2 Chapter 1

Identification and localization of the NR1 sub-unit homologue of the NMDA glutamate receptor in the honeybee brain

2.1: Abstract

The NR1 subunit homologue of the NMDA glutamate receptor was characterized in the honeybee. Sequence analysis suggests that the honeybee NMDA receptor may be act as a coincidence detector molecule similar to its counterpart in the mammalian nervous system. The localization of the expression sites at the mRNA and the protein levels indicates that the receptor is expressed throughout the brain, in neurons and in glial cells.

2.2: Introduction

Glutamate is the major excitatory neurotransmitter in the mammalian brain; several ionotropic and metabotropic glutamate receptors were identified. The ionotropic NMDA receptor was intensively studied in relation to its participation in neural plasticity and memory process (Cain 1997). The functional NMDA receptor is formed by the subunits NR1 and NR2 (Papadakis et al., 2004). The NR1 subunit, common to all NMDA receptor variants, possesses affinity for glycine that modulates the activation of the receptor upon binding of glutamate to the NR1 subunit (Furukawa and Gouaux 2003; Papadakis et al., 2004).

In insects, glutamate is a neurotransmitter at the neuromuscular junction and in the

central nervous system (Bicker et al., 1988; Schürmann et al., 2000; Sinakevitch et al., 2001; Strausfeld 2002). Metabotropic and ionotropic glutamate receptors homologues to the mammalian counterparts were identified in *Drosophila* (Cully et al., 1996; Featherstone et al., 2005; Parmentier et al., 1996; Ultsch et al., 1993; Volkner et al., 2000). A critical role of the NMDA receptor was recently demonstrated in olfactory learning and memory in *Drosophila* (Xia et al., 2005) and in neurohormonal control in the cockroach (Chiang et al., 2002).

In the honeybee *Apis mellifera*, pharmacological evidences indicate the participation of glutamatergic neurotransmission in olfactory memory (Locatelli et al., 2005; Si et al., 2004). However, a comprehensive explanation of the functional role of glutamateergic neurotransmission requires the characterization and localization of glutamate receptors in the CNS of the honeybee.

In this work, we identified the NR1 sub-unit homologue of the NMDA receptor in *A. mellifera*, AmNR1-1. Sequence analysis suggests that the receptor possesses the particular properties known from NMDA receptor in other species. We describe the expression sites of the AmNR1-1 sub-unit at the mRNA and at the protein level.

2.3: Materials and methods

A. mellifera adult bees reared in a flight room were collected while flying outside the hive during the winters of 2003 and 2004.

The cloning of the cDNA was performed as previously described (Leboulle and Müller 2004). The amplification was performed with the direct primer 5'-ATTTAGATAAGGGCGTGAACT-3' and the reverse primer 5'-ACATCTAACAAATTTTTCAGGTAA-3'. The PCR protocol consisted of 30 cycles of

30 s at 95°C, 1 min at 60°C, and 3 min at 72°C, following by 10 min incubation at 72°C. Several clones were sequenced and deposited at GenBank/EMBL: *NR1-1 to NR1-11* (Acc numbers: AM040201 to AM040211).

The comparison of the AmNR1-1 mRNA sequence and of the in situ hybridization probe to the honeybee genome (http://www.ensembl.org/Apis_mellifera/) with the BLASTn algorithm (default parameters) allowed us to retrieve the gene sequence (ENSAPMG00000007726) and to show the specificity of the probe. The tBLASTn analysis was done with default parameters and the results having scores >200 and E-values <10E-10 were considered. The deduced amino acid sequence of AmNR1-1 was compared to the *Drosophila melanogaster* dNR1 (Acc number: X71790) and the human NR1-1 (Acc number: NM000832) sequences by the ClustalW alignment method with the Gonnet series protein weight matrix (default parameters). The search for glycosylation and phosphorylation sites was carried out with the Protean software (DNADTAR, Madison, WI, USA).

A cDNA template was used to generate the sense and anti-sense probes for in situ hybridization by in vitro transcription. It was obtained by PCR using the forward (5′-CATGTATTTCCGTCGCCAAGTC-3′) and the reverse (5′-TTCTGTAAACCAATCCCATAGC-3′) primers. The forward primer was modified at its 5′ extremity by the addition of a T7 promoter sequence (5′-TAATACGATCACTATAGGGCGA-3′) for the synthesis of the sense probe, and the reverse primer was modified at its 5′ extremity by the addition of a T3 promoter sequence (5′-AATTAACCCTCACTAAAGGGACTA-3′) for the synthesis of the anti-sense probe. The PCR protocol consisted of 40 cycles of 30 s at 95°C, 1 min at 63 °C, and 1 min at 72°C, followed by a 5 min incubation at 72°C. Sense and anti-sense RNA probes labeled with digoxigenin were transcribed with T3 and T7 RNA

polymerase, respectively, and the DIG RNA labeling mix according to the manufacture's instructions (Roche, Mannheim, Germany). The reaction products were purified on probeQuant TM G50 micro columns (Amersham Bioscience, Freiburg, Germany). The integrity of the probes was assessed by running samples on a 2% agarose gel stained with ethidium bromide; the yield of digoxiginin incorporation was estimated by Dot blot, according to the instructions provided with the DIG wash and Block Buffer Set (Roche, Mannheim, Germany). Dissected brains were fixed in 4% paraformaldehyde on ice for 10 min, cryoprotected overnight at 4°C with 30% sucrose in 1× PBS, 0.1% Triton X-100 (PBSTr) and embedded in Jung medium (Leica Instruments, Nussloch, Germany). Fourteen micrometers frontal cryosections were collected on superfrost plus slides (Menzel Gläser, Braunschweig, Germany), dried at room temperature (RT) and post-fixed for 10 min in 4% paraformaldehyde. Sections were incubated at 65°C for 2 h in a hybridization solution (5× SSC, 50% formamide, 50 µl/ml heparin, 0.1% Tween-20) and overnight with the probe (1 ng/µl). The next day, the slides were washed in 5× SSC, 0.2× SSC at 65°C and in PBSTr at RT. After blocking in 2% BSA in PBSTr, the slides were probed overnight at 4°C with an anti-digoxiginin antibody coupled to alkaline phosphatase (Roche), diluted 1:500 in the blocking solution. The next day, the slides were washed in PBSTr and developed with the detection buffer (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).

For the immunohistochemistry, the brain were fixed in 4% paraformaldehyde on ice for 2 h and then washed with 1× PBS. Then they were dehydrated-rehydrated in ethanol (50, 70, 90, 99 and 100%), washed with 1× PBS and cryoprotected with 30% sucrose in 1× PBS overnight at 4°C. The brains were embedded and sectioned as described for the in situ hybridization, washed with PBSTr and blocked with 2% BSA in PBSTr. The slides

were incubated with the primary antibodies, anti-NR1 pan (1:200; Upstate, Biomol, Hamburg, Germany) or mab363 (1:200; Chemicon, Hampsshire, United Kingdom) in the blocking solution overnight at 4°C and then incubated with secondary antibodies (1:3000 diluted in the blocking solution; Sigma, Munich, Germany) for 2 h at RT. An anti-rabbit IgG antibody coupled to biotin was used to detect the anti-NR1 pan antibody and an anti-mouse IgG antibody coupled to alkaline phosphatase was used to detect the mab363 antibody. Prior to the detection, "anti-NR1 pan" slides were incubated in Extravidin conjugated with alkaline phosphatase (1:4000 diluted in the blocking solution; Sigma). Finally, the slides were washed PBST and developed with NBT/BCIP.

2.4: Results and discussion

A cloning strategy, based on a previous entry in GenBank/EMBL databases (Accession number: NM.001011573) permitted to confirm the sequence of the AmNR1-1 mRNA. This cloning also led to the identification of truncated forms of the mRNA (called the synthesis of AmNR1-2 to AmNR1-11). The analysis of the sub-unit's structure (see below) showed that the truncated variants AmNR1-2 to AmNR1-9 lack the agonist binding site, the membrane domains and the C-terminal domain, and the variants AmNR1-10 and AmNR1-11 lack the interaction domain with other sub-units. Similar variants, without known activity, were already described in mammals (Campusano et al., 2005; Sugihara et al., 1992). Because the aim of this work to describe potentially functional sub-units of the NMDA receptor, these truncated variants were not taken into further consideration.

The *nmdar1* gene was identified by searching the honeybee genome with AmNR1-1 and a unique locus, comprising 17 exons, matched the mRNA sequence. A tBLASTn

analysis using the human and the *Drosophila* NR1 sub-units against the honeybee genome confirmed that there were no other related sequences in the honeybee. The BLAST hits showing the highest homology matched the nmdar1 gene. Further hits matched genes corresponding to the NR2 and NR3 sub-units of the NMDA receptor, to other non-NMDA ionotropic glutamate receptors, to unrelated proteins, and to unknown genes. The analysis of the amino acid sequences of these unknown genes failed to show that they had the typical signature of NR1 sub-units. Therefore, the NR1 receptor sub-unit is most probably encoded by a unique gene in the honeybee.

The overall amino acid sequence identity between the honeybee AmNR1-1 (953 amino acids) and the *Drosophila* dNR1 (997 amino acids) was of 67.5%, whereas the identity with the human NR1-1 (885 amino acids) was of 48%.

The comparison of the human NR1-1 sub-unit to the honeybee sequence suggested that the latter possesses a similar structure (Fig. 2.1). The first amino acids of the honeybee sub-unit most probably encode the extra-cellular domain interacting with the other sub-unit composing NMDA receptor (39% identical amino acids) (Papadakis et al., 2004). The ligand-binding core is composed of the S1 and S2 domains. The S1 domain terminates the N-terminal extra-cellular region (52% identical amino acids), and the S2 domain is found in an extra-cellular loop (60% identical amino acids). All the amino acids interacting directly with glycine were conserved in AmNR1-1, except in position 695, which was occupied by an alanine instead of a serine (Furukawa and Gouaux 2003). The NR1 sub-unit comprises 4 membrane domains that are highly conserved between honeybee and human. Three of them are transmembrane domains (overall 76% identical amino acids), and the second membrane domain is a re-entrant loop (86% identical amino acids). The re-entrant loop and, in particular, an asparagines conserved in the honeybee sequence (Asp623) influences the divalent cation permeability of the

Figure 2.1: Analysis of AmNR1-1 amino acid sequence. The deduced amino acid sequence of AmNR1-1 was compared to the human NR1-1 sub-unit. The different regions of the honeybee sub-unit are underlined: N-terminal extra-cellular domain (ATD), agonist-binding domain (S1 and S2 domains) and the C-terminal intracellular domain (CTD). Membrane domains (M1 to M4) are highlighted in the boxes. The potential N-glycosylation sites of the extra-cellular domains are indicated in bold. The amino acids of the ligand binding core interacting directly with glycine (∗), the PKC (•), the PKA and PKG (❑) phosphorylation sites, and the RSS motif (amino acids in italic (♋) are indicated under the sequence.

receptor (Featherstone et al., 2005). The C-terminal domain (CTD) of AmNR1-1 sub-unit shared a low homology with the human receptor, with only 23% identical amino acids. Interestingly, potential phosphorylation sites were found in the CTD and the region spanning the membrane domain 2-3, as potential glycosylation sites were located mostly in the extra-cellular part of the sequence. Finally, two retention signal sequence (RSS) motifs were found in the C-terminal half of the sequence. This motif regulates the insertion of NMDA receptors at the cell membrane by retaining the NR1 sub-unit in the endoplasmic reticulum when not assembled in functional receptors (Scott et al., 2001).

The recent characterization of the *Drosophila* NMDA receptor shows that it functions as its mammalian counterpart (i. e. sensitivity to NMDA, glutamate and glycine, and aspartate, but not to AMPA) (Xia et al., 2005). The high degree of conservation of key regions of AmNR1-1 suggests that the honeybee NMDA receptor may express similar properties known from mammals and *Drosophila*, but the characterization of the NR2 sub-unit and functional studies of the honeybee receptor have not yet been performed.

The AmNR1-1 mRNA was detected by in situ hybridisation, by using a probe that yielded no significant hits except for *nmdar1* gene when compared to the honeybee genome. The specificity of the hybridisation was assessed by showing that a negative control, a sense RNA probe, did not yield any signals (data not shown).

AmNR1-1 was detected in all somata regions of the brain (Fig. 2.2). High expression levels were observed in the protocerebral lobe, in the sub-oesophageal ganglion, in the dorsal lobe, in the antennal lobe and in the lobula, the medulla and the lamina of the optic lobe (Fig. 2.2A, B and D). The central body was characterized by pronounced differences in expression levels between cells. A similar heterogeneous distribution was found for intrinsic elements of the mushroom body, the Kenyon cells (Fig. 2.2A-C).

Some clawed Kenyon cells which lying outside the calycal baskets (Rybak and Menzel 1993; Strausfeld 2002), showed intense AmNR1-1 levels, whereas the neighboring cells presented moderate staining or no staining at all. Differences of expression levels were also found in the class I Kenyon cells, located within and above the calyces (Mobbs 1982), but predominant staining was often detected in groups of cells at the top region and in a stack of cells located above the basal ring and the neck of the peduncle (Fig. 2.2A and B).

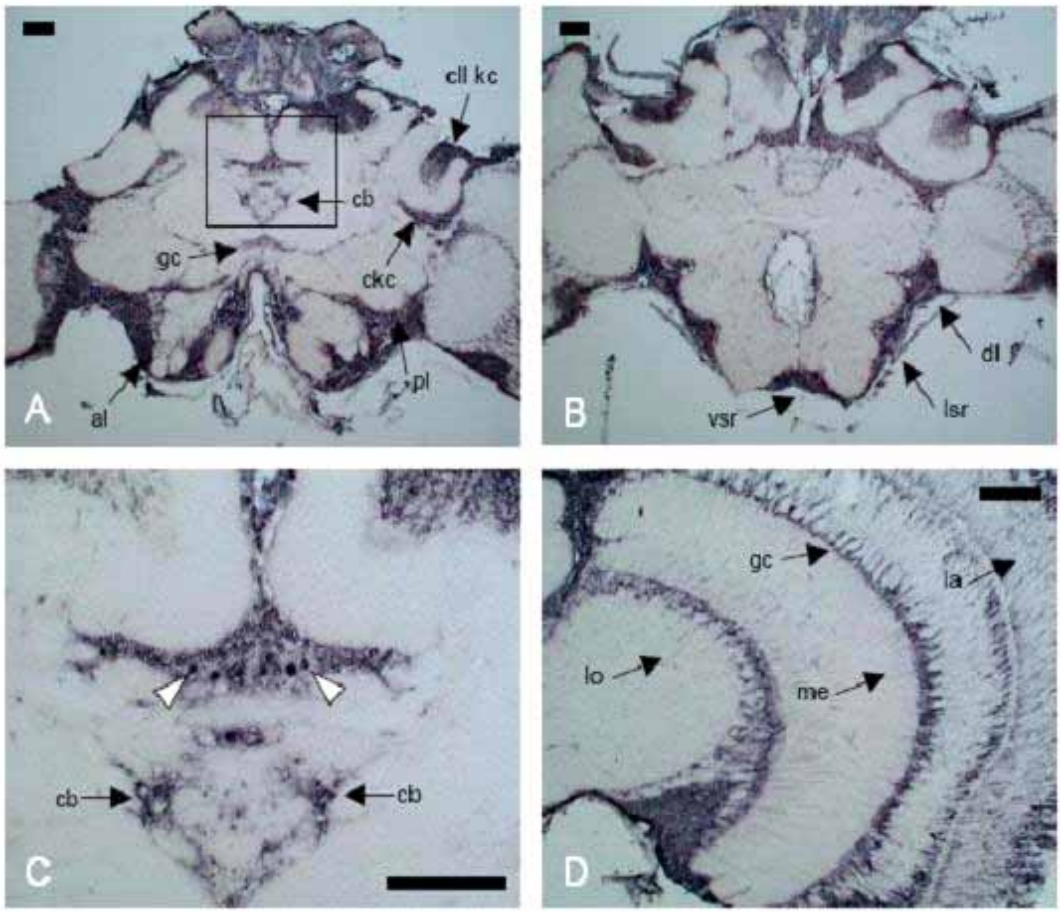

Figure 2.2: Detection of nmdar1 expression sites by in situ hybridisation. Signals were detected in the somata region of the antennal lobes (al), in the protocerebral lobe (pl) (A), in the lateral (lsr) and ventral (vsr) soma rind of the sub-oesophageal ganglion (B) and in the dorsal lobe (dl) (B), in the central body (cb) and in the clawed kenyon cells (ckc)(A-C). Enlargement of the window in A shows some isolated cells with intense staining (white arrow heads) (C). The class I Kenyon cells (clI kc) showed heterogenous signals (A-C). Staining was also found in glial cells (gc). In the optic lobes, strong signals were detected in cell bodes of the lobula (lo), the medulla (me) and the lamina (la) (D). Scale bar = 100 µm.

In insect brains, neuronal somata are grouped in regions that are separated from the neuropil that contains the neural processes and synaptic regions. Therefore, the localization of cell somata within the neuropil may be indicative of glial cells. In the mushroom bodies, glial cells line the peduncles and the lobes (Hahnlein and Bicker 1996). Additionally, they occupy specific regions of the optic lobes (Tix et al., 1997). AmNR1-1 signals were detected at these locations and may thus indicate expression in glial cells (Fig. 2.2A and D).

Immunohistochemical analysis was performed on frontal cryosections using two antibodies specific for different regions of the rat NR1 subunit (Fig. 2.3). The first one (NR1 pan) was directed against the N-terminal part of the rat NR1 protein and shared 84% similar amino acids with the honeybee. The second antibody was directed against the extra-cellular loop of the rat NR1 sub-unit (NR1 mab363) who shared 74% similar amino acids with the honeybee sequence. This antibody was previously used to detect NMDA receptor in the cockroach (Chiang et al., 2002). Immunodetections obtained with the secondary antibody alone served as negative controls and were devoid of staining (data not shown).

Both antibodies yielded signals in all brain neuropils, except in nerve tracts (Fig. 2.3A and B). In the sub-oesophageal ganglion and the dorsal lobe, the most intensive singnals were detected at the periphery of the neuropils (Fig. 2.3A and B). The protocerebral lobe showed one of the most intense signals detected in the brain (Fig. 2.3A-D). In the optic lobes, AmNR1-1 staining was detected in the lobula, the medulla and the lamina, with more intensive signals in specific layers (Fig. 2.3A, B, F and H). In the antennal lobe, the glomeruli showed a specific AmNR1 signal which was more intense in the periphery of each glomerulus, innervated predominantly by receptor cell terminals (Fig. 2.3E and G) (Pareto 1972).

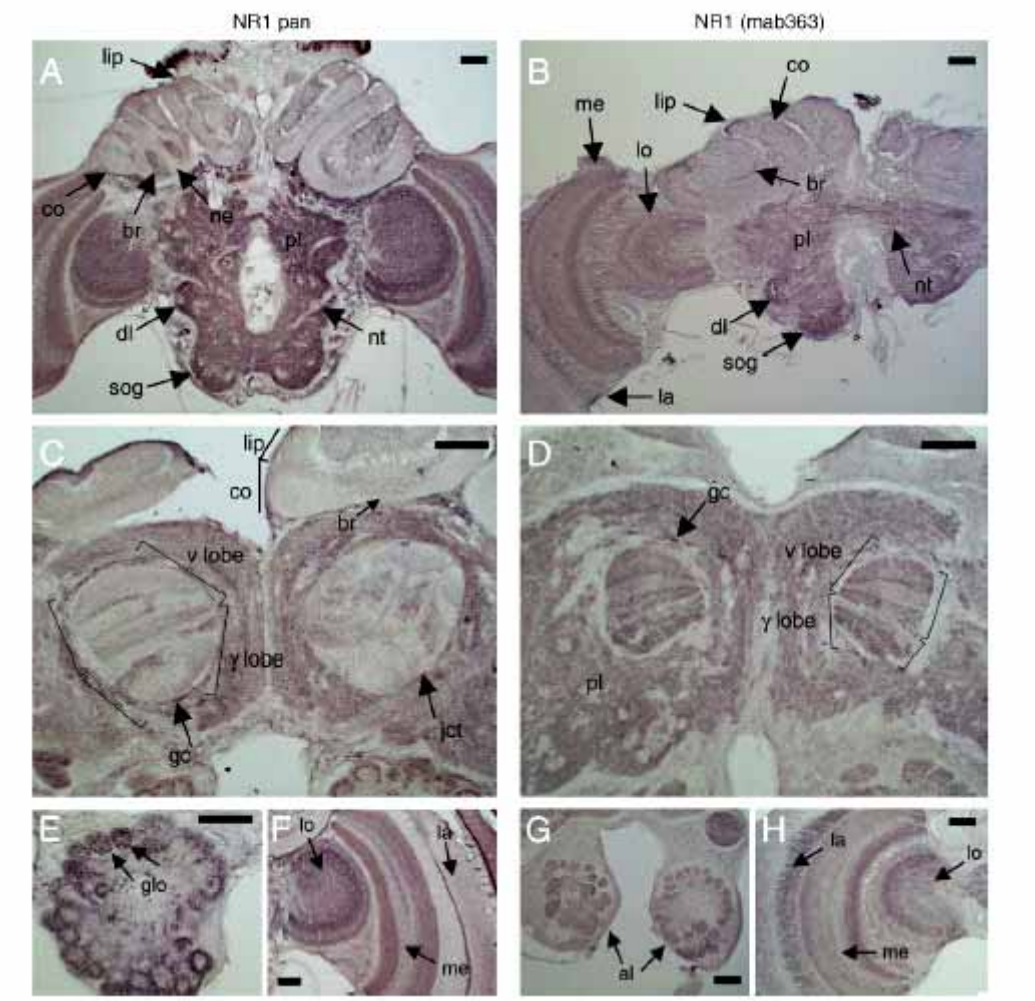

Figure 2.3: Detection of AmNR1 in the honeybee brain. The NR1 sub-unit was detected with the anti-NR1 (NR1-pan) and the mab363 (NR1 (mab363)) antibodies. Signals were found in the protocerebral lobe (pl), the dorsal lobes (dl), the sub-oesophageal ganglion (sog), and in the optic lobe, in the lobula (lo), medulla (me) and lamina (la) (A, B, F and H). In the mushroom bodies signals were detected: in the lip (lip), in the basal ring (br) and in the neck (ne) and only limited signals were found in the collar (co) (A-C). The output region of the mushroom bodies revealed specific patterns in the vertical (v) and gamma (γ) lobes and at the junction (jct) between the vertical and the medial lobes and in glial cells (gc) (C and D). In the antennal lobes (al) signals were found in the glomeruli (glo) (E and G). Scale bar = 100 µm.

The calyces of the mushroom bodies compartmentalize into the lip, the collar and the basal ring, and constitute that main input regions of the mushroom bodies, where Kenyon cells send their dendritic projections (Gronenberg 2001; Mobbs 1982). Kenyon cell axons project ventrally to form the peduncle and bifurcate into two lobes: the medial lobe and the fused vertical and gamma lobes, formerly called the α and β lobes,

respectively (Strausfeld 2002; Vowles 1955). The AmNR1-1 expression was more intense in the lip, the basal ring, at the junction of the neck with the peduncle and the in bundles of axonal processes projection to the dorsal peducle (Fig 2.3A-C). The collar had the weakest expression level. Since it is expected that the NMDA receptor is expressed post-synaptically, the receptors detected in the calyces are presumably expressed by Kenyon cells. Interestingly, the localization of the AmNR1-1 in the calyxes correlates with the high mRNA level detected in class I Kenyon cells innervating the lip and the basal ring. This argues toward a specific immunocytochemical detection of AmNR1-1. Interestingly the AmNR1-1 subunit was also detected in the somata of Kenyon cells (Fig 2.3A and B). The presence of RSS motifs may be indicative of this particular localization.

AmNR1-1 was detected at the output regions of the mushroom bodies, in the vertical and gamma lobes and at the junction between the lobes, where processes are grouped in a columnar pattern (Fig. 2.3C and D). Different AmNR1-1 expression levels were detected in the layers composing the vertical and gamma lobes. The staining patterns obtained with both antibodies were very similar, particularly in the vertical lobes, whereas the distribution of signal intensities in the gamma lobes was not perfectly matching, most probably because the brain section showed in Fig. 2.3C is located more posterior than the section shown in Fig. 2.3D. Staining was also found in particular layers of the medial lobes (data not shown).

It is likely that AmNR1-1 is also expressed in glial cells distributed within and between neuropils (Fig. 2.3C and D). These results corroborate the previous observation made at the mRNA level and are further supported by the fact that glial astrocytes express functional NMDA receptor in mouse (Schipke et al., 2001).

Interestingly, the distribution of AmNR1-1 in the brain correlated well with previous

studies of glutamate immunoreactivity (Bicker et al., 1988). By example, in the vertical and gamma lobes AmNR1-1 was found in the same layers that were previously shown to have Glu-reactivity (Bicker et al., 1988).

In this work, we identified the NR1 sub-unit homologue of the honeybee NMDA receptor and we described its expression sites. It will be an important next step to analyze the functional properties of this receptor and to describe its implications in neurological processes.

Note: This chapter is written according to manuscript. Additional figures for AmNR1 immunoreactivity which are related to this work are presented in the appendix.

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