10 Appendix

10.1: Materials and methods in detail

10.1.1: Special equipment

PCR Mastercycler (Eppendorf, Mastercycler ® gradient, Germany), Spectrophotometer (Ultraspec®II, UV/ Visible Spectophotometer, Pharmacia LKB Biotechnology, Sweden), Gel Electrophoresis chamber, Thermo cycler, UV transilluminator, Brightfield microscope, Confocal microscope (Leica, Wetzlar, Germany), an air pressure injector (PV820 Pneumatic Pico Pump; World Precision Instruments, Inc., USA), Cryostat (Leica, Germany), and Vibratome.

10.1.2: Reagents

Paraformaldehyde, Sodium hydoxide (NaOH), Triethanolamine HCl, Acetic anhydride, Sodium biphosphate (NaH_2PO_4), Sodium chloride (NaCl), Sodium citrate, Potassium biphosphate (KH_2PO_4), Potassium chloride (KCl), Magnesuim chloride ($MgCl_2$), Calcium cloride ($CaCl_2$), Tris HCl, Ethylenediamine tetraacetic acid (EDTA), Dimethyl formamide, Tris-acetate, Tris-borate, Glacial acetic acid, Boric acid, Dithiothreitol (DTT), Polydeoxyadenylic acid, Polyadenylic acid, Agarose, Low melting agarose, Ethidium bromide, Tween 20, Triton X-100, Formamide, Heparin, Yeast RNA, Hering sperm DNA, Chaps, Ficoll, Polyvinylpyrrolidone, Dextran sulfate, Ethanol (different series of ethanol: 50%, 70%, 90%, 99% and 100%), Proteinase K, Glycerol, Bovine serum albumin (BSA), Levimasole, Nitroblue tetrazolium (NBT), 5-bromo-4-chloro-3-indolylphosphate (BCIP) , DAKO glycergel, anti-fade mounting media, 15 mM K_3Fe [(CN_6)] (potassium ferricyanate), 5 mM K_4Fe [(CN_6)] (potassium

ferrocyanate), Triethanolamine (TEA), Triton X-100. Trizol reagent (Invitrogen, Karlsruhe, Germany), oligo-d (T) 18 primer and the Revertaid H minus first strand cDNA synthesis kit (MBI Fermentas, St.Leon-Rot, Germany), Gentherm polymerase (Rapidozym, Berlin, Germany), TOPO TA cloning ® Kit (Invitrogen, Karlsruhe, Germany), PCR DIG probe synthesis Kit (Roche, Mannheim, Germany), DIG RNA labelling mix (Roche, Mannheim, Germany), probeQuant TM G50 micro columns (Amersham Biosciences, Freiburg, Germany), DIG Wash and Block Buffer Set (Roche, Mannheim, Germany), Embedded glue (Leica Instruments, Nussloch, Germany). pBlueBac4.5/V5-His CAT (Invitrogen, Karlsruhe, Germany), pBac(3xP3-EGFPaf), pJ-GFP, pBRJ-LacZ (collected by Dr. Gerard Leboulle). The following primers (Table 2.1) were purchased from TIB MOLBIOL, Tempelhofer Weg, Germany.

Table 10.1: Sequence of primers that was used in this investigation

Name	Sequence (5'-3')
CAT forward primer	GTATGGCAATGAAAGACGGTGAGC
CAT reverse primer	TGAAAACGGGGGCGAAGAAGT
AmNR1 forward primer for PCR	ATTTAGATAAGGGCGTGAACT
AmNR1 reverse primer for PCR	ACATCTAACAAATTTTTCAGGTAA
AmNR1 forward primer for DNA probe	AACACTGACGGTACCGAAGAGGAT
AmNR1 reverse primer for DNA probe	ATGTGCCACGGTAAAAGGTTCTGCT
AmNR1 forward primer for RNA probe	CATGTATTTCCGTCGCCAAGTC
AmNR1 reverse primer for RNA probe	TTCTGTAAACCAATCCCATAGC
T7 promoter sequence	TAATACGACTCACTATAGGGCGA
T3 promoter sequence	AATTAACCCTCACTAAAGGGACTA

Primers were designed by using DNASTAR program (PrimerSelect-DNASTAR, Madison, Wisconsin, USA).

10.1.3: Antibodies

Anti-Digoxygening-AP, Fab fragments (Roche, Penzberg, Germany), Primary antibodies for NMDA receptor subunit R1: NR1-mab363, NR1-mab1485 (Chemicon, Hampshire, UK), and NR1-pan (Upstate; Biomol, Hamburg, Germany), Secondary antibodies: Anti-mouse IgG conjugated with AP, Anti-rabbit IgG conjugated with AP, Anti-mouse IgG conjugated with biotin, Anti-rabbit IgG with biotin (Sigma, Munich, Germany), extravidin conjugated with AP (Sigma, Munich, Germany), Anti-mouse IgG Cy3 and Cy5, Anti-rabbit IgG Cy2 (Jackson Immunoresearch, Cambridgeshire, UK) and AF3 anti-DIG fluorescent antibody (developed in Corey Goodman's lab by Beth Blankemeier).

10.1.4: Buffers and solutions

4% Paraformaldehyde for 10 ml

0.4 g paraformaldehyde was weighed into 8 ml of 1× PBS solution with 2 µl of 10N NaOH. The solution was heated at 65^{o}C until dissolve. Then, it was allowed to cool at room temperature and the pH was adjusted to 7.5. Finally, 1× PBS was added to achieve a final volume of 10 ml.

TEA Buffer

0.1M triethanolamine (TEA) was dissolved in the Millipore water and the pH was adjusted to 8.0.

Acetylation Solution

100 ml of 0.1 M TEA buffer and 250µl (0.25%, v/v) acetic anhydride were mixed at

room temperature just before incubation of the slides.

10× Phosphate Buffered Saline (PBS)

137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4 were dissolved in the Millipore ultra pure water. The pH was adjusted to 7.4 by using HCl and the solution was autoclaved for 20 minutes at 15 psi 1.05 kg/cm^2) on liquid cycle.

10× Tris EDTA (TE)

100 mM Tris-HCl and 50 mM EDTA were dissolved in the Millipore water. The pH was adjusted to 7.4 and autoclaved for 20 minutes at 15 psi (1.05 kg/cm^2) on liquid cycle.

10× TAE Buffer for 1 liter

40 mM Tris base

Glacial acetic acid

1 mM EDTA

48.4 g of Tris base, 11.42 ml of glacial acetic acid and 20 ml of 0.5 M EDTA (pH 8.0) were mixed in the Millipore water and the pH was adjusted to 8.3.

1× TAE Buffer

10× TAE buffer was diluted with Millipore water.

5× TBE Buffer for 1 liter

45 mM Tris base

Boric acid

1 mM EDTA

108 g of Tris base, 55 g of Boric acid and 40 ml of 0.5 M EDTA (pH 8.0) were mixed in the Millipore water and the pH was adjusted to 8.3.

1× TBE Buffer

5× TBE buffer was diluted with Millipore water.

20× SSC

3.0 M NaCl and 0.3 M Sodium citrate were dissolved in the Millipore water. The pH was adjusted to 8.3 and autoclaved as described above.

5× SSC

Diluted in Millipore water from the 20× SSC.

0.2× SSC for 1 liter

Diluted from the 5× SSC.

0.1% 1× PBS-T Solution for 1 liter

100 ml of 10× PBS, 1000 μl of Triton X-100 and 900 ml of the Millipore water were mixed and stirred vigorously to dissolve.

30% Sucrose Solution

3 g sucrose was dissolved into 10 ml of 0.1% 1× PBS-T solutions and shaked vigorously. The solution was stored in the refrigerator.

Proteinase K Buffer

1 M Tris-HCl and 5 M NaCl were dissolved in the Millipore water.

Proteinase K Solution

Lyophilized proteinase K powder was dissolved into proteinase K buffer at a concentration of 20 mg/ml and stored at -20 ^{o}C.

10N NaOH Solution for 1 liter

400 g of NaOH was dissolved in 1 liter of Millipore water and cool down at RT.

2% Blocking Solution

1 g of BSA was added with 50 ml of 1× PBST and shaked vigorously to dissolve and the solution stored at 4^{o}C.

Detection Buffer

0.1 M Tris HCl (pH 9.5), 0.1 M NaCl, 50 mM $MgCl_2$, and 0.1% Tween-20 were

dissolved in the Millipore water and the pH was adjusted at 9.5.

NBT solution for 10 ml

500 mg of NBT powder, 7 ml of dimethyl formamide and 3 ml of Millipore water were mixed and aliquoted in a 1.5 ml microcentrifuge tube. The sample stored at -20^{o}C for several months.

BCIP Solution for 10 ml

250 g of BCIP powder was dissolved in the 10 ml of dimethyl formamide. Aliquoted them in a 1.5 ml microcentrifuge tube and stored at -20^{o}C for several months.

Color Development Solution

66 µl of NBT solution, 66 µl of BCIP solution and 10 ml of detection buffer were mixed and used immediately after making the solution.

50× Denhardts Solution

500 mg of ficoll, 500 mg of polyvinylprrolidone and 500 mg of BSA were dissolved into 50 ml of Millipore water and stored at -20^{o}C.

50% Formamide

100% formamide was diluted in the Millipore water.

50% Dextran Sulfate

100 ml of Millipore water was prewarmed at 60^{o}C and then 50 g of dextran sulfate was added. Vigorously stirred for 30 minutes or until dissolved and cool down at RT. The sampled stored at -20^{o}C.

Hybridization Solution for DNA probe for 500 ml

50% Formamide

5× SSC

5× Denhardts

250 µg/ml yeast RNA

500 µg/ml Hering sperm DNA

50 µg/ml heparin

2.5 mM EDTA

0.1% Tween-20

0.25% Chaps

250 ml of 100% formamide, 125 ml of 20× SSC, 50 ml of 50× denhardts solution, 125 mg of yeast RNA, 250 mg of hering sperm DNA, 25 mg of heparin, 2.5 ml of 0.5 M EDTA, 500 µl of Tween-20 and 1.25 ml of chaps were mixed and stirred gently until dissolved all components and stored at -20^{o}C for several months.

Hybridization Solution for RNA probe for 500 ml

250 ml of 100% formamide, 125 ml of 20× SSC, 25 mg of heparin, 500 µl of Tween-20 and 125 ml of Millipore water were mixed and stirred gently until dissolved all components and stored at -20^{o}C.

6× Bromophenol Blue Solutions for 100 ml

0.125% Bromophenol Blue

30% Glycerol

6× TBE

0.125 g of bromophenol blue, 30 ml of glycerol and 60 ml of 10× TBE buffer were mixed and final volume was adjusted to 100 ml with Millipore water, mixed thoroughly by repeated inversion. The solution was stored in a 50 ml falcon tubes at 4^{o}C and covered with parafilm.

1% Agarose Gel Solution

Agarose powder was dissolved into 50 ml of 1× TAE/TBE buffer with heat.

7% low melting agarose

Low melting agarose powder was mixed with 10 ml of 1× PBS and heated at 70^{o}C to

dissolve.

Poly-D-Lysine solution

10 mg of lyophilizate was dissolved with 10 ml of 1× PBS solution and mixed gently with pipette tip and aliquoted in a 1.5 ml of microcentrifuge tube. The sample stored at -20^{o}C.

10.1.5: Coating of slides with Poly-d-Lysine

Slides were washed with distilled water and then sterilized with 70% ethanol for 10 to 30 minutes. Subsequently, the slides were air dried under the fume hoot and 10 to 15 µl of Poly-d-Lysine solution were pipetted onto each slide and left to dry. Then the slides were rinsed with 1× PBS for 5 to10 minutes and air dried at room temperature (RT) and stored in a slide box at RT or -20^{o}C.

10.1.6: RNase free treatment

To prevent contamination with RNase, all materials that were used for *in situ* hybridization were pre-incubated in 10 N NaOH for 30 minutes and rinsed with Millipore water.

10.1.7: Brain preparation and fixation

Under the binocular microscope cuticle was removed from the bee head with a scalpel and the heads were fixed in 4% paraformaldehyde for 30 minutes on ice. After fixation, the brains were quickly mounted on wax and dissected under a microscope. Dissected brains were then carefully transferred into 4% paraformaldehyde for further fixation for 90 minutes on ice. Subsequently, the brains were washed with 1× PBS 3 times for 15 minutes each, followed by overnight incubation in 30% sucrose (in 1× PBS Triton X-100) at 4^{o}C. The following day, the brains were embedded in the embedding medium

and frozen on the surface of liquid nitrogen. Frozen brains were mounted on cutting chucks and placed into the cryostat chamber for 20 to 30 minutes. Embedded brains were then sliced at 14 µm thickness and mounted on the superfrost ®plus slides. Air-dried the slides (at room temperature for 30 minutes) and were used for *in situ* hybridization for DNA probe.

10.1.8: Detection of NMDA receptor subunit R1 (AmNR1) mRNA by *in situ* hybridization

Preparation of DIG-labeled dsDNA probes

A cDNA template was used to generate a 291 bp fragment of DIG-labeled dsDNA probe using following primer pairs.

Forward primer sequence: 5'- AACACTGACGGTACCGAAGAGGAT -3'

Reverse primer sequence: 5'- ATGTGCCACGGTAAAAGGTTCTGCT -3'

The following components were mixed in a sterile microcentrifuge tube on ice to perform a standard 50 µl each DIG-labeled and unlabeled PCR reaction:

	DIG-labeled PCR	un-labeled PCR
10× conc. PCR buffer with $MgCl_2$-	5 µl	5 µl
PCR DIG labeling mix-	5 µl	-
10× conc. dNTP-	-	5 µl
NMDA-forward primer (10 µM)-	1 µl	1 µl
NMDA-reverse primer (10 µM)-	1 µl	1 µl
DNA template (CAT plasmid 100 ng/µl)-	1 µl	1 µl
Enzyme mix (3.5 u/µl)-	0.75 µl	0.75 µl
H_2O-	36.25 µl	36.25 µl
Total volume	50 µl	50 µl

The following conditions were used to perform the PCR of DIG-labeled probe:

1. 95^{o}C for 3 minutes

2. 40 cycles at 95^{o}C for 30 seconds

3. 66^{o}C for 1 minute

4. 72^{o}C for 1 minute

Final elongation

72^{o}C for 5 minutes

Hold at 4^{o}C.

Gel electrophoresis, purification and quantification of the DIG-labeled dsDNA probes

5 µl of DIG-labeled dsDNA probes were mixed with 5 µl of 6× bromophenol blue and 2 µl of Millipore water on ice. The solution was separated on a 1% agarose gel with 1× TAE/1× TBE buffer at 130 V for 30 minutes. The gel was stained with one drop of ethidium bromide solution and DNA band was visualized using a UV transilluminator. Amplified DIG-labeled dsDNA probes were then purified using probeQuant ^{TM} G-50 micro columns (Amersham Biosciences, Freiburg, Germany) and thereafter the probes were quantified using absorbance at a wavelength 260 nm by spectrophotometer. The sample stored at -80^{o}C.

Preparation of DIG-labeled CAT dsDNA probes

pBlueBac4.5/V5-His CAT (chloramphenicol acetyltransferase, CAT) was used as a template for polymerase chain reaction (PCR) to amplify the 300 bp fragments of DIG-labeled CAT DNA probes. Following sequences were used as forward and reverse primer, respectively.

Forward primer: 5'- GTATGGCAATGAAAGACGGTGAGC-3'

Reverse primer: 5'- TGAAAACGGGGGCGAAGAAGT-3'

The same PCR protocol was applied as mentioned in section 2.9. DIG-labeled CAT DNA probe was purified quantified and checked the expected size as described above. The sample stored at - 80^{o}C. Figure 10.2 shows the construction map of the CAT plasmid.

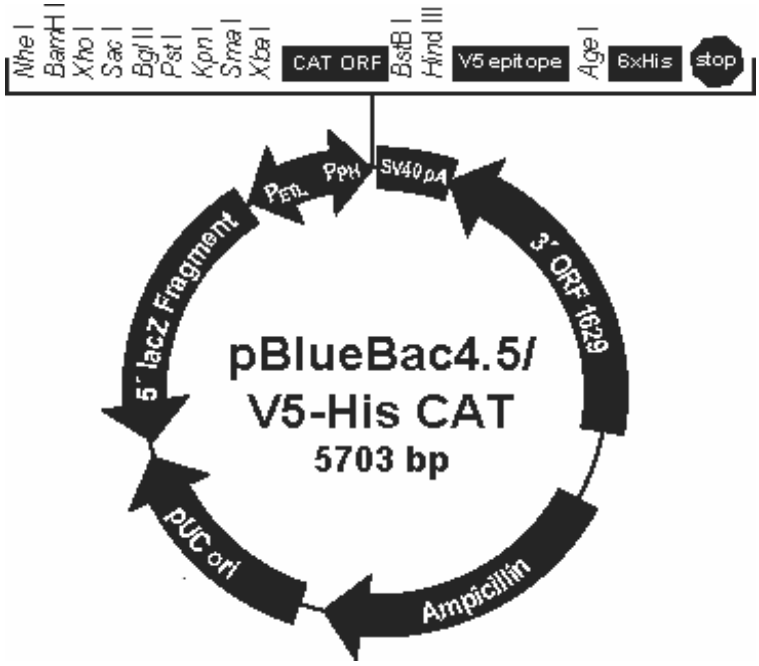

Figure 10.1: Construction map of the CAT plasmid.

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

To generate sense and anti-sense RNA probes, template DNA was obtained by PCR using forward and reverse primers that were modified by the addition of T7 and T3 promoter sequence at the 5' extremity. The sequences of primers are given below.

Forward primer: 5'- CATGTATTTCCGTCGCCAAGTC-3'

Reverse primer: 5'- TTCTGTAAACCAATCCCATAGC-3'

T7 promoter sequence: 5'- TAATACGACTCACTATAGGGCGA-3'

T3 promoter sequence: 5'- AATTAACCCTCACTAAAGGGACTA-3'

Preparation of template DNA by PCR amplification

To perform a standard 50 µl of PCR reaction the following components were mixed in a sterile microcentrifuge tube on ice to amplify the 300 bp fragments:

10× concentrated PCR buffer $(NH_4)_2SO_4$ -	5.00 µl
10 mM dNTP -	1.25 µl
10 µM NMDA forward primer -	2.50 µl
10 µM NMDA reverse primer -	2.50 µl
25 mM $MgCl_2$ -	3.00 µl
cDNA template (50 ng/µl) -	1.00 µl
Taq polymerase 5u/µl -	0.20 µl
H_2O -	34.55 µl
Total Volume	=50.00 µl

PCR was performed using an Eppendorf Mastercycler with a protocol as follows:

1. 95°C for 3 minutes
2. 40 cycles at 95°C for 30 second
3. 63°C for 1 minute
4. 72°C for 1 minute

Final elongation

72°C for 5 minutes

Hold at 4°C.

The PCR product was confirmed by 1% agarose gel electrophoresis in 1× TAE buffer and purified using probeQuant TM G-50 micro columns. Concentration was measured spectrophotometrically at a wavelength of 260 nm and the was sample stored at - 20°C. Purified PCR products were then used as a template DNA to generate 20 µl of DIG labeled sense and anti-sense RNA probes. The reaction mixture for DIG labeling RNA

was as follows:

Anti-sense:	230 ng of purified PCR DNA fragment	
	(contained T3 promoter sequence)-	5 µl
	10× DIG RNA labeling mix-	2 µl
	5× Transcription buffer-	4 µl
	RNA (T3) polymerase-	2 µl
	rRNA sin (RNase inhibitor)-	0.5 µl
	DTT-	1 µl
	H_2O-	5.5 µl
	Total volume	20 µl

Sense:	231 ng of purified PCR DNA fragment	
	(contained T7 promoter sequence)-	5 µl
	10× DIG RNA labeling mix-	2 µl
	5× Transcription buffer-	4 µl
	RNA (T7) polymerase-	2 µl
	rRNA sin (RNase inhibitor)-	0.5 µl
	DTT-	1 µl
	H_2O-	5.5 µl
	Total volume	20 µl

All components for either sense or anti-sense were mixed gently in a clean microcentrifuge tubes and centrifuged briefly at 750 rcf at 4^{o}C for 1 minute. Then the tubes were incubated at 37^{o}C for 2 hours and immediately after completion of *in vitro* transcription probes were placed on ice. Using a probeQuant TM G-50 micro column, the probes were purified and the concentration was measured at a 260 nm wavelength. The probe quality was confirmed on a 1% agarose gel electrophoresis in 1× PBS buffer with one drop of ethidium bromide. Then the probes were diluted with hybridization solution of RNA probe (1:200) and aliquoted into sterile microcentrifuge tubes and stored at - 80^{o}C.

Estimation of yielded DIG-labeled RNA probes by Dot blot

1 µl of newly synthesized experimental DIG-labeled sense and anti-sense RNA probes were diluted in Millipore ultrapure water to get a final concentration of 10 ng/µl and then serial dilutions were made continuously up to 1 ng/µl. 1 µl of each dilution was pipetted onto a nitrocellulose membrane and the nucleic acid spots fixed to the membrane by cross linking with UV light for 3 minutes. The membrane was then transferred to a petri dish that contained 10 ml of DIG washing buffer and incubated at RT for 2 minutes under constant agitation. The membrane was then incubated with 10 ml blocking solution for 30 minutes at RT and incubated with 10 ml Anti-DIG alkaline phosphatase antibody (diluted 1:5000 in the blocking solution) for 30 minutes at RT. The membrane was then washed with 10 ml of washing buffer 2 times for 15 minutes each and equilibrated with 10 ml of detection buffer for 5 minutes. Thereafter incubated with a color development solution [66 µl of NBT (330 µg/ml) and 66 µl of BCIP (165 µg/ml) were diluted in 10 ml of detection buffer] to allow color development in the dark for 3 to 10 minutes or until color precipitation was sufficient. Finally, the membrane was washed with the double distilled water for 5 minutes to stop the color development reaction, and air-dried for several minutes.

10.2: Additional photographs of AmNR1 immunoreactivity in the worker bee brain

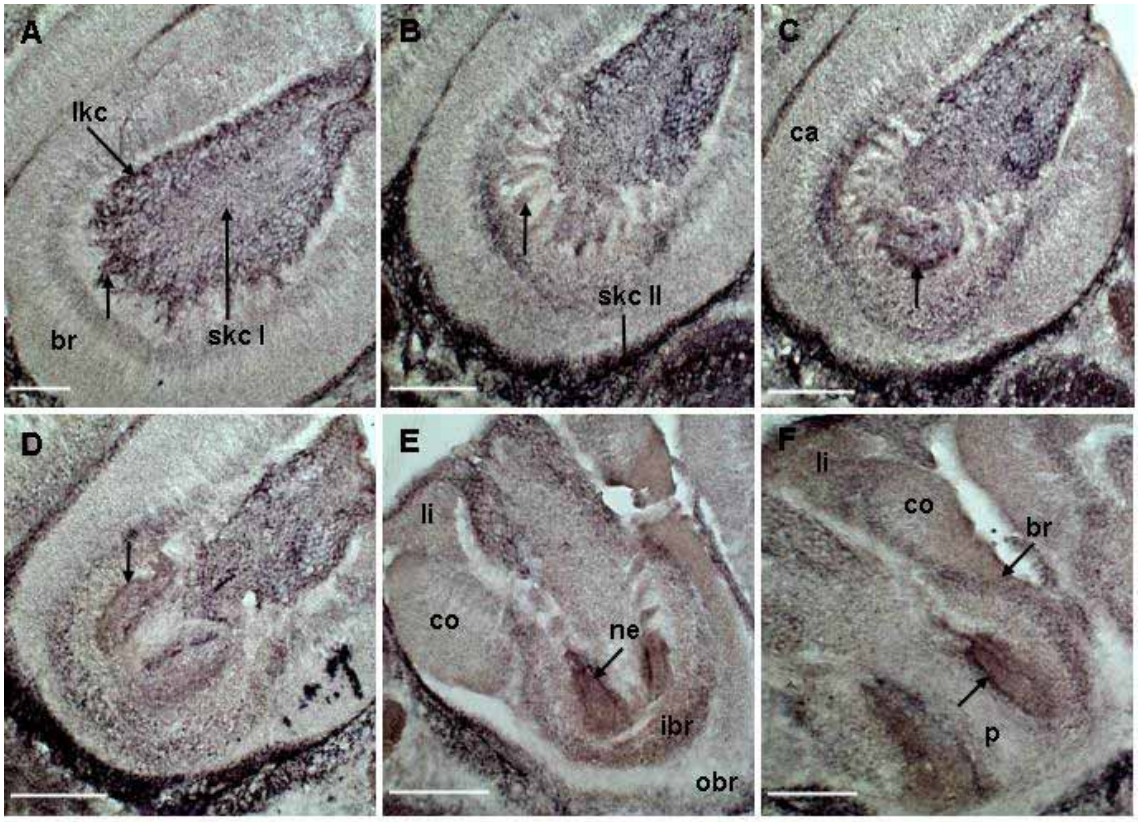

Figure 10.2: Convergence of AmNR1 immunoreactive bundles at the top of the peduncle neck that is forming strata in the peduncle and lobes reveals with NR1-pan antibody. In the panel A, arrow indicates immunoreactive bundles above the basal ring (br). Spoke-like arrangement of immunoreactive bundles at the inner face of the calyx is shown in the panel B (indicate by arrow). C and D: Immunoreactive bundles that converge at the top of the peduncle neck to form a horseshoe-like arrangement of strata indicate by arrow. E: Immunoreactive strata in the neck of the peduncle (ne). F: Immunoreactive strata in the peduncle (p) indicate by arrows. Lip (li), collar (co), basal ring (br), inner basal ring (ibr), outer basal ring (obr), peduncle (p), calyx (ca), large Kenyon cells (lkc), small Kenyon cells class I and II (skc I and skc II). Scale bar = 0.1 mm.

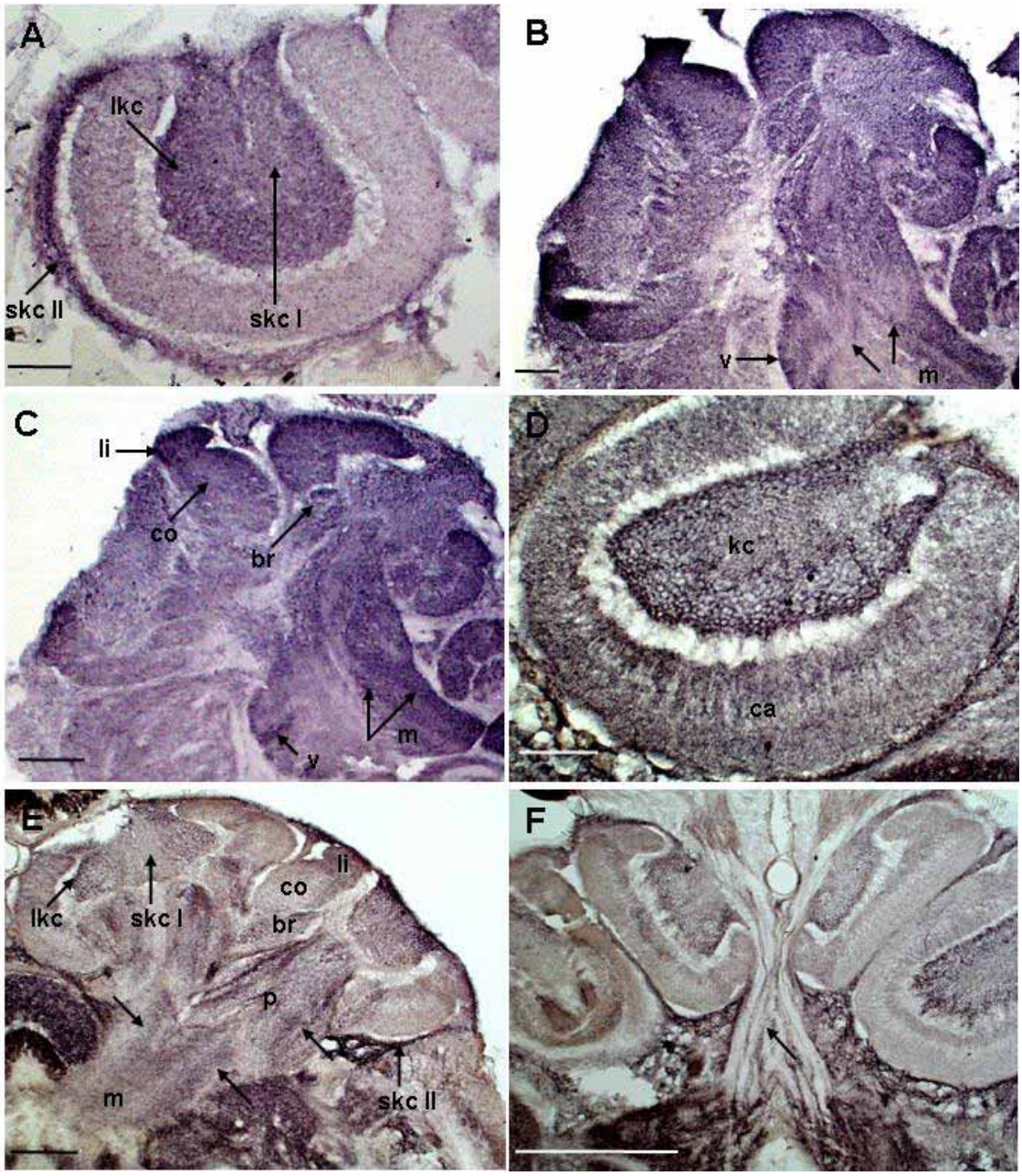

Figure 10.3: AmNR1 immunoreactivity in the mushroom body. A-C: AmNR1 immunoreactivity identified with NR1-mab363 antibody. D-F: Identified with NR1-pan antibody. Projection of AmNR1 immunoreactive strata in the vertical (v) and medial (m) lobes after their divide from the peduncle indicates by double arrows in the panel B. Prominent and broad immunoreactive strata in the vertical lobe and the medial lobe indicates by single and double arrows in the panel C. Immunoreactive strata in the peduncle, medial lobe and in the ocellar tract (indicated by arrows) in the panel E and F revealed with NR1-pan antibody. Large Kenyon cells (lkc), small Kenyon cells class I and II (skc I and skc II), lip (li), collar (co), basal ring (br), Kenyon cells (kc), calyces (ca), peduncle (p), medial lobe (m), and vertical lobe (v). Scale bar = 0.1 mm.

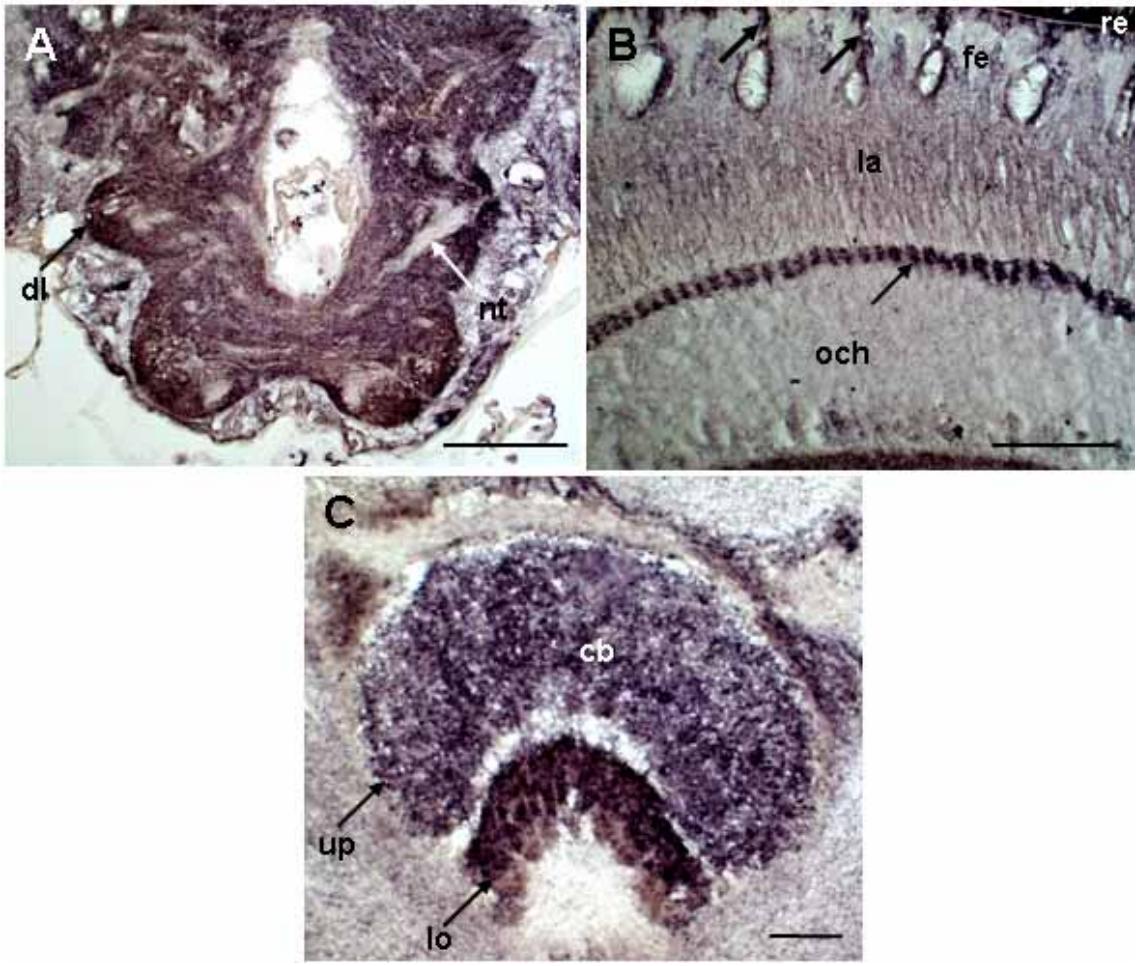

Figure 10.4: AmNR1 immunoreactivity in the different brain regions of the worker bee that reveals with NR1-pan antibody. A: AmNR1 immunoreactive in the suboesophageal ganglion. B: Bundles of AmNR1 immunoreactive photoreceptor axons (indicated by bold arrows) in the fenestrated layer (fe) send their projections in the lamina (la) and the terminal of outer optic chiasma (och, indicated by narrow arrow). C: Differential AmNR1 immunoreactivity in the central brain (cb).Upper part (up), lower part (lo), retina (re), dorsal lobe (dl), and nerve tract (nt). Scale bar = 0.1 mm.

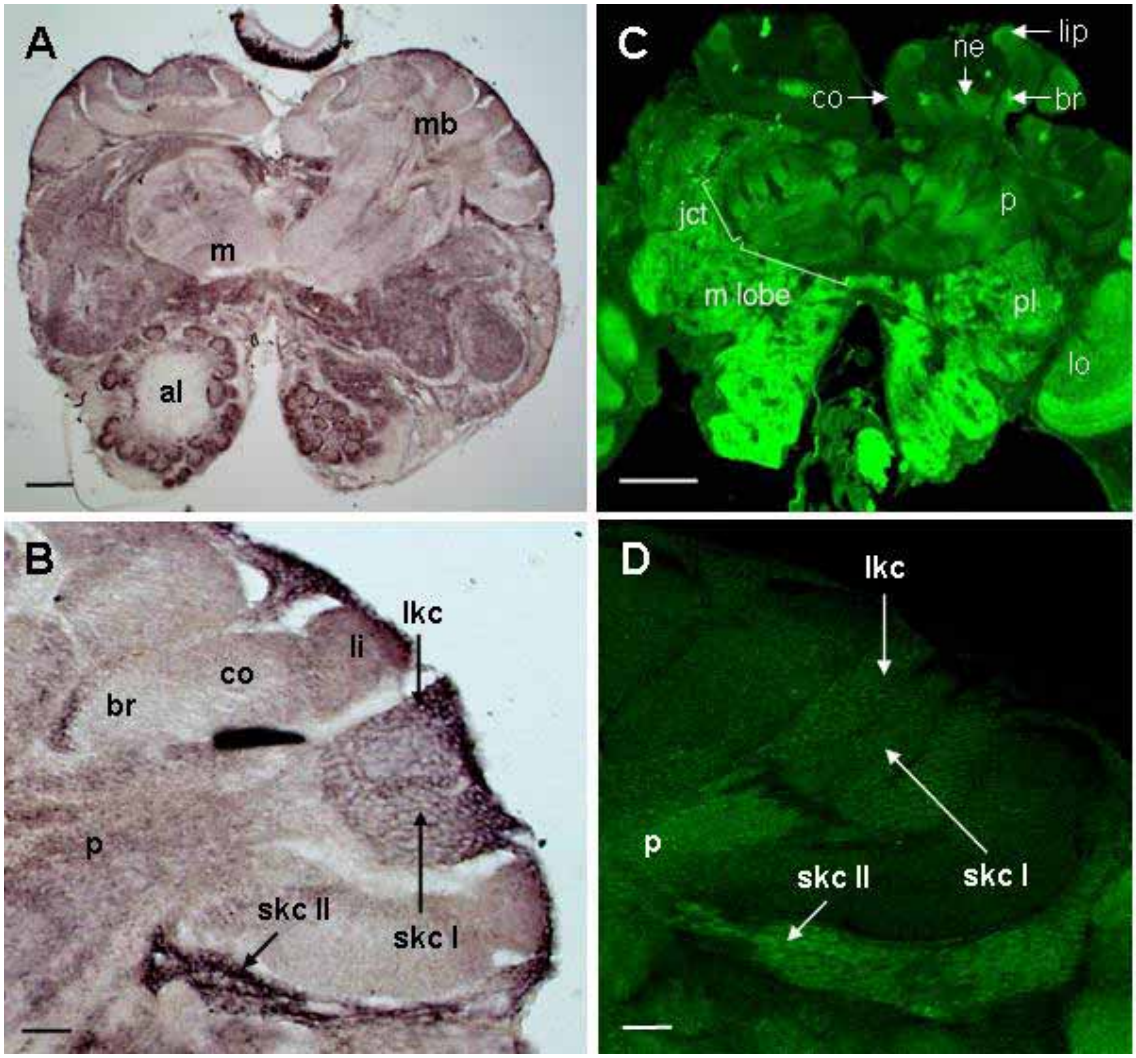

Figure 10.5: Comparative AmNR1 immunoreactivity using to different detection techniques with NR1-pan antibody. A and B: Detection of the expression of AmNR1 protein using immunoenzyme histochemical analysis on brain cryosections. C and D: Detection of the expression of AmNR1 protein using immunofluorescent technique on brain cryo and vibratome sections. Mushroom body (mb), antennal lobe (al), medial lobe (m), lip (li), collar (co), basal ring (br), large Kenyon cells (lkc), small Kenyon cells class I (skc I), small Kenyon cells class II (skc II), peduncle (p), protocerebral lobe (pl), the junction between the vertical and medial lobes (jct), neck of the peduncle (ne), peduncle (p), and lobula of the optic lobe (lo). Scale bar = 0.1 mm.