Regional Distribution and Cellular Localization of β_2-Adrenoceptors in the Adult Zebrafish Brain (*Danio rerio*)

Konstantinos Ampatzis[1,2] and Catherine R. Dermon[1]*
[1]Biology Department, University of Patras, 26500 Rion, Greece
[2]Biology Department, University of Crete, 71409 Heraklion, Crete, Greece

ABSTRACT

The β_2-adrenergic receptors (ARs) are G-protein-coupled receptors that mediate the physiological responses to adrenaline and noradrenaline. The present study aimed to determine the regional distribution of β_2-ARs in the adult zebrafish (*Danio rerio*) brain by means of in vitro autoradiographic and immunohistochemical methods. The immunohistochemical localization of β_2-ARs, in agreement with the quantitative β-adrenoceptor autoradiography, showed a wide distribution of β_2-ARs in the adult zebrafish brain. The cerebellum and the dorsal zone of periventricular hypothalamus exhibited the highest density of [^{3}H]CGP-12177 binding sites and β_2-AR immunoreactivity. Neuronal cells strongly stained for β_2-ARs were found in the periventricular ventral telencephalic area, magnocellular and parvocellular superficial pretectal nuclei (PSm, PSp), occulomotor nucleus (NIII), locus coeruleus (LC), medial octavolateral nucleus (MON), magnocellular octaval nucleus (MaON) reticular formation (SRF, IMRF, IRF), and ganglionic cell layer of cerebellum. Interestingly, in most cases (NIII, LC, MON, MaON, SRF, IMRF, ganglionic cerebellar layer) β_2-ARs were colocalized with α_{2A}-ARs in the same neuron, suggesting their interaction for mediating the physiological functions of nor/adrenaline. Moderate to low labeling of β_2-ARs was found in neurons in dorsal telencephalic area, optic tectum (TeO), torus semicircularis (TS), and periventricular gray zone of optic tectum (PGZ). In addition to neuronal, glial expression of β_2-ARs was found in astrocytic fibers located in the central gray and dorsal rhombencephalic midline, in close relation to the ventricle. The autoradiographic and immunohistochemical distribution pattern of β_2-ARs in the adult zebrafish brain further support the conserved profile of adrenergic/noradrenergic system through vertebrate brain evolution. J. Comp. Neurol. 518:1418–1441, 2010.

INDEXING TERMS: catecholamines; hypothalamus; locus coeruleus; noradrenergic receptors; glia; teleost fish

Adrenoceptors are members of specific membrane G-protein-coupled receptors (GPCRs; Owen et al., 2007) and nine different subtypes of adrenergic receptors (ARs) have been characterized: α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}, and β_1, β_2, β_3 in mammals (Granneman et al., 1991; Bylund, 1992; Nicholas et al., 1996; Civantos-Calzada and Aleixandre-de-Artinano, 2001; Gibbs and Summers, 2005). In fish, as in mammals, the adrenergic system includes the neurotransmitters adrenaline (plus its hormonal form) and noradrenaline, regulating a variety of central and peripheral functions (Santer, 1977; Randall and Perry, 1992), most of which are associated with stress preparing the animal for "fight or flight" responses. The diverse range of adrenergic functions in teleosts, including social, courtship, agonistic (Marrone et al., 1966; Maler and Ellis, 1987; Nechaev, 1991; Winberg and Nilsson, 1992, 1993), and reproductive (Peter and Fryer, 1983) behaviors underlies the wide tissue distribution of adrenergic receptors. For example, various β-adrenoceptors have been identified in many teleost fish tissues such as the heart (Gamperl et al., 1994), branchial tissue (Payan and Girard, 1977), aorta (Klaverkamp and Dyer, 1974), liver (Reid et al., 1992), muscles (Lortie and Moon, 2003), and the brain (Zikopoulos and Dermon, 2005).

Studies in various fish species have shown that some of the mammalian β-adrenergic responses, such as increases in cardiac output and in plasma glucose levels, are

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*CORRESPONDENCE TO: Catherine R. Dermon, Human and Animal Physiology Laboratory, Biology Department, University of Patras, 26500 Rio, Greece. E-mail: dermon@upatras.gr

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also mediated by β-AR signaling in fish (Reid et al., 1992; Gamperi et al., 1994). In general, the signal transduction of β-adrenoceptors is suggested to be similar in mammals and fish (Owen et al., 2007) stimulating adenylyl cyclase through interaction with G-proteins leading to an increase in intracellular production of cyclic AMP, which in turn regulates the activity of protein kinase A, causing dissociation of the regulatory subunit and activation of the catalytic subunit (Smith et al., 1992). However, it has to be considered that the pharmacodynamics and some physiological endpoints between human and fish differ (Owen et al., 2007). The completion of the zebrafish (*Danio rerio*)

Abbreviations

A	Anterior thalamic nucleus
ALLN	Anterior lateral line nerve
AR	Adrenoceptor
ATN	Anterior tuberal nucleus
CC	Cerebellar crest
CCe gr	Gr granular layer of CCe
CCe mol	Mol molecular layer of CCe
CCe	Corpus cerebelli
Chor	Horizontal commissure
CIL	Central nucleus of the inferior lobe
CM	Corpus mamillare
CO	Optic chiasm
CON	Caudal octavolateralis nucleus
CP	Central posterior thalamic nucleus
CPN	Central pretectal nucleus
Cpost	Posterior commissure
Ctec	Tectal commissure
Ctub	Commissure of posterior tuberculum
D	Dorsal telencephalic area
DAO	Dorsal accessory optic nucleus
Dc	Central zone of dorsal telencephalic area
Dd	Dorsal zone of dorsal telencephalic area
DIL	Diffuse nucleus of the inferior lobe
DiV	Diencephalic ventricle
Dl	Lateral zone of dorsal telencephalic area
Dm	Medial zone of dorsal telencephalic area
DOT	Dorsomedial optic tract
DP	Dorsal posterior thalamic nucleus
Dp	Posterior zone of dorsal telencephalic area
DTN	Dorsal tegmental nucleus
DV	Descending trigeminal root
DβH	Dopa-β-hydroxylase
ECL	External cellular layer of olfactory bulb
EG	Granular eminence
ENd	Entopeduncular nucleus, dorsal part
ENv	Entopeduncular nucleus, ventral part
EW	Edinger-Westphal nucleus
FR	Habenulo-interpeduncular tract
GC	Central gray
GL	Glomerular layer of olfactory bulb
Had	Dorsal habenular nucleus
Hav	Ventral habenular nucleus
Hc	Caudal zone of periventricular hypothalamus
Hd	Dorsal zone of periventricular hypothalamus
Hv	Ventral zone of periventricular hypothalamus
IAF	Inner arcuate fibers
ICL	Internal cellular layer of olfactory bulb
IMRF	Intermediate reticular formation
IN	Intermediate nucleus
IO	Inferior olive
ir	Immunoreactivity
IR	Inferior raphe
IRF	Inferior reticular formation
LC	Locus coeruleus
LCa gr	Gr granular layer of LCa
LCa mol	Mol molecular layer of LCa
LCa	Caudal lobe of cerebellum
LFB	Lateral forebrain bundle
LH	Lateral hypothalamic nucleus
LLF	Lateral longitudinal fascicle
LOT	Lateral olfactory tract
LR	Lateral recess of diencephalic ventricle
LVII	Facial lobe
LX	Vagal lobe
MA	Mauthner axon
MAO	Monoamine oxidase
MaON	Magnocellular octaval nucleus
MFB	Medial forebrain bundle
MLF	Medial longitudinal fascicle
MNV	Mesencephalic nucleus of the trigeminal nerve
MON	Medial octavolateralis nucleus
MOT	Medial olfactory tract
NA	Noradrenaline
NI	Nucleus isthmi
NIII	Oculomotor nucleus
NIn	Interpeduncular nucleus
NIV	Trochlear nucleus
NLV	Nucleus lateralis valvulae
NMLF	Nucleus of the medial longitudinal fascicle
NT	Nucleus taeniae
NXm	Vagal motor nucleus
OT	Optic tract
P	Posterior thalamic nucleus
PCN	Paracommissural nucleus
PGl	Lateral preglomerular nucleus
PGm	Medial preglomerular nucleus
PGZ	Periventricular gray zone of optic tectum
PL	Perilemniscal nucleus
PM	Magnocellular preoptic nucleus
PPa	Parvocellular preoptic nucleus, anterior part
PPd	Periventricular pretectal nucleus, dorsal part
PPp	Parvocellular preoptic nucleus, posterior part
PPv	Periventricular pretectal nucleus, ventral part
PSm	Magnocellular superficial pretectal nucleus
PSp	Parvocellular superficial pretectal nucleus
PTN	Posterior tuberal nucleus
PV	Parvalbumin
Rm	Medial raphe nucleus
RV	Rhombencephalic ventricle
SAC	Stratum album centrale
SC	Suprachiasmatic nucleus
SFGS	Stratum fibrosum et griseum superficiale
SGC	Stratum griseum centrale
SGN	Secondary gustatory nucleus
SGT	Secondary gustatory tract
SM	Stratum marginale
SO	Secondary octaval population
SO	Stratum opticum (in TeO)
SRF	Superior reticular formation
T	Tangential nucleus
TelV	Telencephalic ventricle
TeO	Optic tectum
TH	Tyrosine hydroxylase
TL	Longitudinal torus
TLa	Lateral torus
TMC	Mesencephalo-cerebellar tract
TPM	Pretecto-mammillary tract
TPp	Periventricular nucleus of posterior tuberculum
TS	Semicircular torus
TTB	Tecto-bulbar tract
TTBc	Crossed tecto-bulbar tract
TTBr	Uncrossed tecto-bulbar tract
TVS	Vestibulo-spinal tracts
V	Trigeminal nerve
Va	Valvula cerebelli
Val	Lateral division of valvula cerebelli
Vam	Medial division of valvula cerebelli
Vc	Central nucleus of ventral telencephalic area
Vd	Dorsal nucleus of ventral telencephalic area
VII	Facial nerve
VIII	Octaval nerve
Vl	Lateral nucleus of ventral telencephalic area
VL	Ventrolateral thalamic nucleus
VM	Ventromedial thalamic nucleus
VOT	Ventrolateral optic tract
Vp	Postcommissural nucleus of ventral telencephalic area
Vs	Supracommissural nucleus of ventral telencephalic area
Vv	Ventral nucleus of ventral telencephalic area
X	Vagal nerve

genome description allowed the identification of zebrafish β-ARs, showing that the β-ARs in zebrafish and humans demonstrate a high sequence homology (Owen et al., 2007), similar to the high conservation between zebrafish and human α_{2A}-adrenoceptors (Ruuskanen et al., 2005a). In addition, the β_1- and β_2-AR subtypes, which show different potencies on adrenaline and noradrenaline (with adrenaline being more potent than noradrenaline for β_2-ARs), share 54% sequence identity and are expressed in comparable organ tissues in mammals (Hall, 2004; Owen et al., 2007). Moreover, rainbow trout β_2-AR shares a high degree of amino-acid sequence conservation with other vertebrate β_2-AR (Nickerson et al., 2001).

The β_2-adrenoceptor was the first receptor of the GPCR family to be cloned (Dixon et al., 1986) and brain β_2-ARs have been previously studied in many vertebrates, such as mammals (Palacios and Kuhar, 1980; Lorton and Davis, 1987; Flügge et al., 1997), birds (Dermon and Kouvelas, 1988, 1989; Fernández-López et al., 1997; Revilla et al., 1998a), amphibians (Bachman et al., 1998), and marine teleost fish species (Zikopoulos and Dermon, 2005). However, there is no knowledge of the neuroanatomical distribution of β_2-ARs in the adult zebrafish brain, a model organism for neurogenetic and neurotoxicity studies. The present study aimed to determine the localization pattern and the neuronal or glial nature of cellular elements expressing β_2-ARs in adult zebrafish brain. The β-AR localization supplements previous neuroanatomical data on the distribution pattern of α_{2A}-ARs (Ampatzis et al., 2008) and the noradrenaline metabolic pathway enzyme, monoamine oxidase (Anichtchik et al., 2006) that, in relation to known features of the catecholaminergic system (Ma, 1994a,b; Kaslin and Panula, 2001), will add significant knowledge to our understanding of the noradrenergic transmission in the adult zebrafish brain. In addition, the present evidence on β-AR localization will provide further insight to comparative studies between the vertebrate β-ARs and nor/adrenergic system.

MATERIALS AND METHODS

Animals

Adult (n = 26, 7–12 months) wildtype zebrafish (Cyprinidae, *Danio rerio*) of both sexes were used for this study. Experimental animals were kept in aged water at 28.5°C on a 14:10-hour light/dark cycle according to the "Zebrafish Book" (Westerfield, 1995). All experimental procedures were in accordance with the European Communities council directive (86/609/EEC) for the care and use of laboratory animals.

In vitro β-AR autoradiography, [^{3}H]CGP-12177 specific binding

To determine the distribution pattern of β-ARs and compare it with the immunohistochemical labeling of β_2-AR in the adult zebrafish brain, quantitative autoradiographic experiments were performed using [^{3}H]CGP-12177, a selective antagonist (Fernández-López et al., 1997). [^{3}H]CGP-12177 binds equipotently to β_1- and β_2-adrenoceptor subtypes (Revilla et al., 1998a) in many vertebrate species, such as mammals (Morin et al., 1992), birds (Revilla et al., 1998a, 2000), reptiles (Fabbri et al., 1997), amphibians (Fabbri et al., 1997), and bony fish (Niina et al., 1988; Perry and Reid, 1992; Gamperl et al., 1994; Jozefowski and Plytyez, 1998; Fabbri et al., 2001).

Briefly, fish (n = 6) were deeply anesthetized with 0.1% tricaine methane sulfonate (MS-222; Sigma, Deisenhofen, Germany) and their brains were removed carefully and rapidly frozen at −40°C in dry-ice-cooled isopentane and kept at −80°C until use. Coronal transverse sections 10-μm thick were mounted onto gelatin-coated slides. Adjacent sections were prewashed in 50 mM Tris-HCl buffer, pH 7.7 at 4°C for 30 minutes and then were incubated with the radiolabeled nonselective β-AR antagonist [^{3}H]CGP-12177 (52Ci/mol, Amersham, UK), at room temperature for 60 minutes at a concentration of 4 nM. Nonspecific binding was determined in the presence of 10 μM propranolol (Sigma). Free radioligand was removed by two washes in ice-cold Tris-HCl buffer for 5 and 10 minutes, and then sections were dried in a cold air stream. Slides, along with appropriate radioactive standards ([^{3}H]-Microscales; Amersham), were exposed at 4°C for 11 months to tritium-sensitive film (Hyperfilm ^{3}H, Amersham), developed in Kodak D19, fixed with Agfa Acidofix, and cleared in running tap water and followed by distilled water.

Quantitative image analysis

Quantitative analysis of the produced autoradiograms was performed using a computer-based image analysis system comprising a monochrome Sony XC-77CE CCD camera connected to a PowerMac (G4 Macintosh, Apple) via a Scion LG-3 frame grabber (Frederick, MD). NIH Image software (Bethesda, MD) was used for densitometry of various brain nuclei and subsequent expression of specific binding as fmoles per mg of tissue.

Immunohistochemistry

For single and double labeling immunohistochemical experiments, fish were deeply anesthetized with 0.1% tricaine methane sulfonate (MS-222; Sigma-Aldrich) and intracardially perfused with 4% paraformaldehyde (PFA, Sigma-Aldrich) freshly depolymerized in phosphate-buffered saline (PBS, 0.01 M; pH 7.4). The brains were removed extremely carefully and postfixed in 4% PFA in PBS for 2 hours at 4°C, cryoprotected overnight in 20% sucrose in phosphate buffer (PB, 0.1 M; pH 7.4) at 4°C, rapidly frozen in dry-ice-cooled isopentane (2-methyl butane, Sigma-Aldrich) at approximately −35°C, and stored

TABLE 1.
Primary Antibody Characterization

Antibody	Source	Host species	Immunogen	Dilution	Stains
β_2-AR (H-20) Polyclonal	Santa Cruz Biotechnology (sc-569)	Rabbit	Synthetic peptide representing 394-413 amino acids from human β_2-AR	1:50–1:150	β_2-adrenergic receptors (β_2-ARs)
α_{2A}-AR (C-19) Polyclonal	Santa Cruz Biotechnology (sc-1478)	Goat	Synthetic peptide representing 431-450 amino acids from human α_{2A}-AR	1:50–1:150	α_{2A}-adrenergic receptors (α_{2A}-ARs)
HuC/D Monoclonal	Molecular Probes, clone 16A11 (A-21271)	Mouse	Synthetic peptide representing 240-251 amino acids from human HuD	1:100	Neuronal proteins (HuC, HuD, Hel-NI)
TH Monoclonal	Chemicon, clone LNC1 (MAB318)	Mouse	Purified tyrosine hydroxylase (TH) from PC12 cells	1:1,000	Dopaminergic, adrenergic neurons & chromaffine tissue
GFAP Monoclonal	Sigma, clone G-A-5 (G3893)	Mouse	Produced by immunizing mice with native GFAP purified from pig spinal cord.	1:100	Astrocytes, Bergman glia & chondrocytes of elastic cartilage
PV Monoclonal	Swant (235)	Mouse	Parvalbumin purified from carp muscles	1:2,500–1:5,000	Parvalbumin calcium-binding protein

at −80°C until use. Before cutting in a cryostat CM1500 (Leica Instruments, Wetzlar, Germany) at a transverse coronal plane (section thickness 20 μm), the brains were embedded in tissue freezing medium (Jung, Leica Instruments, Germany). All sections were collected serially on gelatin-coated slides, air-dried for 1 hour, and immediately processed for immunohistochemistry.

Single-labeling experiments (n = 14) were performed to determine the detailed distribution pattern of β_2-ARs. For this, serial sections were washed for 10 minutes with 3% H_2O_2 (Sigma-Aldrich) in PBS for 10 minutes at room temperature (RT) to inhibit endogenous peroxidase activity, followed by three washes for 5 minutes each in PBS. Nonspecific protein binding sites were blocked with 0.15% normal horse serum (NHS) with 5% bovine serum albumin (BSA, Sigma-Aldrich) and 0.5% Triton X-100 in PBS for 30 minutes at RT. Sections were then incubated for 20–30 hours at 10°C in a moist chamber with rabbit anti-β_2AR (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:50–1:150 in PBS with 0.5% Triton X-100). The sections were rinsed in PBS three times for 5 minutes each and incubated with a biotinylated antirabbit antibody (Vector, Peterborough, UK; diluted 1:200 in PBS) for 3 hours at RT. Then the sections were washed three times in PBS with 0.5% Triton X-100 and incubated in Vectastain Elite ABC reagent (Vector Laboratories, Burlingame, CA; dilution 1:100A and 1:100B) in PBS with 0.5% Triton X-100n for 1 hour in the dark at RT and washed with PBS, followed by peroxidase-catalyzed polymerization of 3,3′-diaminobenzidine (DAB; Vector) for visualization of the immunoreaction. Methyl green counterstain (Sigma-Aldrich; 1% Methyl Green in ddH_2O) facilitated the identification of brain regions. The sections were dehydrated and cleared with xylene and coverslipped with Entellan (Merck, Darmstadt, Germany).

To identify the phenotype of cell types expressing the β_2 adrenoceptor subtype, double labeling experiments were performed (n = 12) using the primary antibodies described in Table 1. Brain sections were incubated with a mixture of primary antibodies of polyclonal anti-β_2-AR (diluted 1:50 in PBS with 0.5% Triton X-100) with monoclonal anti-HuC/D (diluted 1:100), or with monoclonal anti-TH antibody (diluted 1:1,000), or with polyclonal anti-α_{2A} adrenoceptor antibody (diluted 1:50–1:100), or with monoclonal antibody against GFAP (diluted 1:100), or with monoclonal anti-PV (diluted 1:2,500–1:5,000), for 20–48 hours at 10°C. A mixture of secondary antibodies antirabbit Alexa fluor 488 or 568, antigoat Alexa fluor 488 or 568, and antimouse Alexa fluor 350, 488, or 568 (Molecular Probes, Leiden, The Netherlands; diluted 1:500 in 0.5% Triton X-100 in PBS) were used in these experiments. The secondary antibody cocktail was added to the sections for 3 hours at RT in the dark. The sections were thoroughly rinsed in PBS and coverslipped with fluorescent hard medium (Vector, H-1400).

Antibody details and specificity

The immunohistochemical localization of β_2-adrenoceptors was performed using the rabbit polyclonal β_2-AR antibody (Santa Cruz; Cat. No. sc-569, H20, lot no. C3004) raised against the synthetic peptide TVPSDNIDSQGRNCSTNDSLL mapping amino acids 394–413 at the C-terminus of β_2-AR of human origin that recognizes a protein of about 65 kDa by immunoprecipitation (Davare et al., 2001; Cox et al., 2008). Previous immunohistochemical studies using this primary anti-β_2-AR antibody have provided strong evidence of the neuroanatomical localization of β_2-ARs in the enteric (Nasser et al., 2006) and central nervous system (CNS) of many vertebrates (Davare et al., 2001; Naren et al., 2003; Pullar et al., 2003; Odley et al., 2004), including teleost fish (Zikopoulos and Dermon, 2005) with comparable results between species. In support, agonist-induced changes in receptor binding activity (Liang et al., 2003) and pharmacological studies of desensitization of β_2-AR by the G-coupled receptor kinase pathway (Seibold et al., 2000) were accompanied by immunohistochemically detected changes in the receptor distribution using this polyclonal anti-β_2-AR antibody.

In addition, Western blot analysis protein extracts supports the specificity of this antibody, e.g., recognizes a protein band of 65 kDa in the red porgy brain (Zikopoulos and Dermon, 2005) of ≈64–65 kDa in the zebrafish brain (present study) and of 65 kDa in rat forebrain membranes (Davare et al., 2001; Cox et al., 2008). Zebrafish β_2-AR gene is located in chromosome 21 and consists of 1,113 bp corresponding to a 371 aa protein. Basic Local Alignment Search Tool (BLAST) was performed in order to detect regions with local similarities of the protein sequence of β_2-AR and to calculate the statistical significance of the matches. Zebrafish β_2-AR showed 75% similarity with human (*Homo sapiens*), 77% similarity with mouse (*Mus musculus*), and 76% similarity with rat (*Rattus norvegicus*) β_2-ARs.

The α_{2A}-AR antibody used is a goat polyclonal antiserum (Santa Cruz; Cat. No. sc 1478; lot no. (α_{2A}): C0404) raised against amino acids 431–450 of the carboxy-terminus of the α_{2A}-AR of human origin, Swiss Prot protein accession number P08913. This polyclonal anti-α_{2A}AR antibody has been previously used in studies in the enteric (Nasser et al., 2006) and CNS of many vertebrates (Hou et al., 2002; Manns et al., 2003; Ohshita et al., 2004; Modirrousta et al., 2005), including teleost fish (Zikopoulos and Dermon, 2005) and also zebrafish (Ampatzis et al., 2008) with comparable localization among species. Moreover, a previous study in zebrafish suggested the conservation of α_{2A}-AR compared to mammals (Ruuskanen et al., 2005b), determining the expression of α_{2A}-ARs using mRNA reverse-transcription polymerase chain reaction (RT-PCR) in situ hybridization and receptor autoradiography. The specificity of this antiserum in teleost brain has also been documented by Western blot of brain protein extracts in teleost brain (*Pagrus pagrus*; Zikopoulos and Dermon, 2005; *Danio rerio*; Ampatzis et al., 2008) and by preabsorption with its respective antigen for immunohistochemistry on brain sections (Zikopoulos and Dermon, 2005; Ampatzis et al., 2008). To test the specificity of staining, blocking peptides for α_{2A}- and β_2-AR (SCB; α_{2A}, sc1478P, lot no. G0605; β_2, sc569P, lot no. G0605; dilution 1:50) were used. Additional negative controls, carried out in adjacent sections, included the omission of the primary antibodies and the application of secondary antisera mismatched for species. All control experiments resulted in no immunohistochemical labeling and staining of the α_{2A}- and β_2-AR was completely abolished when preincubated with the relevant blocking peptide.

Antibodies against neuronal and glial markers have been previously characterized and the cellular morphology and the distribution staining pattern observed in the present study is similar to that previously reported in the teleost nervous system (Byrd and Brunjes 2001; Mueller and Wullimann, 2002; Nielsen and Jørgensen 2003; Zupanc et al., 2005; Grandel et al., 2006; Ampatzis et al., 2008). Specifically, mouse anti-HuC/D monoclonal antibody (Molecular Probes, Eugene, OR; Cat. No. A-21271; lot no. 71C1-1; Clone number 16A11) is a monoclonal $IgG_{2b,\kappa}$ antibody against the neuronal (human) HuD 12 amino acids peptide QAQRFRLDNLLN that represents the 240–251 amino acids within the carboxy-terminal domain of the human HuD and recognizes the neuronal proteins HuC, HuD (RNA-binding proteins) and Hel-N1 of Elav family and label the neuronal cell nuclei and perikarya. The presence of Hu-proteins serves as a marker for newly committed postmitotic neuronal cells of the CNS, as well as for mature neurons (Marusich et al., 1994; Barami et al., 1995). This antibody has been shown to specifically label neuronal cells in zebrafish, chick, canaries, and humans and is likely to label neuronal cells in most vertebrate species. In the present study the antibody against HuC/D displayed the same pattern of cellular morphology and distribution with previous studies in zebrafish (Ampatzis and Dermon 2007; Topp et al., 2008). A mouse antiglial fibrillary acidic protein (GFAP; IgG_1 isotype; Clone number G-A-5) was obtained from Sigma (lot no. 033K4869, Cat. No. G3893) and produced by immunizing mice with native GFAP purified from pig spinal cord. GFAP is an intermediate filament protein of 52 kD (432 amino acids) found in astrocytes and radial glia, and some types of ependyma cells in most vertebrates (Levitt and Rakic, 1980; Bodega et al., 1990, 1993, 1994; Eng et al., 2000). GFAP immunostaining through adult zebrafish brain produced an identical cellular and localization pattern to that previously described (Tomizawa et al., 2000; Adolf et al., 2006; Ampatzis and Dermon

2007; Ampatzis et al., 2008). Additional markers used included a monoclonal (IgG1kappa) mouse antityrosine hydroxylase, raised against TH purified from PC12, which recognized and an epitope outside of the regulatory N-terminus of tyrosine hydroxylase. Western blot experiments identified a protein of ≈59–61 kDa that did not react with dopamine-beta-hydroxylase (DβH), tryptophan hydroxylase, dehydropteridine reductase or phenylethanolamine-*N*-methyltransferase (PNMT) (Chemicon International, Temecula, CA; Cat. No. MAB318, lot no. 21121038; Clone number LNC1). Studies in teleost brain have established the specificity of this antibody in teleost brain (Ma, 2003) and the present data are in agreement with the previously described pattern in adult zebrafish brain (Kaslin and Panula 2001; Ampatzis et al., 2008). A mouse monoclonal (IgG_1), antiparvalbumin antibody (Swant, Bellinzona, Switzerland; lot. no. 10-11(F), Cat. No. 235), produced by hybridization of mouse myeloma cells with spleen cells from mice immunized with parvalbumin purified from carp muscles (Celio et al., 1988) has been completely characterized (Celio et al., 1988), and is known to specifically stain the ^{45}Ca-binding spot of parvalbumin (12KD and IEF 4.9) in a two-dimensional immunoblot. The staining pattern observed in adult zebrafish brain in the present study is comparable to that previously established in mammalian (Celio, 1990; Bu et al., 2003), avian (Wild et al., 2005), and teleostean (Alonso et al., 1992; Porteros et al., 1998; Crespo et al., 1999; Ampatzis et al., 2008) brain.

Western immunoblotting

To determine whether the polyclonal β_2-AR subtype-specific antibody, generated against the mammalian adrenoceptor protein, recognized the homologous protein from zebrafish, protein extracts from rat and zebrafish brains were processed by Western blot analysis. Zebrafish (*Danio rerio,* n = 3) and rat (*Rattus norvegicus,* n = 3) brain tissues were homogenized with a Teflon-glass homogenizer in 0.01 M PBS, pH 7.4, containing 1% Triton X-100 (Sigma), 0.5% sodium deoxycholate (Sigma), and 0.05% sodium dodecyl sulfate (SDS; Sigma). The tissue homogenates were incubated on ice for 1 hour and further homogenized by passing through a 22G needle. Remaining cell debris was removed by brief centrifuging at 8,000 rpm for 20 minutes at 4°C. The supernatant was used for protein concentration determination using the Lowry assay and subsequently diluted with loading buffer (2 mL 0.5 M Tris-HCl, 1.6 mL glycerol, 3.0 mL 10% SDS, 0.8 M β-mercaptoethanol, 0.4 mL 0.05% bromophenol blue) accordingly and then boiled for 5 minutes at 95°C. Twenty micrograms of protein were separated electrophoretically in a 10% SDS-polyacrylamide gel. The separated proteins were transferred to nitrocellulose membrane at 400 mA for 2 hours at 4°C that was subsequently stained briefly with Poinceau red to verify protein transfer. After blocking with 5% nonfat dried milk and 1% normal horse serum (NHS) in PBS-Tween (0.05% Tween-20 in 0.01 M PBS) for 60 minutes, the membranes were incubated with the β_2-AR goat polyclonal antibody (Santa Cruz Biotechnology; diluted 1:50–1:200 in PBS-Tween with 2% nonfat dried milk) at 10°C for 24 hours. Following three washes in PBS-Tween for 10 minutes each, the membranes were incubated for 2 hours at RT with secondary antigoat IgG antibodies (Vector ABC Elite kit) diluted 1/2,000 in PBS-Tween, followed by an avidin-biotin-peroxidase solution (Vector ABC Elite kit; diluted 1/1,000 A and 1/1,000 B in PBS-Tween) for 1 hour in the dark at RT. Bands were visualized by the peroxidase-catalyzed polymerization of 0.05% diaminobenzidine solution (DAB; Vector) in 0.01% H_2O_2 buffer solution (pH 7.5). Rat brain homogenates were used as positive control, whereas omission of primary antisera provided the necessary negative controls.

Brain anatomy, microscopy, photomicrograph processing

The nomenclature used was based on the topological zebrafish brain atlas of Wullimann et al. (1996). All microscopic images were captured using a digital camera CFW-1600 (Color CCD, depth 10bit, Scion) adjusted on an optical and fluorescent microscope (Nikon, Eclipse E800) connected to a MacBook Pro (Apple, MacOS 10.5.3). Stacks of optically sliced images were generated using ImageJ software (Rasband, 2004) and further used for identification of double-labeled cells and image processing. Figures were prepared for publication with Adobe Photoshop CS3 (Adobe Systems, San Jose, CA), and graphs using Corel Draw 11 (Dallas, TX), for Macintosh (Apple, MacOS 10.5). All double-labeled images were converted to magenta-green immunofluorescence to make this work more accessible to the ≈10% of male readers who are red-green colorblind.

RESULTS

Regional pattern of β-AR binding sites in adult zebrafish brain

The distribution of β adrenoceptors in the adult zebrafish brain determined by the [^{3}H]CGP-12177 binding was found to be heterogeneous (Fig. 1). Quantification of [^{3}H]CGP-12177 binding sites showed the highest density of β-ARs in the hypothalamus and cerebellar subdivisions (Fig. 1, Table 2) of adult zebrafish brain. Within the forebrain, high densities of β adrenoceptors binding sites were found in the ventral telencephalic, preoptic, and hypothalamic area, including the ventral nucleus of the ventral telencephalic area (Fig. 1A; Vv), lateral nucleus of the ventral telencephalic area (Vl), anterior and posterior part of parvocellular preoptic nuclei (Fig. 1B; PPa, PPp), ventral zone of periventricular hypothalamus (Fig. 1C; Hv), and dorsal zone of periventricular hypothalamus (Fig.

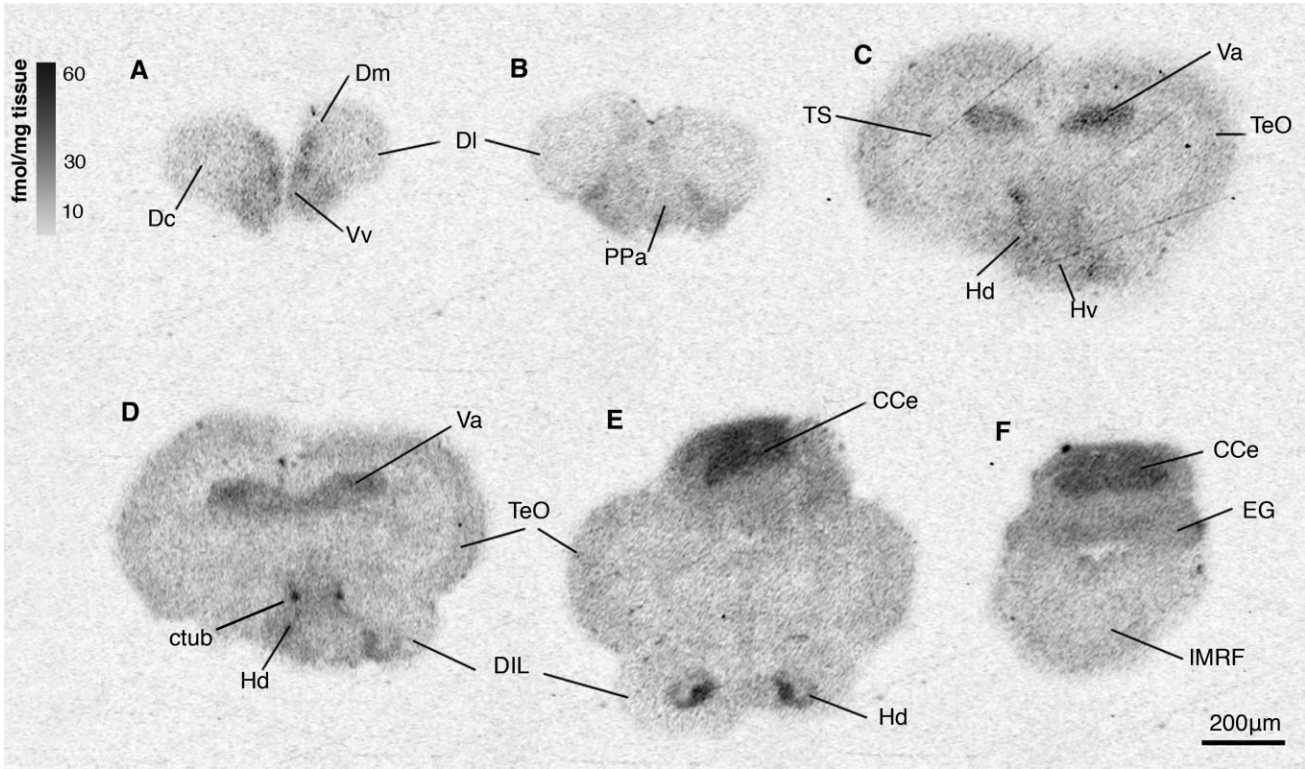

Figure 1. Quantitative autoradiographic images produced from coronal sections at representative rostral (A) to caudal (F) levels, showing the distribution of [^{3}H]CGP-12177 binding sites in the adult brain of *Danio rerio*. Scale bar at the top left indicates the density of specific binding sites in fmoles per mg of tissue. For abbreviations, see list.

1C–E; Hd). Areas exhibiting moderate to low levels of binding sites comprise the olfactory bulbs, the zones of the dorsal telencephalic area (Fig. 1A,B), the dorsal nucleus of the ventral telencephalic area (Vd), the central posterior thalamic nucleus (CP), the periventricular nucleus of posterior tuberculum (TPp), the habenular nuclei (Ha), the ventral part of the periventricular pretectal nucleus (PPv), the lateral torus (TLa), and the diffuse nucleus of the inferior lobe (Fig. 1D,E; DIL).

In the midbrain, the central and superficial layers of the optic tectum (Fig. 1C–E; TeO) and semicircular torus (TS) exhibited moderate levels of [^{3}H]CGP-12177 binding sites while low levels were found in the periventricular gray zone of optic tectum (PGZ) and the longitudinal torus (TL). In the hindbrain, high densities of binding sites characterized valvula cerebelli (Fig. 1C,D; Va), corpus cerebelli (Fig. 1E,F; CCe), granular eminence (Fig. 1F; EG), and caudal lobe of cerebellum (LCa). The reticular formation nuclei (Fig. 1F; SRF, IMRF), and the magnocellular octaval nucleus exhibited moderate levels of [^{3}H]CGP-12177 binding (Table 2).

Western blot analysis and specificity of β_2-AR antibody

The polyclonal rabbit β_2-AR antibody detected a zebrafish protein band migrating at about the same apparent molecular weight as the corresponding rat β-AR protein around 64 kDa (Fig. 2), likely representing the zebrafish and rat homologs of AR. In addition, previous Western blot experiments revealed stained rat and teleostean (*Pagrus pagrus*) brain protein bands of similar molecular weight, specifically reacting with the same anti-β_2-AR polyclonal antibody (Zikopoulos and Dermon, 2005). In addition, Western blot experiments recognized a roughly 45 kDa peptide in zebrafish that is probably a degradation product, as the lysis buffer in both rat and zebrafish proteins extracts did not include all protease inhibitors.

Additionally, preabsorption with excess (×10) of the specific peptide used to raise the β_2-AR antibody completely abolished the labeling in the presence of the primary and secondary antibodies in all brain regions. Taken together, these data support the specificity of the β_2-AR antibody used in the zebrafish brain.

Distribution of β_2-AR immunoreactivity in the zebrafish brain

Mapping β_2-adrenoceptors immunoreactivity, a wide distribution of β_2-adrenoceptors in cell somata and fibers was found throughout the adult zebrafish brain, with high densities of staining in the periventricular (medial) areas of the adult brain. For the telencephalon a characteristic dif-

TABLE 2.
Quantitative Distribution of β-ARs (fmoles/mg Tissue ± SEM) in Adult Zebrafish (*Danio rerio*) Brain

Brain area	[3H]CGP-12177 binding (fmol/mg tissue)
Olfactofy bulbs (OB)	7.86 ± 1.86
Telencephalon	
Medial zone of dorsal telencephalic area (Dm)	13.69 ± 1.53
Lateral zone of dorsal telencephalic area (Dl)	8.53 ± 2.23
Central zone of dorsal telencephalic area (Dc)	7.23 ± 1.14
Posterior zone of dorsal telencephalic area (Dp)	8.39 ± 1.64
Lateral nucleus of ventral telencephalic area (Vl)	27.44 ± 4.05
Dorsal nucleus of ventral telencephalic area (Vd)	13.54 ± 1.00
Ventral nucleus of ventral telencephalic area (Vv)	23.67 ± 2.60
Diencephalon	
Habenular nucleus (Ha)	5.12 ± 0.99
Parvocellular preoptic nucleus, anterior part (PPa)	25.73 ± 2.09
Parvocellular preoptic nucleus, posterior part (PPp)	28.5 ± 4.46
Periventricular pretectal nucleus, ventral part (PPv)	7.23 ± 1.68
Periventricular nucleus of posterior tuberculum (TPp)	11.99 ± 1.77
Central posterior thalamic nucleus (CP)	2.55 ± 0.51
Ventral zone of periventricular hypothalamus (Hv)	22.29 ± 4.69
Dorsal zone of periventricular hypothalamus (Hd)	49.80 ± 7.81
Lateral torus (Tla)	11.65 ± 2.05
Diffuse nucleus of the inferior lobe (DIL)	7.62 ± 1.01
Mesencephalon	
Optic tectum (TeO)	14.51 ± 2.20
Periventricular gray zone of optic tectum (PGZ)	4.88 ± 0.67
Longitudinal torus (TL)	3.01 ± 0.47
Semicircular torus (TS)	11.62 ± 0.90
Rhombencephalon & Cerebellum	
Central gray (GC)	9.62 ± 0.89
Superior reticular formation (SRF)	7.77 ± 1.39
Intermediate reticular formation (IMRF)	7.39 ± 0.37
Magnocellular octaval nucleus (MaON)	16.72 ± 1.01
Granular eminence (EG)	17.89 ± 1.67
Valvula cerebelli (Va)	33.03 ± 2.03
Corpus cerebelli (CCe)	58.04 ± 5.22
Caudal lobe of cerebellum (LCa)	28.52 ± 3.09
Locus coeruleus (LC)	24.22 ± 4.45

ference between ventral and dorsal area was evident, with the ventral telencephalic area expressing higher densities of β_2-ARs than the dorsal (Fig. 3).

Telencephalon

The adult zebrafish telencephalon included low to moderate expression of β_2-adrenoceptors, with the ventral telencephalic area and some of the periventricular locations of dorsal and ventral areas exhibiting the highest density (Fig. 3A–E). In the olfactory bulb a low number of β_2-AR positive fibers and small-sized cells were located in the glomerular layer (Figs. 3A, 4A). Medial, lateral, and dorsal zones of the dorsal telencephalic area (Dm, Dl, Dd) exhibited low to moderate densities of β_2-AR immunoreactivity. Specifically, the medial zone (Dm) included a small number of β_2-adrenoceptor-positive thick fibers extending radically (Figs. 3B–E, 4B). The central zone of the dorsal telencephalic area (Dc) included moderate number of round cells densely stained for β_2-ARs (Figs. 3B–D, 4C). In the ventral telencephalon, only the ventral nucleus of the ventral telencephalic area (Vv) exhibited medium to high numbers of positive cells for β_2-ARs (Figs. 3B,C, 4D). The dorsal nucleus of ventral telencephalic area (Vd) included a moderate number of β_2-AR-labeled cells, while the postcommissural nucleus of ventral telencephalic area (Vp) and the supracommissural nucleus of ventral telencephalic area (Vs) included a small number of cell somata stained (Fig 3B–D). Moreover, β_2-AR-positive cells and fibers were found to be located along the lateral nucleus of ventral telencephalic area (Vl; Fig 3B).

Diencephalon

The superficial pretectal nuclei and the hypothalamus exhibited the highest density of β_2-AR immunoreactivity. In the preoptic area, the anterior and posterior parvocellular preoptic nuclei (Figs. 3D–F, 4E; PPa, PPp) showed moderate density of β_2-AR-immunopositive cells, but low density of positive fibers. In the superficial pretectum, the

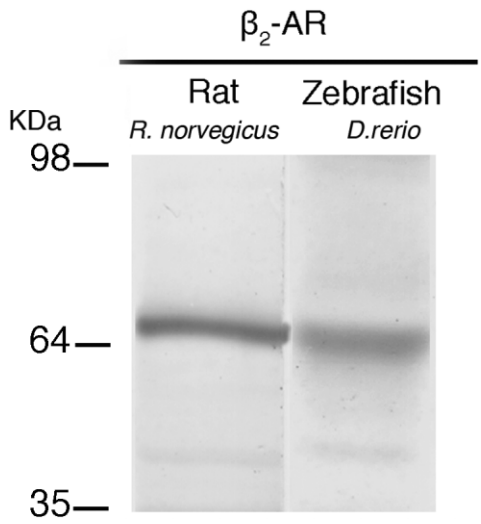

Figure 2. Western blot analysis of the β_2-ARs, using zebrafish (*Danio rerio*) and rat (*Rattus norvegicus*) brains. Note the dense expression of β_2-AR proteins in the rat compared to the zebrafish brain, in agreement with the lower levels of adrenoceptors in the zebrafish brain.

magnocellular and the parvocellular nuclei were characterized by a dense plexus of β_2-AR-stained fibers and a population of strongly stained cell somata outlining the borders of the nuclei (Figs. 3F, 4F; PSm, PSp). In the hypothalamus, high numbers of small round cells and low to moderate levels of characteristic varicose-immunopositive fibers were found in all zones (ventral, dorsal, caudal) of periventricular hypothalamus (Figs. 3F–I, 4H; Hv, Hd, Hc). In the medial preglomerular (PGm) and lateral preglomerular (PGl) nuclei β_2-AR-positive cells and varicose fibers were observed to embrace these nuclei. In the periventricular nucleus of posterior tuberculum (TPp) and the diffuse nucleus of the inferior lobe (Fig. 3G–J, 5D; DIL), only a few β_2-AR-positive cells and fibers were found. In contrast, the adjacent corpus mamillare (CM) included moderate to high numbers of small round β_2-adrenoceptor-immunoreactive cells (Fig. 5D).

Mesencephalon

Optic tectum (TeO), central and ventrolateral nuclei in torus semicircularis (TS), and tegmentum exhibited moderate β_2-AR immunoreactivity. In the optic tectum, immunostained β_2-AR fibers showed a radial orientation, crossing vertically all tectal layers (Figs. 3F–K, 4G; TeO). In addition, most of the β_2-AR-expressing cells were found in the superficial tectal layers, with the ventral and lateral part of the tectum exhibiting more numerous cell labeling. Moreover, a great number of small stained cells expressing β_2-ARs were found in the periventricular gray zone of the optic tectum (Figs. 3G–K, 4G; PGZ). Torus longitudinalis (TL) showed rare immunostaining of β_2-AR cells (Fig. 3G–J). The optic tract (OT) and the ventral optic tract (VOT) included a high density of β_2-AR-immunopositive cells and fibers. Torus semicircularis (TS) included a moderate number of β_2-adrenoceptor-immunopositive cells but only a few stained varicose fibers (Figs. 3I–K, 5A). Motor areas were stained for β_2-ARs in the zebrafish tegmentum specifically; the oculomotor nucleus (NIII) included densely stained large β_2-adrenoceptor cells and few varicose fibers (Fig 5B).

Rhombencephalon and cerebellum

Adult zebrafish rhombencephalic areas included β_2-AR densely labeled cellular structures. Particularly, all cell somata and the proximal dendrites of the locus coeruleus (LC) neurons were strongly stained for β_2-ARs (Fig. 5C). In the trochlear nucleus (Fig. 3J; NIV) large cells and varicose fibers were densely stained. In addition, caudal to NIV, the central gray (GC), exhibited low to moderate levels of β_2-AR-labeled fibers. All reticular formation subdivisions (superior, intermediate, and inferior) included a cluster of cells strongly labeled for β_2-ARs (cell somata and their proximal dendrites). The intermediate reticular formation (IMRF) and inferior reticular formation (IRF) included large and medium-sized stained cells for β_2-ARs (Figs. 3L–M, 5F), while in the superior reticular formation large cells expressed β_2-ARs (Figs. 3J–K, 5E; SRF). The magnocellular octaval nucleus (MaON) and the medial octavolateralis nucleus (MON) were characterized by a high density of large cells strongly labeled for β_2-ARs, in addition to few positive fibers (Fig. 3L). The dorsal and lateral part of the nucleus of vagal lobe (LX) included some β_2-AR-positive cells, while no immunoreactivity was detected in the descending trigeminal root (DV).

In the cerebellum, β_2–AR (Figs. 3I–L, 6A–C) immunoreactivity was present in all cerebellar subdivisions (valvula cerebelli, corpus cerebelli, caudal lobe of cerebellum, and eminentia granularis). Most of the cell somata of the ganglionic cell layer exhibited strong β_2-adrenoceptor staining. Moreover, β_2-adrenoceptor immunoreactivity was also prominent in the dendritic tree of the ganglionic layer cells (Fig. 6). In addition, a few stained small cells were found in the molecular and granular layer of all cerebellar structures.

Neuronal localization of β_2-ARs

Double immunofluorescence experiments with neuronal (Huc/D, PV) and glial (GFAP) markers support the expression of β_2-ARs mainly in neuronal but also in glial elements. Double labeling of β_2-AR/HuC/D showed that a significant number of the positive cells in the telencephalon were found to be neurons, particularly in the central zone of dorsal telencephalic area (Dc). In addition, in diencephalic and mesencephalic areas, such as torus semicircularis (TS) and corpus mamillare (CM), a small to moder-

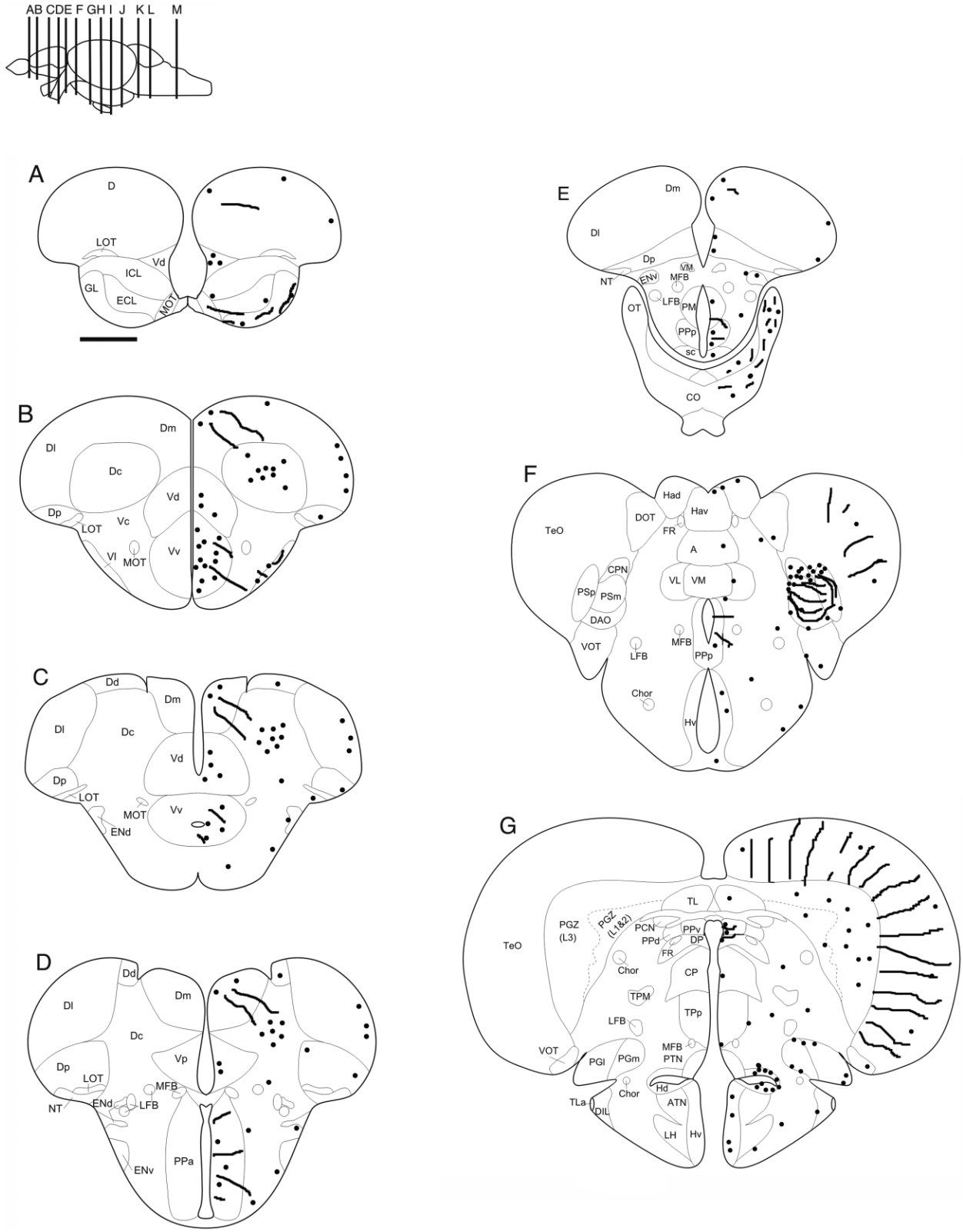

Figure 3. Schematic drawings of transverse sections of the adult zebrafish brain showing at the left the brain nuclei and regions according to Wullimann et al. (1996) and on the right the distribution of β_2-AR-immunoreactive cells (solid circles), fibers (black curved lines), and ganglionic cell layer dendrites (gray curved lines). The anteroposterior level **(A–M)** of the sections is presented in the lateral view of the adult zebrafish brain, on the top of the figure. Scale bars = 200 μm. For abbreviations, see list.

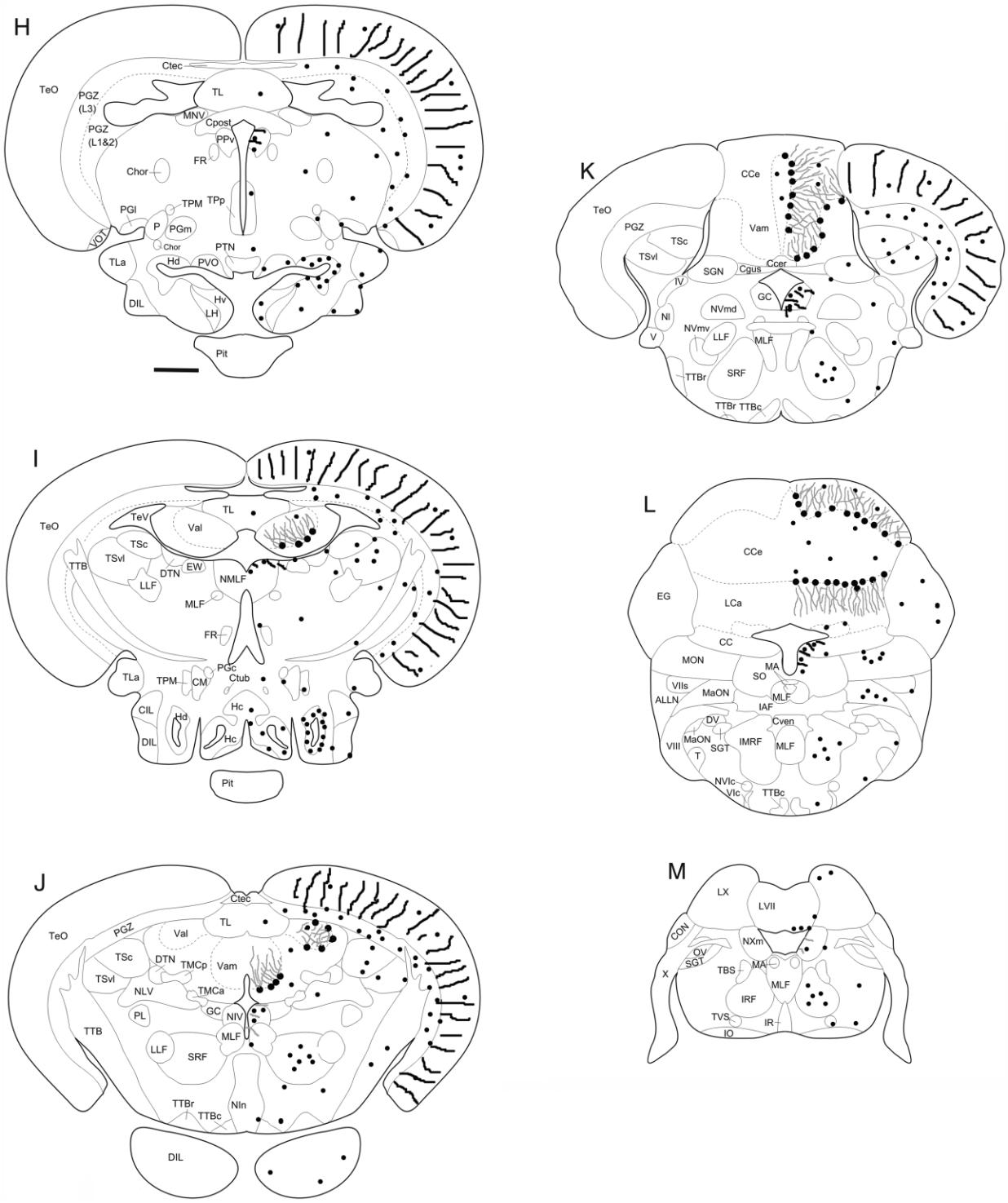

Figure 3. Continued

ate number of neuronal cells were found to express β_2-adrenoceptors. Similarly, in rhombencephalon and mesencephalon the vast majority of the β_2-adrenoceptor-stained cells were double-labeled with HuC/D, such as locus coeruleus, the ganglionic cell layer somata of valvula, corpus, and caudal lobe of cerebellum (Fig. 7A; LCa), the reticular formation nuclei (SRF, IMRF, IRF; Fig. 7B), oculomotor nucleus (NIII), trochlear nucleus (NIV), medial octavolateralis nucleus (MON), and the magnocellular octaval nucleus (MaON).

Additional double-labeling experiments with the neuronal marker tyrosine hydroxylase (TH) (β_2-adrenoceptor/TH)

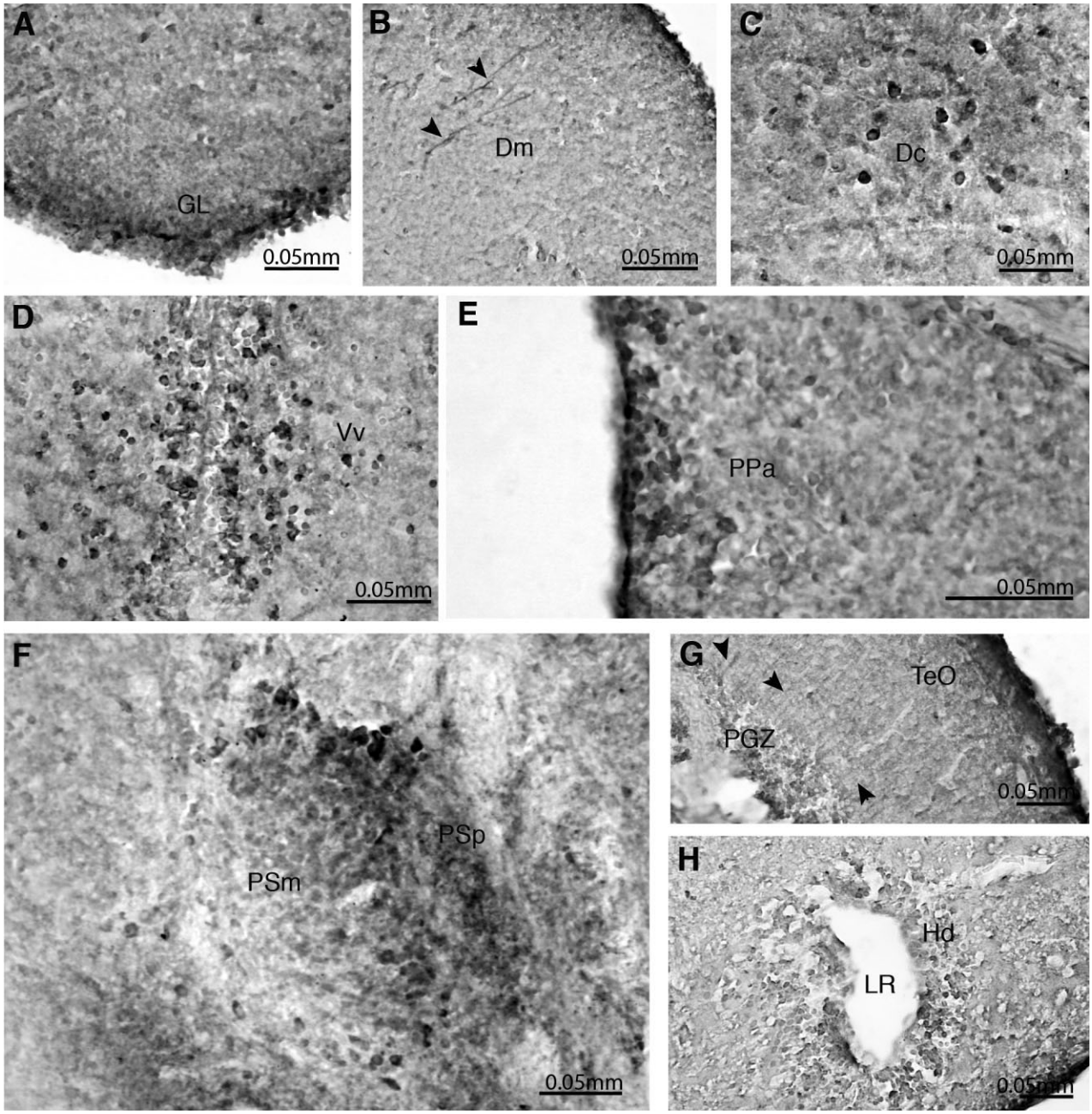

Figure 4. Photomicrographs of coronal sections of zebrafish forebrain and midbrain showing immunoreactivity to β_2-ARs in **(A)** glomerular layer of the olfactory bulb. **B:** Medial zone of the dorsal telencephalic area, arrowheads indicate β_2-AR immunopositive fibers. **C:** Central zone of the dorsal telencephalic area. **D:** Ventral nucleus of the ventral telencephalic area with a high number of stained cells. **E:** Preoptic area. **F:** Parvocellular and magnocellular superficial pretectal nuclei (positive cells and fibers). **G:** Optic tectum, with β_2-AR labeled fibers showing radial orientation. **H:** dorsal zone of periventricular hypothalamus surrounded by positive cells and fibers. For abbreviations, see list.

were performed to reveal the possible relationship of β_2-adrenoceptors in the noradrenergic/dopaminergic interactions. The TH staining pattern observed in the present study is in agreement with the previous studies in adult zebrafish brain (Kaslin and Panula, 2001; Wullimann and Rink, 2001; Ampatzis et al., 2008). The locus coeruleus neurons exhibiting TH immunoreactivity were also found to express β_2-adrenoceptors. In addition, TH immunoreactive cell structures were located in close apposition to the β_2-AR-positive cellular elements in many brain areas, such as dorsal telencephalic area, parvocellular preoptic nuclei, and in periventricular hypothalamus, but did not colocalize (Fig. 7J; Hc). In the central zone of the dorsal telencephalic area (Dc), a TH/β_2-AR double-labeled cell was found (Fig. 7I).

Double labeling of β_2-adrenoceptors and the neuronal marker parvalbumin (PV) showed that in several

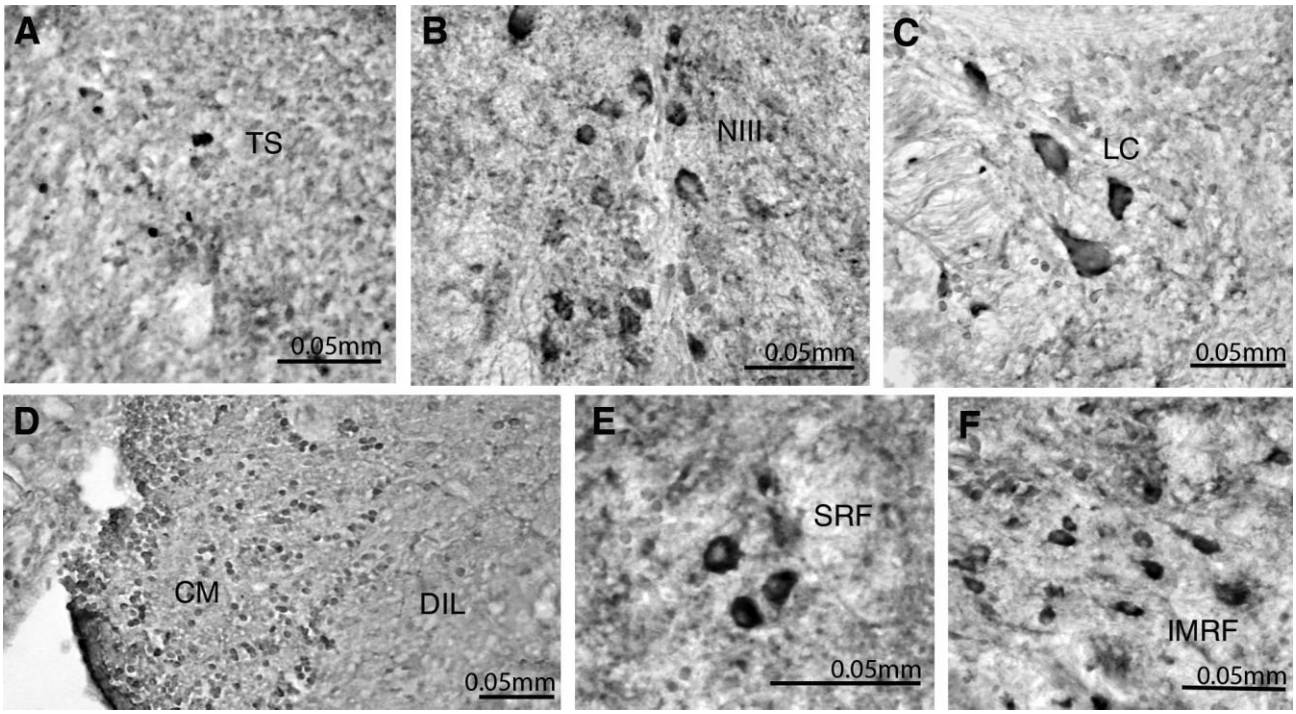

Figure 5. Photomicrographs of selected coronal sections showing the distribution of β_2-ARs in the adult zebrafish mesencephalon and hindbrain. **A:** β_2-AR-immunopositive cells in the torus semicircularis. **B:** β_2-AR immunoreactivity in the oculomotor nucleus. **C:** Strong β_2-AR-immunopositive neurons of the noradrenergic center of the brain, locus coeruleus. **D:** Small size β_2-AR-positive cells in the corpus mamillare. **E,F:** Detail of the strong β_2-AR-immunostained cell somata of the superior and intermediate reticular formation. For abbreviations, see list.

rhombencephalic brain areas parvalbumin-positive cell somata expressed β_2-adrenoceptors. Specifically, double-labeled cells were found in the intermediate reticular formation (Fig. 7E; IMRF) and superior reticular formation (Fig. 7F; SRF). In addition, most of parvalbumin-positive cell somata of the ganglionic cell layer of the cerebellar structures were found to express β_2-adrenoceptors (Fig. 7G,H), suggesting that they are Purkinje cells. However, β_2-ARs were also detected in a subpopulation of PV-negative cells of the ganglionic cell layer (Fig. 7H), possibly representing eurydendroid cells (Alonso et al., 1992). In the forebrain and the mesencephalon, no PV/β_2-AR double-labeled cells were observed; however, in the torus semicircularis (Fig. 7D) a few spare PV-positive cells and fibers were found in close apposition to β_2-adrenoceptor-positive cellular elements.

Localization of β_2-ARs in glial processes

Double-labeled fibers of β_2-AR and GFAP were revealed by double immunohistofluorescence. Astroglial fibers were found to express β_2-ARs in the mesencephalic and rombencephalic midline (Fig. 7C), close to the raphe nuclei (Kaslin and Panula, 2001). In addition, in the central gray, radial glial fibers with mediolateral orientation expressed β_2-adrenoceptors.

Colocalization of β_2-adrenoceptors with α_{2A}-adrenoceptor subtype

Double-labeled cells of β_2- and α_{2A}-ARs characterized several brain areas, supporting the interaction of these receptor subtypes for the function of adrenergic transmission in these areas. Based on the morphology of the cells and the evidence provided by the staining of the neuronal markers, all these double-labeled cells were neurons. Specifically, the cell somata in oculomotor nucleus (Fig. 7K; NIII), in superior, intermediate, and inferior reticular formation nuclei (SRF, IMRF, IRF), in trochlear nucleus (NIV), in magnocellular octaval nucleus (Fig. 7L; MaON), in medial octavolateralis nucleus (MON), and in Purkinje/eurydendroid cells of cerebellar divisions (Va, CCe, LCa) were found to exhibit a diffuse β_2-adrenoceptor staining and a punctuate labeling pattern of α_{2A}-adrenoceptors (Fig. 7N). In addition, the cell somata of the LC were double-labeled, exhibiting a diffuse staining pattern for both β_2- and α_{2A}-adrenoceptors (Fig. 7M).

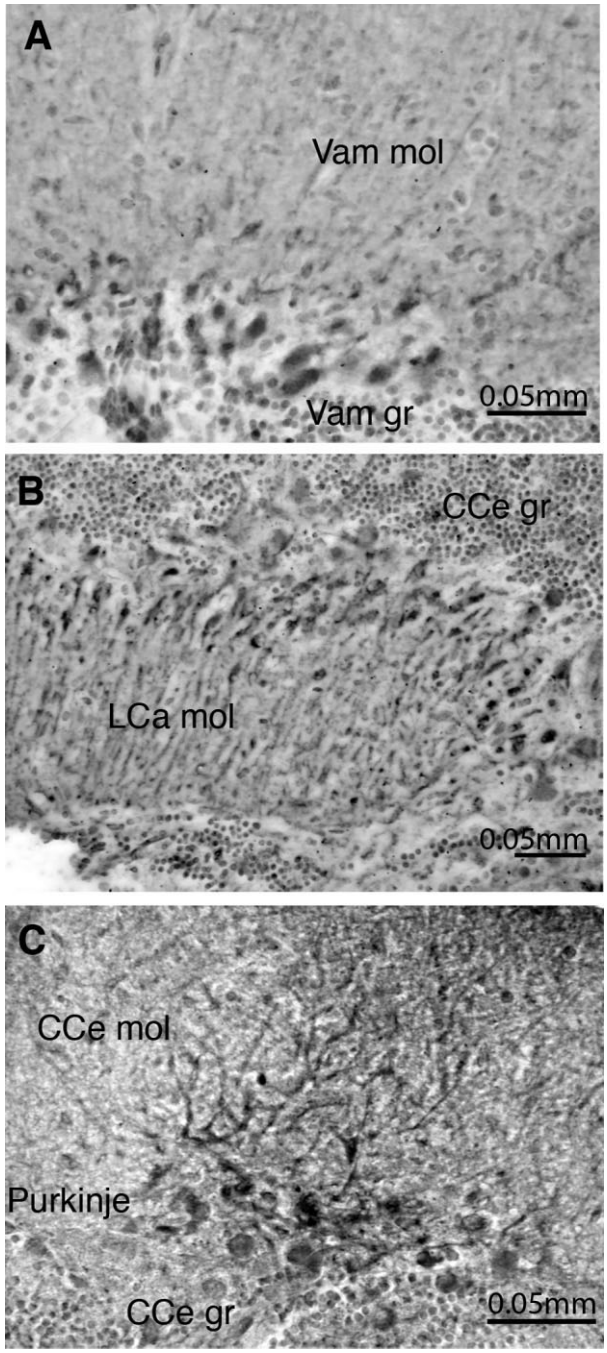

Figure 6. Photomicrographs of selected coronal sections showing the distribution of β_2-ARs in the adult zebrafish cerebellum. **A:** β_2-AR immunoreactivity in the valvula cerebelli. **B:** β_2-AR expression in the Purkinje cell layer and in the molecular layer of the caudal lobe of cerebellum. **C:** High-magnification photomicrograph showing the β_2-AR immunolabeling on the Purkinje and eurydendroid cell somata and dendrites in the corpus cerebellum. For abbreviations, see list.

DISCUSSION

Application of quantitative autoradiographic analysis and immunohistochemistry showed that β_2-AR expression is widely distributed throughout the adult zebrafish brain. In most cases, the areas exhibiting β-AR binding sites are also rich in β_2-AR-immunopositive cells, with β_2-ARs located predominantly on the cell body and/or dendrites. The wide localization pattern of β_2-ARs strongly suggests the significant role of these receptors in mediating the physiological responses to adrenaline/noradrenaline in zebrafish brain. Activation of β_2-adrenoceptor is positively coupled to cAMP formation by stimulating adenylyl cyclase (AC) via G_s protein stimulation. An increase in intracellular cAMP concentration can activate cAMP-dependent kinase (PKA), or directly affect neurotransmitter release. Both presynaptic and postsynaptic membrane β_2-ARs regulate neural excitability, but the present study cannot differentiate between pre- or postsynaptic localization of β-ARs. However, the β_2-ARs, found to colocalize with α_{2A}-ARs on the LC cell somata, most likely represent presynaptic receptors controlling the adrenaline/noradrenaline release. In addition to autoreceptors, presynaptic β_2-AR heteroreceptors are suggested to mediate NA-induced facilitation of glutamate release in mammalian prefrontal cortex (Huang and Hsu, 2006) and hypothalamus (Lee et al., 2007) and therefore play a pivotal role in the regulation of a variety of behavioral functions mediated by these areas. Recently, activation of presynaptic β_2-adrenoceptors has been also shown to elicit long-term upregulation of inhibitory GABAergic transmission at cerebellar interneuron-Purkinje cell synapses (Saitow et al., 2005). On the other hand, intracellular recording studies have demonstrated that postsynaptic β-adrenoceptors activation increases the firing frequency in response to intracellular depolarizing current pulse injection (Kobayashi, 2009). The functional role and the pre- or postsynaptic localization of β_2-adrenoceptors are not known in teleosts. The present localization study suggests the presence of β-AR autoreceptors in the locus coerueus and the existence of β_2-AR heteroreceptors widely distributed in most zebrafish brain regions. Pharmacological and electrophysiological studies are necessary to establish whether such heteroreceptors have a pre- or postsynaptic role similar to that in mammals.

Methodological considerations

To study the quantitative distribution of β-AR in the adult zebrafish brain, the selective antagonist [^{3}H]CGP-12177 was used. [^{3}H]CGP-12177 is considered the most appropriate radiolabeled ligand for the autoradiographic study of β-ARs due to its specificity, high affinity (Staehelin et al., 1983; Revilla et al., 2000), and low levels of nonspecific binding, as it is hydrophilic (Riva and Creese, 1989) and does not bind to nonreceptor sites such as the several lipid domains of the cell membrane.

Furthermore, [^{3}H]CGP-12177 has equal affinity to both β_1- and β_2-AR subtypes (Nanoff et al., 1987) and does not bind to 5-HT_{1b} receptors (Revilla et al., 2000).

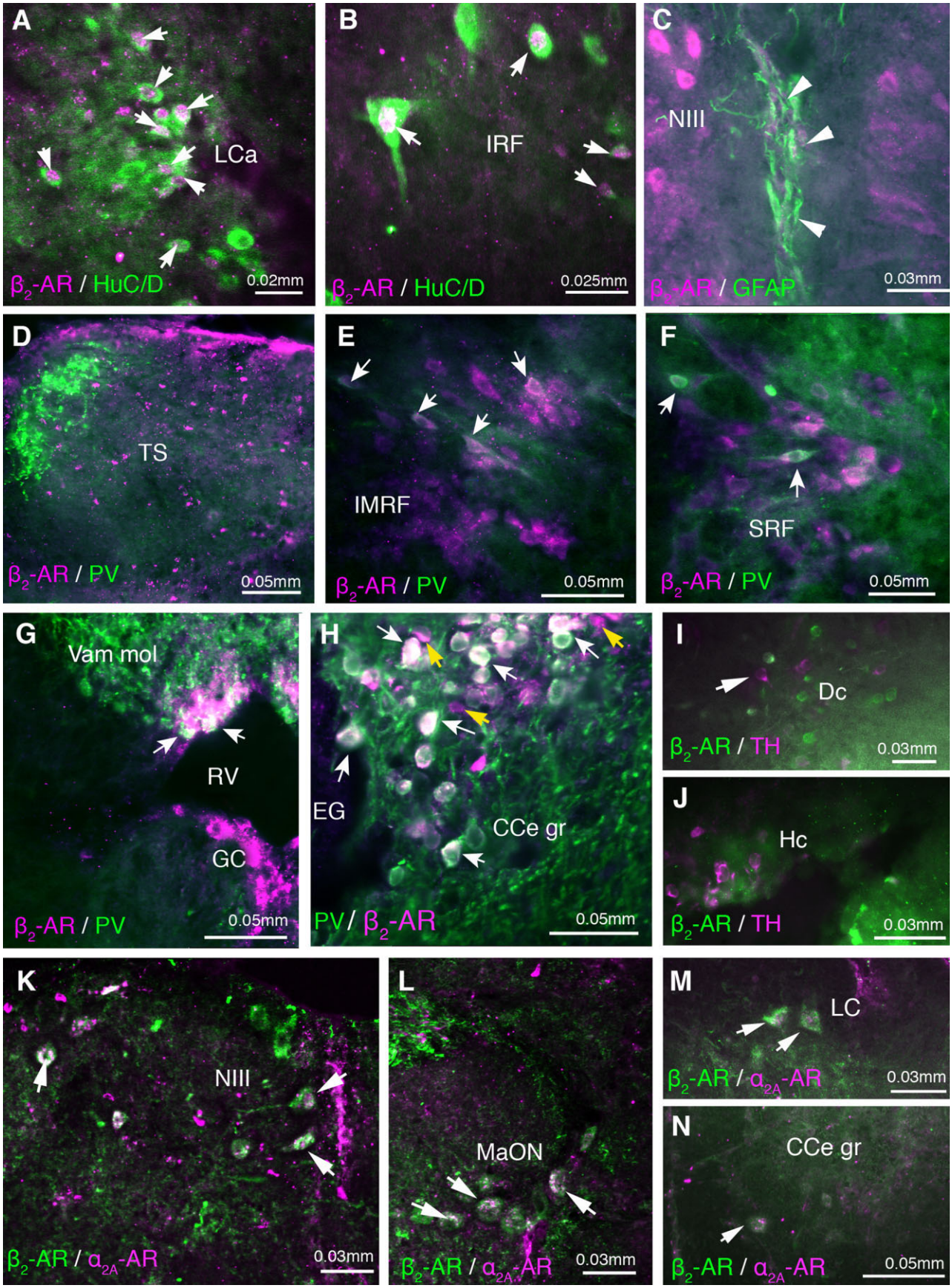
A
LCa
β2-AR / HuC/D
0.02mm
B
IRF
β2-AR / HuC/D
0.025mm
C
NIII
β2-AR / GFAP
0.03mm
D
TS
β2-AR / PV
0.05mm
E
IMRF
β2-AR / PV
0.05mm
F
SRF
β2-AR / PV
0.05mm
G
Vam mol
RV
GC
β2-AR / PV
0.05mm
H
EG
CCe gr
PV/ β2-AR
0.05mm
I
Dc
β2-AR / TH
0.03mm
J
Hc
β2-AR / TH
0.03mm
K
NIII
β2-AR / α2A-AR
0.03mm
L
MaON
β2-AR / α2A-AR
0.03mm
M
LC
β2-AR / α2A-AR
0.03mm
N
CCe gr
β2-AR / α2A-AR
0.05mm

Figure 7

In most brain areas a good correlation of the cellular receptor subtype localization data and density of specific binding sites was found. For example, areas such as preoptic areas, periventricular ventral telencephalon, hypothalamus, LC, cerebellum, and MaON showed strong immunostaining for β_2-ARs as well as a high density of binding sites. However, mismatches between immunohistochemical results and quantitative receptor autoradiography of β_2-ARs are expected, as the immunohistochemistry reveals specific cellular elements expressing the specific receptor subtype, but in contrast the autoradiography demonstrates the average density of β-adrenoceptors diluted by the nonexpressing cells and neuropil, integrated for the whole area measured. Differences between the two methodologies were observed in the reticular formation. In this case, cells were found strongly stained by the antibody against β_2-ARs, but the quantitative analysis of the β-ARs showed a moderate number of receptors. Mismatch was also noted in the periventricular gray zone of optic tectum (PGZ) that included a significant number of medium to large strongly immunostained cells expressing β_2-ARs (55%–67% in total), but low to medium density of β-AR binding sites. Several reasons could account for such a mismatch. First, dilution of the radioligand labeling by other cellular components and neuropil within the area of interest could account for this difference. Second, immunohistochemical detection does not provide evidence for the density of the β_2-adrenoreceptor, as is not a quantitative approach, and it has been suggested that the specific antibody used in the present study labels β_2-ARs in the cytoplasm, nucleus, and plasma membrane of rat hippocampus (Guo and Li, 2007), while autoradiographic ligand binding assays mainly label the functional plasma membrane receptors. Third, the possibility that the primary antibody may crossreact with fragments of AR cannot be excluded. Another mismatch observed, the case of TPp, is characterized by moderate autoradiographic density of β-AR binding sites, accompanied by low β_2-AR immunostaining. This could be partly explained by the fact that the antagonist [^{3}H]CGP-12177 used labels both β_1- and β_2-AR subtypes. In red porgy, both β_1- and β_2-AR subtypes showed a similar wide distribution in most brain areas, with β_2-AR predominance (Zikopoulos and Dermon, 2005).

Distribution pattern of β_2-ARs expression in the zebrafish brain: comparison with other teleosts and nonteleost vertebrates

This study demonstrates for the first time the distribution pattern of β_2-ARs in the adult zebrafish brain by means of in vitro adrenoceptor autoradiography and immunohistochemistry. This is well correlated to the pattern of the noradrenergic intervention in most vertebrate (Smeets and González, 2000) and teleostean brain (Ekström et al., 1986; Meek et al., 1993; Ma, 1994a,b, 1997). In addition, the immunohistochemical labeling of β_2-ARs, summarized in Figure 8, is in general agreement with the regional quantitative ligand-binding pattern. Previous study showed that the β_2-AR subtypes are preferentially found in cerebellum and hindbrain areas, with β_1-ARs in forebrain structures (Minneman et al., 1979). In agreement, our results (both receptor autoradiographic and immunohistochemical data) suggest that this pattern is preserved in the zebrafish brain. It is of great interest that the majority of β_2-AR-positive neurons found in the brain stem nuclei, in most cases, such as the cerebellar ganglionic cell layer and brain stem nuclei (LC, reticular formation nuclei, NIII, NIV, MaON, and MON) were also found to express α_{2A}-ARs. In contrast, nerves such as DV were not found to be double-labeled, that is, nerve fibers expressed α_{2A}-ARs but devoid β_2-ARs. The mammalian β- and α_2-ARs are often coexpressed on the same cell, thus coactivated sharing the same endogenous ligands, and it has been suggested that α_{2A}-AR response is influenced by the presence of β_2-ARs (Bawa-Khalfe et al., 2007). The present data in zebrafish provide initial evidence for such a similar cellular mechanism regulating adrenergic physiological endpoints, mediated by β_2- and α_{2A}-AR converging signals in teleost neurons. Further physiological and pharmacological studies are necessary to validate this hypothesis.

In addition to the neuronal localization of β_2-ARs, evidence is provided for their expression on glial processes and a few spare astrocytic cells by fluorescence double labeling with GFAP. Such astrocytic fibers were mainly found in the mesencephalic and rhombencephalic midline

Figure 7. Immunofluorescent microphotographs of selected transverse sections showing the colocalization of β_2-ARs and the neuronal markers, HuC/D, tyrosine hydroxylase (TH), parvalbumin (PV), and the glial marker (GFAP). **A,B:** Colocalization of the neuronal marker HuC/D with β_2-ARs (A) in cells in the ganglionic cell layer of the caudal lobe of cerebellum (LCa) and (B) in the inferior reticular formation (IRF). **C:** Double-labeled (GFAP/β_2-AR) glial fibers in the rhombencephalon medial to the oculomotor nucleus. **D–H:** Double-labeled PV (green color) and β_2-AR (magenta color) cells (arrows) in TS (D), IMRF (E), SRF (F), cerebellum (G,H) of the adult zebrafish brain. The yellow arrow indicates the eurydendroid cells of the cerebellum. **I–J:** β_2-AR and TH immunofluorescence in the adult zebrafish Hc (J) and Dc (I) forebrain areas. **K–N:** Double labeling of β_2- and α_{2A}-AR in the oculomotor (K), magnocellular octaval nucleus (L), in locus coeruleus neurons (M), and in corpus cerebellum Purkinje cells (N). The white arrows point to double-labeled cells. For abbreviations, see list.

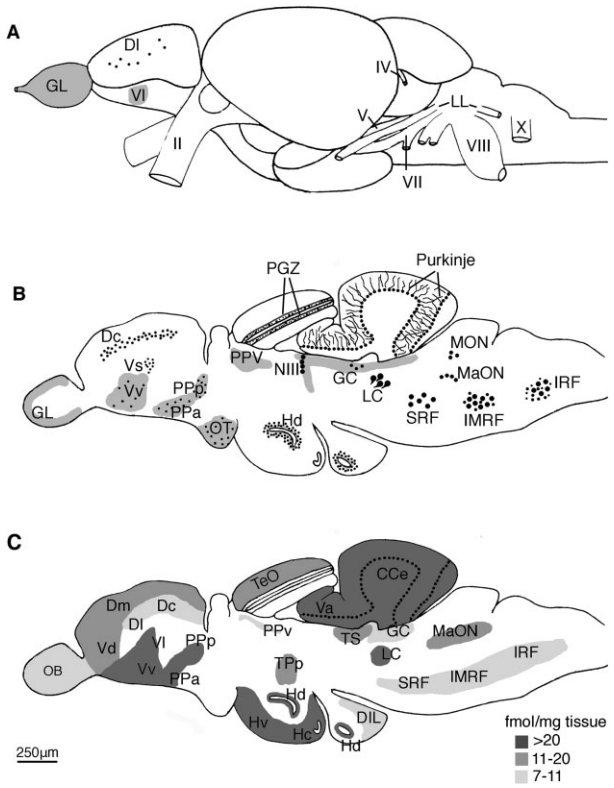

Figure 8. Schematic sagittal drawings of **(A)** lateral view and **(B)** medial view summarizing the main findings of the distribution of β_2-AR containing cells (solid circles), fibers (gray area), and dendrites of ganglionic cell layer of cerebellum (curved lines) close to the midline. **C:** Schematic drawing of medial view illustrating the β-AR binding sites in the adult zebrafish brain (low-medium, 7–11 fmol/mg tissue; light gray, medium, 11–20 fmol/mg tissue; gray) and high (>20 fmol/mg/tissue; dark gray).

near raphe nuclei and within the central gray. Double-labeling experiments showed coexpression with α_{2A}-AR in the fibers located in the same areas and previous studies suggested the significant expression α_{2A}-ARs in the majority of glial fibers within this area (Zikopoulos and Dermon 2005; Ampatzis et al., 2008). It is most likely that the β_2-AR-positive astrocytic fibers represent a subpopulation of the α_{2A}-AR-expressing astrocytic fibers, although direct evidence is not provided in the present study. Furthermore, it is known that both adrenoceptor subtypes studied (α_{2A}- and β_2-AR) are expressed by mammalian astrocytes (Salm and McCarthy, 1989; Aoki and Pickel, 1992; Morin et al., 1997; Milner et al., 1998, 2000; De Keyser et al., 2004), and it has been suggested that astrocytes are innervated by the central noradrenergic system (Gharami and Das, 2003). Taken together, it is suggested that both α_{2A}- and β_2-AR subtypes located to astrocytic fibers have the potential to be activated by synaptically released or circulating noradrenaline/adrenaline, although the functional significance of their expression in zebrafish glial cells is not known at present.

Telencephalon

The present results revealed a small population of β_2-AR-positive cellular elements in the olfactory bulbs of zebrafish. Most of these stained cells and fibers were found in the glomerular layer of the olfactory bulbs (GL). Our results are comparable to the weak monoamine oxidase (MAO) activity (Anichtchik et al., 2006) and the expression of α_2-ARs (Ampatzis et al., 2008) in the zebrafish glomerular layer of the olfactory bulbs. In addition, previous studies in teleosts showed the absence of noradrenergic immunoreactivity in the GL of the olfactory bulbs in *Gasterosteus aculeatus* (Ekström et al., 1986).

The dorsal and ventral telencephalic areas of actinopterygian fish correspond to the pallial and subpallial brain regions, respectively, sharing noradrenergic inputs directly from the noradrenergic center of the brain, the locus coeruleus (LC; Rink and Wullimann, 2004). Comparison of these telencephalic subdivisions of teleosts with those of other vertebrate species is difficult because of the different ontogeny of the actinopterygian telencephalon, known as eversion (Butler, 2000; Wullimann and Mueller 2004). Furthermore, teleostean telencephalic neuroanatomy varies considerably among the species (Wullimann and Mueller, 2004), possibly reflecting the great variety of brain organization in fish.

The medial zone of dorsal telencephalic area (Dm), thought to be homologs to the pallial amygdala (Bradford, 1995; Portavella et al., 2002), and involved in emotional memory processes such as delay and trace avoidance conditioning (Broglio et al., 2005), included moderate levels of β_2-AR-positive cells. Furthermore, the dorsomedial region of the teleostean dorsal telencephalon included high densities of noradrenaline immunoreactivity (Ekström et al., 1986) and moderate levels of cells and fibers expressing α_2-ARs (Ampatzis et al., 2008) and MAO activity (Anichtchik et al., 2006), supporting a role of adrenoceptors in its function. Both the posterior zone of the dorsal telencephalic area (Dp), considered to be homologous to the olfactory cortex (Wullimann and Mueller, 2004), and the lateral zone of the dorsal telencephalon (Dl), involved in spatial learning and short-term memory procedures (Ohnishi, 1997; Lopez et al., 2000; Vargas et al., 2000), showed moderate to low immunoreactivity and moderate in vitro autoradiographic labeling. This AR profile in Dl supports its homology to the hippocampus of land vertebrates (Portavella et al., 2002). The central zone of the dorsal telencephalic area (Dc) was found to have a small distinct cluster of positive β_2-AR cell somata, in contrast to the α_{2A}-ARs localization in fibers (Ampatzis et al., 2008), suggesting possible differential functions of adrenoceptors in

this area. In general, the distribution pattern of autoradiographic labeling β-AR binding in dorsal telencephalon is in agreement with β_2-AR immunoreactivity and β-AR binding studies in other teleost species (Zikopoulos and Dermon, 2005).

High densities of β-AR binding sites as well as β_2-AR immunoreactivity were observed in the medial areas of the ventral zebrafish telencephalon (Vv), proposed to include areas comparable to the nucleus accumbens, substantia innominata, and septal areas of tetrapods (Kaslin and Panula, 2001). Developmental studies during the secondary forebrain neurogenesis revealed highly comparable gene expression patterns characterizing early mice and zebrafish telencephalic organization, underlying the production of GABAergic and cholinergic neurons (Mueller et al., 2008; Wullimann, 2009). According to these studies, ventral and dorsal subdivisions of the dorsal subpallium (adult dorsal nucleus of ventral telencephalic area, Vd) have been interpreted as corresponding to the medial and lateral ganglionic eminences of the mammalian embryonic telencephalon, that is, the striatum and pallidum, respectively, of adult mammals, setting the basis for a neuroanatomically separate adult striatum- and pallidum-like area in the zebrafish. (Mueller et al., 2008; Wullimann, 2009). The moderate density of β_2-ARs in Vd is in accordance with its proposed homology to striatum and pallidum but cannot define finer subpallial subdivisions in zebrafish. The periventricular ventral nuclei of zebrafish telencephalon Vv (ventral subpallium) is considered to represent the septal formation (Wullimann and Mueller, 2004) and the dense staining of β_2-ARs found in the Vv is in agreement with the proposed homology to mammalian septum. In agreement, dense adrenergic/noradrenergic innervation and receptor localization have been reported previously in subpallial areas (particularly septum) in other vertebrates (Dermon and Kouvelas, 1988, 1989; Talley et al., 1996; Bachman et al., 1998). This distribution pattern of β_2-AR is accompanied by high α_{2A}-receptor levels in the zebrafish (Ampatzis et al., 2008), and is comparable to high α_{2A}- and β_2-AR immunoreactivity and binding sites in the red porgy (Zikopoulos and Dermon, 2005) brain.

Diencephalon

The distribution of β-AR binding sites and β_2-AR-positive cells and fibers in zebrafish diencephalic areas was very heterogeneous, in agreement with that described previously in most diencephalic areas of mammals (Palacios and Kuhar, 1980), birds (Dermon and Kouvelas, 1988, 1989; Revilla et al., 1998a), amphibians (Bachman et al., 1998), and fish (Zikopoulos and Dermon, 2005).

Preoptic areas included β_2-AR-positive cells and fibers and showed moderate autoradiographic labeling of β-AR binding sites. Furthermore, strong neuropil staining and small cells positive for MAO were found in preoptic areas of the adult zebrafish (Anichtchik et al., 2006). Moreover, the expression of β-ARs in the preoptic areas is in agreement with the high density of β-AR autoradiographic labeling in the present study and previous results from the marine teleost *Pagrus pagrus* (Zikopoulos and Dermon, 2005), the teleost *Gasterosteus aculeatus* (Ekström et al., 1986), and the chondrosteans *Acipenser baeri* and *Huso huso* (Adrio et al., 2002). Taking into account the role of the preoptic nuclei in the reproductive behavior, thermoregulation, and control of the neuroendocrine system in fish (Butler and Hodos, 1996; Wullimann et al., 1996), the expression of both α_{2A}- (Ampatzis et al., 2008) and β_2-AR types in these nuclei supports the hypothesis of the essential role of adrenergic system in the regulation and modulation of the reproduction and hormone secretion. In support, adult zebrafish hypothalamus showed high density of β-ARs binding sites and strong immunoreactivity for β_2-AR; especially the dorsal zone of the periventricular hypothalamus (Hd), an area suggested to be involved in the sex change of the protandrous marine teleost *Sparus aurata* (Zikopoulos et al., 2001). These results further support that adrenergic receptors are highly expressed in the hypothalamus, as previously suggested for other teleost fish (Ekström et al., 1986; Zikopoulos and Dermon, 2005; Ruuskanen et al., 2005b; Ampatzis et al., 2008), and other vertebrate species (Unnerstall et al., 1984; Dermon and Kouvelas, 1988, 1989; Fernández-López et al., 1997; Revilla et al., 1998a). It is therefore suggested that hypothalamic adrenergic receptors participate in the control of gonadotropin release and sexual differentiation, regulating reproductive behavior in fish.

Mesencephalon

In addition to primary visual input, the optic tectum receives higher-order auditory and lateral line inputs (Meek, 1990) and is involved in multisensory integration processes such as recognition and position of objects, spatial orientation, and motor coordination (Meek, 1990; Guthrie, 1990). The superficial layers of the optic tectum, proposed to be homologous to the mammalian superior colliculus (Butler and Hodos, 1996), receive direct input from retina. Noradrenergic, histaminergic, and serotonergic fibers have been previously reported in optic tectum layers of the adult zebrafish (Kaslin and Panula, 2001). The present study determined moderate labeling for β-AR binding sites and low to moderate densities of stained cells for β_2-AR. However, it is important to note that optic tectum included numerous β_2-AR-positive fibers with radial orientation, resembling periventricular radial glial elements. Alternatively, periventricular stratum neurons show a similar morphology and topography, with a long dendritic tree reaching toward the optic tectum surface. Double-labeling

experiments showed that the β_2-AR fibers did not express GFAP and further experiments, using other glial markers, are necessary to determine whether or not these fibers are of a glial nature. In any case (neuronal or glial), such β_2-AR expression could represent a strategic site of noradrenergic modulation of glutamatergic retinotectal transmission in zebrafish. In agreement, it is known that noradrenergic input, via β-ARs, increase the "signal-to-noise ratio" by facilitation of glutamatergic transmission in mammalian visual cortex, which is believed to be a crucial central function of noradrenaline (Kobayashi et al., 2009). Moreover, the optic tract and the optic chiasm included β_2-AR-immunopositive cell structures, with many stained fibers that were in a close apposition but did not express the astroglial marker GFAP. These results further support an important role of β_2-AR in the visual processing in teleost fish, in agreement with the proposed role of β-ARs in visual processing and adaptation in avian species (Revilla et al., 1998b).

Torus semicircularis (TS) included moderate densities of β_2-AR-positive cellular elements, similar to previous findings in another teleost fish, *Pagrus pagrus* (Zikopoulos and Dermon, 2005). The TS is the main auditory and lateral line center of the teleost midbrain, homolog to the mammalian inferior colliculus (Butler and Hodos, 1996). Monoaminergic innervations of the optic tectum and torus semicircularis have been reported earlier in numerous teleosts (Kah and Chambolle, 1983; Ekström and van Veen, 1984; Ekström et al., 1986; 1990; Hornby et al., 1987; Beltramo et al., 1994; Kaslin and Panula, 2001), and thus our findings further support the hypothesis that the noradrenergic system plays an important role in the integration and modulation of multisensory input in vertebrates. In addition, the periventricular gray zone of optic tectum (PGZ) exhibited numerous small β_2-AR-positive cells, as well as α_{2A}-ARs (Ampatzis et al., 2008), and MAO mRNA activity (Anichtchik et al., 2006), in adult zebrafish. Dense large β_2-AR-positive cells were observed in the oculomotor nucleus (NIII), in agreement with the observations for adrenoceptor localization described in another teleost fish species (Zikopoulos and Dermon, 2005).

Rhombencephalon

The isthmic part of adult zebrafish brain is characterized by a small group of highly stained β_2-AR-positive cell somata, representing the homolog to the mammalian locus coeruleus (LC). LC exhibited high density of β-AR binding sites in agreement to the β_2-AR antibody localization. The LC in adult zebrafish contains a few very large neurons (3–10; Ma, 1994a) that supply most of the noradrenergic input to the brain areas (Ma, 1997). Similarly, in all other teleosts studied, LC consists of a small number of catecholaminergic cells (Ekström et al., 1986; Sas et al., 1990; Rodríguez-Gomez et al., 2000), although in all cases (*Dicentrarchus labrax*; Batten et al., 1993, brown trout; Manso et al., 1993, *Tinca tinca*; Briñón et al., 1998 and *Pagrus pagrus*; Zikopoulos and Dermon, 2005) are more numerous than in zebrafish. The present study provides further evidence for the identification of teleostean LC cell somata by their strong staining of β_2 and α_{2A} adrenergic receptors (Zikopoulos and Dermon, 2005; Ampatzis et al., 2008), in addition to the noradrenaline (Ekström et al., 1986; Meek et al., 1993), TH, DβH immunoreactivity (Ekström et al., 1990; Sas et al., 1990; Meek, 1994; Ma, 1994a,b; Kaslin and Panula, 2001; Ampatzis et al., 2008) and MAO activity (Anichtchik et al., 2006). The adrenergic/noradrenergic nature of LC cells has also been described in amphibians (González and Smeets, 1994), reptiles (Puelles and Medina, 1994), and mammals (Kitahama et al., 1994), demonstrating that is well conserved in vertebrates.

All the reticular formation subdivisions show moderate β-AR binding sites and express substantial β_2-AR immunoreactivity. These results are well correlated with the observed distribution pattern in many vertebrate species (Palacios and Kuhar, 1980; Wanaka et al., 1989; Smeet and González, 2000) including teleosts (Zikopoulos and Dermon, 2005). The reticular formation receiving projections from the torus semicircularis (TS) is involved in fast arousal and sensory-motor control (Schellart, 1990), and β_2-ARs possibly have a role based on their localization pattern in both areas. The rhombencephalic central gray (GC), implicated in several essential physiological processes, including reproductive behavior, visceral animal responses, and analgesia (cf. Van der Bergh et al., 1988), contains high densities of β_2-AR-immunoreactive cell somata and fibers in the adult zebrafish brain. This is similar to the pattern described previously for red porgy β_2-ARs (Zikopoulos and Dermon, 2005).

Cerebellum

The pattern of autoradiographic labeling of β-ARs in the zebrafish cerebellum was consistent with the laminar localization of β-ARs in avian (Dermon and Kouvelas, 1989; Fernández-López et al., 1997), mammalian (Palacios and Kuhar, 1980), and teleostean (Zikopoulos and Dermon, 2005) cerebellum. The ganglionic cell layer, containing the cell somata of the Purkinje and eurydendroid cells, the latter being the efferent elements of teleostean cerebellum (Meek and Nieuwenhuys, 1998) and corresponding to the deep cerebellar nuclei (DCN) of the mammalian cerebellum (Ikenaga et al., 2006), exhibited high density of β-AR binding sites and strong labeling of β_2-adrenoceptors. Cells of the ganglionic cell layer were positive for parvalbumin, a calcium-binding protein expressed by the Purkinje cells, a constant feature throughout the

phylogenetic scale, but not by eurydendroid cells of teleostean cerebellum (Alonso et al., 1992; Porteros et al., 1998; Crespo et al., 1999). Our immunofluorescence data suggest that most of the Purkinje cells and a small population of eurydendroid cell express β_2-ARs. In addition, the β_2-immunopositive cells of ganglionic cell layer were found to be colocalized with the α_{2A} subtype. In contrast, their dendrites located in the molecular layer of the cerebellum were β_2-AR-immunopositive, but not stained for α_{2A} adrenoceptors. This is similar to the localization pattern of the β_2-ARs in the red porgy cerebellum (Zikopoulos and Dermon, 2005) and to the distribution pattern of noradrenaline innervations of stickleback (Ekström et al., 1986). However, very low MAO activity in the adult zebrafish cerebellum was observed (Anichtchik et al., 2006). The characteristic adrenoceptor laminar pattern found in all zebrafish cerebellar subdivisions is in good correlation with the innervation pattern by locus coeruleus fibers (Ma, 1994b). The teleostean cerebellum is involved, as in other vertebrates, in motor coordination (Paul and Roberts, 1979; Roberts et al., 1992), but additionally, the fish cerebellum is known to be involved in spatial and emotional learning (Yoshida et al., 2004; Rodríguez et al., 2005), and in memory processes (Lalonde and Botez, 1990). The colocalization of β_2 and α_{2A} adrenergic receptors on ganglionic cell layer somata suggests an overlapping but distinct role of each receptor type in noradrenergic function, regulating cerebellum motor learning and coordination.

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