Materials and Methods

1. Materials

The following antibodies (Table 1), RNA riboprobes (Table 2), and fluorescent conjugates (Table 3) were obtained and used in this project.

Table 2.1 Antibodies used in this study

Primary Antibody against	Host Species	Dilution	Secondary Antibody	Visualization technique	Supplier	References
Arc	Rabbit	1:1K	B- GaR	DAB	D.Kuhl	(Chowdhury, Shepherd et al. 2006)
ARG-VP	Rabbit	1:10K	B- GaR	DAB	Peninsula labs	(Keverne and Curley 2004)
c-Fos	Rabbit	1:1K 1:20K	B-GaR Alexa Fluor 594 GaR	DAB Fluorescent	Oncogene	(Kruijer, Schubert et al. 1985; Taziaux, Cornil et al. 2006)
DARPP-32	Mouse	1:5K	B-HaM	DAB	BD Bioscience	(Yan, Hsieh-Wilson et al. 1999) (Nishi, Snyder et al. 1997)
Digoxin	Mouse	1:10K	B-HaM	Fluorescent	SIGMA	(Panka, Mudgett-Hunter et al. 1988)
Digoxin	Sheep	1:5K		BCIP/NBT	Roche	(Rigby, Dieckmann et al. 1977)
Egr.1	Rabbit	1:2K	B-GaR	DAB	Santa Cruz	(Lai, Chen et al. 2004)
Fos-B	Rabbit	1:200	B-GaR	DAB	SIGMA, USA	(Dickinson, Amato et al. 1995) (Angel and Karin 1991)
GAD67	Rabbit	1:1K	B-GaR	DAB	Chemicon	(Loscher, Schirmer et al. 2006)
$G\alpha i_2$	Rabbit	1:2K	B- GaR	DAB	WACO	(Shinohara, Kato et al. 1992)
$G\alpha O$	Rabbit	1:50K	B-GaR	DAB	MBL via MoB	(Kumar, Dudley et al. 1999)
Homer	Rat	1:500	B-GaRat	DAB	abcam, UK	(Kammermeier, Xiao et al. 2000)
Jun-B	Rabbit	1:1K	B-GaR	DAB	Santa Cruz	(Maki, Bos et al. 1987)
Narp	Rabbit	1:500	B-GaR	DAB		(Reti, Miskimon et al. 2008)
Oxytocin	Rabbit	1:5K	B-GaR	DAB	Chemicon	(Pittman and Spencer 2005)
pCREB	Rabbit	1:200	B-GaR	DAB	Cell Signaling Technology	(Gonzalez and Montminy 1989)
pSer	Mouse	1:5K	B-HaM	DAB	SIGMA	(Hunter and Cooper 1985)
pThr	Mouse	1:5K	B-HaM	DAB	SIGMA	(Hunter and Cooper 1985)
pThr	Mouse	1:5K	B-HaM	DAB	SIGMA	(Hunter and Cooper 1985)
pTyr	Mouse	1:2K	B-HaM	DAB	SIGMA	(Hunter and Cooper 1985)
vGLUT1	G-Pig	1:2K	B-GaGP	DAB	Chemicon	(Varoqui, Schafer et al. 2002)
vGLUT2	G-Pig	1:2K	B- GaGp	DAB	Chemicon	(Varoqui, Schafer et al. 2002)

Table 2.2 RNA riboprobes

RNA riboprobe	Hapten	Dilution	Secondary Antibody	Visualization	Source of probe	References
GAD65	Digoxigenin	1:80	B- MaDig APShaDig	Alexa Fluor 488-SA BCIP/NBT	C.Derst	(Esclapez, Tillakaratne et al. 1994)
GAD67	Digoxigenin	1:20	B- MaDig APShaDig	Alexa Fluor 488-SA BCIP/NBT	C.Derst	(Esclapez, Tillakaratne et al. 1994)
vGLUT1	Digoxigenin	1:40	B- MaDig APShaDig	Alexa Fluor 488-SA BCIP/NBT	C.Derst	(Barroso-Chinea, Aymerich et al. 2007; Barroso-Chinea, Castle et al. 2007)
vGLUT2	Digoxigenin	1:20	B- MaDig APShaDig	Alexa Fluor 488-SA BCIP/NBT	C.Derst	(Barroso-Chinea, Aymerich et al. 2007; Barroso-Chinea, Castle et al. 2007)
vGLUT3	Digoxigenin	1:80	B- MaDig APShaDig	Alexa Fluor 488-SA BCIP/NBT	C.Derst	(Barroso-Chinea, Aymerich et al. 2007; Barroso-Chinea, Castle et al. 2007)

Table 2.3 Fluorescent conjugates

Conjugate	Dilution	Supplier
AF 488-GaR	1:2000	MoBiTec
AF 594-GaR	1:2000	MoBiTec
AF 488-SA	1:2000	MoBiTec
AF 594-SA	1:2000	MoBiTec

2. Methods

Animals

The social recognition experiments were performed by Prof. Mario Engelmann in Magdeburg. All animal experiments were conducted in accordance with the guidelines of the European Communities Council directive 86/609/EEC and were approved by the Regional Berlin Animals Ethics Committee (LaGeSo No. G 0168/01). Adult (4 months old, 350-400 g) male Wistar rats (Charles River, Sulzfeld, Germany) were used (Engelmann et al., 1995). The animals were housed individually in transparent polycarbonate cages (39x24x16 cm) for at least 1 week prior to testing under controlled

laboratory conditions with 12 h light/12 h dark rhythm (lights on 06:00) and free access to food and water. Male, unfamiliar juveniles of the same rat strain (20-35 days old) were used as social stimuli.

2.1 Animal stimulation

In the juvenile stimulated group, a given juvenile (handled with rubber gloves) was introduced into the home cage of the resident (n=10) for 4 minutes in a normally illuminated quiet room. In carvone stimulation, the subject rats (n=5) were exposed to carvone odour for 4 minutes in a condition similar to the juvenile stimulation. In the control group (n=7), the rats were housed individually for 1 week and not treated at all.

2.2 Fixation of animals by vascular perfusion

Two hours after the stimulation (social or carvone), adults were deeply anaesthetized using a mixture of 0.1 ml/0.06 ml Ketavet and Dormitor (Pharmacia, Bayer Vital, Respectively) and transcardially perfused with physiological saline for 2 minutes followed by 4% formaldehyde, diluted in 0.1 M phosphate buffer pH 7.4 (PB) for 25 minutes. Cage control males in the cohort were sacrificed at the same time of day, as the two other groups were scarified. Brains were removed, post fixed for 20 hours in 4% formaldehyde diluted in PB. Dr. Karin Richter in Magdeburg perfused animals. After sectioning in blocks, the brain blocks were sunk in 15% and then 30% sucrose for 1–2 days. Finally the blocks were frizzed and kept in –80℃ until use. Cryostat sections with 20 and 25 µm thick were obtained for in situ hybridisation and immunocytochemistry, respectively.

2.3 Cresyl violet stain

Slide-mounted sections were left in 70% ethanol overnight, rinsed in water, and stained with cresyl violet (0.2% cresyl violet acetate in 29 mM acetate buffer, pH 4.0) for 30 minutes at room temperature (RT). After rinsing in water, the sections were dehydrated and cover slipped.

2.4 Immunoperoxidase cytochemistry at the light microscopic level

Freely floating sections were rinsed in 0.01 M phosphate buffered saline (PBS) at pH 7.4, treated for 15 minutes with 1% sodium borohydride in PBS to inactivate residual aldehyde groups, and thoroughly washed in PBS.

Thereafter, the sections were pre-treated for 30 minutes in a blocking and permeabilising solution consisting of 10% normal goat serum (NGS; Interchem, Bad Kreuznach, Germany), 0.3% Triton X-100 (Serva, Heidelberg, Germany), and 0.05% phenylhydrazine (Merk, Darmstadt, Germany) in PBS at room temperature.

Subsequently, the primary antibody (Table 1) was applied for 36 hours, diluted in PBS containing 10% NGS, 0.3% Triton X-100, 0.1% sodium azide, and 0.1% thimerosal at 4°C. The sections were thoroughly rinsed in PBS, pre-treated for 1 hour with 0.2% bovine serum albumin in PBS (PBS-A), and exposed for another 24 hours to the secondary antibody (Table 1), diluted in PBS-A, 0.3% Triton X-100, and 0.1% sodium azide at 4°C. After repeated washing in PBS and pre-incubation for 1 hour in PBS-A, the biotinylated secondary antibodies in the sections were complexed for another 12 hours with peroxidase streptavidin (P-SA), (1:20,000 dilution in PBS-A). After further thorough rinses in PBS, pre-incubation for 15 minutes in a solution of 0.05% diaminobenzidine and 10 mM imidazole in 50 mM Tris buffer, pH 7.6, the visualisation of the antigen-antibody complexes were started by adding 0.0015% hydrogen peroxide (25 µl of 0.03% hydrogen peroxide to 500 µl solution) and stopped after 15 minutes at RT by repeated washing with PBS. The sections were mounted on gelatine-coated slides, air-dried for 30 minutes, dehydrated through a graded series of ethanol, transferred into xylene, and cover slipped with Entellan (Merk, Darmstadt, Germany).

2.5 In Situ Hybridisation (ISH)

The mRNA expression of GABAergic and glutamatergic cells in the amygdala was observed by non-radioactive in situ hybridisation. Digoxygenin-labelled antisense riboprobes for vesicular glutamate transporters 1, 2, and 3, glutamic acid decarboxylase GAD65 and GAD67 were used (Table 2). Free-floating (20 µm) sections were rinsed in 0.1 M phosphate buffered saline at pH 7.4 (PBS), immersed for 15 minutes in 1% sodium borohydride, and rinsed in 0.1 M PBS. They were placed for 30 minutes in a hybridisation buffer (HB; 50% formamide, 10 mM Tris, 10 nM phosphate, 600 mM NaCl, 60 mM sodium citrate, 5% dextran sulphate, 10 mM DL-di-thiothreitol, 0.1% ficoll, 0.1% polyvinyl-pyrollidone, 4 mM 2-mercaptoethanol, 200 µg/ml *E. coli* tRNA [Roche, Mannheim, Germany], and 5 mM EDTA, at pH 8.2). They were then incubated in digoxygenin-labelled antisense riboprobes diluted in a hybridisation buffer for 16 hours in a moist chamber (33% formamid in H_2O) at 56°C. On the next day, the sections were

washed off the slides, rinsed in PBS, and washed in 15mM NaCl, 1.5 mM sodium citrate pH 7.0, for 30 minutes at 56°C, followed by RNase A digestion (30 ng/ml, 0.2% BSA in PBS, pH 7.4) for 30 minutes at RT. Subsequently, slices were incubated overnight with sheep anti-digoxygenin Fab fragments conjugated with alkaline phosphatase (Roche, 1:5,000) in 100 mM maleate buffer, 150 mM NaCl, and 1% casein at pH 7.5 at RT. The hybridisation signal was detected by immersing the sections in BCIP/NBT solution (0.1 M Tris, pH 9.5, 150 mM NaCl, 50mM $MgCl_2$, 0.45 mM nitro blue tetrazolium, 0.45 mM 5-bromo-4-chloro-3-indolyl-10-phosphate) for 8 hours at room temperature in the dark. The sections were mounted on gelatine-coated slides, air-dried, and cover slipped with Kaiser's glycerol gelatine (Merck, Darmstadt, Germany).

A combination of in situ hybridisation and immunohistochemistry was done to investigate the co-localisation of mRNA and c-Fos in the amygdala following social stimulation. In this procedure, cryostat slices were incubated for 16 hours at 56°C in a hybridisation solution containing digoxygenin-labelled riboprobes (C. Derst, Department of Neuroanatomy, Charite Berlin, Germany). A second incubation in sodium citrate solution for 30 minutes at 56°C was followed by RNase A digestion. After several washes with PBS, the sections were pre-incubated for 30 minutes in 100 mM maleate buffer, 150 mM sodium azide, and 1% casein at pH 7.5, and incubated overnight at room temperature in a mixture of anti-digoxygenin antibody raised in mice and anti c-Fos antibody from rabbits in a caseinated maleate buffer. Subsequently, after washing with PBS and pre-incubation for 15 minutes in a caseinated maleate buffer, the sections were incubated for 9 hours in biotinylated horse-anti-mouse (B-HaM). After washing in PBS and pre-incubation in 10% PBS-A (bovine serum albumin in PBS) for 60 minutes, the sections were incubated overnight with P-SA diluted 1:20,000 in PBS-A. After washing the sections in PBS, the procedure was followed by catalysed report deposition (CARD) to amplify the signal. CARD signal amplification (Fig. 2) is based on deposition of haptenised tyramine molecules by peroxidase activity (Speel and Hopman et al., 1999). During CARD, the sections were treated with biotinylated tyramine in 50mM Tris and 10mM immidazol in H_2O for 15 minutes, followed by adding H_2O_2 to a final concentration of 0.0015% for 15 minutes.

After thoroughly washing in PBS, the sections were pre-incubated for 1h in PBS-A and incubated overnight with a mixture of fluorescent-labelled secondary antibodies containing Alexa Flour 594-anti-rabbit (AF 594-GaR) and Alexa Flour 488-anti-

streptavidin (AF 488-SA). On the following day, the sections were washed, mounted and cover slipped with DPX.

2.6 ISH/Immunofluorescence double labelling

To visualise immunoreactivity, the co-expression of different proteins or co-localisation of the neuronal activity marker, c-Fos, with mRNAs (Table 2), different fluorescence antibodies (Table 3) were used for single- or double labelling.

In single immunofluorescent staining, tissue sections were incubated in primary antibody overnight, and were developed for 3-4 hours in a dark place at room temperature with a secondary fluorescent-labelled antibody. Then the sections were mounted on gelatine-subbed slides, dehydrated rapidly through a graded series of ethanol, transferred into xylene, and cover slipped with DPX (Fluka Chemie GmbH). For double immunofluorescent labelling, brain sections were incubated with a mixture of two primary antibodies from two different host species overnight. Next, the sections were treated with a mixture of the relevant secondary antibodies, labelled with fluorescent conjugates AF 488, AF594, CY2, and CY3. In the ISH/Immunofluorescent, the RNAs were visualised by Alexa 488 fluorescent-anti-streptavidin (Mobitech, AF 488-SA, 1:2,000). Alexa 594 goat-anti-rabbit (Mobitech, AlexaF 594 GaR, 1:2,000) was used to visualise the neuronal activity markers, c-Fos (Table 3).

2.7 Immunocytochemical analysis

Images of adjacent antibodies labelled and cresyl violet-stained sections were acquired with a digital camera (Camedia C4040, Olympus, Hamburg, Germany) mounted on a Leica LEITZ DMR microscope. Photographic images were optimised in brightness and context by using Adobe Photoshop CS 8.0. In cresyl violet-stained sections, the brain areas of interest were identified based on the location, morphological and cytoarchitectonical criteria according to Paxinos and Watson (Paxinos and Watson,1998 and 2004). Accordingly, the nomenclature of Paxinos and Watson is used throughout this study to refer to anatomical structures of the rat brain.

The boundaries of individual nuclei were delineated on each image. Then, the identified areas on photographs of cresyl violet-stained sections were matched with photographs of adjacent sections stained with c-Fos, Gαo, Gαi2, Egr.1, or Arc. Afterwards the boundaries of each individual nucleus were drawn on each photomicrograph. The

thresholds for detecting immunopositive cells for each IEG were manually defined by using ImageJ software. Then, all c-Fos, Arc and Egr.1 immunopositive cells in identified nuclei on each section were quantified. Nuclei which were only partially present in a section were excluded from the analysis.

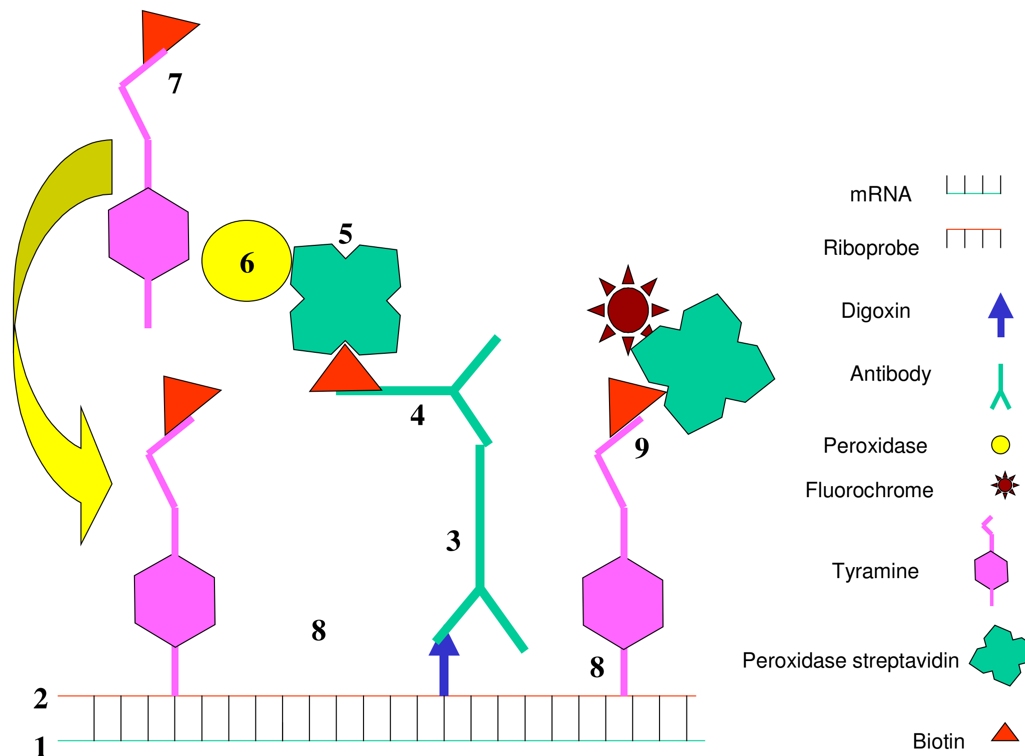

Fig. 2 Schematic representation of in situ hybridization with CARD amplification

Messenger RNA (1) binds digoxigenated mRNA probe (2) which is detected with mouse anti-digoxin (3), biotinylated horse anti-mouse antibody(4) and peroxidase streptavidin conjugated (P-SA) (5). Peroxidase molecule (6) catalytically deposits biotinylated tyramides (7) at the site of reaction (8) and finally fluorochrome or enzyme-labeled streptavidin (9) (in our experiments using AF 488-streptavidin) visualizes the reaction site.

Because the morphology of the various layers of the AOB is quite different and neuronal cell density is highest in the granule layer and lowest in the GL, the effects of juvenile stimulation on c-Fos expression were compared within each cell layer as well as in the anterior and posterior subdivisions. After quantifying c-Fos-ir cells in the anterior and posterior parts of the granule cell layer and mitral cell layer, the related areas were measured. By dividing the number of c-Fos-ir cells by the area, the density of immunoreactivity in each area was measured. For the mitral and granule layers, data

were analysed by a one-way ANOVA followed by the Scheffe's multiple-comparison test. The anterior/posterior distribution of c-Fos-immunopositive cells in the AOB was analysed by Student's T-Test. Differences were considered significant at *P*, 0.05. A Kruskal–Wallis one-way ANOVA on ranks, followed by Mann-Whitney multiple-comparison test analysed the data for the distribution of c-Fos-immunopositive cells in different individual nuclei of the amygdala and the amygdala functional systems.

The analysed data were represented with histograms and box plots. Because the data were not distributed normally, a box plot was used to display data in some cases in order to display graphically a variable's location and spread at a glance, provide some indication of the data's symmetry and asymmetry, show outliers, and compare data sets quickly.

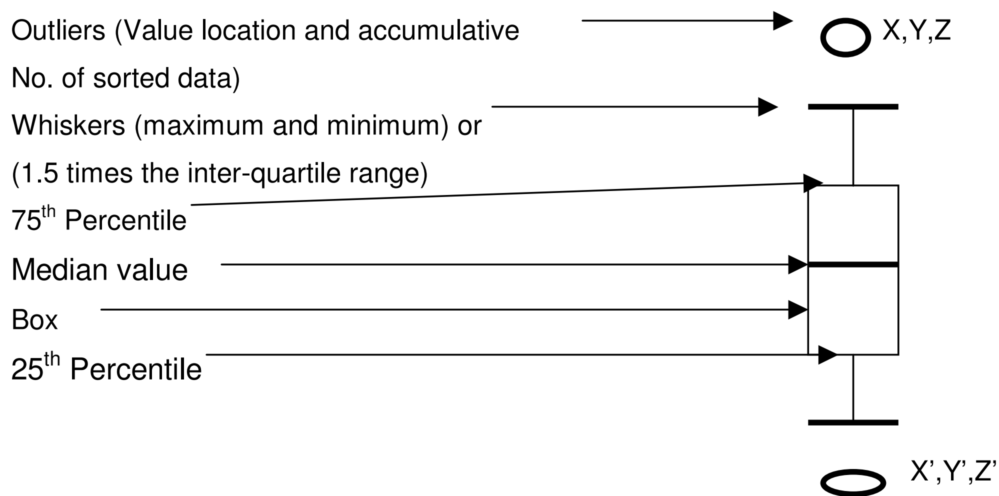

Fig. 2 Box plot diagram displays a variable's location, data symmetry and asymmetry, and outliers
The box itself contains the middle 50% of the data. The upper edge of the box indicates the 75th percentile of the data set, and the lower hinge indicates the 25th percentile. The line in the box indicates the median value of the data. The ends of the vertical lines, or "whiskers", indicate the minimum and maximum data values, unless outliers are present in which case the whiskers extend to a maximum of 1.5 times the inter-quartile range. Outliers ○ indicate the location of values outside the whisker's range, and X, Y, Z, or X', Y', Z' indicate the accumulative number of data in a sorted list.

The box plot interprets as follows:

1. The box itself contains the middle 50% of the data. The upper edge (hinge) of the box indicates the 75th percentile of the data set, and the lower hinge indicates the

25th percentile. The range of the middle two quartiles is called the inter-quartile range.

2. The line in the box indicates the median value of the data.

3. If the median line within the box is not equidistant from the hinges, then the data are asymmetrical.

4. The ends of the vertical lines, or “whiskers”, indicate the minimum and maximum data values, unless outliers are present in which case the whiskers extend to a maximum of 1.5 times the inter-quartile range.

Because the box plot emphasises the tails of a distribution, which are the least certain points in the data set that also hide some of the details of the distribution, in some cases histograms in conjunction with the box plot are shown.