Expression of a Vesicular Glutamate Transporter mRNA in the Brain of the Turtle (*Trachemys scripta elegans*)

（カメの脳における小胞性グルタミン酸トランスポーターmRNAの発現）

2016

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Contents

Introduction

Glutamate, the principal excitatory neurotransmitter in the brain, is involved in normal brain functions, such as learning and memory, and has been implicated in many neurological and psychiatric disorders such as cerebral stroke, Alzheimer's disease, Parkinson's disease, Huntington's disease and epilepsy (Liguz-Lecznar and Skangiel-Kramska, 2007). In these disorders, glutamate induced excitotoxic cell death occur due to anoxia or ischaemia. To evaluate the molecular mechanisms of neuroprotective pathways in ischaemic conditions, turtles have been found to be a useful animal model, because they can inherently survive conditions of oxygen deprivation and post-anoxic reoxygenation without brain damage (Milton and Prentice, 2007). Furthermore, studies on freshwater turtles showed that glutamate is involved in survival strategies under anoxic conditions by controlling ion channels (Bickler et al., 2000; Pamenter et al., 2008). Wilson and Kriegstein (1991) reported that turtle cortical neurons survive glutamate exposures that are lethal to mammalian neurons. In the turtle, glutamate is suggested to be a neurotransmitter, based on pharmacological experiments in which antagonists for ionotropic glutamate receptors injection stopped firing of the neurons (Berkowicz et al., 1994; Larson-Prior et al., 1991, 1995; Muñoz et al., 1998a, 1998b). Pharmacological data also suggest that in the medial cortex of the turtle, glutamate is involved in learning and memory (Muñoz et al., 1998a, 1998b). Despite the importance of glutamate as a neurotransmitter and of the turtle as a research model, the distribution of glutamatergic neurons in the turtle brain remains unknown.

Vesicular glutamate transporters (VGLUTs) mediate glutamate transport into synaptic vesicles at presynaptic terminals, and glutamate released from the vesicles binds to glutamate receptors on postsynaptic membranes (Santos et al., 2009). Three isoforms of VGLUT (VGLUT1-3) have been identified in mammals (Aihara et al., 2000; Fremeau et al., 2001, 2002;

Gras et al., 2002; Herzog et al., 2001; Ni et al., 1994, 1995) and their mRNA distribution in the brain has been investigated extensively in mammals. In mammals, VGLUT1 and VGLUT2 are considered specific biomarkers for glutamatergic neurons, and they show a complementary distribution pattern in the mammalian brain (Fremeau et al., 2004; Hisano et al., 2000; Ni et al., 1994, 1995). VGLUT1 is expressed primarily in the cerebral cortex, hippocampus, and cerebellar cortex, while the highest expression of VGLUT2 is in the thalamus, hypothalamus, amygdaloid nuclei, lower brainstem, and cerebellar nuclei. In the cerebral cortex of rats, VGLUT2 is expressed across layer IV in almost all cortical regions. In some areas, VGLUT2 mRNA is also found in layer VI. Other significant VGLUT2 mRNA expression is found consistently in the subiculum, hippocampus, amygdala, and septum (Hisano et al., 2000). VGLUT3 is distributed sparsely and is found in a discrete subpopulation of non-glutamatergic neurons that synthesise other neurotransmitters, such as acetylcholine, serotonin, and γ-aminobutyric acid (GABA), in the brain (Fremeau et al., 2002; Gras et al., 2002; Herzog et al., 2004). Two isoforms of VGLUT (VGLUT2-3) have been identified in birds and their mRNA distributions have been studied in the brain (Atoji and Karim, 2014; Islam and Atoji, 2008). In birds, VGLUT1 gene has not been identified. Conversely, VGLUT2 mRNA was found to be expressed in the pallium of telencephalon, diencephalon, mesencephalon, lower brainstem, and cerebellar cortex (Islam and Atoji, 2008; Karim et al., 2014). The authors suggested that the distribution areas of avian VGLUT2 mRNA in the brain appear to correspond to those of expression by VGLUT1 and VGLUT2 in mammalian brains. A recent study by *in situ* hybridization from our laboratory has revealed that the VGLUT3 mRNA is expressed in neurons of the caudal linear nucleus of the pigeon brain, a serotonergic nucleus (Atoji and Karim, 2014).

The diversity of reptiles and their evolutionary relationship to mammals make reptilian brains great models to explore questions related to the structural and functional evolution of mammalian brains (Naumann et al., 2015). To this end, comparative studies seek to identify homologies of particular brain regions between reptiles and mammals. Comparative embryological analysis demonstrated conserved expression patterns of several transcription factors in the dorsal part of the reptilian, birds, and mammalian telencephalon, suggesting that those pallial regions are specified as the homologous territories in all amniotes (Butler et al., 2011). In adult mammals and birds, glutamatergic neurons are found in areas of telencephalon called pallium, which has been provided recently by the localization of VGLUT mRNA via *in situ* hybridisation (Broman et al., 2004; Fremeau et al, 2001; Islam and Atoji, 2008; Karim et al., 2014; Ni et al., 1995). From a phylogenetic viewpoint, it is important to do similar studies in the reptilian brain in adult animal. However, none of the VGLUTs has yet been investigated in any reptilian species including the turtle. Therefore, the objective of this study was to determine the distribution of a VGLUT mRNA in the turtle brain by *in situ* hybridisation methods.

Materials and Methods

Animals

Animal handling procedures were approved by the Committee for Animal Research and Welfare of Gifu University. In total, six turtles (*Trachemys scripta elegans*, weighing 560-1385 g) of both sexes were used in this study. The turtles were anaesthetised with an intraperitoneal injection of chloral hydrate (5.4 g/kg body weight). For isolation of total RNA, the brains were dissected quickly, and the pieces were kept in RNA stabilisation solution (RNAlater, Ambion, Austin, TX, USA) and stored at -60°C until use. For *in situ* hybridisation, after deep anaesthesia, turtles were perfused with Ringer's solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Then, the brains were removed and stored in the same fixative for at least 3 days and then transferred to 30% sucrose in 0.1 M PB at 4°C until they had sunk in the solution. The brains were then cut transversely at 40 μm on a cryostat, mounted on poly-L-lysine and MAS-coated slides (Matsunami Glass Ind. Ltd., Japan), and stored at -20°C until *in situ* hybridisation was performed.

RNA isolation to cRNA probe synthesis

Total RNA was isolated from the turtle brain samples (olfactory bulb, cerebrum, optic tectum, and cerebellum) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Briefly, each brain sample was homogenised in 1 mL TRIzol reagent followed by a 5 min incubation at room temperature. Then, 0.2 mL chloroform was added and mixed vigorously. The samples were centrifuged (12,000 g, 15 min, 4°C). The supernatant was collected in a new tube, and 0.5 mL isopropanol was mixed and the samples centrifuged (12,000 g, 15 min, 4°C) to precipitate total RNA. After washing in 75% ethanol, the precipitate was dissolved in diethyl pyrocarbonate-

treated water, the concentration was checked using the Biophotometer plus (Eppendorf AG, Hamburg, Germany), and the samples were stored at -60°C until use.

First-strand complementary DNA (cDNA) from turtle brain total RNA was synthesised using the Superscript III First-Strand Synthesis System (Invitrogen). Briefly, 0.5 µg total RNA was added to a mixture of 2.5 µM oligo(dT) primer and 0.5 mM 2′-deoxyribonucleotide 5′-triphosphates (dNTP) and incubated at 65°C for 5 min and placed on ice. The supplied reaction buffer for the enzyme, 5 mM dithiothreitol, 2 units RNase out, and 10 units Superscript III reverse transcriptase were added to the mixture and incubated at 50°C for 60 min. The reaction was then stopped by heating at 70°C for 15 min, and the synthesised product was preserved at -30°C until use.

Digoxigenin (DIG)-labelled antisense and sense RNA probes were synthesised from purified VGLUT PCR product using the DIG RNA labelling kit (Roche Diagnostics GmbH, Mannheim, Germany) for *in situ* hybridisation. Briefly, a forward primer (5′-GGGGGACAGATTGCAG ATT T-3′; bases 1105-1124 of LC066047) with an attached T7 promoter sequence at the 5′ terminus (5′-TAATAC GACTCACT ATAGGG-3′) and a reverse primer (5′-TTCACTTGTCTGTTCAGGGTC A-3′; bases 1527-1548 of LC066047) with an attached Sp6 promoter sequence at the 5′terminus (5′-ATTTAGGTGAC ACTATA GAA-3′) were used for RT-PCR to obtain the VGLUT PCR product (444 bp) from total RNA extracted from turtle brain. For PCR, 500 ng of the synthesised cDNA were mixed with Takara ExTaq (Takara Bio, Tokyo, Japan) and the supplied dNTP mixture and ExTaq buffer. Then 1 µM forward and reverse primers were added. PCR was performed via 35 cycles of amplification (denaturation at 94°C for 30 s, annealing at 56°C for 40 s, extension at 72°C for 1 min) and a final extension at 72°C for 5 min. The PCR product obtained was purified using the Wizard SV Gel and PCR Clean-up

System (Promega, Madison, WI, USA). Purified PCR product (1 μg) was mixed with reagents from the DIG RNA labelling kit: 2 μL 10× NTP labelling mixture, 2 μL 10× transcription buffer, 1 μL RNase inhibitor, and 2 μL T7 polymerase, for generating the sense RNA probe, or Sp6 polymerase, for generating the antisense RNA probe, and RNase-free water to a final volume of 20 μL. After incubation at 37°C for 4 h, 1.25 μL 8 M LiCl and 49 μL prechilled 100% ethanol were added, and the mixture was precipitated at -20°C overnight. Then, the synthesised RNA transcript was collected by centrifugation (13,000 g, 15 min, 4°C), aliquoted at 100 ng/μL by addition of RNase-free water, and stored at -60°C until use.

In situ hybridisation

The slide-mounted sections were fixed in 4% paraformaldehyde in 0.1 M PB for 30 min (all steps were performed at room temperature unless indicated otherwise) and rinsed three times (10 min each) in phosphate-buffered saline (PBS). The sections were digested with proteinase K (Dako, Glostrup, Denmark) at 15 μg/mL in PBS at 37°C for 30 min. After stopping the digestion by rinsing in cooled PBS, the sections were then acetylated in a solution composed of 1.35% triethanolamine, 0.25% acetic anhydrite, and 0.058% hydrochloric acid in RNase-free water for 10 min. Then, sections were dehydrated through an ethanol series and hybridised with antisense or sense DIG-labelled riboprobes (0.1 μg/μL) dissolved in hybridisation buffer composed of 20% dextran sulphate (Nacalai Tesque, Kyoto, Japan), 50% formamide (Nacalai Tesque), 2% blocking solution (pH 7.5; Roche), 0.01% N-lauroylsarcosine (NLS; Nacalai Tesque), and 0.01% sodium lauryl sulphate (Nacalai Tesque) in 5× standard saline citrate (SSC; pH 7.4. 1× SSC contains 0.15 M sodium chloride and 0.015 M sodium citrate). The sections were first heated on a hot plate (95°C) for 4 min and then incubated at 55°C overnight. After hybridisation, the

sections were rinsed in a solution composed of 50% formamide and 0.01% NLS in 2× SSC at 65°C for 30 min and incubated with RNase A (20 μg/mL; Roche) in NTE buffer (500 mM NaCl, 10 mM Tris, and 1 mM EDTA, pH 8.0) at 37°C for 30 min. After rinsing in Tris-buffered saline (TBS; pH 7.4; 25 mM Tris, 1.37 mM NaCl, and 0.27 mM KCl) containing 0.025% Tween 20 (TBST), the sections were incubated with a blocking solution composed of 1% blocking reagent (Roche) and 2% normal sheep serum in TBS for 60 min. After rinsing in TBST, the sections were incubated with alkaline phosphatase-labelled anti-DIG antibody (Roche; 1:2,000) at 20°C overnight. After rinsing in TBST, the sections were visualised by incubation with a mixture of nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (Roche) in detection buffer (0.1 M NaCl, 50 mM $MgCl_2$, and 0.1% Tween 20 in 0.1 M Tris, pH 9.5) for 24 h in the dark. The sections were then rinsed in PBS and distilled water, dehydrated through a graded ethanol series, cleared with xylene, and coverslipped with Canada balsam.

Image processing

Photomicrographs were taken using a digital camera (Pro 600ES; Pixera Corporation, Los Gatos, CA, USA; or DS-Fi1, Nikon, Tokyo, Japan) mounted on a light microscope. Adjustment of photographs for contrast, brightness, sharpness, layout, and lettering were performed using Adobe Photoshop 7.0J and Adobe Illustrator 10.0J.

Nomenclature

The nomenclature used here is based on turtle brain atlases by Powers and Reiner (1980) for the forebrain and midbrain and by Cruce and Nieuwenhuys (1974) for the rhombencephalon.

The term ‘VGLUT’ mean

Although the sequence of LC066047 suggested homology to VGLUT2 but the expression pattern in the turtle brain did not match with any of the specific VGLUT type of mammals or birds. Thus, VGLUT from this study in the turtle is difficult to categorise simply as VGLUT1 or VGLUT2. So, the term ‘VGLUT’ was used everywhere in this dissertation instead of VGLUT1 or VGLUT2.

Results

VGLUT mRNA distribution

The expression of VGLUT mRNA was investigated in the turtle brain by *in situ* hybridisation using DIG-labelled cRNA probes. Hybridisation signals were observed using the anti-sense probe (Figs. 1-4), whereas the sense probe did not show any specific hybridisation signal in the turtle brain (Fig. 2d). Both male and female turtles were used in this study and there was no differences were found in the VGLUT mRNA distribution regarding to the sex. Within the age range of turtles used in this study, there was no specific difference of VGLUT mRNA distribution. For convenience, the hybridisation signal intensity was divided into three categories: high, moderate, and weak. For example, high levels of expression were observed in the olfactory bulb, several thalamic nuclei, and the nucleus isthmi pars parvocellularis (Figs. 1a, 2a,c, 3a,b). Moderate signal intensities were identified in the nucleus rotundus, nucleus lateralis hypothalami, reticular nuclei, and nucleus cochlearis (Figs. 2a,b,c, 4c,d). Weak expression was noticed in the dorsal ventricular ridge (DVR) (Fig. 1d). VGLUT mRNA signals were observed in neurons but not in any type of glia, ependymal cells of the cerebral ventricle, endothelial cells of the capillaries, or epithelial cells of the choroid plexus. Within neurons, VGLUT mRNA was localised exclusively to cell bodies (Fig. 2b). The detailed patterns of VGLUT mRNA expression in the turtle brain are described below.

Telencephalon

In the telencephalon, high levels of VGLUT mRNA signals were found in the mitral cells of the olfactory bulb (Fig. 1a,b), while no signal was apparent in the anterior olfactory nucleus or olfactory tubercle. High levels of VGLUT mRNA expression were apparent in all four areas of

the cerebral cortex (cortex lateralis, dorsalis, dorsomedialis, and medialis; Figs. 1c,d, 2a,c). In the cortex, VGLUT mRNA was exclusively found in the cellular layer, but not in the superficial molecular layer or subcellular layer. In the four divisions of the DVR, the dorsal area, medial area, ventral area, and central area, weak expression was observed, except in the dorsal area, which showed slightly higher expression of VGLUT mRNA (Fig. 1d). Within the amygdaloid region, the nucleus medialis amygdalae showed intense VGLUT mRNA expression, whereas the nucleus centralis amygdalae showed no signal. The nucleus tracti olfactorii lateralis showed a moderate level of VGLUT mRNA signals (Fig. 2a). Ventral to the DVR nucleus, the commissuralis anterioris showed a moderate level of VGLUT mRNA, whereas the paleostriatum augmentatum did not show VGLUT signals (Fig. 1d).

Diencephalon

Within the epithalamus, strong VGLUT mRNA signals were found in the nucleus habenularis medialis (Fig. 2c). In the dorsal thalamus, high expression was observed in the nucleus dorsolateralis anterior, nucleus dorsomedialis anterior, nucleus geniculatus lateralis pars dorsalis, and the nucleus reuniens, while the nucleus rotundus showed a moderate level of VGLUT mRNA (Figs. 2a,c). The level of expression of VGLUT mRNA in the hypothalamus was generally low. Strong VGLUT mRNA signals were seen in the nucleus periventricularis hypothalami, with moderate expression in the area lateralis hypothalami (Figs. 2a,c).

Mesencephalon

A laminar distribution of VGLUT mRNA was observed in the optic tectum. High levels of expression were found in the stratum griseum centrale and stratum griseum periventriculare, whereas the stratum fibrosum et griseum superficiale showed weak expression (Fig. 3a,c). The

torus semicircularis displayed a high level of VGLUT mRNA (Fig. 3a,b). Deep in the optic tectum, the nucleus isthmi pars parvocellularis showed high expression of VGLUT mRNA, whereas the nucleus isthmi pars magnocellularis showed no VGLUT signal (Fig. 3b). The nucleus mesencephalicus nervi trigemini showed strong expression of VGLUT mRNA (Fig. 3a). Moderate expression was found in the nucleus profundus mesencephali (Fig. 3a).

Rhombencephalon

In the cerebellum, VGLUT mRNA expression was negative in the cerebellar cortex but high in the cerebellar nuclei (Fig. 4a,b). Moderate expression levels were observed in the nucleus vestibularis superior and nucleus vestibularis tangentialis, whereas the nucleus vestibularis ventrolateralis showed strong expression of VGLUT mRNA (Fig. 4b,c). Moderate levels of expression were observed in the nucleus oliva inferior, nucleus cochlearis, nucleus descendens nervi trigemini, reticularis medius, and reticularis inferior, whereas the nucleus vestibularis descendens and raphes inferior showed weak expression of VGLUT mRNA (Fig. 4a,c,d).

Discussion

In the present study, expression of VGLUT mRNA was investigated in the turtle brain by *in situ* hybridisation using DIG-labelled cRNA probes. In this study, strong expression of VGLUT mRNA was found in the mitral cell layer of the olfactory bulb and cerebral cortex, which is characteristic of VGLUT1 mRNA in the mammalian brain. The turtle cerebral cortex and DVR are considered to be homologous to the mammalian neocortex (Butler and Hodos, 2005). In the present study, strong expression of VGLUT mRNA was observed in the diencephalon, mesencephalon, and rhombencephalon, whereas the cerebellar cortex was devoid of expression, which corresponds to the pattern of VGLUT2 mRNA expression in the mammalian brain. In mammals, VGLUT1 and VGLUT2 show a complementary distribution pattern in the brain. In birds, VGLUT1 is not found, and VGLUT2 possesses the attributes of VGLUT1 and VGLUT2 of mammals. In the turtle, in the telencephalon, VGLUT mRNA distribution was similar to that of VGLUT1 in mammals. For the diencephalon, mesencephalon, and rhombencephalon, the distribution was similar to that of VGLUT2 mRNA in mammals in the present study. Although the sequence of LC066047 suggested homology to VGLUT2 but the expression pattern in the turtle brain did not follow any of the specific VGLUT type of mammals or birds. Thus, VGLUT from this study in the turtle is difficult to categorise simply as VGLUT1 or VGLUT2. Furthermore, the cerebellum is a conserved structure among vertebrates and cerebellar cortex is a good candidate for glutamatergic neurons. In the present study, cerebellar cortex was devoid of VGLUT signal. This fact suggests that other isoform of VGLUT may serve the glutamatergic transmission in the cerebellar cortex of turtle. However, to resolve this issue, further examination for the presence of other VGLUT types in the turtle is required.

Comparative perspective of VGLUT mRNA distribution

Olfactory regions

In the present study, within the olfactory bulb, mitral cells exhibited intense VGLUT mRNA expression, while other layers showed no signal. Conversely, in rats, VGLUT2 mRNA is expressed mostly in the glomerular layer of the olfactory bulb and weakly in the mitral cell layer, whereas VGLUT1 mRNA shows a reverse pattern to that of VGLUT2 mRNA (Hisano, 2003). This study suggests that mitral cells of the turtle olfactory bulb are also glutamatergic.

Pallium and subpallium

The reptilian telencephalon that exhibits unique anatomical organization- contains two major divisions, the pallium and the subpallium. The pallium comprises two components: the cortex and dorsal ventricular ridge (DVR). The cortex is divided into medial, dorsomedial, dorsal, and lateral areas, whereas DVR is divided into anterior and posterior parts. The cortex consists of three-layered laminar structures: a tightly packed cell-dense layer and cell-sparse outer and inner plexiform layers (Ulinski, 1990). From an evolutionary viewpoint, evidence from cytoarchitectonic, connectional, developmental studies support the comparison of the medial region of the reptilian cerebral cortex (medial, dorsomedial and part of dorsal cortices) with parts of the mammalian hippocampal formation, the dorsal cortex is thought to resemble at least parts of mammalian neocortex, whereas the lateral cortex has been considered homologous to the piriform cortex (Reiner, 1993). The posterior DVR is considered to be homologous to part of the mammalian amygdala. The anterior DVR and neocortex of mammals share a set of sensory thalamic afferents with striking similarities. The homologous region of the DVR in the mammalian telencephalon has been part of a long debate. Whether the DVR is part of the

pallium rather than part of the more ventrally lying striatum (Lohman and Smeets, 1990) and whether the DVR and the dorsal cortex are homologous in some specified manner to at least part of the mammalian neocortex are the two key stones of the debate (Butler, 1994). Data from the present study do not favor the hypothesis that the reptilian DVR is homologous to mammalian striatum, because in the adult mammalian brain glutamatergic neuron is found to be expressed in the pallium, but not subpallium (Fremeau et al., 2001; Ni et al., 1995). In the present study, in the cerebrum, VGLUT mRNA was expressed in all divisions of the cerebral cortex, the DVR, and the nucleus medialis amygdalae of the turtle brain. This study suggests that the pallial territories in turtles are glutamatergic, as in mammals. Moreover, similar consistent results have been reported in birds in which glutamatergic neurons were demonstrated in the pallial structures, but not in the subpallium (Islam and Atoji, 2008; Karim et al., 2014). Kriegstein et al (1986) postulated that both the cerebral cortex and DVR in turtle contains antigens that cross-react with antisera prepared against mammalian cortex, suggested that cortex and DVR is a pallial structure, well agree with our finding. Recently, many mammalian neocortical layer specific markers have been traced in the turtle cortex and DVR (Dugas-Ford et al., 2012) in which layer specific markers are expressed only in the cellular layer of the turtle cortex, is in the same line with the present findings.

Diencephalon

In the diencephalon, a differential distribution of VGLUT mRNA was found in the habenula, thalamus, and hypothalamus of the turtle brain. In mammals, the strongest expression of VGLUT2 mRNA is found throughout the midline and intralaminar nuclei, including the periventricular and parataenial nuclei. Abundant VGLUT2 mRNA has been observed within the

sensory relay nuclei, including the lateral geniculate complex and the posterior complex. Other associated thalamic nuclei, such as the mediodorsal, anterodorsal, anteromedial, and lateral posterior nuclei, also show significant VGLUT2 mRNA expression in mammalian brain (Barroso-Chinea et al., 2007; Fremeau et al., 2001; Hisano et al., 2000). In reptiles, it has been suggested that the dorsomedial anterior nucleus is homologous to the mammalian paraventricular thalamic (Heredia et al., 2002), the dorsolateral anterior nucleus to the mammalian parataenial nucleus (Ariëns Kappers et al., 1936), the dorsal lateral geniculate nucleus to the mammalian dorsal lateral geniculate nucleus, the nucleus reuniens to the mammalian medial geniculate body, and the nucleus rotundus to the mammalian posterior-pulvinar complex (Butler and Hodos, 2005). In the present study, VGLUT mRNA was found in these turtle nuclei, suggesting similar glutamatergic circuits between the turtle and mammal thalamus. In the turtle thalamic anterior entopeduncular nucleus, VGLUT mRNA expression was not observed, as in the homologous mammalian thalamic reticular nucleus (Barroso-Chinea et al., 2007; Fremeau et al., 2001; Hisano et al., 2000; Kenigfest et al., 2005). In the hypothalamus of mammals, VGLUT2 mRNA is apparently expressed at a lower level than that in the thalamus. Relatively high levels of expression were observed in the mammillary nuclei and the lateral hypothalamic area, and moderate-to-weak expression was observed in the ventromedial, paraventricular, and supraoptic nuclei. In the present study, in the turtle, moderate VGLUT mRNA expression was also found in the lateral hypothalamic area and strong expression in the periventricularis nucleus.

Mesencephalon

The optic tectum holds a central position in the tectofugal pathway of non-mammalian species and is reciprocally connected to the nucleus isthmi. In the present study, abundant VGLUT

mRNA was found in the optic tectum. The optic tectum is homologous to the mammalian superior colliculus (Butler and Hodos, 2005), and high levels of VGLUT2 mRNA are also found in the superior colliculus of mammals (Fremeau et al., 2001). The torus semicircularis is the mesencephalic auditory centre of turtles, and it also showed high VGLUT mRNA expression levels in this study, consistent with the high levels of VGLUT2 mRNA expression in the mammalian mesencephalic auditory centre, the inferior colliculus (Hackett et al., 2011; Ito and Oliver, 2010).

Rhombencephalon

In the mammalian cerebellum, high levels of VGLUT2 mRNA expression were observed in large neurons of all deep cerebellar nuclei, but not in any part of the cerebellar cortex (Hisano et al., 2002). The turtle's cerebellum is an unfoliated single sheet composed of cerebellar cortex and two deep cerebellar nuclei (lateral and medial). VGLUT mRNA expression in the cerebellum of the turtle in this study resembles the VGLUT2 mRNA expression pattern in the mammalian cerebellum. In addition, the nuclei of the turtle brainstem, including the reticular, vestibular, cochlear, and oliva inferior nuclei, expressed VGLUT mRNA, and the expression patterns were similar to VGLUT2 expression in mammals (Broman et al., 2004; Hisano et al., 2002).

Functional implications of glutamatergic neurons in the turtle brain

In the present study, differential distributions of glutamatergic neurons have been investigated. However, little is known of glutamatergic functions in the turtle brain. The dorsomedial region of the reptilian telencephalon appears to be the homologue of the mammalian hippocampal formation. Behavioural studies suggest that the dorsal and medial cortex of turtles

is involved in learning and memory (López et al., 2003a; 2003b). The medial cortex of young turtle brain in vitro, exhibited frequency potentiation, a non-lasting form of synaptic plasticity, and the excitatory postsynaptic potential is abolished by application of AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), indicating that this frequency potentiation is mediated by AMPA type glutamate receptors (Muñoz et al., 1998b). Moreover, in the medial cortex of the turtle brain, tetanic stimulation induces the long-term potentiation, which is also abolished by application of AMPA receptor antagonist (CNQX) (Muñoz et al., 1998a). In turtle, subcutaneous injection of NMDA receptor antagonist MK-801 and dorsal cortex lesions suggest the involvement of the turtle dorsal cortex and the NMDA receptor in the acquisition of a position habit (Avigan and Powers, 1995). In the present study, glutamatergic neurons were detected in the cerebral cortex, including the medial cortex and dorsal cortex. The morphological evidence indicates that glutamatergic neurons are involved in learning and memory in these behavioural studies described above. The present findings may serve as morphological basis for physiological and pharmacological experiments in turtle.

In a nutshell, the present study has investigated the distribution of VGLUT mRNA in the turtle brain by *in situ* hybridisation which reveals many glutamatergic neurons in the turtle brain.

Conclusion

In the present study, the distribution of VGLUT mRNA was evaluated in the turtle brain by *in situ* hybridisation. An anti-sense probe showed differential expression patterns for VGLUT mRNA, whereas the sense probe showed no specific hybridisation. In the telencephalon, high levels of VGLUT mRNA were found in the mitral cells of the olfactory bulb, cerebral cortex, and nucleus medialis amygdalae. Weak expression of VGLUT mRNA was found in the dorsal ventricular ridge, while no expression was apparent in the paleostriatum augmentatum. Within the diencephalon, strong expression was observed in the nucleus habenularis medialis, nucleus dorsolateralis anterior, nucleus dorsomedialis anterior, nucleus geniculatus lateralis pars dorsalis, nucleus reuniens, and nucleus periventricularis hypothalami, while the nucleus rotundus and area lateralis hypothalami showed moderate expression. In the mesencephalon, strong expression was found in the nucleus mesencephalicus nervi trigemini, nucleus isthmi pars parvocellularis, stratum griseum centrale and stratum griseum periventriculare of the optic tectum, and torus semicircularis, whereas moderate expression was seen in the nucleus profundus mesencephali. Within the rhombencephalon, moderate expression was observed in the reticular nuclei, nucleus vestibularis superior, nucleus vestibularis tangentialis, and nucleus oliva inferior, whereas the nucleus vestibularis ventrolateralis showed strong expression. The cerebellar cortex showed no VGLUT mRNA expression, whereas the cerebellar nuclei showed high levels. This study has revealed many glutamatergic neurons in the turtle brain. The present findings provided information about the glutamatergic systems in the turtle brain which would ultimately help turtle to become an ideal animal model. Moreover, their distribution pattern may provide insights in comparisons of the telencephalon among amniotes and provide clues to elucidate the evolution of the brain.

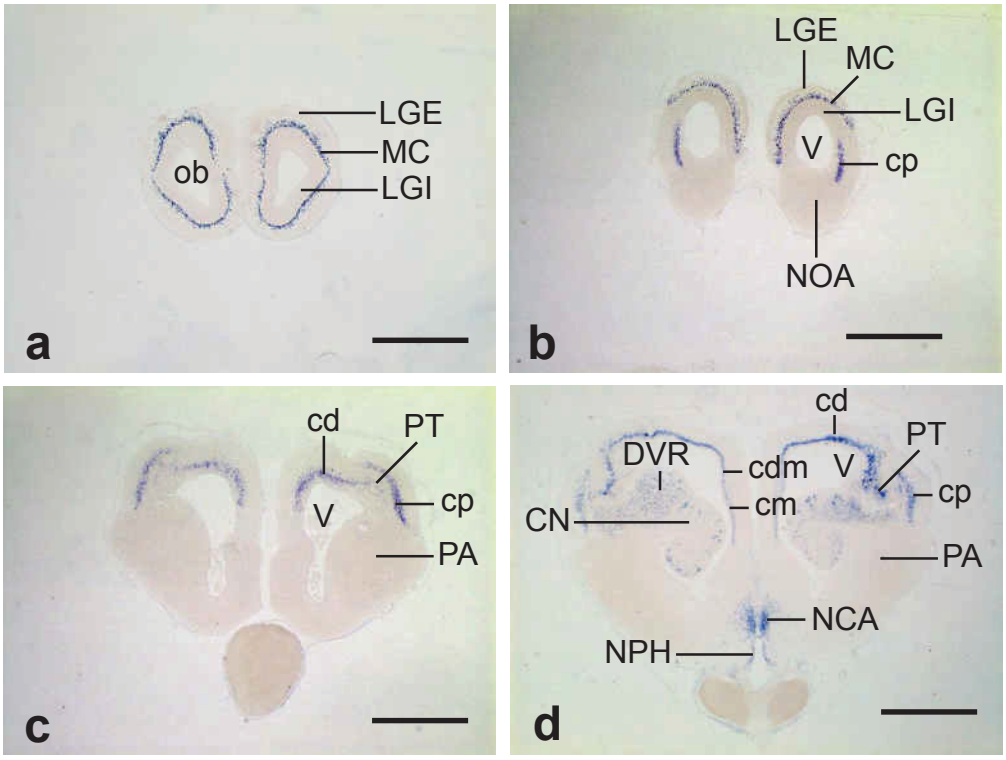

Figure 1. Expression of VGLUT mRNA in the rostral telencephalon of the turtle brain by digoxigenin-labeled (Dig-labeled) cRNA probe (**a**-**d**). Intense signal was found in the MC of ob, and cerebral cortex; moderate in the NCA; weak in the DVR. See list for abbreviations. Scale bar= 2 mm in a-d.

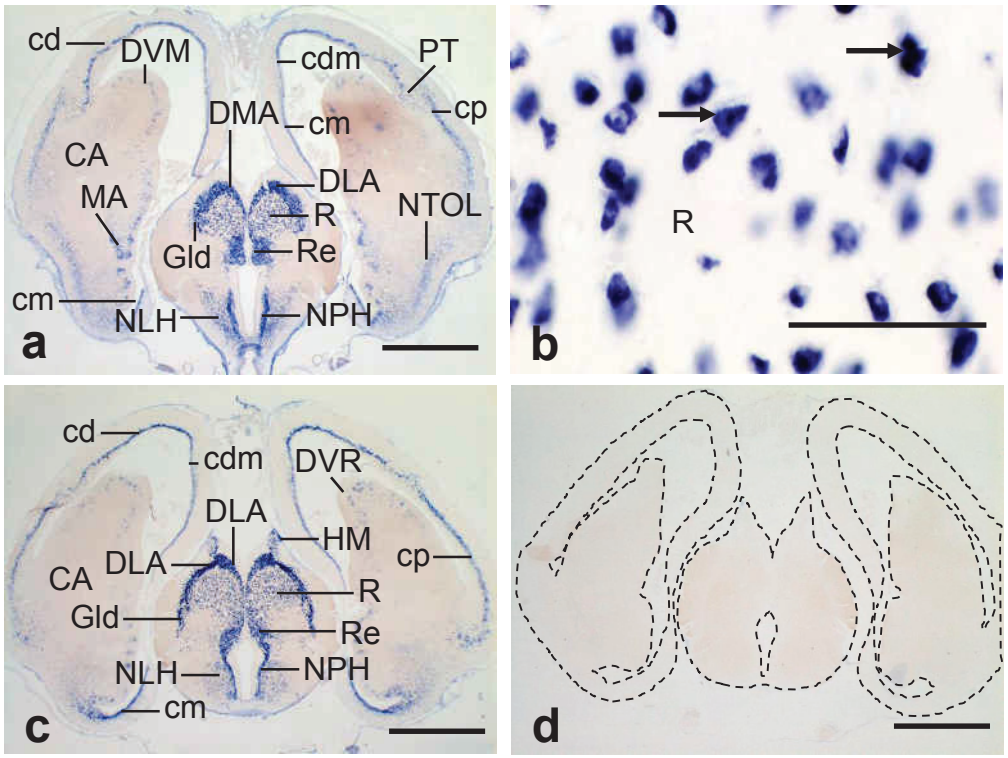

Figure 2. The distribution of VGLUT mRNA labeled neurons in the caudal telencephalon and thalamus of turtle brain with Dig-labeled cRNA probe (**a,c**). High expression was exhibited in the cerebral cortex, MA, DLA, MDA, Gld, Re, HM, and NPH; moderate in the R and NLH. **b**: R in high magnification. Arrows indicate VGLUT mRNA signals located in the cell body of the neurons. **d**: Sense probe did not show any specific signals. See list for abbreviations. Scale bar= 200 mm for a,c,d and 50 μm for (b).

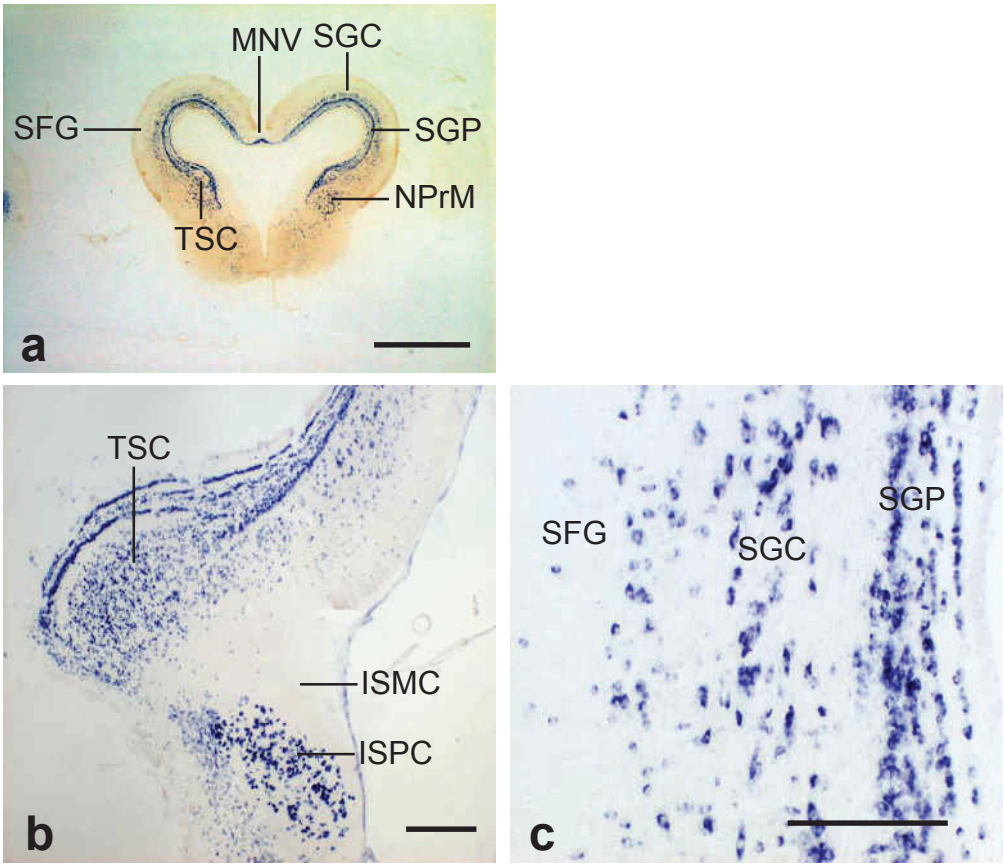

Figure 3. VGLUT mRNA distribution in the mesencephalon of turtle brain with Dig- labeled cRNA probe (**a**). High level of expression was observed in the SGP, SGC, TSC, and MNV. **b**: TSC, ISPC and ISMC with high magnification. **c**: Differential distribution of VGLUT mRNA in the layers of optic tectum. See list for abbreviations. Scale bar= 200 mm for (a), 500 µm for (b), and 100 µm for (c).

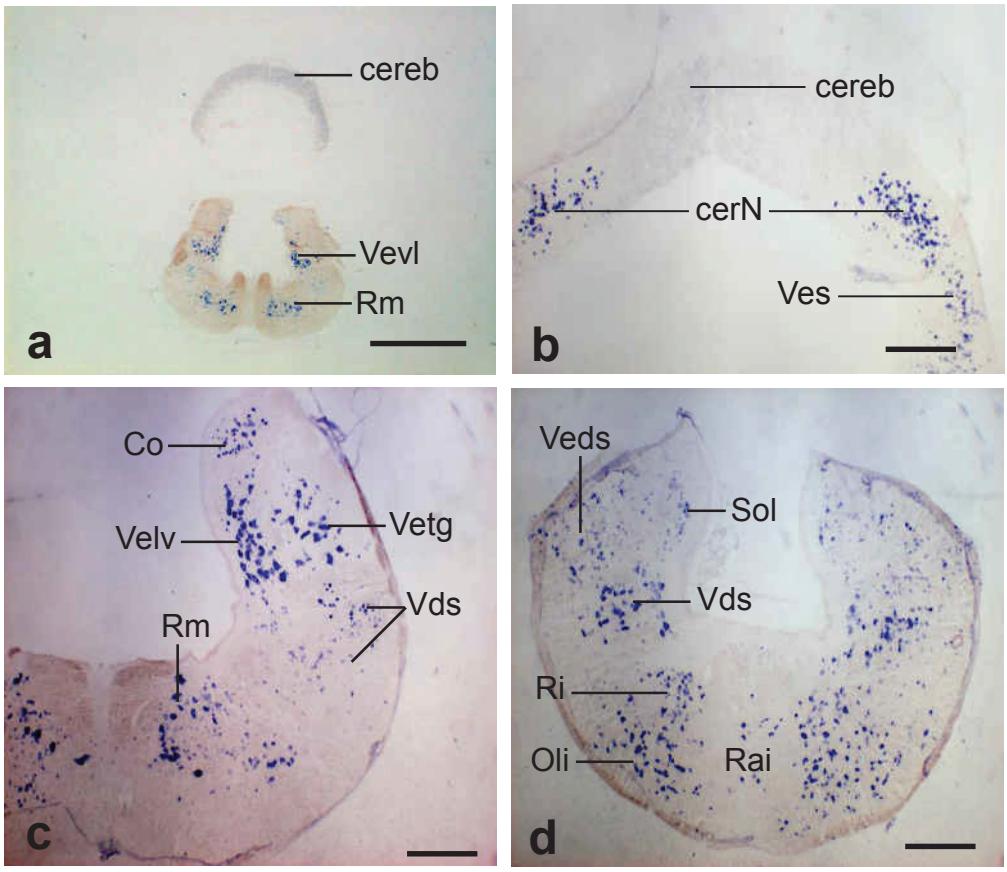

Figure 4. Expression of VGLUT mRNA in the rhombencephalon of the turtle brain (**a**-**d**). High level of expression was observed in the cerN and Vevl. Moderate expression was found in the Co, Oli, Ri, Rm, Vds, Ves and Vetg; weak in the Rai, Sol, and Veds. See list for abbreviations. Scale bar= 2 mm in (a), 500 µm in (b-d).

Acknowledgements

I am grateful to almighty Allah who enables me to accomplish this work and all of His gifts.

Greatest appreciation and sincere thanks to my main supervisor Dr. Yasuro Atoji, Professor, Laboratory of Veterinary Anatomy, Faculty of Applied Biological Sciences, Gifu University for his scholastic guidance, excellent teaching strategy, and active cooperation during my study.

I wish to express my cordial thanks to my assistant advisor Dr. Shouichiro Saito, Associate Professor, Laboratory of Veterinary Anatomy, Faculty of Applied Biological Sciences, Gifu University for his kind and active support during this study period.

My grateful thanks to my secondary advisor Professor Dr. Hideshi Shibata, Tokyo University of Agriculture and Technology, Professor Dr. Yoshio Yamamoto, Iwate University, Professor Dr. Nobuo Kitamura, Obihiro University of Agriculture and Veterinary Medicine, and Professor Dr. Yasutake Shimizu, Gifu University for their valuable suggestions, criticism, and patient revision of this dissertation.

Sincere thanks to Grant-in Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan for grant and Patuakhali Science and Technology University, Bangladesh for allowing me the deputation during this study period.

I feel much pleasure to convey my thanks to the entire members of Laboratory of Veterinary Anatomy, Faculty of Applied Biological Sciences, Gifu University for assistance and cooperation during studying and staying in Gifu.

I would like to extend my thanks to all staff members of the United Graduate School of Veterinary Sciences, Gifu University, Japan for all the support during my study.

I express my gratitude to my family and friends here in Japan, and home in Bangladesh for their inspiration, sacrifice and patience.

Abbreviations

CA	Nucleus centralis amygdalae
cd	Cortex dorsalis
cdm	Cortex dorsomedialis
cereb	Cerebellum
cerN	Nucleus cerebelli
cm	Cortex medialis
CN	Core nucleus of the dorsal ventricular ridge
Co	Nucleus cochlearis
cp	Cortex pyriformis
DLA	Nucleus dorsolateralis anterior
DMA	Nucleus dorsomedialis anterior
DVR	Dorsal ventricular ridge
Gld	Nucleus geniculatus lateralis pars dorsalis
HM	Nucleus habenularis medialis
ISMC	Nucleus isthmi pars magnocellularis
ISPC	Nucleus isthmi pars parvocellularis
LGE	Lamina granularis externa
LGI	Lamina granularis interna
MA	Nucleus medialis amygdalae
MC	Mitral cells
MNV	Nucleus mesencephalicus nervi trigemini
NCA	Nucleus commissuralis anterioris
NLH	Nucleus lateralis hypothalami
NOA	Nucleus olfactorius anterior
NPH	Nucleus periventricularis hypothalami
NPrM	Nucleus profundus mesencephali
NTOL	Nucleus tracti olfactorii lateralis
ob	Bulbus olfactorius
Oli	Oliva inferior

PA	Paleostriatum augmentatum
PT	Pallial thickening
R	Nucleus rotundus
Rai	Nucleus raphes inferior
Re	Nucleus reuniens
Ri	Nucleus reticularis inferior
Rm	Nucleus reticularis medius
SFG	Stratum fibrosum et griseum superficiale
SGC	Stratum griseum centrale
SGP	Stratum griseum periventriculare
Sol	Nucleus of the solitary tract
TSC	Torus semicircularis
V	Ventriculus
Vds	Nucleus descendens nervi trigemini
Veds	Nucleus vestibularis descendens
Ves	Nucleus vestibularis superior
Vetg	Nucleus vestibularis tangentialis
Vevl	Nucleus vestibularis ventrolateralis

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