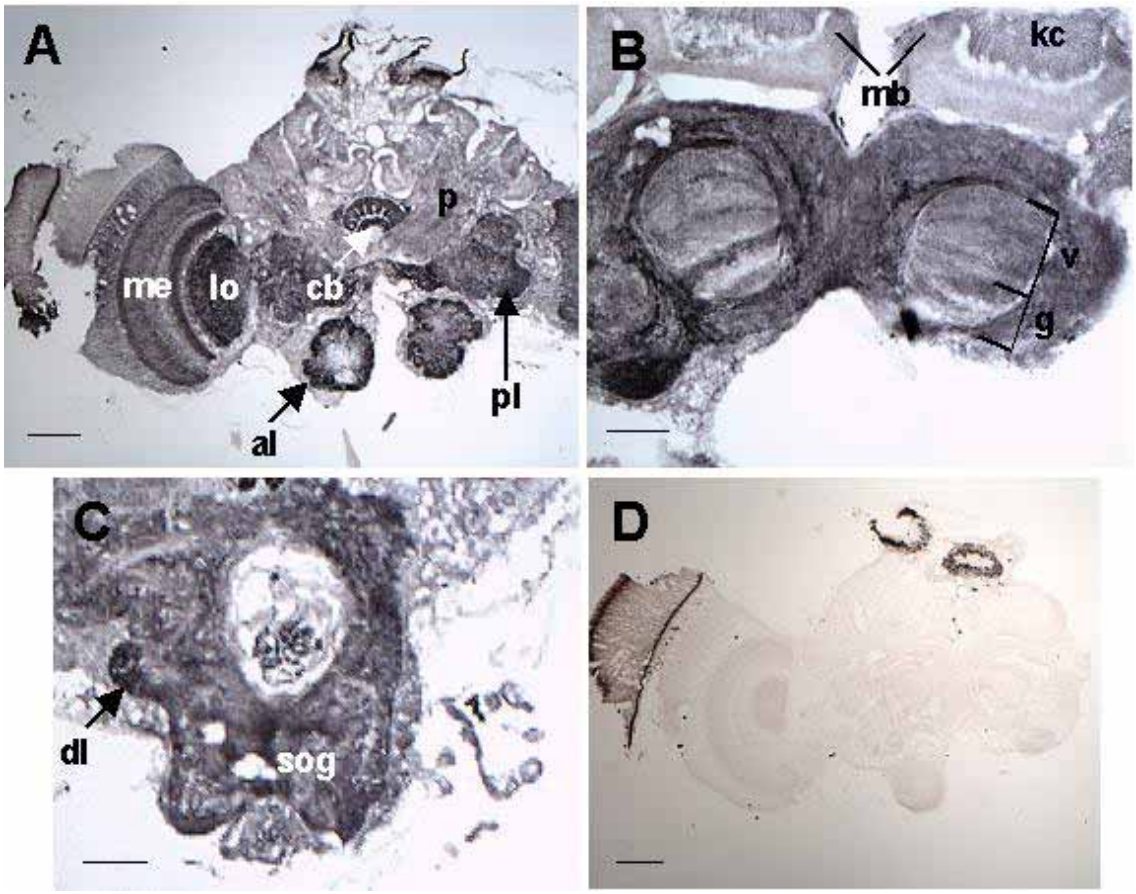

Figure 3.8: AmNR1 immunoreactivity in 7 day old bees realized with NR1-mab363 antibody. A: Frontal section of the bee brain. Vertical (v) and gamma (g) lobes in B. Dorsal lobe (dl) and suboesophageal ganglion in C. D: Control experiment for AmNR1 immunoreactivity. Mushroom body (mb), protocerebral lobe (pl), peduncle (p), lobula (lo), medulla (me), lamina (la), central brain (cb), antennal lobe (al), and kenyon cells (kc). Scale bar = 0.2 mm in A, and D, and 0.1 mm in B and C.

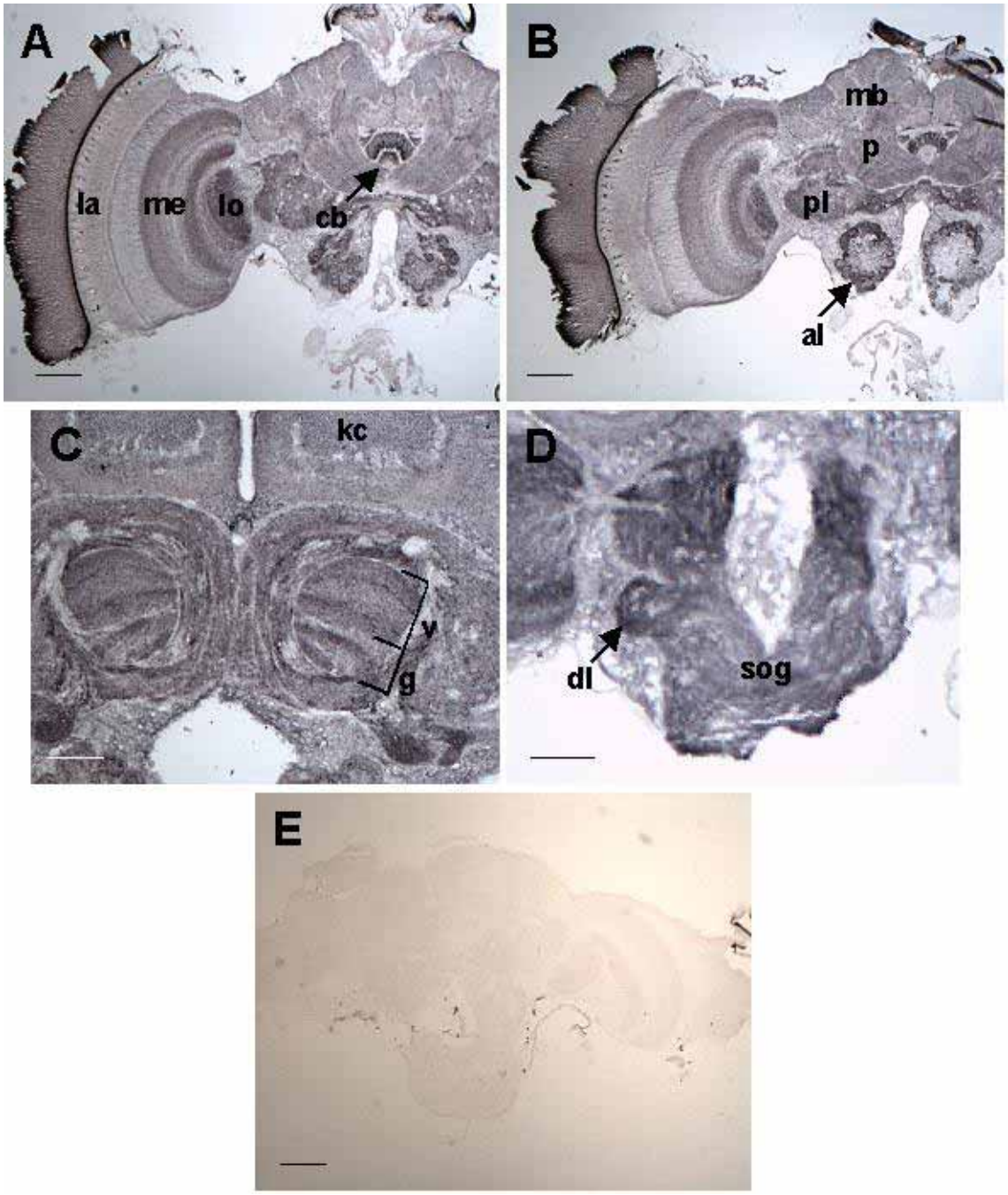

Figure 3.9: AmNR1 immunoreactivity in 15 day old bees realized with NR1-mab363 antibody. A and B: Serial sections from the same bee brain. Vertical (v) and gamma (g) lobes in C. Dorsal lobe (dl) and suboesophageal ganglion in D. E: Control experiment for AmNR1 immunoreactivity where primary antibody was omitted. Mushroom body (mb), protocerebral lobe (pl), peduncle (p), lobula (lo), medulla (me), lamina (la), central brain (cb), antennal lobe (al), and kenyon cells (kc). Scale bar = 0.2 mm in A, B, and E, and 0.1 mm in C and D.

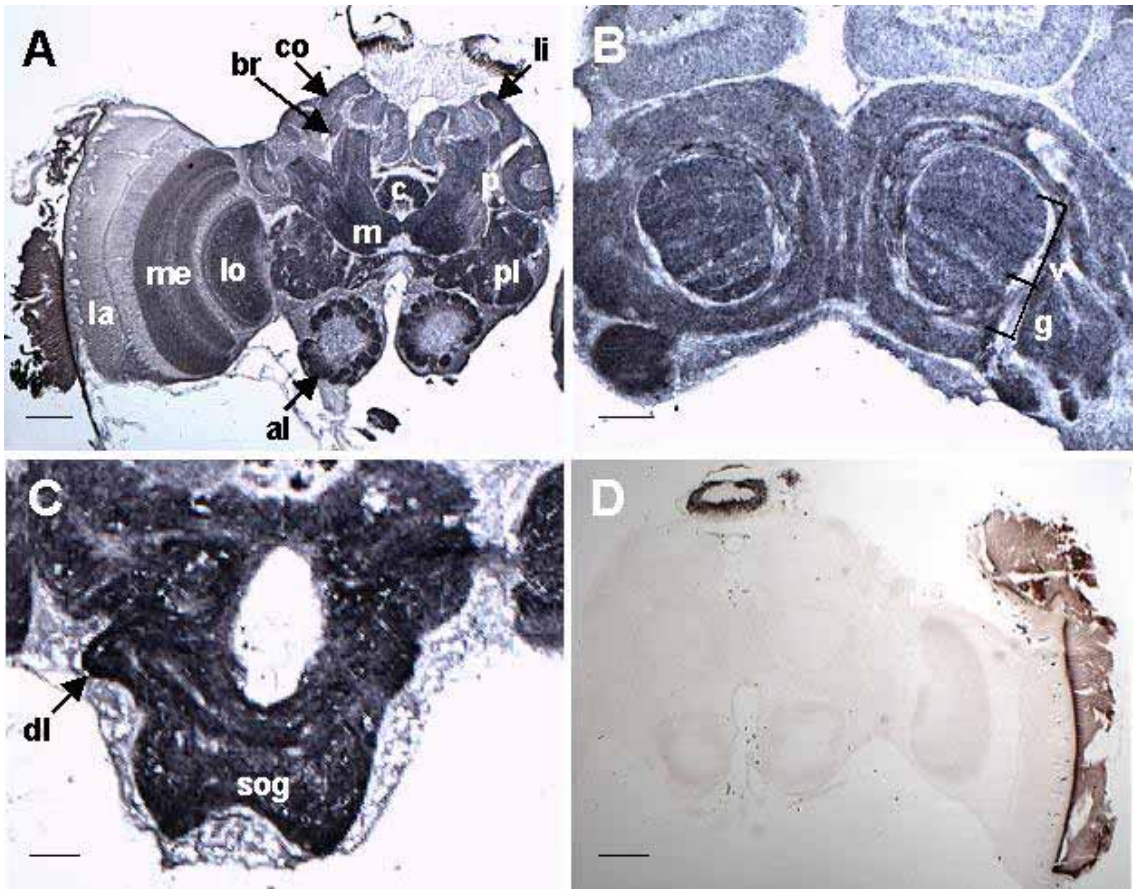

Figure 3.10: AmNR1 immunoreactivity in foraging bees realized with NR1-mab363 primary antibody on brain cryosections. A: Frontal section of the bee brain. Vertical (v) and gamma (g) lobes in B. Dorsal lobe (dl) and suboesophageal ganglion in C. D: Control experiment for AmNR1 immunoreactivity. Lip (li), collar (co), basal ring (br), protocerebral lobe (pl), peduncle (p), lobula (lo), medulla (me), lamina (la), central brain (c), antennal lobe (al), and medial libe (m). Scale bar = 0.2 mm in A, and D, and 0.1 mm in B and C.

3.5: Discussion

In the present investigation, *in situ* hybridization and immunohistochemistry were performed on brain cryosections in order to investigate the developmental stages at which neural cells begin to express the AmNR1 mRNA and protein, and how the expression profile is changed during the development of the central nervous system. In these experiments, four different pupal stages (1 day old, 2-3 day old, 5 day old and late

pupae) and young bees (1 day old and 2 day old bees) were selected for *in situ* hybridization using DIG-labeled sense and anti-sense RNA probes. For immunohistochemistry, four different developmental stages (0 day old, 2 day old, 7 day old, and 15 day old bees) were investigated with NR1-mab363 primary antibody and compared with foragers. Table of experiments are presented in the appendix.

In the pupal stages, high levels of expression of AmNR1 mRNA were detected in all above mentioned pupal stages, except in the late pupae where a little bit less was detected. The expression of AmNR1 mRNA was higher in all pre-adult stages and in the young bees when compared to adult workers. Further interesting differences were also found in the Kenyon cells of the mushroom body. In all pre-adult stages, Kenyon cell neuroblasts expressed high levels of AmNR1 transcripts, whereas there was very low level in the young bees, which was increased in the adult worker bees. This suggests that the AmNR1 gene is expressed differentially at different developmental stages in the honeybee. These results are consistent with the expression of some other genes which are expressed preferentially at different developmental stages of the honeybee, for example: the glutamate transporter gene (Kucharski et al., 2000), nicotinic acetylcholine receptor alpha3 mRNA (Apis α 3 mRNA) (Thany et al., 2003) and the honeybee transferin-gene (Adriana et al., 2004). In *Drosophila*, the metabotropic glutamate receptor (Parmentier et al., 1996) and NMDA receptor subunit R1 (Ultsch et al., 1993) that play a role in the development of embryonic glutamatergic synapses and in central nervous system have also been identified differentially. The metabotropic glutamate receptor was detected in the late embryo and not in the early embryogenesis. In addition, the DNMDA-R1 mRNA was detected at very low level in the larval stages whereas little increase was found in the early pupae which were strongly detected in the late pupae and in the adult fly heads (Ultsch et al., 1993). Further, a comparable regulation

of gene expression was also reported at different developmental stages for the *Drosophila* neuronal acetylcholine receptor subunit encoded by sad, sbd and ard (Sawruk et al., 1990a and b; Hermans-Borgmeyer et al., 1986) and the kainate-selective glutamate receptors (Ultsch et al., 1992). Therefore, high-level expression of AmNR1 mRNA at different developmental stages of the honeybee suggests the AmNR1 gene is developmentally regulated and expressed differentially like other genes of other insects.

Immunohistochemical analysis of the expression of AmNR1 protein at different developmental stages of the honeybee revealed that this protein is expressed differentially and increases with age. For example, the very weak AmNR1 immunoreactivity was detected in 0 day old bees that was increased in 2 day and 7 day old bees but not in the 15 day old bees. It was raised again in the foragers. This stands in gross contrast to the results from *in situ* hybridization that revealed high level expression of AmNR1 mRNA in the pupal stages and in the young bees (1 day and 2 days old). Similarly, in *Drosophila*, the ionotropic glutamate receptor (DgluR-1B) mRNA was identified in the embryos that showed moderate DgluR-1B mRNA expression but no corresponding protein (Völkner et al., 2000). Age related increase of AmNR1 protein in the honeybee suggests that it may be involved in behavioral maturation underlying learning and memory.

Here, the localization of AmNR1 mRNA and protein are described at different developmental stages. These results will allow us for further study about the functional involvement of this receptor in the developing brain neurogenesis.

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