Localization of the NMDA glutamate receptor subunit R1 in the honeybee, *Apis mellifera* neurons

Inaugural-Dissertation to obtained the academic degree Doctor rerum naturalium (Dr. rer. nat) Submitted to the Department of Biology, Chemistry and Pharmacy of Freie Universität Berlin

by

Mst. Thangima Zannat from Bogra

August, 2007

Reviewer: Prof. Dr. R. Menzel
Reviewer: Prof. Dietmar Kuhl

Date of defence: 27.11.2007

Chapter 1 is based on the following manuscript:

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

Mst. Thangima Zannat, Fernando Locatelli, Jürgen Rybak, Randolf Menzel, Gérard Leboulle

The contribution of the different authors was as follows:

I prepared PCR, probes and also performed *in situ* hybridization and immunohistochemistry. Dr. Gérard Leboulle performed cDNA cloning, computational analysis, analysis of AmNR1-1 amino acid sequence and wrote the manuscript. Results and the manuscript were discussed with Dr. Fernando Locatelli, Jürgen Rybak, and Prof. Dr. Randolf Menzel.

For immunofluorescent study, I performed all experiments and watched when Dr. Fernando Locatelli and Dr. Gérard Leboulle were studied fluorescent signal with the confocal microscope. I got fluorescent photographs as files from them, and figure 5.2 from Dr. Gérard Leboulle.

Contents

1 I	1 Introduction		
1.1	Thesis outline	1	
1.2	General introduction	1	
	1.2.1: Honeybee in research	1	
	1.2.2: Brain structure of the honeybee	2	
	1.2.3: Honeybee and learning and memory	5	
	1.2.4: Glutamate receptors	6	
1.3	The aim of this thesis	11	
	References	12	

2	Chapter 1	
Identification and localization of the NR1 sub-unit homologue of the NMDA		
gluta	glutamate receptor in the honeybee brain	
2.1	Abstract	22
2.2	Introduction	22
2.3	Materials and methods	23
2.4	Results and discussion	26
	Acknowledgement	35
	References	36

3	Chapter 2	
Dev	elopmental expression of the NMDA glutamate receptor subunit R1	gene in
the	central nervous system of the honeybee	
3.1	Abstract	39
3.2	Introduction	39
3.3	Materials and methods	41
	3.3.1: Bees	41
	3.3.2: Preparation of DIG-labeled sense and anti-sense RNA probes	41
	3.3.3: Brain preparation and <i>in situ</i> hybridization	42
	3.3.4: Immunohistochemistry	42
3.4	Results	43

	3.4.1:Expression of AmNR1 mRNA at different developmental stages	43
	3.4.2: Expression of AmNR1 protein at different developmental	49
	stages	
3.5	Discussion	54
	References	57

4 Chapter 3

The expression of the 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

	0	
4.1	Abstract	61
4.2	Introduction	61
4.3	Materials and methods	64
	4.3.1: Animal's maintenance	64
	4.3.2: Preparation of DIG-labeled dsDNA probe	64
	4.3.3: Preparation of DIG-labeled CAT dsDNA probe	65
	4.3.4: In situ hybridization	65
	4.3.5: Immunohistochemistry	67
	4.3.6: Immunofluorescent and double staining of AmNR1 and DLG	67
4.4	Results and discussion	68
	4.4.1: Synthesis of probes	68
	4.4.2: Localization of AmNR1 transcript in the adult bee brain	69
	4.4.3: Localization of AmNR1 transcript in two different seasonal	74
	bees	
	4.4.4: Localization of AmNR1 protein in different seasonal stages and	75
	hives bees	
	4.4.5: Comparisons and co-localization of AmNR1 immunoreactivity	83
	with DLG	
	References	87

5 General Discussion	
5.1 : Chapter 1	91
5.2 : Chapter 2	93
5.3 : Chapter 3	95
References	99

6 Conclusions and Outlook		103
7 Summary		104
8 Acknowledgements		106
9	CV	107
10	Appendix	
10.1	Materials and methods in detail	108
	10.1.1: Special equipments	108
	10.1.2: Reagents	108
	10.1.3: Antibodies	110
	10.1.4: Buffers and solutions	110
10.1.5: Coating of the slides with Poly-d-Lysine		115
10.1.6: RNase free treatment		115
	10.1.7: Brain preparation and fixation	115
	10.1.8: Detection of NMDA receptor subunit R1 (AmNR1) mRNA	116
	by in situ hybridization	
	Preparation of DIG-labeled dsDNA probes	116
	Gel electrophoresis, purification and quantification of the	117
	DIG-labeled dsDNA probes	
	Preparation of DIG-labeled CAT dsDNA probes	117
	Preparation of DIG-labeled sense and anti-sense RNA probes	118
	Preparation of template DNA by PCR amplification	119
	Estimation of yielded DIG-labeled RNA probes by Dot blot	121
10.2	Additional photographs of AmNR1 immunoreactivity in the worker	122
	bee brain	
10.3	Table of experiments	126

Figure index

Figure 1.1	Schematic diagram of adult honeybee brain	4
Figure 1.2	Organization of the mushroom body of honeybee	5
Figure 2.1	Analysis of AmNR1-1 amino acid sequence	28
Figure 2.2	Detection of <i>nmdar1</i> expression sites by <i>in situ</i> hybridization	30
Figure 2.3	Detection of AmNR1 in the honeybee brain	32
Figure 3.1	Gel electrophoresis and dot blot for DIG-labeled probes	44
Figure 3.2	Expression of AmNR1 transcript in young pupae hybridized	45
	with DIG-labeled AmNR1 anti-sense RNA probes	l
Figure 3.3	In situ hybridization to AmNR1 transcript in 5 day old pupae	46
	with DIG-labeled AmNR1 anti-sense RNA probes	l
Figure 3.4	Expression of AmNR1 transcript in the mushroom body and	
	vertical lobe of 5 day old and late pupae with DIG-labeled	47
	AmNR1 anti-sense RNA probes	l
Figure 3.5	AmNR1 mRNA expression in the young bee revealed by in	
	situ hybridization with DIG-labeled AmNR1 anti-sense RNA	48
	probes	l
Figure 3.6	Expression of AmNR1 immunoreactivity in 0 day old bee	l
	realized with NR1-mab363 primary antibody on brain	50
	cryosections	1
Figure 3.7	Expression of AmNR1 immunoreactivity in 2 day old bees	l
	realized with NR1-mab363 primary antibody on brain	51
	cryosections	1
Figure 3.8	Expression of AmNR1 immunoreactivity in 7 day old bees	l
	realized with NR1-mab363 primary antibody on brain	52
	cryosections	<u> </u>
Figure 3.9	Expression of AmNR1 immunoreactivity in 15 day old bees	l
	realized with NR1-mab363 primary antibody on brain	53
	cryosections	<u> </u>
Figure 3.10	Expression of AmNR1 immunoreactivity in adult foraging	l
	bees realized with NR1-mab363 primary antibody on brain	54
	cryosections	
Figure 4.1	Analysis of PCR DIG-labeled probes by gel electrophoresis	67
Figure 4.2	In situ hybridization to AmNR1 transcripts hybridized with	71

	DIG-labeled AmNR1 DNA probes	
Figure 4.3	In situ hybridization to AmNR1 transcript hybridized with	72
	DIG-labeled AmNR1 anti-sense RNA probes	
Figure 4.4	Expression of AmNR1 transcript in the mushroom body	73
Figure 4.5	Seasonal basis expression of AmNR1 transcript in the	
	worker bee brain revealed by in situ hybridization with	74
	DIG-labeled AmNR1 anti-sense RNA probes	
Figure 4.6	AmNR1 immunoreactivity in the winter bee realized with	76
	NR1-mab363 primary antibody	
Figure 4.7	AmNR1 immunoreactivity in the spring bee realized with	77
	NR1-mab363 primary antibody	
Figure 4.8	AmNR1 immunoreactivity in the summer bee realized with	78
	NR1-mab363 primary antibody	
Figure 4.9	AmNR1 immunoreactivity in the garden bee (hive A)	79
	realized with NR1-mab363 primary antibody	
Figure 4.10	AmNR1 immunoreactivity in the garden bee (hive B)	80
	realized with NR1-mab363 primary antibody	
Figure 4.11	AmNR1 immunoreactivity in the flight room bee (hive A)	81
	realized with NR1-mab363 primary antibody	
Figure 4.12	AmNR1 immunoreactivity in the flight room bee (hive B)	82
	realized with NR1-mab363 primary antibody	
Figure 4.13	Immunostaing of AmNR1 and DLG in the worker bee brain	84
Figure 4.14	Comparative immunoreactivity of AmNR1 and DLG in the	85
	mushroom body on brain vibratome sections	
Figure 4.15	Co-localization of AmNR1 and DLG proteins in the bee	86
	brain	
Figure 5.1	Diagram summarizing the expression profile of AmNR1	
	transcripts at different developmental stages and also in the	95
	adult bee brain	
Figure 5.2	Identification of several AmNR1 mRNA variants.	97
Figure 10.1	Construction map of the CAT plasmid	118
Figure 10.2	Convergence of AmNR1 immunoreactive bundles at the top	
	of the peduncle neck that is forming strata in the peduncle	122
	and lobes reveals with NR1-pan antibody	
Figure 10.3	AmNR1 immunoreactivity in the mushroom body	123

Figure 10.4	AmNR1 immunoreactivity in the different brain regions of the	124
	worker bee that reveals with NR1-pan antibody	
Figure 10.5	Comparative AmNR1 immunoreactivity using to different	
	detection techniques with NR1-pan antibody on brain	125
	cryosections	

Table index

Table 10.1	Sequence of primers that was used in this investigation	109
Table 10.2	Experiments performed with adult bees	126
Table 10.3	Experiments performed in the developmental stages of the	129
	honeybee	
Table 10.4	Experiments performed with adult worker bees collected	133
	from different seasonal stages and hives	

Abbreviations

AmCO	Apis mellifera carnica catalytic subunit of PKA
AmNR1	Apis mellifera NMDA glutamate receptor subunit R1
AMPA	Alpha-amino-3hydroxy-5-methylisoxazole
AP	Alkaline phosphatase
CAT	Choloramphenicol acetyl transferase
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
cGMP	Cyclic guanosine 3',5'-monophosphate
CNS	Central nervous system
CREB	cAMP response element binding protein
DIG	Digoxigenin
DLG	Protein discs-large
DNA	Deoxyribonucleic acid
DNMDA-R1	Drosophila NMDA receptor subunit R1
dsDNA	Double stranded DNA
ELISA	Enzyme linked immunoabsorbent assay
EST	Expressed sequence tag
FMRF-amide	Phe-Met-Arg-NH ₂ -amide
GABA	Gamma-aminobutyric acid
GCCK	Gastrincholecystokinin
GlyR	Glycine receptor
GPCRs	G-protein coupled receptors
ITRs	Inverted terminal repeats
K cell	Kenyon cell
LTD	Long-term depression
LTP	Long-term potentiation
MB	Mushroom body
mGluRs	Metabotropic glutamate receptors
mRNA	Messenger RNA
NMDA	N-methyl-D-aspartate
NMDA-R1	NMDA receptor subunit R1
ORN	Olfactory receptor neuron
PER	Proboscis extension response

PCR	Polymerase chain reaction
РКА	Protein kinase A
РКС	Protein kinase C
PKG	Protein kinase G
PSD	Postsynaptic density protein
qRT-PCT	Real-time quantitative reverse-transcription polymerase chain reaction
rcf	Relative centrifugal force
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PCR	Reverse transcription-polymerase chain reaction
Gr-hive A	Garden bees collected from the hive A
Gr-hive B	Garden bees collected from the hive B
Fl-hive A	Flight room bees collected from the hive A
Fl-hive B	Flight room bees collected from the hive B