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GENE EXPRESSION OF IONOTROPIC GLUTAMATE RECEPTOR SUBUNITS IN THE TECTOFUGAL PATHWAY OF THE PIGEON

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Abstract—The tectofugal pathway in birds consists of four stations, the retina, optic tectum, rotundal nucleus, and entopallium, and it conveys visual information via three ascending pathways. These pathways consist of retinotectal, tecto-rotundal and rotundo-entopallial cells, all of which are glutamatergic. The present study examined the localization of ionotropic glutamate receptors (iGluRs) to identify the target areas of glutamatergic projections in the tectofugal pathway in pigeons. Nine subunits of iGluRs were analyzed using *in situ* hybridization as follows: AMPA receptors (GluA1, GluA2, GluA3, and GluA4), kainate receptors (GluK1, GluK2, and GluK4), and NMDA receptors (GluN1 and GluN2A). Hybridization signals of subunits showed various intensities in different cells. In the optic tectum, a strong to moderate expression was observed in layer 10 (GluA2, GluA3, GluK4, and GluN1) and layer 13 (GluA2, GluK4, GluN1, and GluN2A). The rotundal nucleus intensely expressed GluA3, GluA4, GluK1, and GluK4. In the entopallium, an intense to moderate expression of GluK1 and GluK4, and a moderate to weak expression of AMPA and NMDA receptors were observed. Furthermore, the parvocellular and magnocellular parts of the isthmic nuclei showed a strong expression of GluA2, GluA3, GluK4, and GluN1. The present findings demonstrate the expression of iGluRs in glutamatergic projection targets of the tectofugal pathway in birds and suggest a diversity of iGluRs in the transmission of visual information. © **2015 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: AMPA receptor, kainate receptor, NMDA receptor, isthmic nucleus, vGluT2, GAD65.

INTRODUCTION

In birds, visual information is conveyed from the eyes to the telencephalon via the tectofugal and thalamofugal pathways. The tectofugal pathway includes two relay stations, one in the optic tectum (OT) and the other in the rotundal nucleus (Rt). OT is the first relay station and receives afferents from retinal ganglion cells to the superficial layers. Based on electrophysiological and anatomical evidence, ganglion cells appear to be glutamatergic (Dye and Karten, 1996; Islam and Atoji, 2009; Atoji, 2015). OT has reciprocal connections with the deeper nuclei of the parvocellular (Ipc) and magnocellular (Imc) parts of the isthmic nuclei (Wang et al., 2004, 2006). Stimulation of Ipc neurons elicits action potentials to tectal cells (Wang et al., 2000, 2006), and Ipc neurons express mRNA for the vesicular glutamate transporter 2 (vGluT2) (Islam and Atoji, 2008; Karim et al., 2014; González-Cabrera et al., 2015), a marker of glutamatergic neurons (Fremeau et al., 2001). Furthermore, OT receives descending projections from the apical part of the hyperpallium and the arcopallium of the telencephalon (Zeier and Karten, 1973; Dubbeldam et al., 1997; Manns et al., 2007). Neurons in the two telencephalic areas strongly express vGluT2 mRNA (Islam and Atoji, 2009; Karim et al., 2014). Within OT, excitatory postsynaptic potentials have been recorded in tectal neurons (Wang et al., 2000; Luksch and Golz, 2003) and immunohistochemical studies have demonstrated numerous varicosities or axon terminals immunoreactive for vGluT2 in this region (Atoji, 2011). This electrophysiological and morphological evidence suggests the presence of glutamatergic synapses in OT. Layer 13 neurons in OT send ascending axons to Rt (Karten et al., 1997; Hellmann and Güntürkün, 2001) and express vGluT2 mRNA (Islam and Atoji, 2008). In turn, Rt neurons project to the entopallium via glutamatergic efferents (Atoji and Karim, 2014). Therefore, the tectofugal pathway relays information from the retina to the entopallium via the glutamatergic projection neurons.

Ionotropic glutamate receptors (iGluRs) are classified into three types that include α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, kainate (KA) receptors, and *N*-methyl-D-aspartate (NMDA) receptors, and their subunits have been identified (Collingridge and Lester, 1989; Collingridge et al., 2009). The iGluRs primarily mediate fast excitatory neurotransmission in the central nervous system and can be distinguished by their differential pharmacological properties such as activation, desensitization kinetics, selective permeability, and single-channel properties (Wollmuth and Sobolevsky,

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; DEPC, diethyl pyrocarbonate; DIG, digoxigenin; Ei, internal portion of the entopallium; Ep, perientopallium; EPSPs, excitatory postsynaptic potentials; Ex, external portion of the entopallium; GABA, γ-amino butyric acid; GAD, glutamic acid decarboxylase; HA, hyperpallium; iGluR, ionotropic glutamate receptor; Imc, magnocellular part of the isthmic nucleus; Ipc, parvocellular part of the isthmic nucleus; KA, kainate receptor; NLS, N-lauroylsarcosine; NMDA, *N*-methyl-D-aspartate receptors; OT, optic tectum; PB, phosphate buffer; PBS, phosphate-buffered saline; Rt, rotundal nucleus; RT-PCR, real-time polymerase chain reaction; SSC, standard saline citrate; TBS, Tris-buffered saline; TBST, Tris-buffered saline Tween 20; vGluT2, vesicular glutamate transporter 2.

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2004; Mayer, 2005; Lerma, 2006; Perrais et al., 2010; Traynelis et al., 2010). AMPA receptors mediate most basal excitatory synaptic transmission and play a role in synaptic integration, signaling, and plasticity. NMDA receptors trigger many forms of synaptic plasticity because of their permeability to calcium and their selective activation by a depolarizing potential. KA receptors mediate currents and actions of the intracellular second messengers and are involved in axonal navigation or a rapid switch from KA receptor- to AMPA receptor-mediated synaptic transmission in long-term potentiation.

Theiss et al. (1998) have described the distribution of AMPA receptor subunits in the pigeon tectofugal pathway. Their immunohistochemical study on OT has shown that GluA1 is located in layers 2–5, 9, 10, and 13, whereas GluA2/3 is found in layers 9, 10, and 13. GluA4 was not detected in any layers. Expression of mRNAs for AMPA receptor subunits was reported in the pigeon OT by *in situ* hybridization using radioisotope-labeled probes. GluA2 mRNA expression was prominent in all neuronal cell layers, whereas the expression of GluA1, GluA3, and GluA4 mRNAs was weaker (Ottiger et al., 1995; Islam and Atoji, 2008). Dye and Karten (1996) reported that non-NMDA antagonists CNQX and DNQX, as well as NMDA antagonist AP5, eliminated the postsynaptic field potential in the pigeon OT. However, the expression patterns of KA and NMDA receptor subunits have not been elucidated, although these subunits are expected to be involved in glutamatergic transmission in the pigeon OT. GluA4 immunoreactivity and GluA2 mRNA expression have been observed in Rt, and GluA2/3 immunoreactivity and GluA2 and GluA3 mRNAs expression have been observed in the entopallium (Ottiger et al., 1995; Theiss et al., 1998), suggesting that AMPA receptor subunits are strongly expressed in the Rt and the entopallium. However, as in OT, the localization of KA and NMDA receptors remains unknown in Rt or entopallium.

In contrast to the excitatory glutamate, γ-amino butyric acid (GABA) is the major inhibitory neurotransmitter, and GABA and glutamic acid decarboxylase (GAD), an enzyme involved in GABA synthesis, have been widely used to identify GABAergic neurons in the brain. In the tectofugal pathway in birds, GAD65 mRNA is expressed in layers 3–5 and 9–10 of OT and is weakly expressed in the entopallium (Sun et al., 2005). GABAergic neurons appear to modify or regulate visual information in major glutamatergic circuits in the tectofugal pathway.

In the present study, *in situ* hybridization using RNA probes was carried out to examine the detailed localization of mRNAs of AMPA receptor subunits (GluA1, GluA2, GluA3, and GluA4), KA receptor subunits (GluK1, GluK2, and GluK4), and NMDA receptor subunits (GluN1 and GluN2A) in the tectofugal pathway including Imc and Ipc in the pigeon. In addition, vGluT2 and GAD65 mRNA expression was examined.

EXPERIMENTAL PROCEDURES

Animals

Adult pigeons (*Columba livia*, four males and two females, 283–405 g) were housed in cages (one animal per cage) with food and water accessible *ad libitum*. Experimental protocols including animal handling procedures were approved by the Committee for Animal Research and Welfare of Gifu University.

Real-time polymerase chain reaction (RT-PCR)

Stocked OT cDNA, which had been made in a previous study (Atoji, 2015) and kept at -60 °C, was used for RT-PCR. The cDNA was mixed with Takara Ex Taq (Takara Bio, Tokyo, Japan), dNTP mixture, EX Taq buffer, forward and reverse primers. Primers for vGluT2, iGluR subunits, GAD65, and β-actin (positive control) were designed based on pigeon cDNA sequences registered in a GenBank database and the primer sequences are shown in Table 1. PCR reactions were carried out by 35 cycles of amplification (denaturation at 94 °C for 30 s, annealing at 56 °C for 40 s, extension at 72 °C for 1 min) and a final extension was done at 72 °C for 5 min. PCR products were measured in gel electrophoresis (1.8% agarose) with lader markers.

Probe preparation

Antisense and sense RNA probes for vGluT2, iGluRs, and GAD65 were labeled with digoxigenin (DIG) using a DIG RNA labeling kit (Roche Diagnostics GmbH, Mannheim, Germany). Namely, forward primer attached T7 promoter sequence in 5′ terminal (5′-TAATACGACT CACTATAGGG-3′) and reverse primer attached Sp6 promoter sequence in 5′ terminal (5′-ATTTAGGTGACAC TATAGAA-3′) were used for RT-PCR to obtain PCR products of vGluT2, iGluRs, and GAD65 from total RNA extracts (Table 1). PCR products were purified using Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI, USA) and were mixed with reagents of a DIG RNA labeling kit (10× NTP labeling mixture, 10x transcription buffer, RNase inhibitor, polymerase T7 for sense or Sp6 for antisense RNA probe, and diethyl pyrocarbonate (DEPC)-treated water). After incubation at 37 °C for 4 h, 8 M LiCl and 100% ethanol were added into the mixture. Then, the mixture was incubated at −20 °C overnight. Synthesized RNA transcripts were collected by centrifugation and were aliquoted into 100 ng/μl with DEPC-treated water.

In situ hybridization

The procedure for non-radioactive *in situ* hybridization was described previously (Atoji, 2015). Animals were deeply anesthetized with sodium pentobarbital (50 mg/kg) and perfused with Ringer's solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Brains were removed from the skull and were stored in the same fixative for 2–3 days. They were transferred in 0.1 M PB containing 30% sucrose at 4 °C for 3–4 days. Serial cross sections were cut at 30–40-μm-thickness on a cryostat and were mounted onto glass slides coated with poly-L-lysine and (3-aminopropyl) triethoxysilane. After fixation with 4% paraformaldehyde in 0.1 M PB at room temperature for 30 min, the sections were washed in phosphate-buffered saline (PBS) three times. Proteinase