Vesicular Glutamate Transporter 2 and Glutamate Receptors as Cues to the Glutamatergic Circuits in the Brain of the Zebra Finch *(Taeniopygia guttata)*

（ゼブラフィンチ脳におけるグルタミン酸作動性回路に関わる2型小胞性グルタミン酸トランスポーターとグルタミン酸受容体）

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Contents

General Introduction

Songbirds, much like human, learn their vocalizations by imitating adult conspecifics (Marler, 1997). Birdsong learning is a widely used model for studying the neural mechanisms of learning and memory. In the most commonly studied songbird species, the zebra finch, only males sing and females not sing. The male is the sex that most often demonstrates vocal learning. In male zebra finches, song production and maintenance involve networks of interconnected brain nuclei, known as the song system (Nottebohm et al., 1976; Wild, 1997; Brainard and Doupe, 2002; Zeigler and Marler, 2004; Mooney, 2009), which consist of two pathways (Fig. 1). The posterior forebrain pathway, or motor pathway, connects the HVC (letter-based proper name; Reiner et al., 2004), the robust nucleus of the arcopallium (RA), and the tracheosyringeal motor nucleus of the hypoglossal nerve (nXIIts) (Nottebohm et al., 1976; Wild, 1993). Additionally, the RA also projects to the dorsomedial nucleus of the intercollicular complex (DM) (Wild et al., 1997). The anterior forebrain pathway is a loop that projects from area X through a thalamic relay (medial nucleus of the dorsolateral thalamus, DLM) to the lateral magnocellular nucleus of the anterior nidopallium (LMAN) and then back to area X (Bottjer et al., 1989; Vates et al., 1997; Luo et al., 2001). The posterior and anterior forebrain pathways interact via connection through the HVC to area X and the LMAN to the RA (Bottjer et al., 1989; Vates et al., 1997; Zeigler and Marler, 2004; Fig. 1).

In addition to these two pathways, an auditory pathway is involved in audition and auditory learning in songbirds. The ascending auditory pathway has been characterized in pigeons (Karten, 1967, 1968; Boord, 1968) and in songbirds (Kelley and Nottebohm, 1979; Vates et al., 1996; Krützfeldt et al., 2010a, b; Wild et al., 2010). This pathway is

generally the same for both songbirds and non-songbirds. The cochlear nerve projects to both the magnocellular (NM) and angular (NA) nuclei that in turn project to the superior olivary nucleus (OS) via separate routes: NM → laminar nucleus (NL) →OS and NA → OS. Thereafter, the pathway from OS to field L passes through a single route: OS → dorsal part of the lateral mesencephalic nucleus (MLd) → ovoidal nucleus (Ov) → field L (Fig. 2). The field L complex in songbirds project to caudomedial nidopallium (NCM), HVC shelf and RA-cup regions (Kelley and Nottebohm, 1979; Vates et al., 1996). Thus, the auditory and vocal pathways interact via connection through the field L to HVC-shelf or to RA-cup region (Fig. 2). The NCM and caudomedial mesopallium (CMM) are thought to contain the neural substrate for tutor song memory (Bolhuis et al., 2000; Bolhuis and Gahr, 2006; Gobes and Bolhuis, 2007) and these two regions are reciprocally connected (Vates at al., 1996). In the descending motor pathway which extends from the telencephalon to the tracheosyringeal motor nucleus in the brainstem, the DM receives afferents from the RA, and the retroambigual nucleus (RAm) receives afferents from the RA and DM (Wild, 1993; Kubke et al., 2005; Wild et al., 2009). The tracheosyringeal motor nucleus receives excitatory inputs from the RA and RAm (Kubke et al., 2005).

Excitatory and inhibitory transmitters (glutamate and GABA) and their receptor activation are involved in the modification of neural circuits in song control nuclei for altering song behavior (Basham et al., 1996; Mooney and Prather, 2005; Sizemore and Perkel, 2008). Electrophysiological studies investigating neurotransmission in the song system indicate that γ-aminobutyric acid (GABA) evokes inhibitory potentials in the HVC and RA (Luo and Perkel, 1999; Rosen and Mooney, 2006). Furthermore, immunohistochemical studies found that GABA is localized in somata and axon terminals in song nuclei, such as the HVC, RA, LMAN, and area X (Grisham and

Arnold, 1994; Luo and Perkel, 1999; Pinaud and Mello, 2007). GABA receptors have been identified in these nuclei as well (Thode et al., 2008). In contrast, it is reported that Hebbian-like processes of synaptic change are coupled with NMDA receptor activation in specific song nuclei, and pharmacological blockades of NMDA receptors can impair vocal learning (Basham et al., 1999, Heinrich et al., 2002). Pharmacological and electrophysiological studies have identified ionotropic glutamate receptors in the HVC, LMAN, RA, and caudomedial nidopallium (Mooney and Konishi, 1991; Basham et al., 1999; Pinaud et al., 2008). A previous study determined the presence of AMPA, kainate and NMDA receptors (cDNA sequence and mRNA) in the vocal nuclei or areas of the adult male zebra finch brain (Wada et al., 2004). In conjunction with data from electrophysiological studies, these finding indicate a role for the glutamatergic neurons and circuits in the song system. However, the glutamatergic system has not yet been considered in detail in the songbird brain. Thus, evaluation of the mRNA expression of the vesicular glutamate transporter (VGLUT) and various glutamate receptors in the brain or unexplored brain regions and nuclei are necessary.

The storage and release of glutamate in excitatory circuits in the mammalian brain is regulated by the vesicular glutamate transporters (VGLUTs) and glutamate receptors (Collingidge et al., 1989; Fremeau et al., 2004a, 2004b, 2001; Gras et al., 2002; Herzog et al., 2001; Kaneko and Fujiyama, 2002; Kaneko et al., 2002; Takamori, 2006; Takamori et al., 2000, 2001). VGLUTs accumulate glutamate into synaptic vesicles of glutamatergic neurons at the presynaptic terminals, and glutamate released from the vesicles binds to glutamate receptors on postsynaptic membranes (Newpher and Ehlers, 2008; Santos et al., 2009). Three types of VGLUTs have been identified in mammals: VGLUT1, VGLUT2, and VGLUT3. The mRNA for VGLUT1 and VGLUT2 are present in the majority of glutamatergic neurons in the brain, whereas VGLUT3 is sparsely

distributed and is found in a discrete subpopulation of non-glutamatergic neurons (Ni et al., 1994; Bellocchio et al., 1998; Fremeau et al., 2001; Herzog et al., 2001; Gras et al., 2002). VGLUT1 and VGLUT2 have been considered as specific biomarkers for glutamatergic neurons. In birds, chicken VGLUT2 (JF320001) and VGLUT3 (XM_425451) genes sequences have been registered in a gene database, but the VGLUT1 gene has not been found. Islam and Atoji (2008) first cloned a cDNA sequence for pigeon VGLUT2 (FJ428226) and mapped that VGLUT2 mRNA is distributed in the neuronal cell bodies of the pallium of the telencephalon, in many nuclei in the thalamus, midbrain, discrete brainstem nuclei, and in granule cells of the cerebellar cortex. In both in mammals and birds, VGLUT2 mRNA distribution has been found in the somata of neurons, and thus its expression could utilized to identify the origin of glutamatergic projections in neuronal circuits. On the other hand, VGLUT2 immunoreactivity is preferentially observed in the excitatory presynaptic terminals of asymmetric synapses in rats (Fremeau et al., 2001; Kaneko et al., 2002), and pigeons (Atoji, 2011), indicating the projection terminals of the glutamatergic neurons in the neuronal circuits. The expression of VGLUT2 mRNA and protein in the brain has not yet been described in any songbird species.

Neurons receiving glutamatergic afferents express the mRNA of ionotropic glutamate receptor subunits in the soma. Therefore, the projection targets of glutamatergic neurons in the neuronal circuits could also be identified using the expression patterns of these mRNAs. In mammalian brains, ionotropic glutamate receptors are widely distributed and are defined according to the binding of selective agonists as α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate, or N-methyl-D-aspartate (NMDA) type receptors (Collingridge and Lester, 1989, Conti et al., 1994; Muñoz et al., 1999). In birds, the mRNAs of AMPA-type receptors are

expressed in the pigeon brain (Ottiger et al., 1995; Islam and Atoji, 2008), and the mRNAs of AMPA, kainate and NMDA receptors are expressed in the telencephalic song nuclei (LMAN, HVC, RA and area X) and related areas (DLM and DM) of the zebra finch brain (Wada et al., 2004). However, the distributions of glutamate receptor subunits in the auditory nuclei or areas of the telencephalon, thalamus and lower brainstem remain unclear in the zebra finch.

In the present study, the origins and putative targets of glutamatergic neurons in the zebra finch brain were examined with a particular focus on nuclei or areas within auditory and song systems. VGLUT2 mRNA and the mRNAs of five ionotropic glutamate receptor subunits (at least one subunit from each type of ionotropic glutamate receptor: GluA1, GluA4, GluK1, GluN1, and GluN2A) were evaluated using in situ hybridization, and VGLUT2 protein was assessed by immunohistochemical analysis.
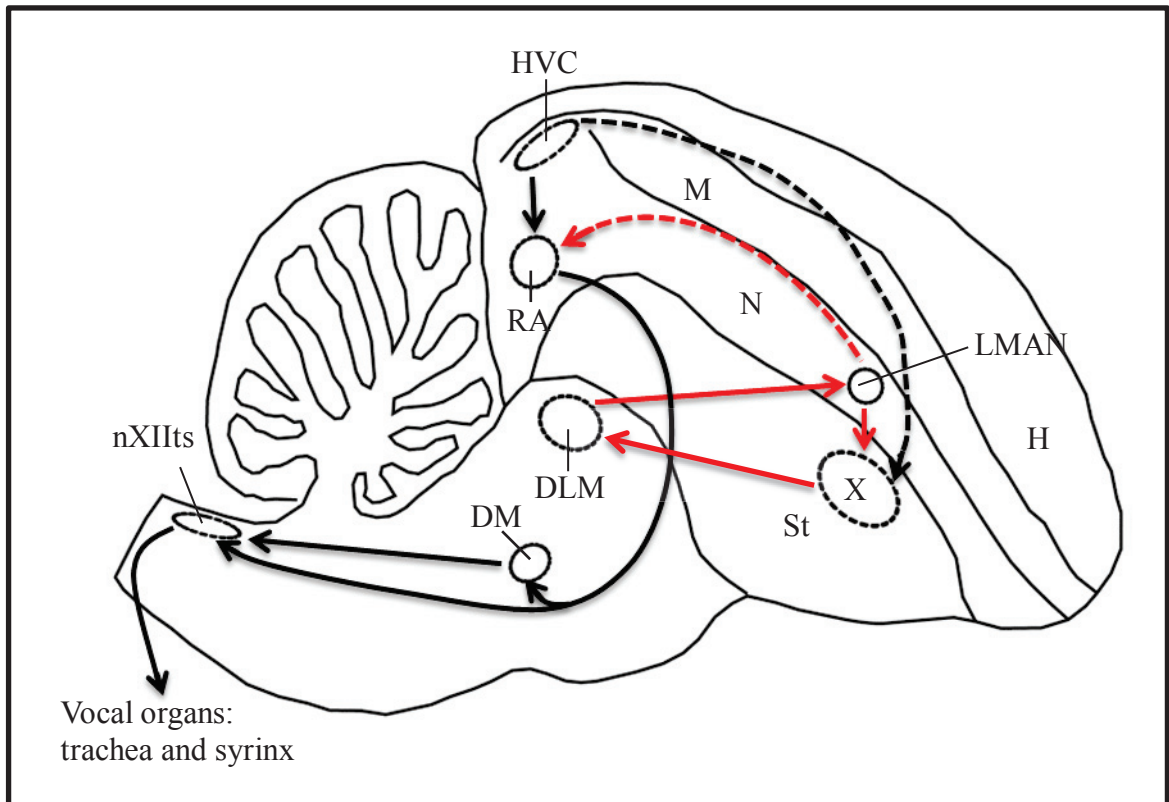
Fig. 1. Schematic longitudinal section of zebra finch brain showing the song pathways with known connections. Black arrows represent the connections of the motor or posterior forebrain pathway (Nottebohm et al., 1976; Wild et al., 1997); red arrows represent the connections of the anterior forebrain pathway (Bottjer et al., 1989; Vates and Nottebohm, 1995; Vates et al., 1997; Luo et al., 2001), and dashed line arrows show connection between the two pathways (Bottjer et al., 1989; Vates et al., 1997; Zeigler and Marler, 2004). DLM, medial nucleus of the dorsolateral thalamus; DM, dorsomedial nucleus of the intercollicular complex; H, hyperpallium; HVC, letter-based proper name; LMAN, lateral magnocellular nucleus of the anterior nidopallium; M, mesopallium; N, nidopallium; RA, robust nucleus of arcopallium; St, striatum; nXIIts, tracheosyringeal motor nucleus of the hypoglossal nerve; X, area X.
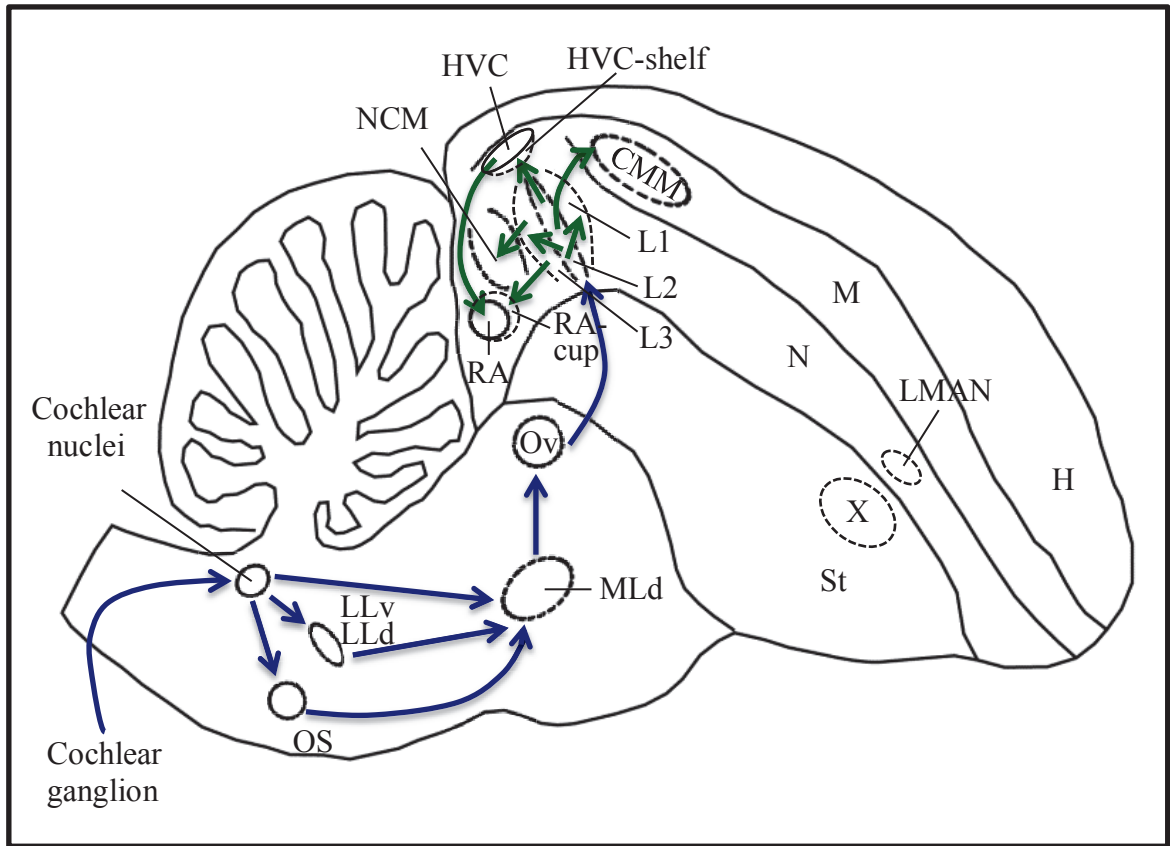
Fig. 2. Schematic longitudinal section of zebra finch brain showing the auditory pathways, with the known connections. Blue color arrows show the major ascending auditory pathway, which ends in field L2 (Karten, 1967, 1968; Kelley and Nottebohm, 1979; Krützfeldt et al., 2010a, b; Wild et al., 1993, 2010); green color arrows show some connections in auditory brain regions and with the HVC shelf and RA-cup regions (Vates et al., 1996; Kelley and Nottebohm, 1979). The field L complex project to caudomedial nidopallium, HVC-shelf and RA-cup regions (Kelley and Nottebohm, 1979; Vates et al., 1996). CMM, caudomedial mesopallium; H, hyperpallium; HVC, letter-based proper name; LLd, dorsal nucleus of the lateral lemniscus,; LLv, ventral nucleus of the lateral lemniscus; LMAN, lateral magnocellular nucleus of the anterior nidopallium; M, mesopallium; MLd, dorsal part of the lateral mesencephalic nucleus; N, nidopallium; NCM, caudomeial nidopallium; Ov, ovoidal nucleus; OS, superior olivary nucleus; RA, robust nucleus of arcopallium; St, striatum; X, area X.

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Chapter 1

Gene sequence and distribution of zebra finch vesicular glutamate transporter 2 mRNA

1.1. Introduction

Glutamate, a neurotransmitter used by a majority of excitatory connections in the mammalian brain and glutamatergic transmission is critical for controlling neural activity. Glutamate is loaded into synaptic vesicle by means of vesicular glutamate transporters before its exocytotic release. Three types of VGLUTs have been identified in mammals: VGLUT1 (Ni et al., 1994; Bellocchio et al., 1998), VGLUT2 (Fremeau et al., 2001; Herzog et al., 2001), and VGLUT3 (Fremeau et al., 2002; Gras et al., 2002; Schäfer et al., 2002; Takamori et al., 2002). VGLUT1 and VGLUT2 mRNAs are mostly present in glutamatergic neurons, and VGLUT3 mRNA is expressed not only in other types of neurons that use acetylcholine, serotonin, and γ-aminobutyric acid (GABA) as neurotransmitters, but also in astrocytes (Takamori et al., 2000; Bai et al., 2001; Gras et al., 2002; Herzog et al., 2004; Kawano et al., 2006). The identification of VGLUT1 and VGLUT2 are major breakthrough in search for molecular marker for glutamatergic neurons. In general, VGLUT1 mRNA is massively present in excitatory glutamatergic neurons from the cerebral and cerebellar cortices, and hippocampus, whereas most glutamatergic neurons from the diencephalon and rhombencephalon preferentially express VGLUT2 mRNA (Bai et al., 2001; Fremeau et al., 2001; Herzog et al., 2001). Together, VGLUT1 and VGLUT2, with their complementary distributions, seem to account for most of the known glutamatergic neurons of brain (Fremeau et al., 2001; Varoqui et al., 2002). In birds, Islam and Atoji (2008) cloned a cDNA sequence

for pigeon VGLUT2 (FJ428226) and demonstrated that VGLUT2 mRNA is distributed in the cell bodies of glutamatergic neurons in the pigeon brain. In rats and pigeons, VGLUT2 mRNA distribution has been found in the somata of neurons, and thus its expression could utilized to identify the origin of glutamatergic projections in neuronal circuits.

In songbirds, pharmacological or electrophysiological studies indicate a pivotal role for the glutamatergic neurons or circuits in the song system (Basham et al., 1996; Mooney and Prather, 2005; Sizemore and Perkel, 2008). However, distribution of glutamatergic neurons in the brain of songbirds has not been identified before. In the present study, I determined the cDNA sequence of zebra finch VGLUT2 mRNA and then demonstrated the distribution of its mRNA-expressing glutamatergic neuron in the zebra finch brain including auditory and song systems by in situ hybridization histochemistry.

1.2. Materials and Methods

Animals

Ten adult male zebra finches (*Taeniopygia guttata*, body weight: 11-22g and age: 4-7 months) were used in the present study. I examined only males, because usually song control nuclei are larger in volume, cell size and cell number relative to those of female (Nottebohn and Arnold, 1976; Nordeen et al., 1987) and most often demonstrates vocal learning. Animal handling procedures were approved by the Committee for Animal Research and Welfare of Gifu University. Two animals were used for the reverse transcription-polymerase chain reaction (RT-PCR), eight animals were used for in situ hybridization. For isolation of total RNA, the telencephalon,

thalamus, optic tectum, cerebellum and lower brainstem were dissected out quickly and kept in RNA stabilization solution (RNA*later*, Ambion, Austin, TX, USA) and stored at -60°C until use. For in situ hybridization, fresh brains were quickly removed and immediately frozen on powdered dry ice. Serial transverse or longitudinal sections were cut at 30 μm thickness on a cryostat, thaw-mounted onto the 3-aminopropyltriethoxysilane coated slides, and stored at -30°C until use.

RNA isolation, cDNA synthesis and PCR amplification

Total RNA was isolated from the zebra finch brain samples (telencephalon, thalamus, optic tectum, cerebellum and lower brainstem) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Briefly, each brain sample was homogenized in TRIzol reagent followed by 5 minutes incubation at room temperature. Then appropriate volume of chloroform was added and mixed vigorously. The sample was then centrifuged at 12,000g for 15 minutes at 4°C. The supernatant fluid was collected, mixed with same volume of isopropanol, and centrifuged at 12,000g for 15 minutes at 4°C to precipitate total RNA. After washing in 75% ethanol, the precipitate was dissolved into diethyl pyrocarbonate treated water, checked the concentration by Biophotometer plus (Eppendolf AG, Hamburg, Germany), and preserved at -60°C until use.

First-strand complementary DNA (cDNA) was synthesized using Superscript III First-Strand Synthesis System (Invitrogen). Briefly, 0.5 μg of total RNA was mixed with 2.5 μM of oligo-dT primer and 0.5 mM of 2′-deoxyribonucleotide 5′-triphosphates (dNTP) mixture, incubated at 65°C for 5 minutes and put on ice. Supplied reaction buffer of the enzyme, 5 mM of dithiothreitol, 2 units of RNase out and 10 units of Superscript III reverse transcriptase were added to the mixture and incubated at 50°C

for 60 minutes, then the reaction was stopped by heating at 70°C for 15 minutes and the synthesized product was preserved at -30°C until use.

For polymerase chain reaction (PCR), 500 ng of the synthesized cDNA was mixed with Takara Ex Taq (Takara Bio Inc., Tokyo, Japan), supplied dNTP mixture and EX Taq buffer, then 1 μM of appropriate forward and reverse primers were added. The primers for VGLUT2 were designed based on the cDNA sequences of the pigeon VGLUT2 (FJ428226), chicken VGLUT2 (JF320001), and the partial cDNA sequence of zebra finch VGLUT2 obtained in the present study. β-actin was selected as a positive control and its primers were designed based on chicken β-actin (NM_205518). The primers is shown in Table 1. PCR was performed by 35 cycles of amplification (denaturation at 94°C for 30 seconds, annealing at 57°C for 40 seconds, extension at 72°C for 1 minute) and a final extension at 72°C for 5 minutes. Obtained PCR product was refined by a Wizard SV gel and PCR clean-up system (Promega, Madison, WI, USA) and the refined sample was forwarded for sequencing.

Sequence analysis

The sequences of respective cDNA fragments were analyzed by ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster, CA, USA). The obtained zebra finch nucleotide and encoded amino acid sequences of VGLUT2 were compared with the nucleotide and amino acid sequences of the other birds and mammals. The following sequences were used for VGLUTs: chicken VGLUT2 (JF320001), chicken VGLUT3 (XP_425451), zebra finch VGLUT3 (XP_002190363), pigeon VGLUT2 (FJ428226), human VGLUT1 (NP_064705), human VGLUT2 (NM_020346), human VGLUT3 (NP_647480), rat VGLUT1 (NP_446311), rat VGLUT2 (NM_053427), rat VGLUT3 (NP_714947), mouse VGLUT1 (NP_892038), mouse VGLUT2 (NM_080853), and mouse VGLUT3 (NP_892004).

In situ hybridization

Slide-mounted sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 minutes at room temperature, rinsed 3 times in 4x standard saline citrate (SSC; pH 7.4; 1x 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 minutes, and immersed in 100% ethanol twice for 5 minutes. Hybridization was performed by incubating the sections at 41°C for overnight with the following buffer : 4x SSC, 50% deionized formamide, 0.12M phosphate buffer (pH 7.4), 1% Denhardt's solution (Nacalai Tesque, Kyoto, Japan), 250 µg/ml yeast tRNA (Roche, Mannheim, Germany), 10% dextran sulfate (Nacalai Tesque), and 20 mM dithiothreitol. The buffer contained ^{35}S-dATP (46.25 TBq/mmol; PerkinElmer Life Science, Waltham, MA, USA) labeled oligonucleotide probe at the concentration of approximately 1-2 x 10^7 dpm/ml. The probe was labeled at 3'-end with ^{35}S-dATP by terminal deoxynucleotidyl transferase (Takara) before hybridization. After hybridization, sections were washed in 1x SSC (pH 7.4), then dehydrated through a graded ethanol series (70%–100%), and exposed to X-ray films (Fuji Medical X-Ray Film, Tokyo, Japan) for 7 days. After X-ray film autoradiography, 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 development, the sections were fixed, washed and dehydrated. Some sections were counterstained with 0.1% cresyl violet.

Oligonucleotide probes

Antisense and sense oligo DNA probes of VGLUT2 were designed based on the zebra finch VGLUT2 cDNA sequence obtained in the present study, and synthesized

commercially (Rikaken, Nagoya, Japan). Zebra finch VGLUT2 anti-sense probe (VGLUT2-AS) was complementary to bases 1,707-1,742 (Table 1). Sense probe (VGLUT2-S) was complementary to the antisense probe. The sequence of the zebra finch VGLUT2-AS probe region shows homology against VGLUT2 cDNA sequence of pigeon (bases 1,699-1,734; FJ428226) with 100%, chicken (bases 1,699-1,734; JF320001) with 94%, rat (bases 1,699-1,737; NM_053427) and mouse (bases 1,699-1,737; NM_080853) with 69% and human (bases 1,699-1,737; NM_020346) with 78%, and less than 52% homology with any other non-VGLUT2 related sequences in a gene bank data base.

Image processing

Photographs at low-power magnification were taken with a scanner (Epson GT-9300UF, Tokyo, Japan). Photomicrographs at high-power magnification were taken with a digital camera (Nikon, DS-Fi1, Tokyo, Japan) mounted on a light microscope. Adjustment of photographs for contrast, brightness and sharpness, layout, and lettering were performed using Adobe Photoshop 7.0J (Tokyo, Japan) and Adobe Illustrator 10.0J (Tokyo, Japan).

Nomenclature

The nomenclature used here is based on available avian brain atlases, including pigeon (Karten and Hodos, 1967), Digital Atlas of the Zebra Finch (*Taeniopygia guttata*) Brain (Karten et al., 2013), as well as a recent publication on zebra finch neuroanatomy (Jarvis et al., 2013).The revised avian brain terminology recommended by the avian brain Nomenclature Forum (Reiner et al., 2004).

1.3. Results

The initial analysis of VGLUT2 expression in the different brain regions utilized reverse transcription of RNA followed by DNA amplification (RT-PCR) and sequencing. In situ hybridization with VGLUT2 oligonucleotides probe was subsequently used for distribution of VGLUT2 mRNA in zebra finch brain.

RT-PCR and cDNA sequence of VGLUT2

High level expressions of VGLUT2 mRNA were observed in the telencephalon, thalamus, optic tectum, cerebellum, and lower brainstem of the zebra finch by RT-PCR (Fig. 3.1). A cDNA sequence of 1,779 base pairs containing 8 base pairs of 5′ untranslated region, 1,746 base pairs of a single open reading frame and 25 base pairs of 3′ untranslated region was obtained for zebra finch VGLUT2 gene from PCR products. The open reading frame sequences of zebra finch VGLUT2 showed 94% identity for pigeon (FJ428226) and chicken (JF320001), and 81%, 82%, 83% identity for rat (NM_053427), mouse (NM_080853) and human (NM_020346)VGLUT2, respectively. The open reading frame sequences encoded 581 amino acids (Fig. 3.2). This encoded amino acids showed 99% identity for pigeon (ACJ64118) and chicken (ADX62354, Fig. 2), and 94% for human (NP_065079), rat (NP_445879) and mouse (NP_543129) VGLUT2 amino acids.

Distribution of VGLUT2 mRNA

In situ hybridization, an antisense probe showed a differential expression VGLUT2 mRNA in the adult male zebra finch brain, including many nuclei or areas in auditory and song systems (Figs. 3.3A-F; 3.4A-D, Table 2). The hybridization signal intensity

was evaluated as follows: mesopallium (Fig. 3.3 A, B), nidopallium (Fig. 3.3 C), and tracheosyringeal motor nucleus of the hypoglossal nerve (Fig. 3.4A) were high, moderate, or weak, respectively. A sense probe of VGLUT2 mRNA did not show specific hybridization signal in X-ray film autoradiogram (Fig. 3.4E). Detail patterns of VGLUT2 mRNA expression in the zebra finch brain were described below.

As previously found in the pigeon brain, within the telencephalon, we found that in the zebra finch, the pallium expressed high VGLUT2 mRNA levels whereas the subpallium (striatum and pallidum) was devoid of it (Figs. 3.3A-D, 3.4A-D). Within the pallium, VGLUT2 mRNA expression was highest in the mesopallium, and intermediate but still high in the nidopallium, hyperpallium, arcopallium, and hippocampus (Figs. 3.3A-C, 3.4A-D). The labeled mesopallial regions, and the relative expression in them to the nidopallium and hyperpallium are consistent with a recent revised view of avian brain organization (Jarvis et al., 2013; Chen et al., 2013); that is this study is using the same terminology of Jarvis et al. (2013), as opposed to Reiner et al. (2004). Within the zebra finch auditory pathway, as in pigeons, the auditory nuclei show similar expression as their surrounding brain subdivisions. For example, the caudomedial mesopallium (CMM) has similar high expression as the surrounding mesopallium and the caudomedial nidopallium (NCM) has similar expression as the intermediate levels in the surrounding nidopallium (Fig. 3.4A, 3.5A, C). Moderate expression was seen in the interfacial nucleus (NIf), fields L1 and L3, but field L2a and entopallium showed weak expression (Figs. 3.4C, 3.5B). In contrast, in the zebra finch song nuclei, VGLUT2 mRNA expression patterns differed from the surrounding brain subdivisions. In all three major pallial song nuclei (HVC, RA, and LMAN) VGLUT2 mRNA levels were higher than the respective surrounding brain subdivisions (Figs. 3.3B, E, F, 3.4C, D, 3.6A, B, D). In addition, the HVC shelf and RA cup region showed weak expression of VGLUT2

mRNA (Fig. 3.6B, D). Cresyl violet-stained section indicated silver grains were localized on the cell bodies of neurons in the HVC (Fig. 3.6C). In the striatum, however, the area X was devoid of VGLUT2 mRNA similar to the surrounding striatum (Fig. 3.3B, 3.4C). But, weak expression is found in the septal commissural nucleus and pallial commissural nucleus, but septal nuclei are devoid of VGLUT2 mRNA (Fig. 3.3C).

Within the diencephalon, VGLUT2 mRNA expression was very high in the anterior portion of nucleus dorsolateralis anterior thalami, pars medialis (aDLM), which is a song nucleus part of medial nucleus of the dorsolateral thalamus (DLM) (Wada et al., 2004; Horita et al., 2012), and high in the surrounding dorsal thalamus (Figs. 3.3D, 3.4B, 3.6D). VGLUT2 mRNA expression was high in the ovoidal nucleus (Ov) and moderate in the rotundal nucleus and triangular nucleus (Figs. 3.3C-D, 3.4B, 3.5D). In the hypothalamus, the VGLUT2 mRNA signals was weak (Fig. 3.3D). In the pretectum, signal intensity of VGLUT2 mRNA was high to moderate in the pretectal and subpretectal nuclei, respectively (Figs. 3.3D, 3.5D).

Differential expression of VGLUT2 was found in the mesencephalon and rhombencephalon. Laminar distribution of VGLUT2 mRNA was observed in the optic tectum (Figs. 3.3D-F, 3.7A). Emulsion-coated sections indicated a high density of VGLUT2 labeled cells in layers 8 and 13 and a moderate density in layers 4, 11 and 15 (Fig. 3.7B). VGLUT2 mRNA showed high differential expression in the dorsomedial nucleus of the intercollicular complex (DM), a song nucleus (Jarvis and Nottebohm, 1997) compared with the adjacent midbrain (Fig. 3.7A). Nuclei of the descending motor pathway showed high or weak expression of VGLUT2 mRNA. In particular, high expression in the dorsal part of the lateral mesencephalic nucleus (MLd) (Figs. 3.3E, F, 3.7A), and weak expression in the retroambigual nucleus (RAm) and tracheosyringeal

motor nucleus of the hypoglossal nerve (nXIIts) (Figs. 3.4A, B, 3.7G, H). The parvocellular isthmic nucleus (Ipc) and intercollicular nucleus showed high expression of VGLUT2 mRNA, but magnocelluar isthmic nucleus was devoid of it (Figs. 3.3E, F, 3.7A). The ventral tegmental area (VTA) showed moderate expression of VGLUT2 mRNA (Fig. 3.4B). High signal was found in the principal sensory trigeminal nucleus. Moderate expression of VGLUT2 was also found in the vestibular nuclei (Fig. 3.7F). In the ascending auditory pathway, high expression was found in the ventral and dorsal nuclei of the lateral lemniscus (LLv and LLd) (Fig. 3.7D), and cochlear nuclei magnocellularis, angularis and laminar nucleus (NM, NA and NL) (Fig. 3.7F). The superior olivary nucleus (OS) and inferior olivary nucleus revealed weak expression of VGLUT2 mRNA (Fig. 3.7E, G). In the cerebellum, high VGLUT2 mRNA signal was found in the granular layer, but the Purkinje cell layer, molecular and white matter were devoid of VGLUT2 mRNA signals (Figs. 3.3E-F, 3.4A-B, 3.7C).

1.4. Discussion

In the present study, I determined the cDNA sequences of the zebra finch VGLUT2 and mapped the distribution of VGLUT2 mRNA in the brain of adult male zebra finch. In agreement with the high expression of VGLUT2 mRNA by RT-PCR, VGLUT2 mRNA-expressing neurons are widely distributed in the zebra finch brain and show a characteristic distribution pattern in many nuclei or areas of the brain including the auditory and song systems.

Comparison of zebra finch VGLUT2 gene with other birds and mammals

The nucleotide and deduced amino acid sequences of zebra finch VGLUT2 show a high degree of similarity in nucleotide and amino acid sequences in between the avian and mammalian VGLUT2 subtype. Whereas, the zebra finch VGLUT2 amino acid sequence shows low similarity (73 - 74%) with VGLUT3 amino acid sequences of birds and mammals (chicken: XP_425451, zebra finch: XP_002190363, human: NP_647480, rat: NP_714947, and mouse: NP_892004). Although the VGLUT1 subtype has not been identified in birds, zebra finch VGLUT2 amino acids has a 77% identity to human (NP_064705), rat (NP_446311), and mouse (NP_892038) VGLUT1. Therefore, the zebra finch VGLUT gene obtained in this study is strongly suggested to be a member of VGLUT2 subfamily in vertebrate VGLUT family.

Comparison of distribution of VGLUT2 mRNA other birds and mammals

Islam and Atoji (2008) used similar in situ hybridization techniques as in our current study to map the distribution of VGLUT2 mRNA in the central nervous system of a non-songbird species, the pigeon. The author found that VGLUT2 mRNA is highly expressed in the telencephalic pallium, thalamic nuclei, many brainstem nuclei, and the cerebellar cortex, but that is absent in the striatum and pallidum. The general expression patterns of VGLUT2 in the pigeon brain, including the auditory areas and primary sensory regions (field L and entopallium), are similar to those in the zebra finch. However, no differential expression patterns in the areas of the telencephalon where song nuclei are found in zebra finches were observed in pigeons. This is likely due to inherent variations between the two species and suggests that glutamatergic neurons exist in song control nuclei.

In the mammalian cerebrum, VGLUT1 and VGLUT2 mRNAs exhibit a

complementary expression patterns in the cortex but are not expressed in the subpallium except for weak VGLUT2 mRNA expression in the septal nuclei, nucleus of the diagonal band, and globus pallidus (Ni et al., 1994; Hisano et al., 2000; Fremeau et al., 2001). The cerebral cortex and hippocampus show a predominance of VGLUT1 mRNA expression whereas the diencephalon, brainstem, and deep cerebellar nuclei primarily express VGLUT2 mRNA. The cerebellar cortex exhibits an intense expression of VGLUT1 mRNA in granule cells, but does not express VGLUT2 mRNA. In contrast, in the pigeon and zebra finch, VGLUT2 mRNA is expressed in the entire pallium of the telencephalon, thalamus, optic tectum, cerebellar cortex and brainstem (Islam and Atoji, 2008; present study). These finding suggests a predominance expression of VGLUT2 mRNA in glutamatergic neurons in the avian brain whereas complementary utilization of VGLUT1 and VGLUT2 mRNA occurs in the mammalian brain.

1.5. Summary

In the present study, I identified a full length open reading frame cDNA sequence of the zebra finch VGLUT2 gene and demonstrated the distribution of its mRNA in the zebra finch brain including auditory and song systems by in situ hybridization histochemistry. The nucleotide and deduced amino acid sequences of zebra finch VGLUT2 share a high similarity to the other birds and mammals, which are higher than that of the VGLUT1 or VGLUT3 subtypes. In situ hybridization, within the telencephalon, the pallium expressed high VGLUT2 mRNA levels whereas the subpallium was devoid of it. Within the diencephalon, VGLUT2 mRNA signal was high in the thalamus than in the hypothalamus. Rich VGLUT2 mRNA expression was noted in the optic tectum and granular layer of the cerebellum. These results suggest that the

identified cDNA sequence of zebra finch VGLUT2 is comparable with that of VGLUT2 in other birds and mammals. The general distribution pattern of VGLUT2 mRNA-expressing glutamatergic neurons in the zebra finch and pigeon brains are similar. The distribution of zebra finch and pigeon VGLUT2 mRNA in the brain appear to correspond to those of expression by VGLUT1 and VGLUT2 in mammalian brains, that is VGLUT1 is mainly express in excitatory glutamatergic neurons from the cerebral and cerebellar cortices, whereas most glutamatergic neurons from the diencephalon and rhombencephalon preferentially express VGLUT2 mRNA.

Interestingly, high VGLUT 2 mRNA-expressing glutamatergic neurons are found in telencephalic, thalamic and midbrain auditory or song nuclei in the zebra finch brain. The nuclei of the ascending auditory pathway including NM, NA, NL, OS, MLd, and Ov showed high distribution of VGLUT2 mRNA-expressing glutamatergic neurons. The telencephalic auditory areas, field L subfields and the caudomedial nidopallium (NCM) exhibit mRNA signal of VGLUT2. Therefore, it seems that glutamatergic neurons are existed in the auditory pathway in the zebra finch brain.

VGLUT2 mRNA was seen in the cell bodies of neurons in the LMAN, HVC and RA, but area X devoid of VGLUT2 mRNA expression. In all three major pallial song nuclei VGLUT2 mRNA levels were higher than the respective surrounding brain subdivisions. In addition, the HVC-shelf and RA-cup region showed weak expression of VGLUT2 mRNA. In the descending motor pathway, VGLUT2 mRNA was detected in the DM, RAm and nXIIts. The present in situ hybridization assays for VGLUT2 mRNA confirm the presence of glutamatergic neurons in the HVC, RA and LMAN.

TABLE 1. List of primers and probes for PCR amplification and in situ hybridization

Primers	
Forward primers (source of sequence)	Reverse primers (source of sequence)
VGLUT2	
5'-CTGCAGGAATGGAGTCGGTA-3'(chicken)	5'-CGTGGATCATGCCGACTGTT-3'(zebra finch)
5'-GGGGGACAAATTGCCGACTT-3'(pigeon)	5'-TCGCTTGTCTGTTCAGGGTCT-3'(pigeon)
5'-ACTGGGATCCTGAAACAGTC-3'(pigeon)	5'-AAGTCGGCAATTTGTCCCCC-3' (zebra finch)
5'-ACCTTGTCTGGAATGGTATG-3'(chicken)	5'-CTGAGTGCAAACAATCACAATG-3'(chicken)
β-actin	
5'-TGCGTGACATCAAGGAGAAG-3'(chicken)	5'-CTTCTC CTTGATGTCACGCA-3' (chicken)
Probes	
Anti-sense probes (zebra finch)	Sense probes (zebra finch)
VGLUT 2	
5'-TCCTTCCTTGTAGTTGTATGAGTCTTGT ACTTCCTC-3	5'-GAGGAAGTACAAGACTCATACAACTACAA GGAAGGA-3'

TABLE 2. Regional intensity of VGLUT2 mRNA in the zebra finch brain.

Regions	mRNA intensity
Telencephalon	
Olfactory bulb	+++
Hyperpallium	++
Mesopallium	+++
Hippocampal formation	++
Nidopallium	++
Lateral magnocellular nucleus of the anterior nidopallium	+++
HVC	+++
HVC shelf region	+
Field L1	++
Field L2	+
Field L3	++
Nucleus interface of the nidopallium	++
Caudal nidopallium	++
Entopallium	+
Arcopallium	+++
Robust nucleus of the arcopallium	+++
RA cup region	+
Nucleus taeniae of the amygdalae	+++
Striatum	-
Area X	-
Globus pallidus	-
Lateral septal nucleus	-
Medial septal nucleus	-
Pallial commissural nucleus	+
Septal commissural nucleus	+
Diencephalon	
Thalamus	
Dorsolateral anterior nucleus of the thalamus	+++
Lateral part of dorsolateral anterior nucleus of the thalamus	+++
Medial part of dorsolateral anterior nucleus of the thalamus	+++
Anterior nucleus of DLM	+++
Dorsomedial posterior nucleus of the thalamus	+++
Ovoidal nucleus	+++
Rotundus nucleus	++
Triangular nucleus	++
Uvaeform nucleus	++
Lateral habenular nucleus	+
Medial habenular nucleus	++
Pretectal nucleus	+++
Subpretectal nucleus	++

TABLE 2 (Continued)

Regions	mRNA intensity
Hypothalamus	
Preoptic area	-
Supraoptic area	-
Tuberal area	-
Mammillary area	+
Mesencephalon	
Ventral tegmental area	++
Interpeduncular nucleus	-
Optic tectum	- to +++
Dorsal part of lateral mesencephalic nucleus	+++
Dorsomedial nucleus of the of the intercollicular complex	+++
Intercollicular nucleus	++
Isthmic nucleus, magnocellular part	-
Isthmic nucleus, parvocellular part	+++
Substantia nigra	+
Isthmo-opticus nucleus	++
Rhombencephalon	
Cerebellum	
Molecular layer	-
Purkinje cell layer	-
Granular layer	+++
Cerebellar nuclei	++
Pontine and medullary regions	
Principal sensory trigeminal nucleus	+++
Lateral pontine nucleus	++
Medial pontine nucleus	+
Locus coeruleus (A8)	+
Ventral nucleus of the lateral lemniscus	+++
Dorsal nucleus of the lateral lemniscus	++
Superior olivary nucleus	+
Magnocellular nucleus	+++
Angular nucleus	+++
Laminar nucleus	+++
Vestibular nuclei	++
Pontine reticular nucleus giganticellular part	+
Raphe nucleus	+
Inferior olivary nucleus	+
Retroambigual nucleus	+
Tracheosyringeal nucleus of the hypoglossal nerve	+

Hybridization intensity is evaluated as follows: mesopallium (3+, Fig. 3.3B), hyperpallium (2+, Fig.3.3A), and tracheosyringeal nucleus of the hypoglossal nerve (1+, Fig. 3.4A).
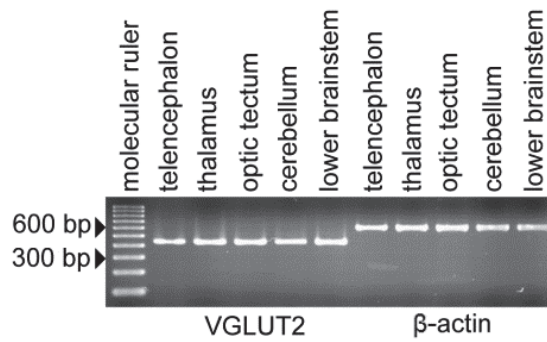
Fig. 3.1. Detection of VGLUT2 mRNA in RT-PCR. Single band (450bp) in each lane shows expression of VGLUT2 mRNA in telencephalon, thalamus, optic tectum, cerebellum, lower brainstem. β-actin (600bp) is used as a control.

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Zebrafinch  MESVKQRILTPGKEGLKNFAGKSLGQLYRVLEKRQKPGDTIELTEDGKPMEVPEKKAPLCDCTCFGLPRRYIIAIMSGLGFCIS  84
Pigeon      MESVKQRILTPGKEGLKNFAGKSLGQLYRVLEKRQKPGDTIELTEDGKPVEMPEKKAPLCDCTCFGLPRRYIIAIMSGLGFCIS  84
Chicken     MESVKQRILTPGKEGLKNFAGKSLGQLYRVLEKRQKPGDTIELTEDGKPMEMPEKKAPFCDCTCFGLPRRYIIAIMSGLGFCIS  84
Human       MESVKQRILAPGKEGLKNFAGKSLGQIYRVLEKKQDTGETIELTEDGKPLEVPERKAPLCDCTCFGLPRRYIIAIMSGLGFCIS  84
            ********* **************** ****** *   * ********* * ** *** ************************

Zebrafinch  FGIRCNLGVAIVDMVNNSTIHRGGKIIKEKAKFNWDPETVGMIHGSFFWGYIITQIPGGYISSRLAANRVFGAAILLTSSLNML  168
Pigeon      FGIRCNLGVAIVDMVNNSTIHRGGKIIKEKAKFNWDPETVGMIHGSFFWGYIITQIPGGYISSRLAANRVFGAAILLTSSLNML  168
Chicken     FGIRCNLGVAIVDMVNNSTIHRGGKIIKEKAKFNWDPETVGMIHGSFFWGYIITQIPGGYISSRLAANRVFGAAILLTSSLNML  168
Human       FGIRCNLGVAIVDMVNNSTIHRGGKVIKEKAKFNWDPETVGMIHGSFFWGYIITQIPGGYIASRLAANRVFGAAILLTSTLNML  168
            ************************* ********************************** ************** ****

Zebrafinch  IPSAARVHYGCVIFVRILQGLVEGVTYPACHGIWSKWAPPLERSRLATTSFCGSYAGAVIAMPLAGILVQYTGWSSVFYVYGSF  252
Pigeon      IPSAARVHYGCVIFVRILQGLVEGVTYPACHGIWSKWAPPLERSRLATTSFCGSYAGAVIAMPLAGILVQYTGWSSVFYVYGSF  252
Chicken     IPSAARVHYGCVIFVRILQGLVEGVTYPACHGIWSKWAPPLERSRLATTSFCGSYAGAVIAMPLAGILVQYTGWSSVFYVYGSF  252
Human       IPSAARVHYGCVIFVRILQGLVEGVTYPACHGIWSKWAPPLERSRLATTSFCGSYAGAVIAMPLAGILVQYTGWSSVFYVYGSF  252
            **********************************************************************************

Zebrafinch  GIVWYMFWLLVSYESPAKHPTITDEERRYIEESIGESANLLGAMEKYKTPWRKFFTSMPVYAIIVANFCRSWTFYLLLISQPAY  336
Pigeon      GIVWYMFWLLVSYESPAKHPTITDEERRYIEESIGESANLLGAMEKYKTPWREFFTSMPVYAIIVANFCRSWTFYLLLISQPAY  336
Chicken     GIVWYMFWLLVSYESPAKHPTITDEERRYIEESIGESANLLGAMEKYKTPWRKFFTSMPVYAIIVANFCRSWTFYLLLISQPAY  336
Human       GMVWYMFWLLVSYESPAKHPTITDEERRYIEESIGESANLLGAMEKFKTPWRKFFTSMPVYAIIVANFCRSWTFYLLLISQPAY  336
            * ********************************************* ***** **************************

Zebrafinch  FEEVFGFEISKVGILSAVPHLVMTIIVPIGGQIADFLRSRQIVSTTNVRKIMNCGGFGMEATLLLVVGYSHSKGVAISFLVLAV  420
Pigeon      FEEVFGFEISKVGILSAVPHLVMTIIVPIGGQIADFLRSKQILSTTTVRKIMNCGGFGMEATLLLVVGYSHSKGVAISFLVLAV  420
Chicken     FEEVFGFEISKVGILSAVPHLVMTIIVPIGGQIADFLRSKQILSTTTVRKIMNCGGFGMEATLLLVVGYSHSKGVAISFLVLAV  420
Human       FEEVFGFEISKVGMLSAVPHLVMTIIVPIGGQIADFLRSKQILSTTTVRKIMNCGGFGMEATLLIVVGYSHTRGVAISFLVLAV  420
            ************* ********************** ** *** ***************** ****** ***********

Zebrafinch  GFSGFAISGFNVNHLDIAPRYASILMGISNGVGTLSGMVCPIIVGAMTKNKTREEWQYVFLIAALVHYGGVIFYGIFASGEKQP  504
Pigeon      GFSGFAISGFNVNHLDIAPRYASILMGISNGVGTLSGMVCPIIVGAMTKNKTREEWQYVFLIAALVHYGGVIFYGIFASGEKQP  504
Chicken     GFSGFAISGFNVNHLDIAPRYASILMGISNGVGTLSGMVCPIIVGAMTKNKTREEWQYVFLIAALVHYGGVIFYGIFASGEKQP  504
Human       GFSGFAISGFNVNHLDIAPRYASILMGISNGVGTLSGMVCPIIVGAMTKNKSREEWQYVFLIAALVHYGGVIFYAIFASGEKQP  504
            *************************************************** ********************** *********

Zebrafinch  WADPEQTSEEKCGFIHEDELAEETGDITQNYVNYGTTKSYGATTQVNGGWPNGWEKKEEFVQEEVQDSYNYKE-GDYS      581
Pigeon      WADPEQTSEEKCGFIHEDELAEETGDITQNYVNYGTTKSYGATTQVNGGWPTGWEKKEEFVQEEVQDSYNYKE-GDYS      581
Chicken     WADPEQTSEEKCGFIHEDELAEETGDITQNYVNYGTTKSYGATTQVNGGWPNGWEKKEEFVQQDVQDSYNYKE-GDYS      581
Human       WADPEETSEEKCGFIHEDELDEETGDITQNYINYGTTKSYGATTQANGGWPSGWEKKEEFVQGEVQDSHSYKDRVDYS      582
            ***** ************** ********* ************ ***** ***** ********* **  ***
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Fig. 3.2. Deduced amino acid sequence of zebra finch VGLUT2 shows high similarity to the chicken (ADX62354), pigeon (ACJ64118) and human (NP_065079) VGLUT2. Identical amino acids are indicated by asterisks and the number of amino acids is shown at the right edge.
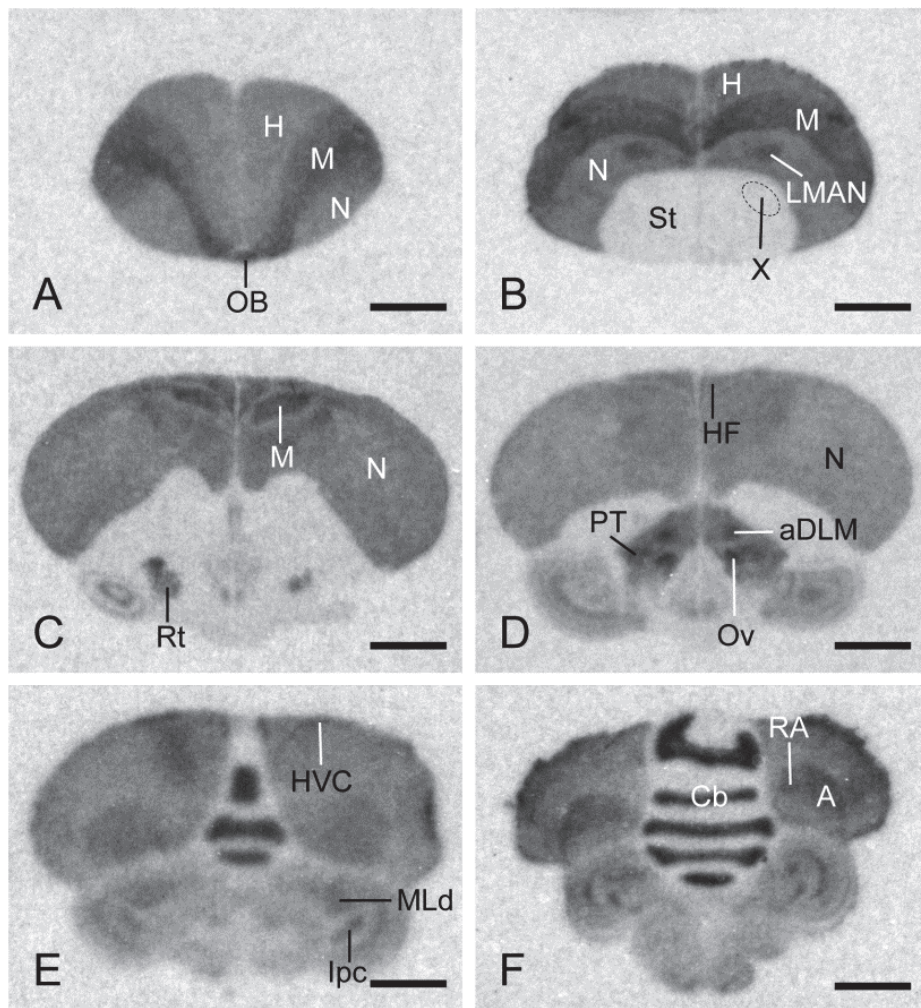
Fig. 3.3. In situ hybridization X-ray film autoradiograms show VGLUT2 mRNA distribution in transverse sections of the zebra finch brain (A-F). VGLUT2 mRNA highly expressed in the olfactory bulb (OB), mesopallium (M), lateral magnocellular nuclus of the nidopallium (LMAN), HVC, robust nucleus of the arcopallium (RA) of the telencephalon; in the anterior nucleus of the dorsal lateral medial thalamus (aDLM), ovoidal nucleus (Ov) of the thalamus; in the nucleus mesencephalicus lateralis, pars dorsalis (MLd) of the mid brain, and in the granular layer of the cerebellum. For other abbreviations, see list. Scale bars = 2 mm in A-F.
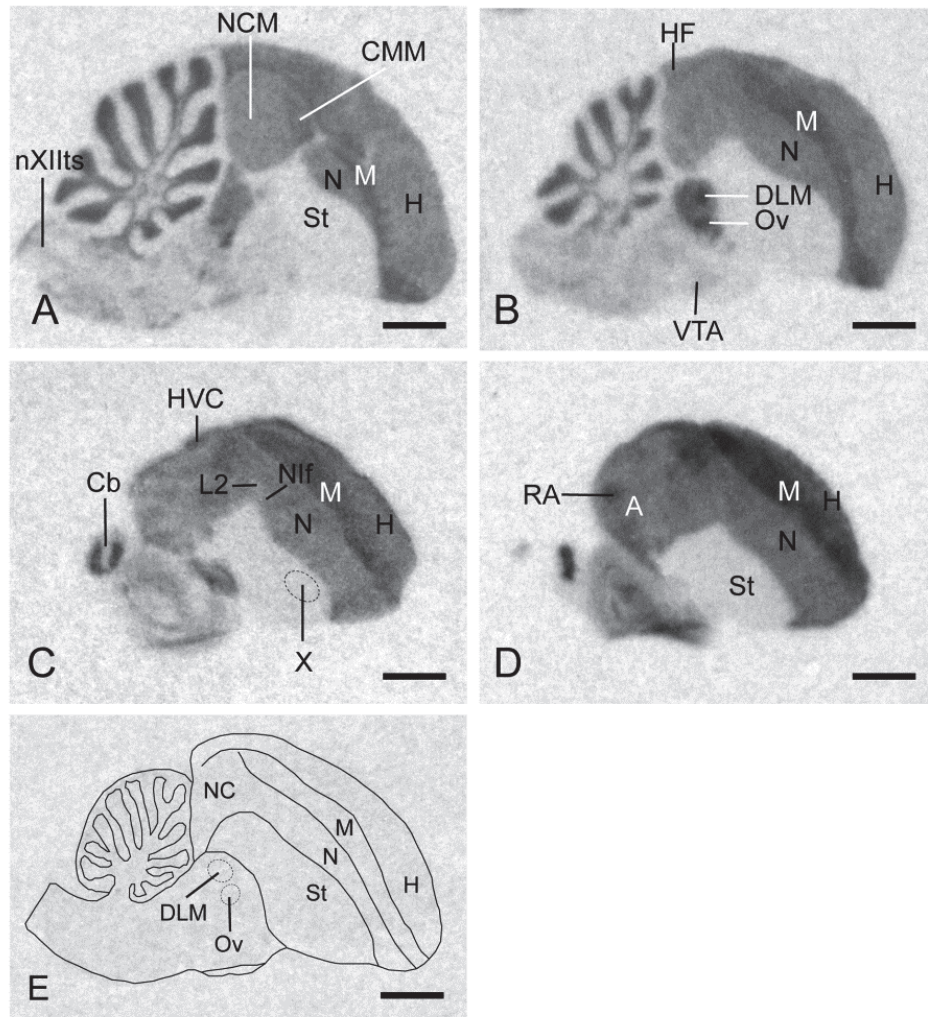
Fig. 3.4. In situ hybridization X-ray film autoradiograms show expression of VGLUT2 mRNA in longitudinal sections of the zebra finch brain (A-D). E: A sense probe shows no specific hybridization signal in the brain. For other abbreviations, see list. Scale bars = 2 mm in A-E.
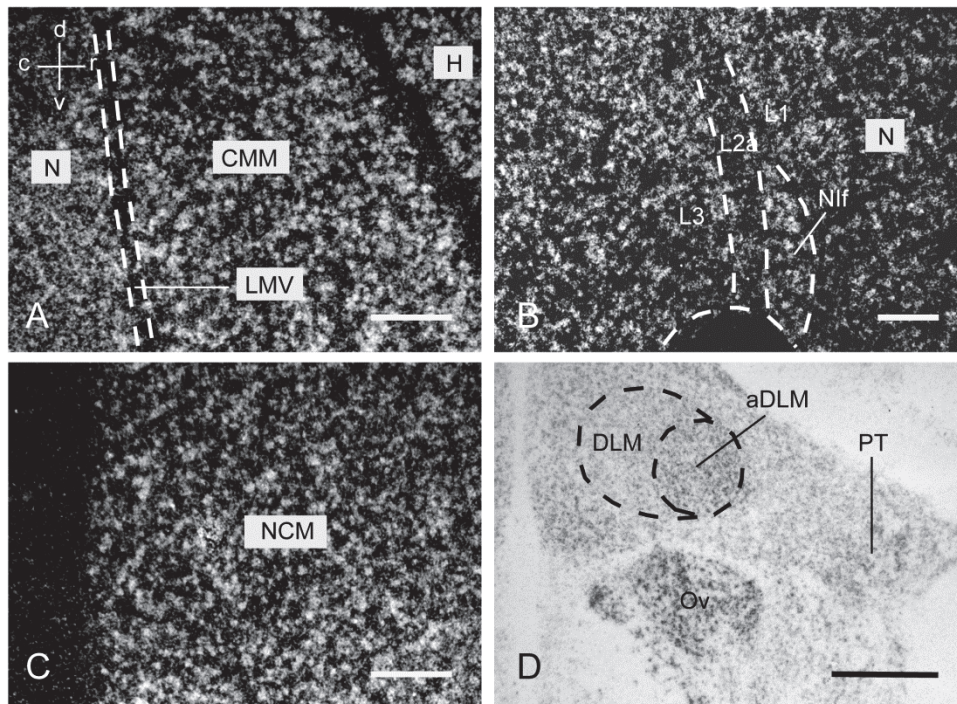
Fig. 3.5. Emulsion-coated sections show expression of VGLUT2 mRNA in neurons of auditory areas of the telencephalon and thalamus under darkfield (A-C) and brightfield (D) illuminations. Photomicrographs of A-C are taken from the longitudinal sections and D from transverse section. A: Caudomedial mesopallium (CMM) shows intense expression of VGLUT2 mRNA. B: Moderate signal appears in the NIf, filed L3 and L1, and weak signal is in the field L2a. C: Many labeled neurons are observed in caudomedial nidopallium (NCM). D: VGLUT2 mRNA expression in thalamic nuclei. The anterior part of DLM (aDLM) and Ov showed intense VGLUT2 mRNA. LMV: lamina mesopallium ventralis, For other abbreviations, see list. Scale bars = 200 μm in A-D.
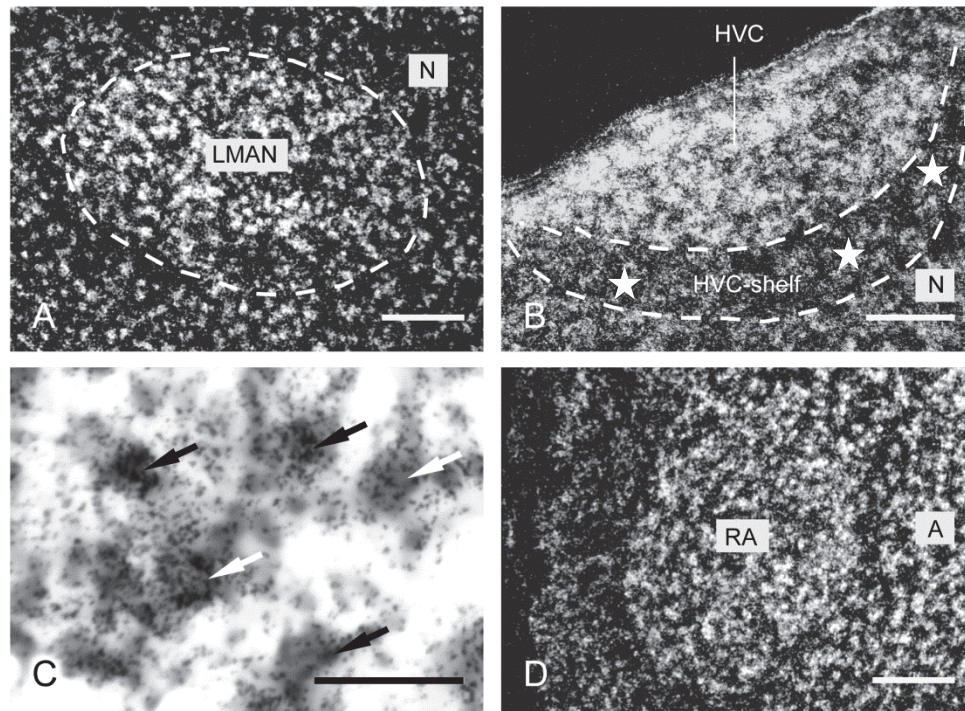
Fig. 3.6. Emulsion-coated sections show expression of VGLUT2 mRNA in neurons of telencephalic song nuclei under darkfield (A, B, D) and brightfield (C) illuminations. Photomicrographs of A, D are captured from the transverse sections and B, C from the longitudinal sections. A: LMAN shows intense expression of VGLUT2 mRNA. B: Many labeled neurons are observed in HVC and few labeled neurons are seen in HVC shelf (arrow heads). C: Cresyl violet-stained section. Many silver grains are localized in the cell body of neurons of the HVC (arrows). D: VGLUT2 mRNA expression in RA. HVC-shelf: HVC shelf region. For other abbreviations, see list. Scale bars = 200 μm in A, B, D; 50μm in C.
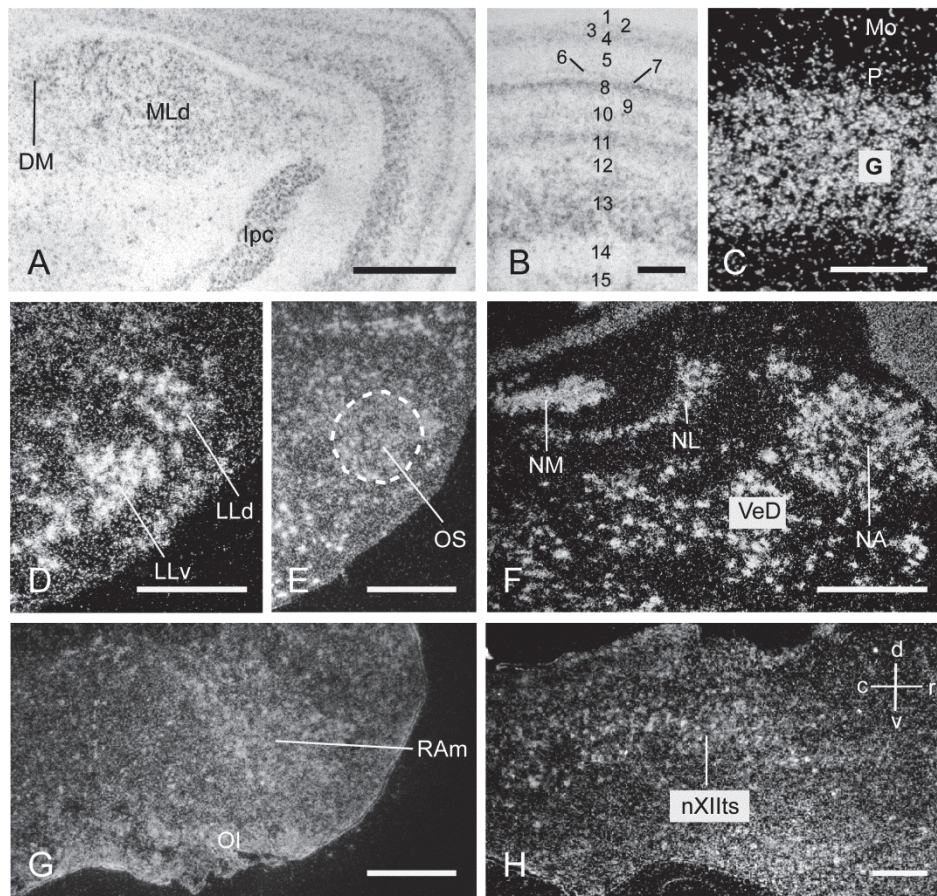
Fig. 3.7. Emulsion-coated sections show expression of VGLUT2 mRNA in neurons of the brainstem and cerebellum under bright-field (A-B) and dark-field (C-H) illuminations. A: VGLUT2 mRNA expression in the mesencephalic nuclei and optic tectum. B: Differential distribution is found in the layers of the optic tectum. C: VGLUT2 mRNA expression in the cerebellar cortex. The granular layer (G) shows high expression of VGLUT2 mRNA. No signals are found in the Purkinje cell layer (P) or molecular layer (Mo). D-F: Labeled neurons are observed in the ventral (LLv) and dorsal (LLd) nuclei of the lateral lemniscus (D), OS (E) and NM, NA and NL (F). G: VGLUT2 mRNA expression in the retroambigual nucleus (RAm). H: VGLUT2 mRNA expression in nXIIts in a longitudinal section. VeD: descendens vestibular nucleus. For other abbreviations, see list. Scale bars = 250 μm in A, B, E, G, H; 150μm in D, F; 50μm in C.

Chapter 2

Immunohistochemistry of zebra finch vesicular glutamate transporter 2

2.1 Introduction

Vesicular glutamate transporters (VGLUTs) mediate glutamate transport into synaptic vesicles at the presynaptic terminals of glutamatergic neurons. In the previous chapter, I confirmed that VGLUT2 mRNA was distributed in neuronal cell bodies in the pallium of the telencephalon, in many nuclei in the ascending auditory and song systems, and in granule cells of the cerebellar cortex using in situ hybridization. But, in situ hybridization histochemistry does not reveal the projection targets of glutamatergic neurons. In contrast, immunohistochemistry using anti-VGLUT2 antibody is available for detection of glutamatergic targets in mammals at both light and electron microscopic levels (Fremeau et al., 2001; Hisano et al., 2002; Kaneko et al., 2002; Raju et al., 2006; Hackett and de la Mothe, 2009; Ge et al., 2010). In general, all layers in the cerebral cortex are immunoreactive for VGLUT1, but layers I and IV are labeled with VGLUT2 in slightly lower density. In the hippocampus, all strata except pyramidal and granular layers stain for VGLUT1, but an outer part of the granular layer in the dentate gyrus labels selectively for VGLUT2 (Bellocchio et al., 1998; Sakata-Haga et al., 2001; Hisano et al., 2002; Kaneko et al., 2002; Varoqui et al., 2002). The caudate-putamen stains with both VGLUT1 and VGLUT2. In the thalamus and hypothalamus, different nuclei essentially reveal immunoreactivity of either VGLUT1 or VGLUT2 (Fremeau et al., 2001; Kaneko et al., 2002, Barroso-Chinea et al., 2007a). In the midbrain, VGLUT2 stains intensely the superior and inferior colliculi and periaqueductal gray, but VGLUT1 does not. In the cerebellum, VGLUT1 stains only parallel fibers whereas VGLUT2 labels only climbing fibers. Nevertheless, mossy fibers in the glomeruli are immunoreactive for both VGLUT1

and VGLUT2. In pigeons, VGLUT2 immunoreactivity is intense in the pallium, striatum, dorsal thalamus, hypothalamus and cerebellar cortex (Atoji, 2011).VGLUT2 immunoreactivity is preferentially observed in the excitatory presynaptic terminals of asymmetric synapses in rats (Fremeau et al., 2001; Kaneko et al., 2002), and pigeons (Atoji, 2011). Thus, protein expression considers the projection terminals of glutamatergic neurons in the neural circuits. The localization of VGLUT2 protein has not yet been described in any songbird species.

The present determined the molecular weight of zebra finch VGLUT2 protein and demonstrated localization of VGLUT2 protein in the zebra finch brain special focus on the auditory and song systems.

2.2. Materials and Methods

Animals

Six adult male zebra finches (*Taeniopygia guttata*, body weight: 11-22g and age: 4-7 months) were used for Western blot and immunohistochemistry. Animal handling procedures were approved by the Committee for Animal Research and Welfare of Gifu University.

SDS-PAGE and Western blot

A zebra finch for Western blot was anesthetized with sodium pentobarbital (50 mg/Kg). The telencephalon and cerebellum were dissected and lysed in CelLytic (Sigma-Aldrich, St. Louis, MO, USA). Each lysate was then centrifuged at 15,000 g for 10 minutes at 4°C and the supernatant was collected. The supernatant containing 10 μg of total protein fraction was mixed with the same volume of 2x sample buffer (Nacalai Tesque), and

2-mercaptoethanol and sodium dodecyl sulfate (SDS) were added to yield a final concentration of 1% each. This mixture was heated to 95°C for 5 minutes, immediately chilled on ice, and then separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins in the gel were transferred to polyvinylidene difluoride membrane. The membrane was blocked with 5% skim milk in Tris-HCl buffered saline (pH 7.4) containing 0.05% Tween 20 (TBST) for 60 minutes at room temperature followed by 1% normal goat serum in TBST for 60 minutes at room temperature, and then incubated with a rabbit anti-VGLUT2 antibody against a synthetic peptide EEFVQEEVQDSYNYKEGDYS which corresponds to residues 562-581 of the pigeon VGLUT2 (1:10,000, Atoji, 2011, see discussion for specificity) (Table 2) for 60 minutes at room temperature. After washing with TBST, the membrane was incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP, Kirkegaard & Perry Laboratories, Inc, Gaithesburg, MD, USA) (1:5,000) for 60 minutes at room temperature. After washing the membrane, the HRP was visualized by 3,3'-diaminobenzidine tetrahydrochloride (DAB, 20 mg/100ml) containing 0.003% H_2O_2 in 0.1 M Tris-HCl buffer at pH 7.4.

Immunohistochemistry

Five zebra finches were anesthetized with sodium pentobarbital (50 mg/Kg) and perfused with Ringer's solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed and stored in the same fixative for two days. They were transferred to 30% sucrose in phosphate-buffered saline (PBS) at 4°C for one day and cut transversely or sagittally at 50 µm on a cryostat. Sections were pretreated with 50% methanol containing 0.3% H_2O_2 for 30 minutes. After washing in PBS, they were pre-incubated with 1% normal goat serum for 60 minutes at room temperature. After washing thoroughly in PBS, the sections were incubated in PBS containing a rabbit

anti-VGLUT2 antibody (1:10,000, Atoji, 2011, see Discussion for specificity) (Table 3) and 0.3% Triton X-100 for two days at 4°C, followed by a biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA, USA; 1:500) for 60 minutes at room temperature, and finally incubated in avidin-biotin-horseradish peroxidase complex (ABC Elite Kit; Vector Laboratories, Inc.) for 60 minutes at room temperature. The VGLUT2-peroxidase complex was visualized by DAB (20 mg/100ml) containing 0.003% H_2O_2 in 0.1 M Tris-HCl buffer at pH 7.4. Sections were then mounted, dehydrated, and cover slipped with DPX.

Two immunohistochemical controls for anti-VGLUT2 antibody were carried out. First, non-immune rabbit serum was incubated instead of the primary antibody. Second, sections were incubated with the primary antibody that had been pre-absorbed with the immunogen peptide (EEFVQEEVQDSYNYKEGDYS, 10 μg/ml), which corresponds to C-terminal 20 amino acids of pigeon VGLUT2 (Islam and Atoji, 2008).

Image processing

Photographs at low-power magnification were taken with a scanner (Epson GT-9300UF, Tokyo, Japan). Photomicrographs at high-power magnification were taken with a digital camera (Nikon, DS-Fi1, Tokyo, Japan) mounted on a light microscope. Adjustment of photographs for contrast, brightness and sharpness, layout, and lettering were performed using Adobe Photoshop 7.0J (Tokyo, Japan) and Adobe Illustrator 10.0J (Tokyo, Japan).

Nomenclature

The nomenclature used here is based on available avian brain atlases, including pigeon (Karten and Hodos, 1967), Digital Atlas of the Zebra Finch (*Taeniopygia guttata*) Brain (Karten et al., 2013), as well as a recent publication on zebra finch neuroanatomy (Jarvis et

al., 2013).The revised avian brain terminology recommended by the avian brain Nomenclature Forum (Reiner et al., 2004).

2.3. Results

Western blot by VGLUT2 antibody

A clear band of VGLUT2 immunoreactivity was calculated to be approximately 61.2 kDa in both lanes of the telencephalon and cerebellum (Fig. 4.1). The band intensity was strong in the telencephalon and moderate in the cerebellum. The molecular weight of the zebra finch VGLUT2 consisting of 581 amino acids was estimated to be 64.36 kDa.

Immunohistochemistry for VGLUT2

VGLUT2 immunoreactivity was observed throughout the adult male zebra finch brain (Figs. 4.2-4.5) and it was localized in neuropil at a microscopic level (Fig. 4.2E, F) except in the arcuate hypothalamic nucleus where some neuronal cell bodies were positive (Fig. 4.4C). Immunoreactive neuropil appeared to be fine granules, varicosities or puncta. These immunoreactive structures were readily seen in weakly stained areas or nuclei (Fig. 4.2F), but it was somewhat difficult to detect immunoreactive varicosities or fine granules in strongly stained nuclei where background showed highly homogeneous immunoreactivity (Fig. 4.2E). Intensity of immunoreactivity was evaluated as follows: caudal nidopallium (Figs. 4.2C, 4.3E), arcopallium (Figs. 4.2A, C), and area X (Fig. 4.2C) were intense or strong, moderate, weak, respectively. No labeling was seen in large fiber tracts, e.g., septopallio-mesencephalic tract, lateral forebrain fascicle, anterior commissure, optic chiasma, or dorsal supraoptic decussation. In control sections, specific immunoreactivity was not observed when sections were incubated with pre-absorbed antibody (Fig. 4.2B) or

with non-immune rabbit serum.

The telencephalon basically showed VGLUT2 immunoreactivity except for the unstained globus pallidus (Figs. 4.2A, C, D, 4.3A-F). Intense immunostaining was seen in the olfactory bulb, apical part of the hyperpallium, mesopallium (M), hippocampal formation (HF), dorsolateral corticoid area, nucleus taeniae of the amygdala, and septum. In the nidopallium, caudomedial (NCM) revealed strong immunoreactivity, but other song regions of HVC, field L complex, LMAN, interfacial nucleus (NIf), and the visual entopallium were weakly positive (Figs. 4.2C-F, 4.3A-F, 4.4A). The remaining nidopallium, including basorostral pallial nucleus, showed moderate immunostaining. RA and area X were weakly immunopositive (Figs. 4.2C, D, F, 4.3A, F).

In the diencephalon, medial thalamus showed intensely immunoreactive (Figs. 4.3B-D, 4.4B). On the other hand, lateral thalamus was generally weak; it included sensory-relay nuclei, e.g., ventral part of the lateral geniculate nucleus, DLM, rotundal nucleus, Ov and pretectal nucleus (Figs. 4.2C, 4.3B-D, 4.4B). The habenular nuclei revealed moderate immunostaining (Fig. 4.3D). In the hypothalamus, the median eminence showed strong immunoreactivity, particularly more intense in a lateral part (Fig. 4.3C-D). The arcuate nucleus near the median eminence contained strongly immunostained cell bodies (Fig. 4.4C).

In the midbrain, the gray and superficial fiber stratum and central gray stratum of the optic tectum were moderately immunoreactive (Figs. 4.3C-F, 4.4D). Fine varicosities were distributed in the two strata. The central white stratum and optic stratum were devoid of immunoreactivity. Intense immunoreactivity was observed in lateral mesencephalic nucleus (MLd) and interpeduncular nucleus (Fig. 4.5A). DM and mesencephalic lentiform nuclei were weakly immunopositive. In the magnocellular isthmic nucleus, moderately immunoreactive puncta surrounded cell bodies, but the parvocellular isthmic nucleus was

not immunostained.

In the lower brainstem, the isthmo-optic nucleus showed moderate immunoreactivity. OS (Fig. 4.5B) and dorsal and ventral nuclei of the lateral lemniscus were moderately immunostained. Immunoreactive varicosities were numerously found in the three nuclei. In the medulla oblongata, intense immunoreactivity was seen in NM, NA, and NL (Fig. 4.5C). The three nuclei revealed pericellular localization of immunoreactive puncta against immunonegative cell bodies and neuropil (Fig. 4.5D). In the cerebellum, glomeruli in the granular layer were intensely stained (Fig. 4.5E). The molecular layer showed strongly homogeneous immunostaining. The Purkinje cell layer was devoid of immunoreactivity. Moderate immunoreactivity was seen in the retroambigual nucleus. The tracheosyringeal motor nucleus of the hypoglossal nerve showed weak immunoreactivity.

The VGLUT2 immunoreactive density in major nuclei and areas of the brain is shown in Table 4.

2.4. Discussion

The present study investigated the localization of axon terminals of VGLUT2 mRNA-expressing glutamatergic neurons in the zebra finch brain by immunohistochemistry, using an anti-VGLUT2 antibody. The regional differences of VGLUT2 immunoreactivity in the brain indicate many glutamatergic terminals are existed in the zebra finch brain including auditory and song pathways.

Characterization of VGLUT2 antibody

The molecular weight of the human and rat VGLUT2, which is deduced from 582 amino acid sequence, is 64.4 kDa and 65 kDa, respectively (Takamori et al., 2002). The

molecular weight of the pigeon VGLUT2 consisting of 581 amino acids is estimated to be 64.3 kDa (accession number: FJ428226). In the present Western blot, the molecular weight of zebra finchVGLUT2 was calculated to be 61.2 kDa. The molecular weight of the zebra finch VGLUT2 consisting of 581 amino acids is estimated to be 64.36 kDa, which is in good agreement with that of the human and rat. The anti-VGLUT2 antibody used in the present study recognizes C-terminal 20 amino acids (residues 562- 581) of the pigeon VGLUT2, i.e., EEFVQEEVQDSYNYKEGDYS (Atoji, 2011), and this amino acid sequence is same in the zebra finch VGLUT2 (residues 562- 581). Immunostaining with immunogen peptide also showed no immunoreactivity in the zebra finch brain. These results indicate that the antibody used in this study recognizes zebra finch VGLUT2.

Comparison of expression of VGLUT2 immunoreactivity with other birds and mammals

High levels of VGLUT2 immunoreactivity have also been reported in the pallium and subpallium of the telencephalon, dorsal thalamus, hypothalamus, and cerebellar cortex, but not in the globus pallidus of the pigeon brain (Atoji, 2011). In the brainstem of the pigeon, a high level of VGLUT2 immunoreactivity is evident in the interpeduncular nucleus, MLd, isthmo-optic nucleus, NM, NA and NL. The present findings regarding VGLUT2 immunoreactivity in the zebra finch brain are consistent with previous findings of studies of pigeons.

In mammals, the distribution of VGLUT1 and VGLUT2 immunoreactivity has been reported in the brain (Bellocchio et al., 1998; Sakata-Haga et al., 2001; Hisano et al., 2002; Kaneko et al., 2002; Varoqui et al., 2002). All layers in the cerebral cortex are immunoreactive for VGLUT1, but layers I and IV are labeled with VGLUT2 in slightly lower density. In the hippocampus, all strata except pyramidal and granular layers stain for VGLUT1, but an outer part of the granular layer in the dentate gyrus labels selectively for

VGLUT2. The caudate-putamen stains with both VGLUT1 and VGLUT2. In the thalamus and hypothalamus, different nuclei essentially reveal immunoreactivity of either VGLUT1 or VGLUT2. In the thalamus, VGLUT1 stains the mediodorsal, laterodorsal, ventromedial nuclei, lateral and medial geniculate nuclei, whereas VGLUT2 is immunopositive in the lateral and medial habenular nuclei, anterior nucleus, lateral and medial geniculate nuclei, ventrolateral, paraventricular, parafascicular nuclei. VGLUT1 neurons in the cortex project to the striatum and thalamus, while VGLUT2 neurons in the thalamus send efferents to the cortex and striatum (Fujiyama et al., 2001; Varoqui et al., 2002; Raju et al., 2006; Barroso-Chinea et al., 2007a). In the hypothalamus, VGLUT2 immunoreactivity is much stronger and larger than VGLUT1. VGLUT2 labels strongly the preoptic area, anterior, lateral, dorsal, posterior hypothalamic areas, arcuate nucleus, and median eminence, while VGLUT1 stains moderately the ventromedial nucleus and mammillary nuclei. In the midbrain, VGLUT2 stains intensely the superior and inferior colliculi and periaqueductal gray, but VGLUT1 does not. In the cerebellum, VGLUT1 stains only parallel fibers whereas VGLUT2 labels only climbing fibers. Nevertheless, mossy fibers in the glomeruli are immunoreactive for both VGLUT1 and VGLUT2. The complementary immunoreactivity of VGLUT1 and VGLUT2 in mammals appears to be similar as mRNA expression of VGLUT1 and VGLUT2 does. Thus, total immunoreactive patterns of VGLUT1 and VGLUT2 in mammalian brain are consistent with VGLUT2 immunoreactivity in the pigeon and zebra finch brain.

2.5. Summary

The VGLUT2 immunoreactivity is preferentially observed in the glutamatergic presynaptic terminals of asymmetric synapses in both rats (Bellocchio et al., 1998; Fremeau et al., 2001; Kaneko et al., 2002), and pigeons (Atoji, 2011). In the Chapter 1

author showed that VGLUT2 mRNA-expressing glutamatergic neurons are widely distributed in the zebra finch brain including many nuclei of auditory and song systems. In the present study, author identified molecular weight of zebra finchVGLUT2 protein and mapped the expression pattern of VGLUT2 protein in the zebra finch brain to determine the projection targets of VGLUT2 mRNA-expressing glutamatergic neurons. High levels of VGLUT2 immunoreactivity are found in the pallium and subpallium of the telencephalon, dorsal thalamus, hypothalamus, and cerebellar cortex, but not in the globus pallidus like pigeon brain (Atoji, 2011). In the thalamus, weak immunoreactivity was seen in the sensory relay nuclei e.g., rotundal nucleus, Ov, and DLM. In the brainstem, a high level of VGLUT2 immunoreactivity is evident in the auditory nuclei MLd, NM, NA and NL. VGLUT2 protein expression was detected in the telencephalic song nuclei HVC, RA, LMAN and also in the area X. VGLUT2 immunohistochemistry revealed a regional difference in the brain including auditory and song systems. The present findings suggest many glutamatergic axon terminals are exist in the different regions of the zebra finch brain including the auditory and song systems.

TABLE 3. Antiboby characterization

Antigen	Immunogen	Manufacturer	Specificity	Dilution
Vesicular glutamate transporter 2 (VGLUT2)	Synthetic peptide from pigeon VGLUT2 (EEFVQEEVQDSYN YKEGDYS: 562-581)	Y. Atoji, Gifu University JCN 519:2887–2905, 2011, rabbit polyclinal, JCN antibody database PubMed ID 21618220	Detects a single band of 65KDa in WB of lysates from pigeon telencephalon and cerebellum	1:10,000

TABLE 4. Regional intensity of VGLUT2 immunoreactivity in the zebra finch brain.

Regions	Immunohistochemical intensity
Telencephalon	
Olfactory bulb	+++
Hyperpallium	+++
Mesopallium	+++
Hippocampal formation	+++
Nidopallium	++
Lateral magnocellular nucleus of the anterior nidopallium	+
HVC	+
HVC shelf region	+
Filed L1	+
Field L2	+
Field L3	+
Nucleus interface of the nidopallium	+
Caudal nidopallium	+++
Entopallium	+
Arcopallium	++
Robust nucleus of the arcopallium	+
RA cup region	-
Nucleus taeniae of the amygdalae	+++
Striatum	+++
Area X	+
Globus pallidus	-
Lateral septal nucleus	+
Medial septal nucleus	+
Pallial commissural nucleus	++
Septal commissural nucleus	+
Diencephalon	
Thalamus	
Dorsolateral anterior nucleus of the thalamus	+++
Lateral part of dorsolateral anterior nucleus of the thalamus	++
Medial part of dorsolateral anterior nucleus of the thalamus	+
Anterior nucleus of DLM	+
Dorsomedial posterior nucleus of the thalamus	++
Ovoidal nucleus	+
Rotundus nucleus	+
Triangular nucleus	+
Uvaeform nucleus	+
Lateral habenular nucleus	+
Medial habenular nucleus	++
Pretectal nucleus	+
Subpretectal nucleus	-

TABLE 2 (Continued)

Regions	Immunohistochemical intensity
Hypothalamus	
Preoptic area	+++
Supraoptic area	+++
Tuberal area	+++
Mammillary area	++
Mesencephalon	
Ventral tegmental area	++
Interpeduncular nucleus	+++
Optic tectum	++
Dorsal part of lateral mesencephalic nucleus	++
Dorsomedial nucleus of the of the intercollicular complex	+
Intercollicular nucleus	++
Isthmic nucleus, magnocellular part	+
Isthmic nucleus, parvocellular part	-
Substantia nigra	+
Isthmo-opticus nucleus	++
Rhombencephalon	
Cerebellum	
Molecular layer	+++
Purkinje cell layer	-
Granular layer	+++
Cerebellar nuclei	++
Pontine and medullary regions	
Principal sensory trigeminal nucleus	+
Lateral pontine nucleus	++
Medial pontine nucleus	++
Locus coeruleus (A8)	+
Ventral nucleus of the lateral lemniscus	++
Dorsal nucleus of the lateral lemniscus	++
Superior olivary nucleus	++
Magnocellular nucleus	+++
Angular nucleus	+++
Laminar nucleus	+++
Vestibular nuclei	++
Pontine reticular nucleus giganticellular part	+
Raphe nucleus	++
Inferior olivary nucleus	++
Retroambigual nucleus	++
Tracheosyringeal motor nucleus of the hypoglossal nerve	+

Immunohistochemical intensity is evaluated as follows: caudal nidopallium (3+, Fig. 4.2C, D), arcopallium (2+, Fig. 4.2D), and area X (1+, Figs. 4.2C, 4.3A). For other abbreviations, see list.
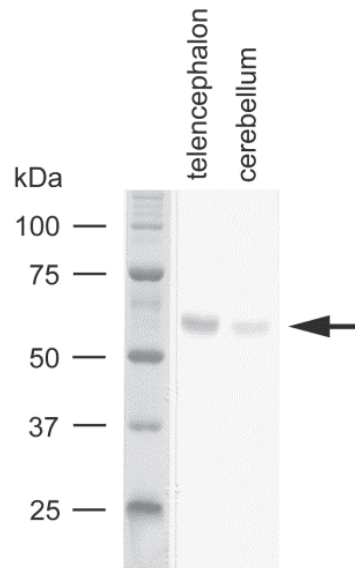
Fig. 4.1. Molecular weight of VGLUT2 in Western blotting. A single band is found in each lane of the telencephalon and cerebellum and the two bands align at the same molecular weight (arrow).
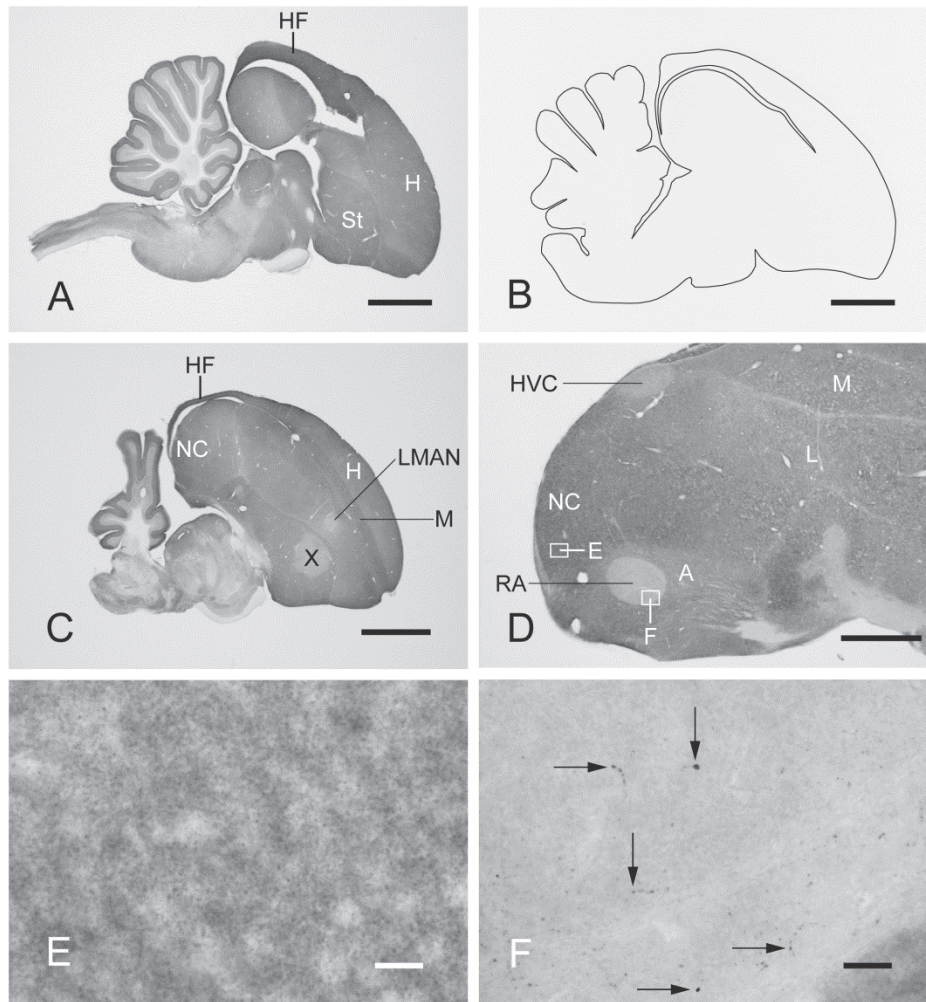
Fig. 4.2. Immunohistochemical localization of VGLUT2 in longitudinal sections of the zebra finch brain (A-F). A, C, D: All regions except HVC, area X, RA, and LMAN show intense or moderate VGLUT2 immunoreactivity in the telencephalon. B: Control immunostaining with a pre-absorbed VGLUT2 antibody by an immunogen peptide shows no specific reaction in a sagittal section. E: Enlargement of a box in D. Caudal nidopallium (NC) shows intense immunoreactivity due to a large number of VGLUT2 immunoreactive granules or varicosities. F: Enlargement of a box in D. Immunoreactive varicosities are small in number in RA. L: field complex. For other abbreviations, see list. Scale bars = 2 mm in A-C; 1mm in D; 25 μm in E, F.
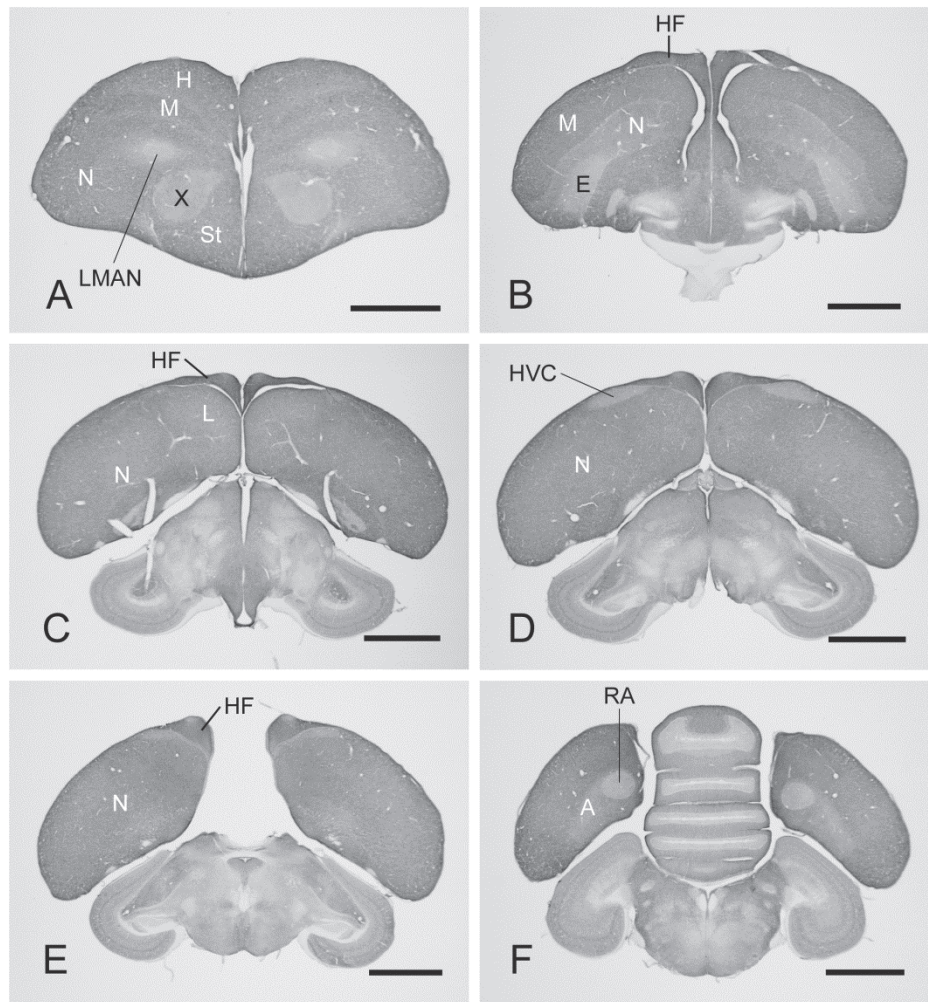
Fig. 4.3. Immunohistochemical localization of VGLUT2 in transverse sections of the telencephalon (A-F). L: field L complex. For other abbreviations, see list. Scale bars = 2 mm.
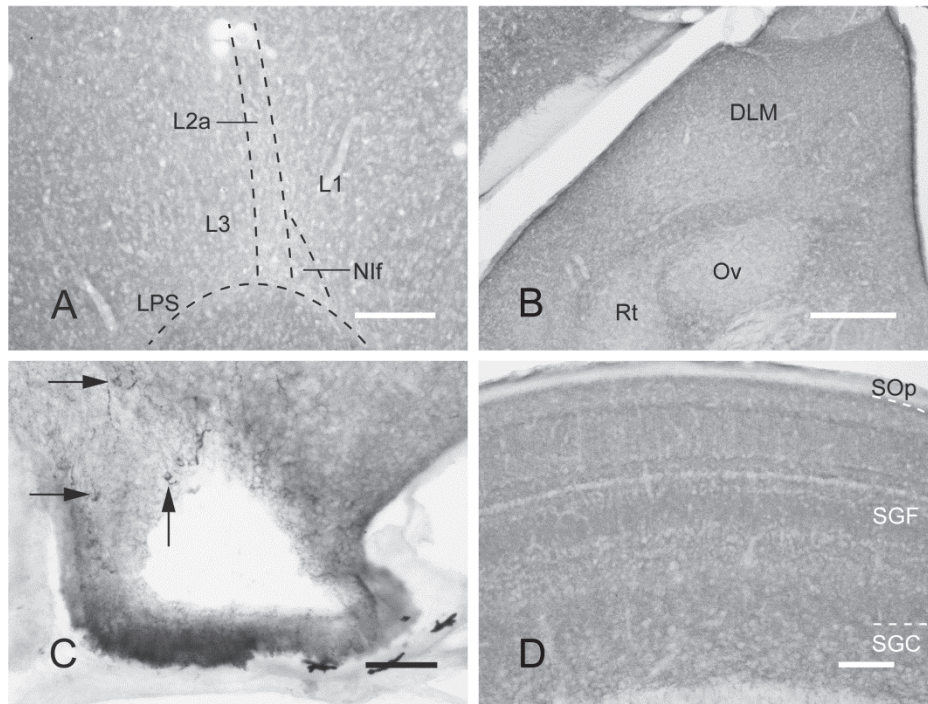
Fig. 4.4. Photomicrographs of immunohistochemical localization of VGLUT2. A: Field L complex and NIf. B: Weak immunoreactivity is seen in DLM, Ov and Rt in the thalamus. C: The tuberal area in the hypothalamus shows strong VGLUT2 immunoreactivity, especially in the median eminence. Some neurons (arrows) in the arcuate nucleus are immunoreactive. D: VGLUT2 immunoreactivity in the optic tectum. LPS: pallial-subpallial lamina; SGC: central gray stratum; SGF: gray and superficial fiber stratum; SOp: optic stratum. For other abbreviations, see list. Scale bars = 400 μm in B; 200 μm in A; 100 μm in C, D.
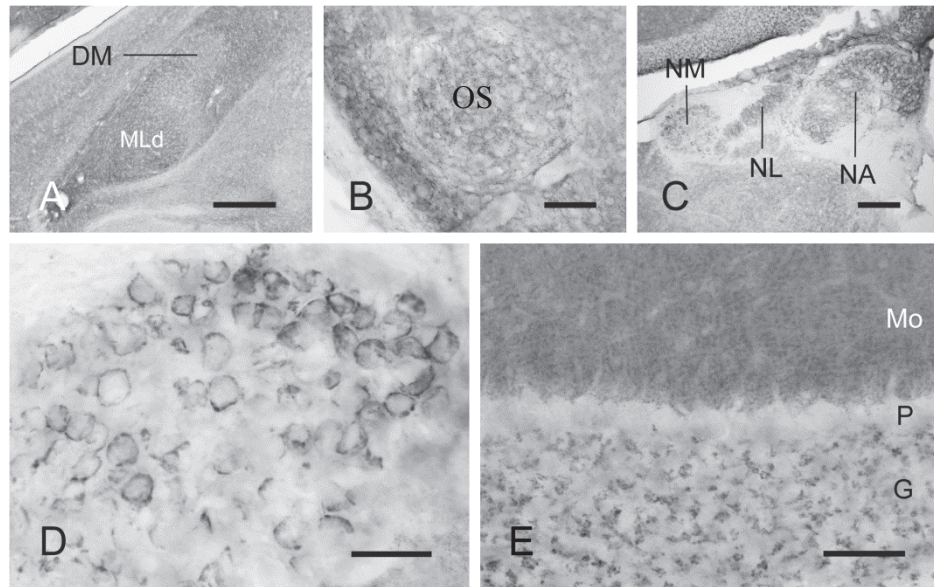
Fig. 4.5. Photomicrographs of immunohistochemical localization of VGLUT2 in the midbrain, lower brainstem, and cerebellum. A: Deep nuclei in the optic tectum. B: The superior olivary nucleus. C: Strong immunoreactivity is seen in NM, NA, and NL. D: Immunoreactive puncta surround neuronal cell bodies in NM. E: Cerebellar cortex. Immunoreactivity is found in cerebellar glomeruli in the granular layer (G). The molecular layer (Mo) shows homogeneous immunostaining. Purkinje cell layer (P) is devoid of immunoreactivity. For other abbreviations, see list. Scale bars = 500 μm in A; 200 μm in C; 100 μm in B; 50 μm in D, E.

Chapter 3

Distribution of zebra finch glutamate receptor subunits mRNA

3.1. Introduction

Excitatory synaptic transmission in brain is primarily mediated by the neurotransmitter glutamate. Glutamate released from presynaptic terminals binds to glutamate receptors on postsynaptic membrane (Newpher and Ehlers, 2008). In mammals, two main types of glutamate receptors are ionotropic glutamate receptors and metabotropic glutamate receptors. The ionotropic glutamate receptors contain glutamate-gated cation channels and directly cause excitation. Ionotropic glutamate receptors are pharmacologically classified as AMPA (amino-3-hydroxy-5-methylisoxazole-4- propionic acid), NMDA (N-methyl-D-aspartic acid), and kainate sensitive glutamate receptors (Collingridge and Lester, 1989). Each type of glutamate receptor is assembled by several subunits e.g., AMPA type assembled as four subunits (GluA1-4), kainate type five subunits (GluK1-5) and NMDA type seven subunits (GluN1, GluN2A-D, GluN3A-B). Neurons receiving glutamatergic afferents express the mRNA of glutamate receptor subunits in the soma. Therefore, the projection targets of glutamatergic neurons in the neuronal circuits could be identified using the expression patterns of these mRNAs. The glutamate receptor subunits mRNA are widely distributed in the mammalian brain (Petralia and Wenthold, 1992; Conti et al., 1994; Huntley et al., 1994; Muñoz et al., 1999). In birds, AMPA type receptors are expressed in the pigeon brain (Ottiger et al., 1995; Islam and Atoji, 2008). Gene sequences of AMPA, kainate and NMDA glutamate receptors were analyzed fully or partially in the zebra finch and reported their mRNA expression in vocal areas of the zebra finch brain (Heinrich et al., 2002; Wada et al., 2004). However, the distributions of glutamate receptor

subunits mRNA in the auditory areas of the telencephalon, thalamus and lower brainstem remain unclear in the zebra finch.

The aim of the present study is to confirm the distribution of mRNAs for five glutamate receptor subunits (at least one subunit from each type of glutamate receptor: GluA1, GluA4, GluK1, GluN1, and GluN2A) in the zebra finch brain including ascending auditory pathway and song system using in situ hybridization.

3.2. Materials and Methods

Animals

Eleven adult male zebra finches (*Taeniopygia guttata*, body weight: 11-22g and age: 4-7 months) were used in the present study. Animal handling procedures were approved by the Committee for Animal Research and Welfare of Gifu University. One animal was used for the reverse transcription-polymerase chain reaction (RT-PCR), ten animals were used for in situ hybridization. Animals were anesthetized with sodium pentobarbital (50 mg/kg). For isolation of total RNA, the telencephalon was dissected out quickly and kept in RNA stabilization solution (RNA*later*, Ambion, Austin, TX, USA) and stored at -60°C until use. For in situ hybridization, fresh brains were quickly removed and immediately frozen on powdered dry ice. Serial transverse or longitudinal sections were cut at 30 μm thickness on a cryostat, thaw-mounted onto the 3-aminopropyltriethoxysilane coated slides, and stored at -30°C.

RNA isolation, cDNA synthesis and PCR amplification

Total RNA isolation and first-stand cDNA synthesis procedures were same as describe in chapter 1. To amplify cDNA sequence for glutamate receptor subunits (GluA1, GluA4, GluK1, GluN1, and GluN2A), the primers for glutamate receptor subunits, AMPA type 1

and 4 (GluA1 and GluA4), kainate type 1 (GluK1), and NMDA type 1 and 2A (GluN1 and GluN2A), were designed based on the cDNA sequences of the zebra finch, pigeon, and chicken (Wada et al., 2004; FJ428225, XM_416697, NM_206979, XM_425252, Table 1).

Sequence analysis:

The sequences of respective cDNA fragments were analyzed by ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster, CA, USA). The obtained zebra finch nucleotide and encoded amino acid sequences of glutamate receptor subunits were compared with the nucleotide and amino acid sequences of the other birds and mammals. The following sequences were used for glutamate receptor subunits sequence analysis: chicken GluA1 (NM_001001774), chicken GluA4 (NM_001113186), chicken GluK1 (XM_004938378), chicken GluN1 (NM_206979), chicken GluN2A (XM_425252), zebra finch GluA1 (Wada et al., 2004; AB042749), zebra finch GluA4 (Wada et al., 2004; AB042752), zebra finch GluK1 (XM_002187819), zebra finch GluN1 (NM_001076693), zebra finch GluN2A (XM_002194918), pigeon GluA1 (FJ428225), pigeon GluA4 (Z29920), human GluA1 (NM_001258023), human GluA4 (NM_000829), human GluK1 (NM_000830), human GluN1 (NM_001185090), human GluN2A (NM_001134407).

In situ hybridization

In situ hybridization procedure was same as describe in chapter 1.

Oligonucleotide probes

Antisense and sense oligo DNA probes of zebra finch GluA1, GluA4, GluK1, GluN1 and GluN2A were designed based on obtained a partial sequences of zebra finch GluA1, GluA4, GluK1, GluN1 and GluN2A cDNA sequences, respectively (Table 1), and were

synthesized commercially (Rikaken, Nagoya, Japan).

Image processing

Photographs at low-power magnification were taken with a scanner (Epson GT-9300UF, Tokyo, Japan). Photomicrographs at high-power magnification were taken with a digital camera (Pro 600ES, Pixera Corporation, Los Gatos, CA, USA or Nikon, DS-Fi1, Tokyo, Japan) mounted on a light microscope. Adjustment of photographs for contrast, brightness and sharpness, layout, and lettering were performed using Adobe Photoshop 7.0J (Tokyo, Japan) and Adobe Illustrator 10.0J (Tokyo, Japan).

Nomenclature

The nomenclature used here is based on available avian brain atlases, including pigeon (Karten and Hodos, 1967), Digital Atlas of the Zebra Finch (*Taeniopygia guttata*) Brain (Karten et al., 2013), as well as a recent publication on zebra finch neuroanatomy (Jarvis et al., 2013).The revised avian brain terminology recommended by the avian brain Nomenclature Forum (Reiner et al., 2004).

3.2. Results

In situ hybridization was performed to examine distribution of mRNAs for GluA1, GluA4, GluK1, GluN1, and GluN2A in the zebra finch brain. Antisense probes revealed differential expression of glutamate receptor subunits mRNA in the brain (Fig. 5.1A-H). Sense probes for five subunits did not exhibit specific hybridization signal in X-ray images and the image on GluN1 was shown as a representative (Fig. 5.1I).

GluA1 mRNA signals was high in the mesopallium, hippocampal formation, area X,

striatum, and septal nuclei of the telencephalon (Fig. 5.1A, B). GluA1 mRNA signal was weak in the dorsal thalamus and ovoidal nucleus (Ov) in the diencephalon (Fig. 5.1A, B). Moderate or weak expression of GluA1 was observed in the optic tectum, orsomedial nucleus of the intercollicular complex (DM) and dorsal part of the lateral mesencephalic nucleus (MLd). In the cerebellar cortex, moderate expression of GluA1 mRNA was observed in the Purkinje cell layer and granular layer (Fig. 5.1A). GluA4 mRNA was expressed moderately in the hyperpallium, mesopallium and nidopallium (Fig. 5.1C). Area X showed weak GluA4 mRNA expression (Fig. 5.1C). Weak or moderate expression of GluA4 mRNA was noted in medial nucleus of the dorsolateral thalamus, Ov and rotundal nucleus in the diencephalon (Fig. 5.3C). Weak signal was found in the cochlear nuclei magnocellularis (NM), angularis (NA) and laminaris (NL) of the lower brainstem (Fig. 5.3E).

GluK1 mRNA was expressed moderately in the robust nucleus of the arcopallium (RA) and medial part of the entopallium and weakly in lateral part of the entopallium and field L2 of the telencephalon (Fig. 5.1D, E; 5.2C; 5.3A). Moderate or weak signal was found in Ov, optic tectum (Fig. 5.1D), and MLd. GluK1 mRNA was highly expressed in the Purkinje cell layer and granular layer of the cerebellum.

GluN1 mRNA signal was high in the striatum, especially area X (Fig. 5.1F, G; 5.2D). Moderate GluN1 mRNA expression was found in the hyperpallium, mesopallium, nidopallium and fields L1 and L3, but field L2 was devoid of GluN1 mRNA (Fig. 5.1F). HVC and RA showed weak GluN1 mRNA expression (Fig. 5.1F). GluN1 mRNA was expressed moderately or weakly in the optic tectum, DM, and MLd (Fig. 5.1G; 5.3D). In the cerebellar cortex, the granular layer expressed GluN1 mRNA moderately (Fig. 5.1F). GluN2A mRNA signal was moderate in the hyperpallium, mesopallium, nidopallium, and area X (Fig. 5.1H; 5.3B). The telencephalic auditory areas, caudomedial mesopallium

(CMM), field L complex and caudomedial nidopallium (NCM) showed moderate or weak expression of GluN2A mRNA (Fig. 5.3B). LMAN and HVC showed weak GluN2A mRNA expression (Fig. 5.1H, 5.2A-B). GluN2A mRNA signal was moderate or weak in the optic tectum, MLd, and dorsal and ventral nuclei of the lateral lemniscus.

Signal density of ionotropic glutamate receptor subunit mRNAs in auditory and song nuclei is shown in Table 6.

3.3. Discussion

Five glutamate receptor subunits mRNAs revealed differential expression in the telencephalic and midbrain vocal nuclei or areas of the zebra finch, which was similar with previous report on the zebra finch done by Wada et al. (2004). Many areas or nuclei expressing glutamate receptor subunit mRNAs match those which show expression of VGLUT2 immunoreactivity, that is sites of glutamatergic axon terminals (Chapter 2). For example, striatum of the telencephalon shows high VGLUT2 immunoreactivity as well as high signals for GluA1, GluN1 mRNAs. In the auditory and song nuclei show high or moderate hybridization signal for glutamate receptor subunit mRNAs (Wada et al., 2004; present study) and weak labeling of VGLUT2 protein (Chapter 2). The present finding suggests many glutamatergic projections exist in the zebra finch brain including auditory and song systems.

Comparison of distribution of glutamate receptor subunit mRNAs with other birds and mammals

In pigeons, GluA1 mRNA is expressed in most areas in the telencephalon (Islam and Atoji, 2008). Particularly, high expression is seen in the olfactory bulb, densocellular part

of the hyperpallium, mesopallium, arcopallium and striatum, and the nidopallium shows weaker expression than these areas. GluA2 and GluA3 mRNAs show similar distribution patterns and the expression in the mesopallium is higher than the nidopallium and visual Wulst (Ottiger et al., 1995). Weak expression is seen in the telencephalon for GluA4 mRNA (Ottiger et al., 1995). In the zebra finch, Wada et al. (2004) reported that AMPA, kainate and NMDA subunits show regional differences of their mRNA expression. The pallium of the telencephalon shows high expression for GluA1, GluA2, and GluN1, while it reveals moderate or weak expression for GluA3, GluA4, GluK1-5, GluN2A, and GluN2B. Telencephalic song nuclei RA highly express GluK1 but HVC, LMAN and area X show weak expression of GluN2A. The subpallium expresses strong mRNAs for GluA1, GluA2, and GluN1, but does not for GluA3-4 and GluK1. In the present study, expressions of GluA1, GluA4, GluN1, GluN2A in vocal areas or nuclei of the zebra finch brain, agrees with that of Wada et al. report (2004). In addition present study also demonstrated that the glutamate receptor subunits mRNA was expressed in auditory areas or nuclei of the telencephalon (NCM), thalamus (Ov), and lowerbraintem (lateral lemniscus nuclei and cochlear nuclei).

In mammalian AMPA types, GluA1-3 mRNA signals are intense in all cortical layers of the rat cerebrum and GluA4 mRNA signal is weak (Sato et al., 1993). GluK5 mRNA is highly expressed in layers II and IV and GluK1-4 mRNAs are weak in the layers II and IV in the rat (Wisden and Seeburg, 1993). In NMDA types, GluN1 mRNA is expressed homogeneously throughout the whole brain of the rat and mouse (Wisden and Seeburg, 1993; Laurie and Seeburg, 1994). GluN2A mRNA is highly expressed in layers II-VI of the neocortex, hippocampus, and cerebellum, but is weakly expressed in the striatum, septum, thalamus, and hypothalamus (Watanabe et al., 1993). GluN2B mRNA shows a similar expression pattern of GluN2A in the cortex, while no expression is seen in the cerebellum

(Watanabe et al., 1993). Therefore, similar to the mammalian brain, the regional differences of glutamate receptor subunits mRNA, suggest that many glutamatergic projection areas are exist in the avian brain including auditory and song systems.

3.5. Summary

In the present study, I confirmed the distribution of five glutamate receptor subunit (GluA1, GluA4, GluK1, GluN1 and GluN2A) mRNA-expressing neurons in the zebra finch brain. These glutamate receptor subunits mRNA showed differential expression in the telencephalic and midbrain song nuclei or areas of the zebra finch similar to Wada et al. result in the zebra finch (2004). In addition, I mapped the distribution of five glutamate receptor subunits mRNA in the auditory system. The field L and NCM showed moderate expression of GluN2A mRNA. At least one of five glutamate receptor subunit mRNAs was expressed in nuclei (NM, NA, NL, LLv, MLd and Ov) of the ascending auditory pathway. GluN2A mRNA signals were observed in nuclei (RAm and nXIIts) of the descending motor pathway. The present finding indicate that glutamatergic projections are existed in the nuclei of auditory and song systems.

TABLE 5. Primers and probes for PCR amplification and in situ hybridization

Primers	
Forward primers (source of sequence)	Reverse primers (source of sequence)
GluA1 5'-GTGGGAGGTAACTTGGATTC-3'(zebra finch)	5'-GGCAACCAGCATGGCTAGTC-3'(pigeon)
GluA4 5'-ATGGAGCAAGGGATCCAGAG-3'(zebra finch)	5'-GCGACCTGAGAGGGATCTTG-3'(zebra finch)
GluK1 5'-GAGCTCATCAAAGCCCCTTC-3'(chicken)	5'- ACCATCCCATTCCACTCTCC-3'(zebra finch)
GluN1 5'- ATAAGGACGCGCGGAGGAAG-3(zebra finch)	5'-AAGGGATCTGTGCGTCTCGG-3'(chicken)
GluN2A 5'-CAACTACCCTGATATGCACC-3'(zebra finch)	5'-GTACTCCATGAATGCAGCTG-3'(chicken)
Probes	
Anti-sense probes (zebra finch)	Sense probes (zebra finch)
GluA1 5'-GATATAGAAAACCCCCGCCACATTGCTGAGACTCAG-3'	5'-CTGAGTCTCAGCAATGTGGCGGGGGTTTTCTATATC-3'
GluA4 5'-CTGCTAACTAGGAACAGGACCACACTGACACCAATG-3'	5'-CATTGGTGTCAGTGTGGTCCTGTTCCTAGTTAGCAG-3'
GluK1 5'-GTCCTTGTTGCTGTCTGTCATGTTAAGGCCACTGTA-3'	5'-TACAGTGGCCTTAACATGACAGACAGCAACAAGGAC-3'
GluN1 5'-GGGTCAGGTTCTGCTCTACCACTTTTTCTATCCTGC- 3'	5'-GCAGGATAGAAAAAGTGGTAGAGCAGAACCTGACCC-3'
GluN2B 5'-CCAGCTGGCTGCTCATGACTTCGTTCTTCTCGTTGT-3'	5'-ACAACGAGAAGAACGAAGTCATGAGCAGCCAGCTGG-3'

TABLE 6. Hybridization intensity of glutamate receptor subunit mRNAs in auditory and song nuclei of the zebra finch brain.

Regions	GluA1	GluA4	GluK1	GluN1	GluN2A
field L2	-	-	+	-	-
HVC	-	-	-	+	+
Lateral magnocellular nucleus of the anterior nidopallium	+	-	-	+	+
Caudomedial mesopallium	++	++	-	++	++
Field L2	-	-	+	-	+
Caudomedial nidopallium	+	++	-	+	++
Robust nucleus of the arcopallium	-	-	++	+	+
area X	+++	+	-	+++	++
Rotundus nucleus	+	+++	+	-	-
Ovoidal nucleus	+	+	++	-	+
Dorsal part of the lateral mesencephalic nucleus	+	+	+	+	+
Dorsomedial nucleus of the intercollicular complex	+	+	-	+	-
Ventral nucleus of the lateral lemniscus	-	-	-	-	+
Dorsal nucleus of the lateral lemniscus	-	-	-	-	++
Magnocellular nucleus	-	++	-	-	-
Angular nucleus	-	+	-	-	-
Laminar nucleus	-	+++	-	-	-
Inferior olivary nucleus	-	-	-	+	+
Retroambigual nucleus	-	-	+	-	++
Tracheosyringeal nucleus of the hypoglossal nerve	-	-	+	-	+

Hybridization intensity is evaluated as follows: area X (3+, Fig. 5.1F; 5.2D), robust nucleus of the arcopallium (2+, Fig. 5.1D), and Lateral magnocellular nucleus of the anterior nidopallium (1+, Fig. 5.2A).
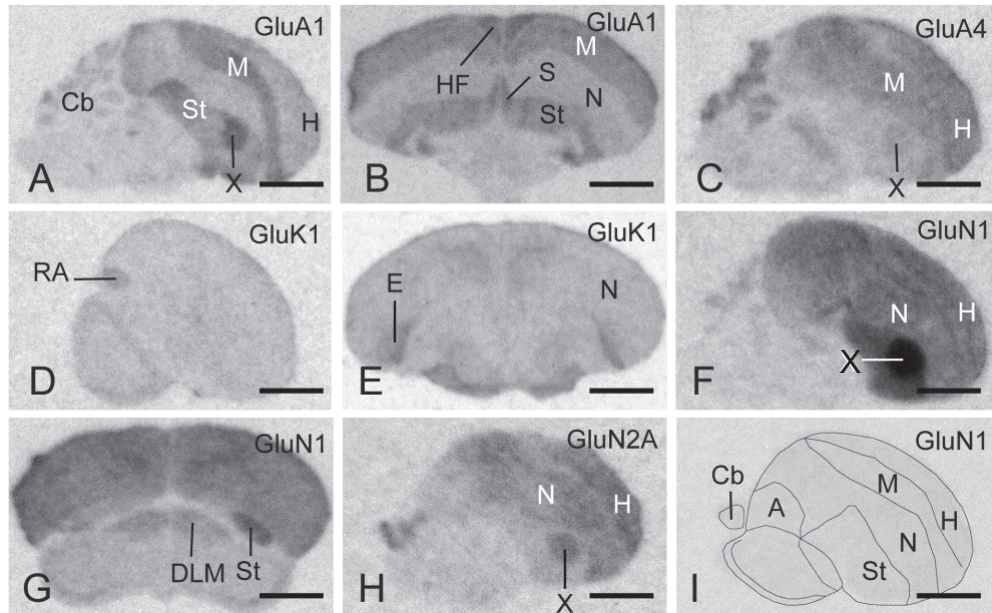
Fig. 5.1. Ionotropic glutamate receptor subunit mRNAs in the zebra finch brain. A-H: X-ray film autoradiograms show differential expression of GluA1 (A, B), GluA4 (C), GluK1 (D, E), GluN1 (F, G) and GluN2A (H) mRNAs in longitudinal sections. I: A sense probe shows no specific hybridization signal in an X-ray film autoradiogram. J-Q: S: septum. For other abbreviations, see list. Scale bars = 2mm in A-I.
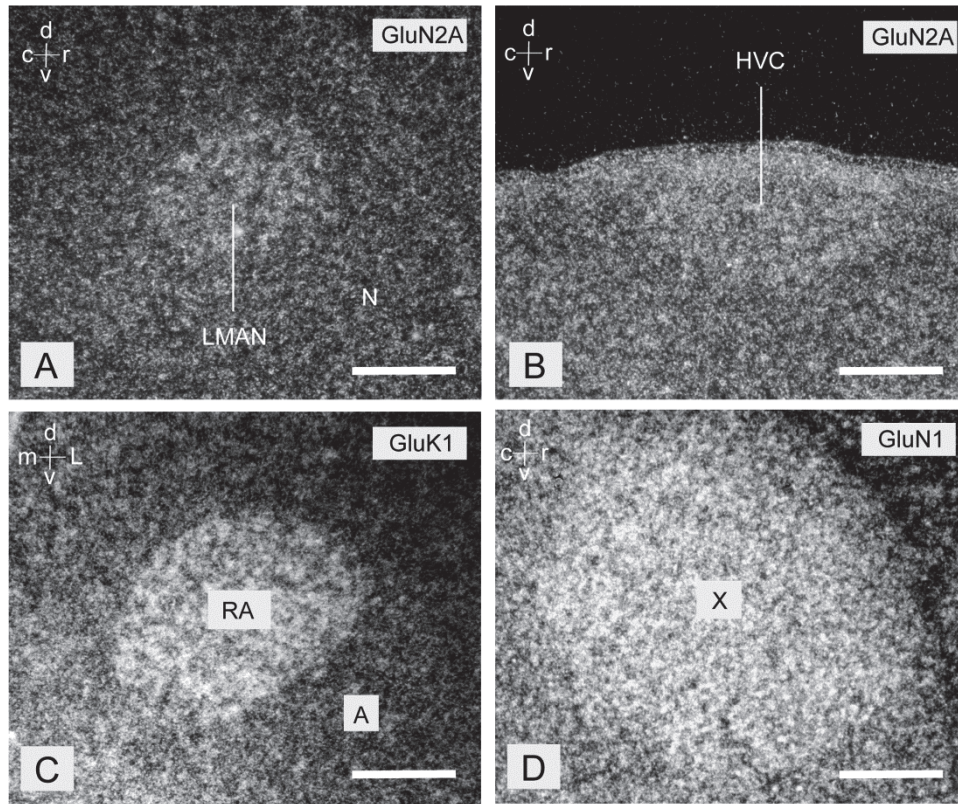
Fig. 5.2. Emulsion-coated sections show expression of glutamate receptor subunits mRNAs in neurons of the telencephalic song nuclei under dark-field illuminations. Photomicrograph of A,B,D are captured from the longitudinal sections and C from the transverse section. A-B: LMAN and HVC showed weak expression of GluN2A mRNA. C: RA showed moderate expression of GluK1 mRNA. D: GluN1 mRNA was highly expressed in the area X. For other abbreviations, see list. Scale bars = 300 μm in A-D.
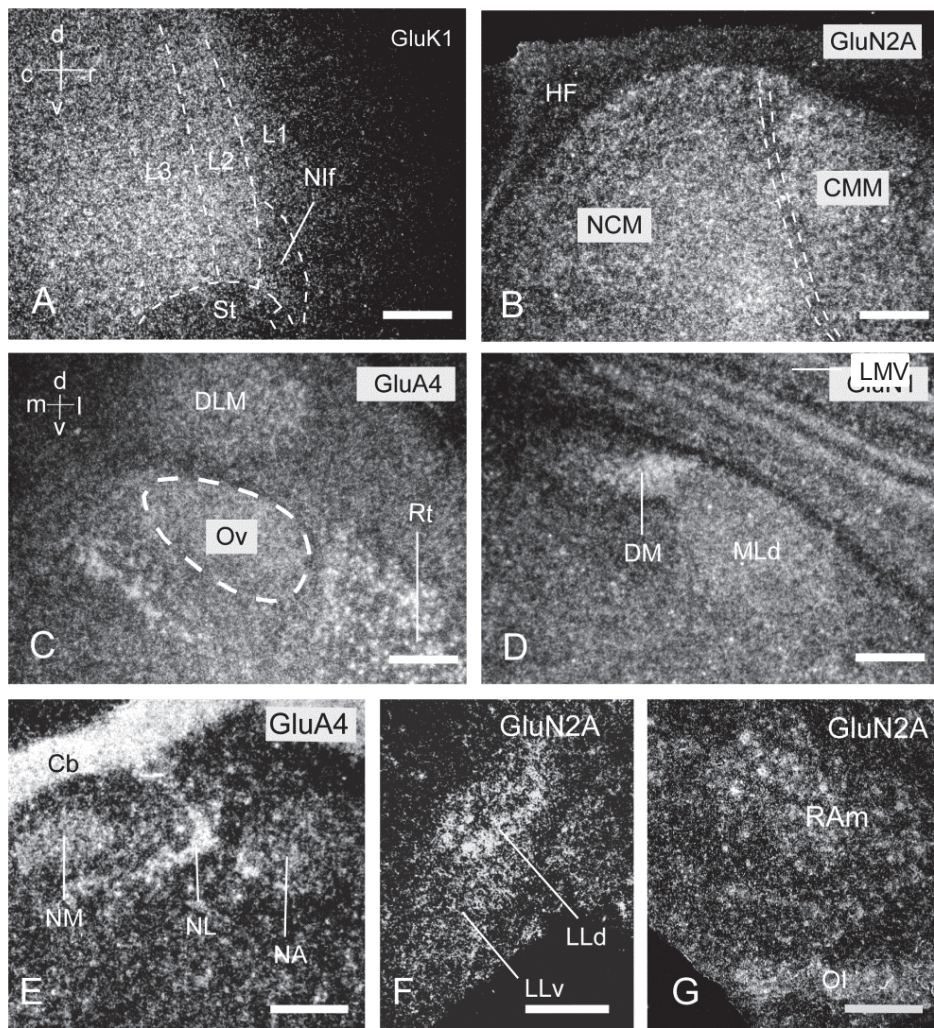
Fig. 5.3. Emulsion-coated sections show expression of glutamate receptor subunits mRNAs in telencephalon, thalamus, midbrain and lower brainstem of the zebra finch under dark-field illuminations. Photomicrographs of A, B are captured from the longitudinal sections and C-G from the transverse sections. A: The NIf, field L complex showed expression of GluK1 mRNA. B: The caudomedial mesopallium (CMM) and caudomedial nidopallium (NCM) showed positive signal for GluN2A mRNA. C, E: GluA4 mRNA expression in thalamic nuclei (C) and cochlear nuclei NM, NA, NL (E). D: GluN1 mRNA expression in the mesencephalic nuclei and optic tectum. F-G: GluN2A mRNA expressing neurons are seen in the ventral (LLv) and dorsal (LLd) nuclei of the lateral lemniscus (F) and retroambigual nucleus (G). LMV: lamina mesopallium ventralis. For other abbreviations, see list. Scale bars = A-D 300μm and E-F in 250μm.

General Discussion

Glutamatergic circuits are not well established in songbird brains. In the present study, a cDNA sequence of zebra finch VGLUT2 gene is determined and demonstrated distribution of VGLUT2-mRNA expressing glutamatergic neurons and the expression of VGLUT2 protein in the adult male zebra finch brain including auditory and song systems. Additionally, the distribution of mRNA for the glutamate receptor subunits GluA1, GluA4, GluK1, GluN1, and GluN2A were identified in the zebra finch brain especially in the auditory and song nuclei or areas of the telencephalon, thalamus, and lower brainstem. VGLUT2 mRNA expression indicate the origin of glutamatergic projections and localization of VGLUT2 protein indicates the glutamatergic projection terminals in the neural circuits. The projection targets of glutamatergic neurons in the neuronal circuits could also be identified using the expression patterns of glutamate receptor subunit mRNAs. Therefore, present findings consider morphological cues to the glutamatergic circuits in the songbird brain and provide insights on comparisons between birds and mammals.

Comparison of distribution of VGLUT2 mRNA, glutamate receptor subunit mRNAs, and VGLUT2 immunoreactivity with other birds and mammals

Studies on the expression of VGLUT2 mRNA and protein in avian brains are limited. In pigeon, VGLUT2 mRNA-expressing neurons are widely distributed in the whole brain except subpallium (Islam and Atoji, 2008). In the present study, zebra finch VGLUT2 mRNA express in the pallium, thalamus, midbrain and granular layer of the cerebral cortex, this is consistent with that of pigeon. However, no differential expression patterns in the areas of the telencephalon where song nuclei are found in zebra finches were

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observed in pigeons. In zebra finch, VGLUT2 mRNA was highly expressed in the telencephalic song nuclei HVC, LMAN, and RA. This is likely due to inherent variations between the two species and suggests that glutamatergic neurons exist in song control nuclei. High levels of VGLUT2 immunoreactivity have also been reported in the pallium and subpallium of the telencephalon, dorsal thalamus, hypothalamus, and cerebellar cortex, but not in the globus pallidus of the pigeon brain (Atoji, 2011). In the brainstem of the pigeon, a high level of VGLUT2 immunoreactivity is evident in the interpeduncular nucleus, MLd, isthmo-optic nucleus, NM, NA and NL. In the present study of the zebra finch, VGLUT2 immunoreactivity is high in the pallium and subpallium in the telencephalon, dorsal thalamus and optic tectum. The cerebellar cortex shows intense VGLUT2 immunostaining in glomeruli and molecular layer. VGLUT2 protein labeling regions is also representing expression of glutamate receptors mRNAs (Wada et al., 2004; present study). Particularly, GluA1, GluA2, GluA4, GluN1 and GluN2A mRNAs are expressed in the pallium and subpallium of the telencephalon, thalamus, midbrain, and several nuclei of the brainstem. The general brain expression of VGLUT2 mRNA and protein expression pattern of the pigeon is similar to that of the zebra finch. The regional difference of VGLUT2 mRNA-expressing glutamatergic neurons and VGLUT2 immunoreactivity as well as glutamate receptor subunit mRNA-expressing neurons indicates the existence of many glutamatergic circuits in the zebra finch brain.

In the mammalian cerebrum, VGLUT1 and VGLUT2 mRNAs exhibit a complementary expression patterns in the cortex but are not expressed in the subpallium except for weak VGLUT2 mRNA expression in the septal nuclei, nucleus of the diagonal band, and globus pallidus (Ni et al., 1994; Hisano et al., 2000; Fremeau et al., 2001). The cerebral cortex and hippocampus show a predominance of VGLUT1 mRNA expression whereas the diencephalon, brainstem, and deep cerebellar nuclei primarily express

VGLUT2 mRNA. The cerebellar cortex exhibits an intense expression of VGLUT1 mRNA in granule cells, but does not express VGLUT2 mRNA. In contrast, in the pigeon and zebra finch, VGLUT2 mRNA is expressed in the entire pallium of the telencephalon, thalamus, optic tectum, cerebellar cortex and brainstem (Islam and Atoji, 2008; present study). These finding suggests a predominance expression of VGLUT2 mRNA in glutamatergic neurons in the avian brain whereas complementary utilization of VGLUT1 and VGLUT2 mRNA occurs in the mammalian brain.

In the mammalian cerebrum, all layers of the cortex are immunoreactive for VGLUT1, but layers I and IV exhibit a slightly lower density of labeling for VGLUT2 (Bellocchio et al., 1998; Kaneko et al., 2002; Varoqui et al., 2002). In the hippocampus, all strata except for the pyramidal and granular layers are immunoreative for VGLUT1, but the outer part of the granular layer in the dentate gyrus selectively expresses VGLUT2. The globus pallidus exhibits weak VGLUT2 immunoreactivity but little immunoreactivity for VGLUT1 (Kaneko et al., 2002), whereas the caudate-putamen is immunoreactive for both VGLUT1 and VGLUT2. Prominent expression of VGLUT2 immunoreactivity has been observed in the thalamus and hypothalamus (Varoqui et al., 2002; Barroso-Chinea et al., 2007). In the cerebellum, VGLUT1 staining is evident only in parallel fibers, whereas VGLUT2 labeling exists in climbing fibers but mossy fibers in the glomeruli are immunoreactive for both VGLUT1 and VGLUT2. The expression patterns of VGLUT2 immunoreactivity in the pigeon and zebra finch telencephalon are similar to that of VGLUT1 in the mammalian telencephalon. However, in the thalamus, hypothalamus and climbing fibers of the cerebellar cortex, the expression pattern of VGLUT2 immunoreactivity is similar between birds and mammals. That is, the entirety of the immunoreactive patterns of VGLUT1 and VGLUT2 in the mammalian brain are consistent with VGLUT2 immunoreactivity in pigeon and zebra finch brains.

In the mammalian cerebrum, cortical layers also show a unique expression of ionotropic glutamate receptors. GluA1-3 mRNA signals are intense in all cortical layers of the rat cerebrum (Sato et al., 1993), and GluK5 mRNA is highly expressed in layers II and IV and GluK1-3 and GluK4 mRNAs are weak in the layers II and IV in the rat (Wisden and Seeburg, 1993). But GluN1mRNA is expressed homogeneously throughout the whole brain of the rat and mouse (Wisden and Seeburg, 1993; Laurie and Seeburg, 1994) and GluN2A mRNA is highly expressed in layers II-VI of the neocortex, hippocampus, and cerebellum (Watanabe et al., 1993). In the present study, VGLUT2 mRNA-expressing glutamatergic were found in the pallium, but not found in the subpallium like mammals. Whereas, VGLUT2 protein and mRNA for GluA1, GluN1 and GluN2A were expressed both in the pallium and subpallium of the zebra finch. The principal pallial neurons have excitatory projections to the striatum in birds (Veenman et al., 1995) and mammals (Broman et al., 2004). The striatum showed positive signals for GluA1-3, GluK5 and GluN1 receptors in birds and mammals (Sato et al., 1993; Wiseden and Seeburg, 1993; Ottiger et al., 1995; Wada et al., 2004; Islam and Atoji, 2008 and present study). The present results demonstrate the regional differences of VGLUT2 mRNA and protein as well as GluR mRNA in the zebra finch brain and suggest many glutamatergic projections and circuits exist in the avian brain like mammals.

Glutamatergic circuits in the auditory system

In mammalian auditory pathway, medial geniculate body receives inputs from inferior colliculus that comes from auditory nuclei of the hindbrain, and in turn, projects to the auditory cortex (Butler and Hodos, 2005). Ito and Oliver (2010) showed VGLUT1 mRNA-expressing glutamatergic neurons of auditory cortex, and VGLUT2 mRNA-expressing glutamatergic neurons of superior olivary complex and of ventral

cochlear nucleus are project to the inferior colliculus. VGLUT1 and VGLUT2 immunoreactivity and GluA1-4, GluK1, GluN1 mRNAs are distributed in the medial geniculate nucleus of thalamus, and cochlear nuclei, superior olivary complex, nucleus of the trapezoid body, nucleus of the lateral lemniscus of the lower auditory system in mammals (Laurie and Seeburg, 1993; Wisden and Seeburg, 1993; Kaneko et al., 2002; Sato et al., 1993; Ito et al., 2011).

In birds, the main ascending auditory pathway in the brainstem of the zebra finch brain is similar to that in other birds (Vates et al., 1996; Krützfeldt et al., 2010a, b; Wild et al., 2010) and contains GABAergic and glutamatergic neurons. GABA-positive neuritis and puncta are identified in most nuclei of the ascending auditory pathway, including the NM, NA, NL, OS, MLd and Ov (Pinaud and Mello, 2007). Electrophysiological studies have revealed that the cochlear nucleus NM receives highly active excitatory glutamatergic inputs that originate from spiral ganglion neurons (Warchol and Dallos, 1990; Born et al., 1991). In the pigeon, the spiral ganglion expresses VGLUT2 mRNA (unpublished data). The present findings demonstrate that the NM and NA moderately or weakly express GluA4 mRNA and that cell bodies in these two nuclei are surrounded by intense VGLUT2 immunoreactivity in the pericellular puncta. Pericellular VGLUT2 immunoreactivity has been observed in the asymmetric large axon terminals of the NM and NL of pigeons (Atoji, 2011). The NM projects to the NL (Krützfeldt et al., 2010a) and expresses VGLUT2 mRNA-expressing glutamatergic neurons, whereas NL expresses GluA4 mRNA in the present study. It appears that in the zebra finch, the NM and NA receive glutamatergic inputs from the cochlear nerve and, in turn, the NM sends glutamatergic projections to the NL. Both the NA and NL send ascending projections to the OS, the lateral lemniscus nuclei, and the MLd (Krützfeldt et al., 2010a, b). The OS, ventral and dorsal nuclei of the lateral lemniscus, NL, and NA display VGLUT2 mRNA expression. In this study, the OS was

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negative for mRNAs of AMPA, kainate, and NMDA receptor subunits. However, this does not necessarily indicate an absence of ionotropic glutamate receptors in the OS as this nucleus contains immunoreactive varicosities for VGLUT2. In the owl, the immunostaining of GluA2/3 and GluA4 is observed in neurons within the OS (Levin et al., 1997) and it is possible that other ionotropic glutamate receptor subunits are present in the OS of the zebra finch. The ventral and dorsal nuclei of the lateral lemniscus express VGLUT2 mRNA and glutamate receptor GluN2A mRNA (present study). The lateral mesencephalic nucleus (MLd) is known to express GluA1, GluA4, GluN1, and GluN2A mRNAs (Wada et al., 2004; present study), which suggests that glutamatergic axons project to this region from the NA and NL. The Ov receives input from the MLd (Karten, 1967). In the present study, MLd was highly distributed VGLUT2 mRNA-expressing glutamatergic neurons and send afferents to the Ov. The Ov exhibited mRNA signal for GluA1, GluA4, GluK1, and GluN2A and also positive for VGLUT2 immunoreactivity. It is likely that the main ascending relay nuclei from the NM and NA to the Ov contain, at least, glutamatergic projections.

The telencephalic auditory areas, field L subfields and the caudomedial nidopallium (NCM) receive inputs from the Ov (Karten et al., 1967; Wild et al., 1993; Vates et al., 1996). The field L subfields (L1, L2, L3) are connected to each other as well as to the NCM and caudomedial mesopallium (CMM) (Vates et al., 1996), and these telencephalic areas exhibit mRNA signals for GluK1, GluN1 and GluN2A (present study). In the present study, the CMM and NCM exhibited high or moderate expression of VGLUT2 mRNA, and high levels of the VGLUT2 protein were localized, whereas the field L subfields showed moderate or weak expression for VGLUT2 mRNA and protein. Furthermore, the Ov highly expressed VGLUT2 mRNA-expressing glutamatergic neurons. Together the current results demonstrate that glutamatergic axons project from the thalamus to the telencephalon in the

auditory system of the zebra finch brain. The telencephalic auditory areas showed GABA-immunoreactivity and mRNA signals for glutamic acid decarboxylase (zGAD65, Pinaud et al., 2008). This study also demonstrated that GABAergic interneurons inhibit the excitatory response. Therefore, it seems that both glutamatergic and GABAergic neurons play pivotal roles in the auditory pathway in zebra finch brains.

Glutamatergic circuits in the song system

Glutamatergic circuits were found in the song system to the zebra finch brain. In the telencephalic song nuclei, HVC, RA, and LMAN have been suggested to contain both glutamatergic and GABAergic neurons. AMPA currents have been identified in these nuclei (Stark and Perkel, 1999). The HVC contains two types of excitatory neurons that project either to area X or the RA as well as one type of inhibitory interneuron (Mooney, 2000; Wild et al., 2005). Projections from the HVC to the RA are sensitive to AMPA and NMDA agonists, and area X is responsive to NMDA agonists (Mooney, 2000; Sizemore and Perkel, 2008). GABAergic interneurons in the HVC receive collaterals from RA projection neurons and depress projection neurons to both the RA and area X (Mooney and Prather, 2005; Wild et al., 2005). The RA expresses glutamate receptor subunit mRNAs, including GluA2, GluK1, and GluN2A, and area X displays positive signals for GluA1, GluN1, and GluN2A mRNAs (Wada et al., 2004; present study). In the present study, intense hybridization signals for VGLUT2 mRNA were observed in the cell bodies of neurons in the HVC, and weak VGLUT2 immunoreactivity was observed in the RA and area X. These results suggest that projection neurons in the HVC are glutamatergic. The RA consists of projection neurons and interneurons (Mooney and Konishi, 1991; Spiro et al., 1999; Stark and Perkel, 1999). The projection neurons send long axons to the dorsomedial nucleus of the intercollicular complex (DM), retroambigual nucleus (RAm),

and racheosyringeal motor nucleus of the hypoglossal nerve (nXIIts) as well as collaterals to other projection neurons within the RA. Interneurons within the RA are GABAergic, receive excitatory input from the HVC and LMAN, and make inhibitory contacts with projection neurons (Spiro et al., 1999). In the present study, intense hybridization signals for VGLUT2 mRNA were observed in the cell bodies of neurons in the RA whereas the DM was positive for GluA1, GluK1, and GluN2A mRNA signals (Wada et al., 2004; present study). Moreover, this study identified the expression of GluK1 and GluN2A mRNA in the RAm and nXIIts as well as VGLUT2 immunoreactivity in the DM, RAm, and nXIIts, which suggests that projection neurons in the RA are glutamatergic. It has been shown that the LMAN evokes excitatory inputs in the RA via NMDA-type receptors (Mooney, 1992; Spiro et al., 1999; Sizemore and Perkel, 2008). GABA[A] receptor mRNA-expressing neurons and GABA immunoreactive neurons have also been observed in the LMAN (Grisham and Arnold, 1994; Pinaud and Mello, 2007; Thode et al., 2008), although questions about whether glutamatergic and GABAergic neurons in this region interact remain unanswered. The evidence shows that the LMAN projects to area X (Vates and Nottehohm, 1995) and that the RA and area X are thought to be the target nuclei of glutamatergic projection neurons. The current study confirmed high distribution of VGLUT2 mRNA-expressing glutamatergic neurons in the LMAN, which suggests that projection neurons in this nucleus are glutamatergic. It is known that area X sends projections to the DLM, and DLM projects back to the LMAN (Bottjer et al., 1989; Vates et al., 1997; Luo et al., 2001). The present study found that the DLM expresses VGLUT2 mRNA as well as mRNAs for GluA1, GluA2, and GluN2D (Wada et al., 2004). However, although AMPA and NMDA receptors in the DLM likely receive glutamatergic inputs from unidentified areas, this does not include area X because projection neurons from area X to the DLM are GABAergic (Grisham and Arnold, 1994; Luo and Perkel, 1999, 2002).

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Accordingly, this study did not find VGLUT2 mRNA expression in area X. Taken together with previous studies investigating ionotropic glutamate receptor subunit mRNAs using in situ hybridization (Wada et al., 2004), the present in situ hybridization assays for VGLUT2 mRNA and the immunohistochemistry analyses for VGLUT2 support the presence of glutamatergic neurons, their target neurons, and glutamatergic terminals in the HVC, RA and LMAN. Additionally, it appears that glutamatergic neurons and GABAergic neurons co-exist independently in the HVC, RA, and LMAN.

In the descending motor pathway which extends from the telencephalon to the tracheosyringeal motor nucleus in the brainstem, the DM receives afferents from the RA, and the RAm receives afferents from the RA and DM (Wild, 1993; Kubke et al., 2005; Wild et al., 2009). The tracheosyringeal motor nucleus receives excitatory inputs from the RA and RAm (Kubke et al., 2005). The current study found that the mRNA expressions of ionotropic glutamate receptor subunits; VGLUT2 immunoreactivity in the DM, RAm, and nXIIts; and the hybridization signal for VGLUT2 mRNA are high in the DM and weak in the RAm. Therefore, these results support the idea that the descending motor pathway is glutamatergic. A weak hybridization signal for VGLUT2 mRNA was also observed in nXIIts, but the targets of glutamatergic projections from this nucleus remain unclear, because the tracheosyringeal motor nucleus, which innervates innervating the syringeal muscles, appears to be cholinergic (Cookson et al., 1996).

Functional implications of glutamate in song system

Pharmacological or electrophysiological studies suggest that glutamate plays an important role for learning or memory in the song system of the zebra finch. Aamodt et al. (1996) reported that systematical injection of NMDA receptor antagonist MK-801 just prior to tutoring impairs vocal learning to copy the first tutor's song significantly. Infusion

of NMDA receptor antagonist amino-5-phosphopentanoic acid into LMAN before exposure of tutor's songs impaired song learning as well (Basham et al., 1996). Blocking of NMDA receptors by injecting NMDA receptor antagonist (2R)-amino-5-phosphonopentanoate into HVC before tutoring prevented dendritic spine enlargement of HVC neurons and disrupted copying of the tutor song or imitative learning (Roberts et al., 2012). In slice preparations, Ding and Perkel (2004) reported long-term potentiation in area X neurons depending on the activation of NMDA receptors. Long-term potentiation in LMAN neurons was also dependent on NMDA receptors (Boettiger and Doupe, 2001). The present study that glutamatergic neurons are localized in song nuclei provides morphological basis to support functions via NMDA receptors in these studies.

Conclusions

The distribution of glutamatergic neurons and their putative projection terminals in the brain of songbirds has not been identified before. The present study is determined the cDNA sequence of zebra finch VGLUT2 gene in the zebra finch brain, and demonstrated origins of glutamatergic neurons and putative targets of them in the zebra finch brain by in situ hybridization for VGLUT2 mRNA and ionotropic glutamate receptor subunit mRNAs and immunohistochemistry for VGLUT2 protein.

The nucleotide sequence of zebra finch VGLUT2 cDNA showed a single open-reading frame of 1746 base pairs in the total length of 1779 base pairs. The in situ hybridization show VGLUT2 mRNA-expressing glutamatergic neurons are widely distributed in the zebra finch brain including the telencephalic pallium, many thalamic nuclei, optic tectum, several mesencephalic nuclei and a discrete brainstem nuclei. The target nuclei of the VGLUT2 mRNA-expressing glutamatergic nuclei show immunoreactivity for VGLUT2 and hybridization signals for glutamate receptor subunit mRNAs. Therefore, the present findings suggest the presence of many glutamatergic circuits in the zebra finch brain including auditory and song systems.

Glutamatergic circuits were found in the auditory pathways to the zebra finch brain. The cochlear nerve projects to both the nuclei angularis (NA) and magnocellularis (NM) that in turn project to the superior olivary nucleus (OS). The pathway from OS to field L passes through a single route: OS → dorsal part of the lateral mesencephalic nucleus (MLd) → ovoidal nucleus (Ov) → field L (Vates et al., 1996; Krützfeldt et al., 2010a, b; Wild et al., 2010). In the present study, high VGLUT2 mRNA signal was seen in the ascending auditory nuclei NM, NA, nucleus laminaris (NL), ventral nucleus of the lateral lemniscus (LLv), MLd, and Ov. Weak mRNA signal was seen in the OS and dorsal nucleus

of the lateral lemniscus (LLd). VGLUT2 immunoreactivity was intense in the NM, NA, NL and MLd, moderate in the OS, LLv, LLd, and weak in the Ov. On the other hand, at least one of five ionotropic glutamate receptor subunit mRNAs (GluA1, GluA4, GluK1, GluN1 and GluN2A) was expressed in the auditory nuclei or areas in the present study, except OS. The OS was negative for mRNAs of AMPA, kainate, and NMDA receptor subunits. But the OS contains immunoreactive varicosities for VGLUT2 in zebra finch. Immunostaining of GluA2/3 is observed in neurons within the OS in the owl (Levin et al., 1997) and it is possible that other ionotropic glutamate receptor subunits are present in the OS of the zebra finch. It is likely that the main ascending relay nuclei from the NM and NA to the Ov contain, at least, glutamatergic projections (Fig. 6).

In songbirds, the field L complex project to caudomedial nidopallium (Kelley and Nottebohm, 1979; Vates et al., 1996). The field L subfields (L1, L2, L3) are connected to each other as well as to the NCM and caudomedial mesopallium (CMM) (Vates et al., 1996), and these telencephalic areas show mRNA signals for VGLUT2 (present study). VGLUT2 immunoreactivity was weak or moderate in the field L2 and NCM. These telencephalic areas exhibit at least one of five ionotropic glutamate receptor subunit mRNAs (present study). The Ov show high density of VGLUT2 mRNA-expressing glutamatergic neurons. Thus, glutamatergic axons project from the thalamus to the telencephalon in the auditory system of the zebra finch brain (Fig. 6). These results supported that the glutamatergic circuits play a pivotal role in the songbird auditory pathway.

Glutamatergic circuits were found in the song system to the zebra finch brain. The telencephalic song nuclei HVC, RA and LMAN contained large number of VGLUT2 mRNA-expressing glutamatergic neurons in the present study. But, area X was devoid of VGLUT2 mRNA expression. High VGLUT2 mRNA signals were found in the anterior

portion of nucleus dorsolateralis anterior thalami, pars medialis (aDLM), which is a song nucleus part of medial nucleus of the dorsolateral thalamus (DLM). The telencephalic song nuclei including area X, weak VGLUT2 protein expression were detected. In song nuclei, GluN2A mRNA signal was found in the HVC, RA, LMAN and area X. The distribution patterns of VGLUT2 RNA and differential expression of VGLUT2 protein as well as glutamate receptor subunits mRNAs indicated glutamatergic networks embracing in the song pathway (Fig. 7).

In the descending motor pathway which extends from the telencephalon to the tracheosyringeal motor nucleus in the brainstem, the dorsomedial nucleus of the intercollicular complex (DM) receives afferents from the RA, and the retroambigual nucleus (RAm) receives afferents from the RA and DM (Wild, 1993; Kubke et al., 2005; Wild et al., 2009). The tracheosyringeal motor nucleus receives excitatory inputs from the RA and RAm (Kubke et al., 2005). In the present study, the nuclei of the descending motor pathway display VGLUT2 mRNA-expressing glutamatergic neurons. The DM, RAm, and nXIIts show hybridization signal for glutamate receptor subunit mRNAs as well as positive VGLUT2 immunoreactivity. Therefore, these results support the idea that the descending motor pathway is glutamatergic.

The morphological distribution of VGLUT2 and glutamate receptor subunit mRNAs, and localization of VGLUT2 protein in auditory and song pathways support that glutamatergic circuits (Figs. 6, 7) are involved in song production and vocal learning in songbirds.
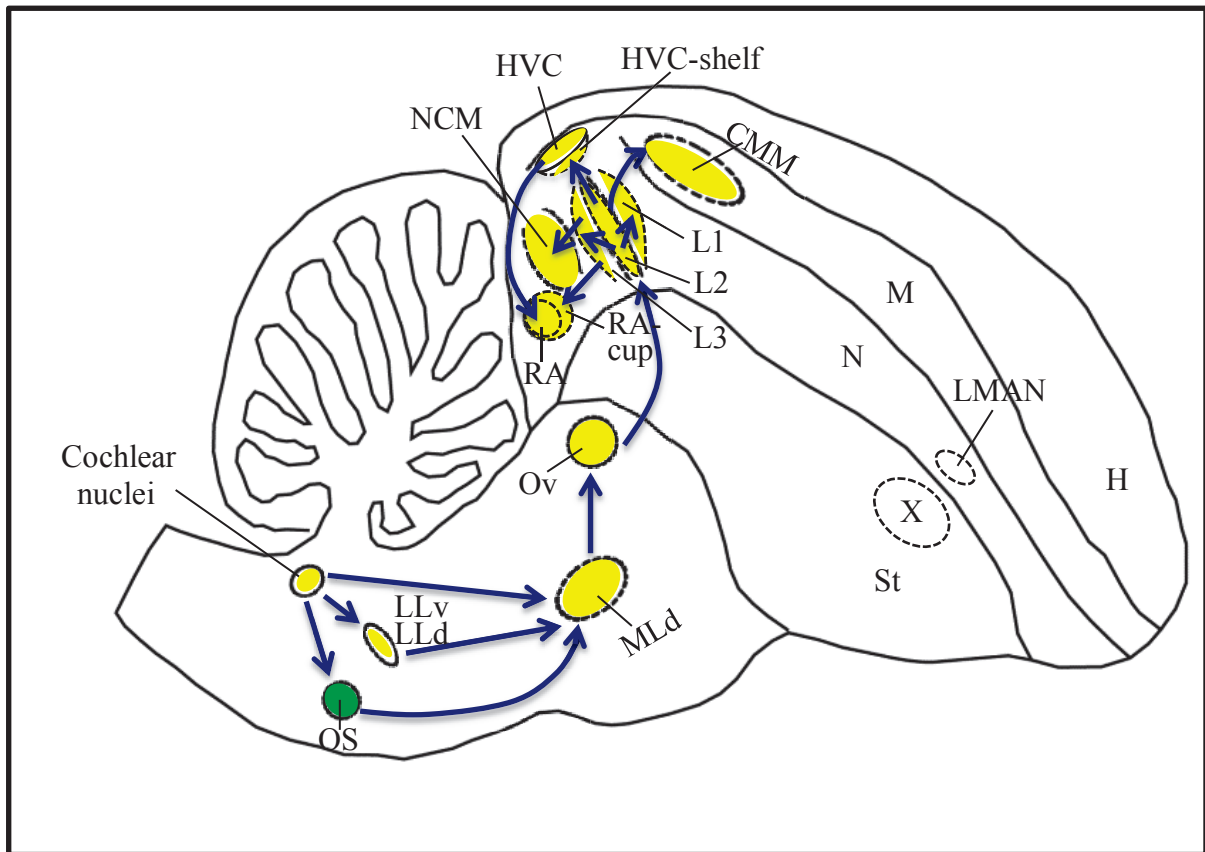
Fig. 6. Schematic longitudinal section of zebra finch brain showing the glutamatergic circuits in the ascending auditory pathway based on VGLUT2 and glutamate receptor subunits genes expressions. Yellow nuclei show hybridization signal for VGLUT2 and glutamate receptor subunits mRNAs, and immunoreactivity for VGLUT2. Green nucleus shows hybridization signal for VGLUT2 mRNA and immunoreactivity for VGLUT2. H, hyperpallium; LMAN, lateral magnocellular nucleus of the anterior nidopallium; LLv, ventral nucleus of the lateral lemniscus; LLd, ventral nucleus of the lateral lemniscus; M, mesopallium; MLd, dorsal part of the lateral mesencephalic nucleus; N, nidopallium; Ov, nucleus ovoidalis; OS, superior olivary nucleus; RA, nucleus robustus arcopallii; HVC, letter-based proper name; St, striatum; nXIIts, nucleus nervi hypoglossi, pars tracheosyringealis; X, area X.
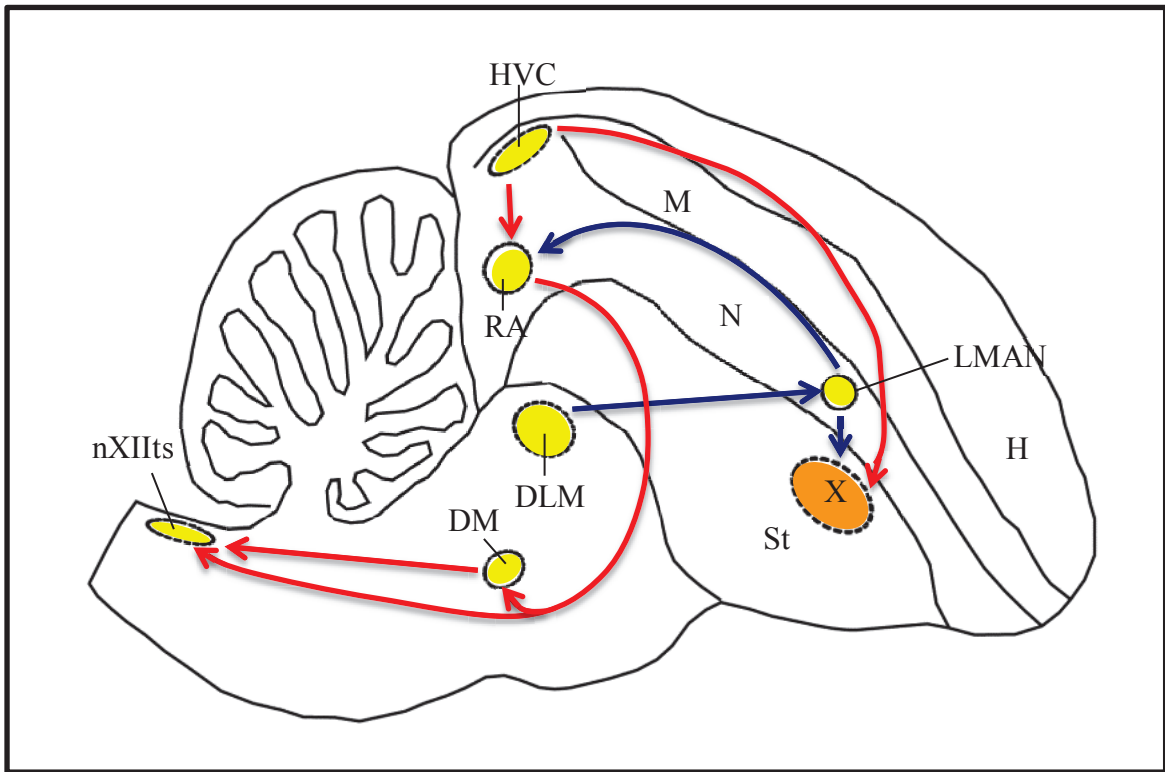
Fig. 7. Schematic longitudinal section of zebra finch brain showing the glutamatergic circuits in the song pathway based on VGLUT2 and glutamate receptor subunits genes expressions. Red arrows represent the glutamatergic circuits of the motor or posterior forebrain pathway and blue arrows represents the glutamatergic circuits of the anterior forebrain pathway. Yellow nuclei show hybridization signal for VGLUT2 and glutamate receptor subunits mRNAs, and immunoreactivity for VGLUT2. Orange area shows hybridization signal glutamate receptor subunits mRNA and immunoreactivity for VGLUT2. DLM, medial part of the dorsolateral anterior nucleus of the thalamus; DM, dorsomedial nucleus of the intercollicular complex; H, hyperpallium; LMAN, lateral magnocellular nucleus of the anterior nidopallium; M, mesopallium; N, nidopallium; RA, robust nucleus of the arcopallium; HVC, letter based proper name; St, striatum; nXIIts, tracheosyringeal nucleus of the hypoglossal nerve; X, area X.

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Abbreviations

A   arcopallium

aDLM   anterior nucleus of the dorsal lateral medial thalamus

Cb   cerebellum

CMM   caudomedial mesopallium

DLM   medial part of the dorsolateral anterior nucleus of the thalamus

DM   dorsomedial nucleus of the intercollicular complex

E   entopallium

H   hyperpallium

HF   hippocampal formation

HVC   letter-based proper name

Ipc   parvocellular isthmic nucleus

L1,2,2a,3   field L1, L2, L2a, L3

LLd   dorsal nucleus of the lateral lemniscus

LLv   ventral nucleus of the lateral lemniscus

LMAN   lateral magnocellular nucleus of the anterior nidopallium

M   mesopallium

MLd   dorsal part of the lateral mesencephalic nucleus

N   nidopallium

NA   angular nucleus

NC   caudal nidopallium

NCM   caudomedial nidopallium

NIf   interfacial nucleus

NL   laminar nucleus

NM	magnocellular nucleus
nXIIts	tracheosyringeal motor nucleus of the hypoglossal nerve
OB	olfactory bulb
OI	inferior olivary nucleus
OS	superior olivary nucleus
Ov	ovoidal nucleus
RA	robust nucleus of the arcopallium
RAm	retroambigual nucleus
Rt	rotundus nucleus
St	striatum
X	area X

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