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Letter to the Editor

Distribution of vesicular glutamate transporter 2 and glutamate receptor 1 and 2 mRNA in the pigeon retina

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ABSTRACT

Glutamate is an excitatory neurotransmitter in the central and peripheral nervous systems of the vertebrate. The previous studies show the presence of mRNAs of AMPA-type glutamate receptors, GluR1 and GluR2, in the optic tectum of the pigeon, suggesting glutamatergic input from the retina. The present study examined localization of vesicular glutamate transporter 2 (VGLUT2) and GluR1 and GluR2 to confirm source of glutamatergic neurons in the pigeon retina by in situ hybridization histochemistry. VGLUT2 mRNA expressed in the inner nuclear layer and ganglion cells, while GluR1 and GluR2 mRNAs were observed in the inner nuclear layer, ganglion cells, and superficial layers of the optic tectum. The results suggest that photoreceptor cells, bipolar cells and ganglion cells are glutamatergic in the avian retina as in mammals.

The visual system in birds is composed of tectofugal and thalamofugal pathways. The tectofugal pathway conveys visual input via the rotundal nucleus to the entopallium, whereas the thalamofugal pathway sends visual information to the visual Wulst. Glutamate, which is a major excitatory amino acid neurotransmitter in the central nervous system of the vertebrate, appears to be involved in neurotransmission of the visual system of the avian brain on the basis of morphological analysis (Theiss et al., 1998; Islam and Atoji, 2008). In the mammalian retina, glutamate is considered to be used a neurotransmitter acting in a vertical pathway of visual information, i.e., photoreceptor cells–bipolar cells–ganglion cells (Brandstätter and Hack, 2001; Land et al., 2004). In birds, as in mammals, glutamate is a candidate neurotransmitter in photoreceptor, bipolar, and ganglion cells in the retina, as revealed in two studies using an immunohistochemical technique with anti-glutamate antibody (Kalloniatis and Fletcher, 1993; Sun and Crossland, 2000). Glutamate immunoreactivity is usually located in the inner segments of the photoreceptor and cell bodies of photoreceptor, bipolar, and ganglion cells. However, it should be noted that the mere localization of glutamate in these three types of neurons does not identify glutamatergic neurons, because it does not distinguish the neurotransmitter from its metabolites or precursor to γ-aminobutyric acid. It is necessary to confirm the localization of glutamate in synaptic terminals in order to identify glutamatergic neurons in terms of immunohistochemistry using anti-glutamate antibody.

Vesicular glutamate transporters (VGLUTs) accumulate glutamate into synaptic vesicles in axon terminals. Three types of VGLUTs have been identified based on biochemical and genetic studies in mammals (Ni et al., 1994; Aihara et al., 2000; Bai et al., 2001; Takamori et al., 2002). VGLUT1 and VGLUT2 express in glutamatergic neurons, whereas VGLUT3 appears to be localized in a subset of cholinergic and serotoninergic neurons (Bellocchio et al., 1998; Hisano et al., 2000; Fremeau et al., 2001, 2002; Gras et al., 2002; Herzog et al., 2004). Immunohistochemical and in situ hybridization histochemical studies indicate VGLUT1 and VGLUT2 are very good marker for the identification of glutamatergic neurons in the mammalian brain (Hisano, 2003; Fremeau et al., 2004). In birds, VGLUT2 can also identify glutamatergic neurons in the brain and spinal cord (Islam and Atoji, 2008).

The postsynaptic actions of glutamate are mediated by at least three major pharmacologically distinct classes of ionotropic glutamate receptors that are defined according to their selective agonists into *N*-methyl-D-aspartate (NMDA)–type, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type, and kainate-type receptors (Collingridge and Lester, 1989). AMPA-type glutamate receptors are assembled as GluR1, GluR2, GluR3, and GluR4 (Keinänen et al., 1990) in mammals. Among birds, GluR1-4 mRNAs have been also cloned in the pigeon (Ottiger et al., 1995; Islam and Atoji, 2008).

In the present study, we performed in situ hybridization histochemistry using VGLUT2, GluR1, and GluR2 mRNAs to investigate localization of glutamatergic neurons in the pigeon retina.

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Nine adult pigeons (*Columba livia*, 4 males and 5 females, weighing 310–370 g) were used in the present study. The animal handling procedures were approved by the Animal Experimental Committee of the Faculty of Applied Biological Sciences, Gifu University. Eight pigeons were used for in situ hybridization and one female pigeon was used for Nissl staining. After the animals were anesthetized by sodium pentobarbital (50 mg/kg), the fresh brains and eyeballs were quickly removed and immediately frozen on powdered dry ice. The serial coronal sections were cut at 30-μm thickness on a cryostat, thaw-mounted onto 3-aminopropyltriethoxysaline-coated slides, and stored at −30 °C. For Nissl staining, a pigeon was anesthetized with sodium pentobarbital (50 mg/kg) and perfused with Ringer's solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4. The eyeballs were dissected and placed in 30% sucrose for three days. Sections were cut at 30-μm thickness on a cryostat and were stained with 0.1% cresyl violet.

The slide-mounted sections were warmed to room temperature, fixed in 4% paraformaldehyde in PB for 15 min (all steps were performed at room temperature unless otherwise indicated), rinsed three times (5 min each) in 4× standard saline citrate (SSC; pH 7.4; 1× SSC contains 0.15 M sodium chloride and 0.015 M sodium citrate), and dehydrated through a graded ethanol series (70–100%). Sections were then defatted with chloroform for 3 min, and immersed in 100% ethanol (twice for 5 min each time) before hybridization. Hybridization was performed by incubating the sections with the following buffer at 41 °C overnight: 4× SSC, 50% deionized formamide, 1.2 M phosphate buffer (pH 7.4), 1% Denhardt's solution (Nacalai, Kyoto, Japan), 0.025% yeast tRNA (Roche, Mannheim, Germany), 10% dextran sulfate (Nacalai). The buffer contained probes labeled with [^{35}S] dATP (46.25 TBq/mmol; PerkinElmer Life Science, Waltham, MA, USA; approximately $1–2\times10^7$ d.p.m/ml, 0.3 ml/slide). After hybridization, sections were rinsed in 1× SSC (pH 7.4) for 10 min followed by rinsing three times in 1× SSC at 55 °C for 20 min, dehydrated through a graded ethanol series (70–100%), and exposed to X-ray films (Fuji Medical X-Ray Film, Tokyo, Japan) for 7 days. Then the sections were coated with NTB-2 emulsion (Eastman Kodak Company, Rochester, NY, USA) diluted 1:1 with distilled water and exposed at 4 °C for 4 weeks in tightly sealed dark boxes. After being developed in D-19 developer (Eastman Kodak Company), the sections were fixed, washed with tap water, and dehydrated. Some sections were counterstained with 0.1% cresyl violet to allow morphological identification.

The anti-sense and sense oligo cDNA probes of VGLUT2, GluR1 and GluR2 were designed based on the pigeon VGLUT2 (GenBank accession no. FJ428226), GluR1 (GenBank accession no. FJ428225), and GluR2 (GenBank accession no. Z29713), respectively, and were synthesized commercially (Rikaken, Nagoya, Japan). The pigeon VGLUT2 anti-sense (pVGLUT2-AS), GluR1 anti-sense (pGluR1-AS), and GluR2 anti-sense (pGluR2-AS) probes were as follows: pVGLUT2-AS: 5′-TCCTTCCTTGTAGTTGTATGAGTCTTGTACTTCCTC-3′ (nucleotides; 1273–1309), pGluR1-AS: 5′-CTTCCGGAGTCCTTGCTTC CACATTCCCCTTTATCG-3′ (nucleotides; 2360–2396), and pGluR 2-AS: 5′-AATTGTGATAACCTGATCAATGTCGTTGACTTTATCGCGCT C-3′; (nucleotides 570–615).

The sense probes pVGLUT2-S, pGluR1-S, and pGluR2-S were complementary base sequences to the corresponding anti-sense probes. Computer-assisted homology searches (NCBI-BLAST) showed that each probe had less than 52% homology with any chicken sequences registered in the GenBank. The probes were labeled at the 3′-end with [^{35}S] dATP (46.25 TBq/mmol; PerkinElmer Life Science) by using terminal deoxynucleotidyl transferase (Takara, Tokyo, Japan).

X-ray film images were scanned into a computer by using an image scanner (Epson GT 8700F, Nagano, Japan). Photomicrographs were taken with a digital camera (Pro 600ES, Pixera Corporation, Los Gatos, CA, USA) on a light microscope with adjustment of contrast, brightness, and sharpness. Layout of micrographs and lettering were performed by using Adobe Photoshop 7.0J and Adobe Illustrator 10.0J (Tokyo, Japan).

We examined expression of VGLUT2 mRNA in the retina and of GluR1 and GluR2 mRNAs in the retina, thalamus, and optic tectum. Cells with hybridization signals detectable against the background were considered to be positive, and mRNA density was classified as high, moderate, or weak. No labeling was detected when sense probes were used (Fig. 1I and J).

X-ray film images revealed VGLUT2, GluR1 and GluR2 mRNA expression in the retina (Fig. 1A–C). The pigment layer of the retina showed non-specific binding for anti-sense and sense probes, and as a result a darker, non-specific layer was seen outside a specific layer in X-ray films. Fig. 1D shows retinal layers in a Nissl-stained section. Emulsion-coated sections confirmed hybridization signals in the retinal layers with different labeling intensity. VGLUT2 mRNA signal was expressed highly in the ganglion cell layer and moderately in the inner nuclear layer (Fig. 1E and F), and the other layers were negative for hybridization signals. Weak GluR1 mRNA expression was found in the ganglion cell layer and moderate expression was found in the inner nuclear layer (Fig. 1G), whereas both the ganglion cell layer and inner nuclear layers showed weak GluR2 mRNA expression (Fig. 1H).

In the thalamus, GluR1 mRNA was weakly expressed in the rotundal nucleus but not in the optic principal nuclei consisting of the nucleus lateralis anterior (LA), nucleus dorsolateralis anterior thalami, pars lateralis (DLL), and nucleus dorsolateralis anterior thalami, pars magnocellularis (DLAmc) of the thalamus (Fig. 2A). GluR2 mRNA expression was weak in the optic principal nuclei but not in the rotundal nucleus (Fig. 2B). The optic tectum expressed GluR1 and GluR2 mRNAs, and GluR2 labeling showed higher intensity than GluR1 mRNA expression (Fig. 2C and D). Differential laminar distribution was found both for GluR1 and GluR2 mRNAs in the optic tectum. Weak GluR1 mRNA labeling was found in layers 4, 6, 8, 11, 15, and moderate labeling was found in layer 10 (Fig. 2E), whereas GluR2 mRNA expression was weak in layers 4, 6, 8, 11, and was moderate in layers 10 and 13 in the emulsion-coated sections (Fig. 2F).

The present study demonstrated the existence of VGLUT2 mRNA in neurons of the inner nuclear layer and ganglion cells but not in photoreceptor cells in the pigeon retina. Lack of VGLUT2 mRNA expression in the photoreceptor cells does not immediately indicate that they are non-glutamatergic. The inner nuclear layer expressed GluR1 and GluR2 mRNAs in the present study. Photoreceptor cells probably release glutamate as a neurotransmitter to neurons in the inner nuclear layer. As VGLUT2 mRNA expression was homogeneous in the inner nuclear layer, signals are considered to be localized in bipolar cells. If VGLUT2 signals were expressed in horizontal cells or amacrine cells, laminar labeling would be expected in the inner nuclear layer. However, laminar labeling was not found in the present study. GluR1 and GluR2 mRNAs were expressed in the ganglion cell layer. Bipolar cells appear to be glutamatergic. Ganglion cells in the pigeon retina project to the optic tectum and dorsal thalamic region (Gamlin and Cohen, 1988). An immunoelectron microscopic study using anti-glutamate antibody demonstrated glutamate immunoreactivity axon terminals, which contain clear, spherical synaptic vesicles, in layers 3, 5, and 7 of the pigeon optic tectum (Morino et al., 1991). GluR1 and GluR2 mRNAs are expressed mainly in the superficial half of the optic tectum, i.e., layers 4, 6, 8, and 11 (Ottiger et al., 1995; Islam and Atoji, 2008; present study). GluR1 is immunohistochemically localized in neurons of layers 2–7, 9 and 10 in the pigeon optic tectum (Theiss et al., 1998). The localization