VGLUT1 and VGLUT2 mRNA expression in the primate auditory pathway

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1. Introduction

The storage and release of glutamate in excitatory circuits in the brain is regulated in part by the vesicular glutamate transporters (VGLUTs) (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). Of the three known transporters, the VGLUT1 and VGLUT2 isoforms are the most densely expressed in sensory pathways. VGLUT1 appears to be the main isoform expressed by neurons in the cortex, while VGLUT2 appears to be dominant in the thalamus and brainstem. The regional differences in expression of these transporters are of interest, as there is evidence that they may be localized in synapses with different release probabilities and trafficking mechanisms, and therefore represent functionally distinct circuits (De Gois et al., 2005; Kaneko and Fujiyama, 2002; Mohrmann et al., 2008; Santos et al., 2009; Varoqui et al., 2002). For example, in the cortex of adult animals, including the auditory cortex, VGLUT1 mRNA is strongly expressed by most neurons in layers II–VI, whereas VGLUT2 is expressed by a subset of neurons, mainly in the middle layers (De Gois et al., 2005; Fremeau et al., 2001; Graziano et al., 2008; Herzog et al., 2001). During postnatal development, VGLUT1 levels increase and VGLUT2 levels decrease to adult levels over similar periods, and remain co-expressed by subsets of neurons in adults, especially in the middle layers (De Gois et al., 2005). Similarly, the laminar distributions of VGLUT1 and VGLUT2 immunoreactive (-ir) terminals are partly complementary in that VGLUT1-ir terminals are concentrated in layers I–III and VGLUT2-ir is most dense in layer IV (Fremeau et al., 2001; Fujiyama et al.,...
2004; Graziano et al., 2008; Kaneko et al., 2002). The concentration of VGLUT2-ir in layer IV terminals suggests that these inputs are thalamic in origin (Hur and Zaborszky, 2005). Yet, some terminals in layer IV contain both proteins (Graziano et al., 2008), suggesting that a subpopulation of neurons projecting to layer IV expresses both transporters. These findings are consistent with the observation that VGLUT1 and VGLUT2 mRNA are co-expressed by most neurons in the primary sensory relay nuclei of the rat thalamus, including the medial geniculate, lateral geniculate, and ventro-posterior nuclei (Barroso-Chinea et al., 2008, 2007; Herzog et al., 2001). Otherwise, VGLUT2 mRNA expression in the thalamus is generally stronger and more broadly distributed compared to VGLUT1. Thus, it can be concluded that the two transporters are co-expressed in some thalamic and cortical circuits and complementary in others.

As in the thalamus, VGLUT2 mRNA expression in the brainstem is also strong among glutamatergic neurons in most nuclei (Berube-Carriere et al., 2009; Fremeau et al., 2001; Geisler et al., 2007; Graziano et al., 2008; Herzog et al., 2001; Hisano et al., 2002; Islam and Atoji, 2008; Nair-Roberts et al., 2008; Stornetta et al., 2002; Wang and Morales, 2009), whereas VGLUT1 is strong in only a few (e.g., vestibular and cochlear nuclei, lateral reticular, external cuneate nuclei). Neither transporter appear to be expressed in GABAergic nor monoaminergic populations (e.g., Purkinje cells, substantia nigra, locus coeruleus, raphe nuclei). Comparable findings are available for the mouse in the Allen Brain Atlas database (Lein et al., 2007) (http://mouse.brain-map.org).

Detailed studies of gene expression in auditory nuclei are lacking, but gleaming from the sources listed above, VGLUT2 expression is strong in excitatory neurons in the principal auditory nuclei. VGLUT1 expression is weaker or absent in many of these nuclei, but appears to be strong in the dorsal and ventral cochlear nuclei. In contrast to gene expression patterns, VGLUT1 and VGLUT2 protein expression overlaps spatially in most nuclei, but is contained within circuits that are largely segregated, and therefore likely to subserve different functional roles (Altschuler et al., 2008; Ito et al., 2009; Kaneko et al., 2002; Zhou et al., 2007).

Given high sequence homology between humans and mice for these genes, it is reasonable to expect similar expression patterns in primates. To date, however, exploration of the VGLUT expression in primates has been limited to VGLUT1 and VGLUT2 immunoreactivity (-ir). Rubio et al. (2008) studied VGLUT1-ir in the dorsal cochlear nucleus of the rhesus monkey, and found that the laminar and subregional distribution of VGLUT1-ir terminals was comparable to patterns in rats and mice (Kaneko et al., 2002; Zhou et al., 2007). In macaque auditory cortex, the regional and laminar distribution of VGLUT2-ir terminals is well established. Using mRNA expression in the auditory pathway are largely complementary, but overlapping in some structures, in line with predictions based on previous studies in other species. The similarity to patterns of expression in mice and rats suggests that the expression of these genes is highly conserved in rodents and primates.

2. Methods

2.1. Animals

Three adult owl monkeys (Aotus trivirgatus) were used for the present studies. All surgical procedures were carried out according to the NIH Guidelines for the care and use of laboratory animals (NIH publication 86–23) under approved protocols from the Vanderbilt Animal Care and Use Committee.

2.2. Tissue preparation for histology

Animals were deeply anesthetized with a lethal dose of sodium pentobarbital (80 mg/kg) and perfused transcardially with 0.9% saline in 0.1 M phosphate buffer (PB) followed by 4% paraformaldehyde (PFA) in 0.1 M PB. The brain was removed and postfixed for 3–6 h in 4% PFA in 0.1 M PB. The thalamus and brainstem were separated from the cerebral hemispheres, blocked, and cryoprotected in 30% sucrose in 0.1 M PB for 3–5 days at 4 °C. The block was cut into 40 μm frozen coronal sections on a sliding microtome and sections were stored at −20 °C in cryoprotectant solution (30% glycerol, 30% ethylene glycol, 40% 0.1 M phosphate-buffered saline). Brain sections were saved in 12 series, with alternating series of sections processed for Nissl staining with thionin, cytochrome oxidase (CO) (Wong-Riley, 1979), in situ hybridization for vesicular glutamate transporter 1 (VGLUT1) and vesicular glutamate transporter 2 (VGLUT2) mRNA. Selected sections from auditory cortex were counterstained with green fluorescent Nissl stain (Neurotrace 500/525, 1% in DH2O, Invitrogen Corp, Carlsbad, CA) to show laminar details.

2.3. In situ hybridization (ISH)

Two adjacent series in each animal were processed for localization of VGLUT1 and VGLUT2 mRNA. Digoxigenin (DIG)-labeled sense and antisense riboprobes for VGLUT1 and VGLUT2 were prepared from a macaque cDNA library through RT-PCR and conventional TA cloning techniques, and labeled using a DIG-UPL labeling kit (Roche Diagnostics, Indianapolis, IN). The macaque VGLUT2 riboprobe was also used in the previous study (Takahata et al., 2010). Another VGLUT2 riboprobe was newly prepared from a galago cDNA library for the same position of VGLUT2 mRNA as macaques and used for one owl monkey case. The coding sequences of these genes are well conserved and the homology is high among
species (approximately 90% between human and mouse). On the other hand, the homology between VGLUT1 and VGLUT2 mRNA is around 70%. Therefore, our preparation can reliably detect specific signals for each gene. The forward and reverse primers used for VGLUT1 were ccgctcattatgcctca and cgatgggcacgatgatggtc respectively, which targeted position 204–1093 of human VGLUT1 (AB032436). The forward and reverse primers for VGLUT2 were gccatgtagctagttgct (fl indicates a or g) respectively, targeting position 693–1888 of human VGLUT2 (NM_020346). The sense probes served as a negative control, and detected no signals stronger than the background reactivity. ISH was carried out as previously described (Takahata et al., 2010, 2006). Briefly, free-floating sections were soaked in 4% PFA/0.1 M PB (pH 7.4) overnight at 4 °C and treated with 10 μg/ml proteinase K for 30 min at 37 °C. After acetylation with 0.25% acetic anhydride in 0.9% triethanolamine and 0.12% hydrochloric acid, the sections were incubated in hybridization buffer (pH 7.5) containing 5× standard saline citrate (SSC; 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0), 50% formamide (FA), 0.1% N-lauroylsarcosine (NLS), for 20 min each. Hybridized mRNA signals were visualized by alkaline phosphatase (AP) immunohistochemical staining using a DIG detection kit (Roche Diagnostics). Sections were acetylated and treated with 10 μg/ml RNase A in RNase A buffer (10 mM Tris–HCl, 10 mM ethylenediamine-N,N,N′,N′′-tetraacetatic acid (EDTA), 500 mM NaCl; pH 8.0) for 15 min at 37 °C and sections were washed again in 2× SSC, 0.1% NLS, followed by 0.2× SSC, 50% FA, 0.1% NLS, for 20 min each. Hybridized mRNA signals were visualized by alkaline phosphatase (AP) immunohistochemical staining using a DIG detection kit (Roche Diagnostics). Sections were mounted onto gelatin-subbed glass slides and dehydrated through a graded ethanol series (70% for 5 min, 90% for 10 min, 100% for 10 min), cleared in xylene (5 min), and then coverslipped with Permount.

3. Results

The distribution of VGLUT1 and VGLUT2 mRNA varied regionally in the brainstem and thalamus, and generally co-varied with patterns of CO expression. The lower power images of coronal sections at different rostral—caudal levels (Fig. 1) show that VGLUT2 mRNA was most densely expressed by neurons in the auditory nuclei (i.e., cochlear nucleus, inferior colliculus, medial geniculate), as well as the inferior olive, cerebellum, central gray, lateral geniculate, and pulvinar nuclei. VGLUT1 mRNA levels within neurons were present at reduced levels in most of the auditory nuclei, but remained strong in the ventral cochlear nuclei, cerebellum, and motor tract. The higher power images (Fig. 2) show VGLUT1 and VGLUT2 signals within neurons of the medial superior olive, inferior colliculus, and medial geniculate complex. In these subcortical structures, VGLUT1 was typically expressed at lower levels within neurons and exhibited an incomplete subcellular distribution of the signal, compared to VGLUT2. At higher magnification, additional differences in expression were found among the major auditory nuclei and their subdivisions. These results are discussed below.

3.1. Cochlear nucleus (CN)

In the dorsal cochlear nucleus (DCN), laminar divisions were somewhat difficult to resolve, as noted by Moore (1980), but the combined architectonic markers used in this study suggested the presence of at least 3 layers (Fig. 3). In Nissl sections, the outermost (molecular) layer contained axons and small cells similar to that observed in other species (Fig. 3A). A second layer could be divided into two domains based on CO expression (Fig. 3B). The deeper domain contained larger neurons and corresponds most closely to the fusiform cell layer. Interestingly, VGLUT2 mRNA expression in the DCN was largely confined to cells in this layer (Fig. 3B). In contrast, VGLUT1 mRNA was rather weakly expressed in this zone and elsewhere in the DCN, with expression at levels just above

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**Fig. 1.** Low magnification images of the owl monkey brainstem and thalamus at the level of the cochlear nucleus (left), inferior colliculus and superior olivary complex (middle), and medial geniculate (right). At each level, VGLUT1 and VGLUT2 mRNA expression is illustrated in adjacent sections to show their respective distribution patterns. Scale bar = 1 mm.
background (Fig. 3G). In the deepest third layer, there was a thin line of cells that expressed VGLUT2 mRNA.

In the anteroventral (AVCN) and posteroverentral (PVCN) divisions, VGLUT1 and VGLUT2 mRNA were densely expressed by the large neurons that were distributed rather evenly throughout both divisions (Fig. 3H–L). Their distribution matched that of neurons stained for Nissl (Fig. 3B, C). Although dual-label ISH was not performed in this study, the similarity in the distributions of neurons containing VGLUT1 and VGLUT2 mRNA suggests that most of these neurons in both nuclei express the mRNA for at least one or possibly both transporters (see Section 4).

### 3.2. Superior olivary complex (SOC)

The SOC of the owl monkey contained nuclei identified herein as the medial (MO), lateral (LSO), medial (MNTB) and ventral (VNTB) nuclei of the trapezoid body, as well as a several perioliival nuclei, which were not defined (Fig. 4). For example, the regions labeled as MNTB and VNTB did not have homogeneous architecture, and so may also include other divisions, such as the dorsomedial (DMPO) and ventromedial (VMPO) perioliival nuclei. In the MO, the central line of principal cells stained darkly for Nissl substance and CO, and the neuropil was also darkly stained by CO. The intermediate (INLL) and ventral (VNLL) nuclei had similar features, and overall the architectonic appearance of the LL was comparable to the New World squirrel monkey (Emmers and Akert, 1963). The cells of the DNLL and VNLL generally did not express either VGLUT1 or VGLUT2 mRNA (Fig. 5C–L), although one or two cells in the DNLL were usually found in each section that contained moderate levels of VGLUT2, and less often VGLUT1. The relative absence of VGLUT mRNA labeling in the DNLL and VNLL is consistent with the dominance of inhibitory neurons in those nuclei, and matches findings in the rat (Ito and Oliver, 2010). In contrast, numerous cells in the INLL and nearby sagulum strongly expressed VGLUT2, and frequently VGLUT1 at reduced levels.

### 3.3. Lateral lemniscus and sagulum

The three major divisions of the lateral lemniscus (LL) most commonly identified were well delineated in the owl monkey. Due to the plane of section, it was necessary to obtain a series of images to view each division. The dorsal nucleus (DNLL) was most prominent in rostral sections (Fig. 5). Its neurons stained darkly for Nissl substance and CO, and the neuropil was also darkly stained by CO. The intermediate (INLL) and ventral (VNLL) nuclei had similar features, and overall the architectonic appearance of the LL was comparable to the New World squirrel monkey (Emmers and Akert, 1963). The cells of the DNLL and VNLL generally did not express either VGLUT1 or VGLUT2 mRNA (Fig. 5C–L), although one or two cells in the DNLL were usually found in each section that contained moderate levels of VGLUT2, and less often VGLUT1. The relative absence of VGLUT mRNA labeling in the DNLL and VNLL is consistent with the dominance of inhibitory neurons in those nuclei, and matches findings in the rat (Ito and Oliver, 2010). In contrast, numerous cells in the INLL and nearby sagulum strongly expressed VGLUT2, and frequently VGLUT1 at reduced levels.

### 3.4. Inferior colliculus

On the basis of Nissl and CO staining (Cant and Benson, 2006; Morest and Oliver, 1984), we identified three major divisions of the inferior colliculus in the present study: central (ICc), dorsal cortex (DC), and lateral nucleus (LN) (Fig. 6). Although the architectonic appearance of each division was also heterogeneous, we made no attempt to further subdivide them. The central nucleus of the inferior colliculus (ICc) stood out from the surrounding structures with dense cell packing and generally elevated CO expression (Fig. 6A–D). Neurons in the ICc that strongly expressed VGLUT2 mRNA were broadly distributed throughout (Fig. 6G, H). VGLUT1 mRNA was expressed weakly by cells distributed in a pattern similar to that of VGLUT2 in the ICc (Fig. 6E, F). Neurons that were the most intensely reactive for either signal tended to be clustered in a smaller dorsolateral zone that was darkly labeled by CO.

In the DC and cell sparse region corresponding to the LN, CO expression levels were reduced compared to the ICc (Fig. 6C, D).
Fig. 3. VGLUT mRNA expression in the cochlear nucleus (CN). Adjacent series of sections of the cochlear nucleus at the level of the DCN (left columns); PVCN (middle columns), and AVCN (right columns). Sections illustrated at each level were processed for Nissl (A–C), cytochrome oxidase (CO) (D–F), VGLUT1 mRNA (G–I), and VGLUT2 mRNA (J–L) by in situ hybridization. Scale bars = 500 μm.
observed no clear indication of periodicities in LN in the distributions of CO or other markers used in this study, as found in rodents (Chernock et al., 2004). VGLUT1 mRNA expression was very weak in the LN and DC (Fig. 6E, F). In contrast, VGLUT2 mRNA was expressed in neurons distributed rather evenly throughout (Fig. 6G, H), except rostrally in the DC which was broken up by commissural axons. In general, VGLUT2 expression levels within LN and DC neurons were moderate compared to neurons in the ICc, although a few large cells in the LN were darkly stained.

3.5. Medial geniculate complex

As shown in Fig. 7, the medial geniculate complex (MGC) was divided into three major divisions, as delineated in sections stained for Nissl (Fig. 6A). The criteria used to characterize them have been described in previous studies of New World primates (de la Mothe et al., 2006; Morel and Kaas, 1992). The three divisions recognized in this study are the ventral (V, MGv), medial or magnocellular (M, MGm), and posterodorsal (PD, MGpd). The anterodorsal division

Fig. 4. VGLUT mRNA expression in the superior olivary complex (SOC). Adjacent sections processed for Nissl (A), CO (B), VGLUT1 mRNA (C, D) and VGLUT2 mRNA (E, F). MSO, medial superior olive; LSO, lateral superior olive; MNTB and LNTB, medial and lateral nuclei of the trapezoid body. Scale bars = 500 μm.

Fig. 5. VGLUT mRNA expression in the lateral lemniscus (LL) and sagulum (Sag). Adjacent sections through the dorsal nucleus (DNLL, black arrow) and sagulum (gray arrow) (A–D), intermediate nucleus (INLL, black arrow) (E–H), and ventral nucleus (VNLL, black arrow) (I–L). Sections illustrated at each level were processed for Nissl (A, E, I), cytochrome oxidase (CO) (B, F, J), VGLUT1 mRNA (C, G, K), and VGLUT2 mRNA (D, H, L) by in situ hybridization. GC, substantia griseum centralis. Scale bars = 500 μm.
(MGad) is not illustrated. The MGC is flanked dorsally and medially by the lateral geniculate nucleus (LGN), inferior pulvinar (PI), medial pulvinar (PM), posterior (Po), suprageniculate (Sg), and limitans (Lim) nuclei. By comparing with the distribution of cells in the Nissl preparation, it appears that most of these neurons express VGLUT1 and VGLUT2 mRNA, but as in other nuclei, expression levels were different (Fig. 6E-H; see also Fig. 2). VGLUT2 mRNA was broadly expressed by neurons in all divisions, with slightly stronger expression in the MGv and dorsal MGm and extending into Po and Sg. VGLUT1 mRNA expression was distributed in a similar manner across divisions, but expression levels were much lower than for VGLUT2, due in part to a restricted subcellular distribution (Fig. 2E). Thus, it appears that most glutamatergic cells probably express both transcripts, but expression density is greater for VGLUT2 and
Fig. 7. VGLUT mRNA expression in the medial geniculate complex (MGC) and adjoining nuclei of the posterior thalamus. The posterodorsal (PD), medial or magnocellular (M), and ventral (V) divisions of the MGC are indicated in panel A, stained for Nissl. Also indicated are the locations of the lateral geniculate (LGN), inferior pulvinar (PI), medial pulvinar (PM), posterior (Po), suprageniculate (Sg), and limitans (Lim) nuclei. CO expression illustrated in panels C and D. VGLUT1 mRNA (E, F). VGLUT2 mRNA (G, H), shown at different magnifications. Scale bars = 500 μm.

Fig. 8. VGLUT1 and VGLUT2 mRNA expression in auditory cortex. VGLUT1 mRNA (A–C) is expressed in neurons spanning layers II–VI. VGLUT2 mRNA (E–G) expression is strongest in layer IIIb, weaker in layers II, IIIa, and IV, and very low or absent in layers V and VI. Paired panels (C–D) and (G–H) are images of the same sections through A1, dual-labeled for mRNA by in situ hybridization (C, G) and fluorescent Nissl stain (D, H) to show laminar details. Scale bars = 500 μm.
varies between divisions. Note that VGLUT1 and VGLUT2 mRNA were also contained in neurons of the LGN, but VGLUT1 levels were greater than in the MGC (data not shown).

3.6. Auditory cortex (area A1)

In the auditory cortex (Fig. 8), VGLUT1 mRNA was strongly expressed by neurons from layers II–VI, including the smaller cells of layer IV. In contrast, VGLUT2 mRNA was most densely expressed in pyramidal neurons of layer IIIb, and at lower levels in layers II, IIIa, and IV. Expression in layers V and VI was very low for VGLUT2 mRNA. These laminar patterns were similar across core, belt and parabelt regions, suggesting that there were no major differences between regions of auditory cortex with respect to the expression of these genes.

3.7. Other areas

To facilitate comparison with studies in other species, VGLUT mRNA expression was also illustrated for portions of the cerebellum, inferior olivary complex, and hippocampus (Fig. 9). Overall, expression of VGLUT1 and VGLUT2 in these structures was complementary. In the cerebellum, VGLUT1 expression was strong in the granular layer and relatively weak in the molecular layer. VGLUT2 was moderate in those layers, which contrasts from studies in most mammals where VGLUT2 levels are typically very low or absent. An exception is the study by Danik et al. (2005) in which about 60% of neurons co-expressed both transcripts in adult animals. No cells expressed signals for either VGLUT1 or VGLUT2 mRNA in the pyramidal cell layer of the cerebellum. In the inferior olivary complex, VGLUT2 signals were strong in most neurons and VGLUT1 expression was very low. In the hippocampus, VGLUT1 mRNA is strongly expressed by pyramidal neurons and granule cells across divisions, whereas VGLUT2 mRNA expression is very low. These results compare well with those in rodents (Danik et al., 2005; Fremeau et al., 2001; Hioki et al., 2003; Hisano et al., 2002).

4. Discussion

The main purposes of this study were to determine the distribution of VGLUT1 and VGLUT2 mRNA expressing neurons in cortex and subcortical nuclei of the primate auditory pathway and compare those results to those derived from species that have been previously studied. In both mice and rats, previous studies have repeatedly found that their expression was complementary in some domains, and overlapping in others (Barroso-Chinea et al., 2008, 2007; Fremeau et al., 2004a, 2001; Graziano et al., 2008;...
Herzog et al., 2001; Ito and Oliver, 2010). Perhaps the most widely cited difference has been that VGLUT1 is expressed at higher levels in structures including the cortex, cerebellum and hippocampus, whereas VGLUT2 expression is strongest in thalamus, brainstem and deep cerebellar nuclei. A less emphasized finding from the same studies, however, is that VGLUT1 is not absent from the thalamus and brainstem, but variably present at lower levels compared to VGLUT2, and even overlapping in some nuclei (e.g., MGC, LGN, VP). Similarly, VGLUT2 is not absent in cortex, but expressed mainly by neurons in the middle cortical layers.

These patterns are generally consistent with the findings of the present study. VGLUT2 mRNA was most intensely expressed by neurons located in nuclei of the primary (lemniscal) pathways and layers IIb–IV in auditory cortex. VGLUT1 mRNA expression was widely and strongly expressed in layers II–VI in cortex and ventral cochlear nuclei, at moderate levels in the medial geniculate, and at low levels compared to VGLUT2 in the other primary auditory nuclei. Thus, with respect to our principal hypothesis, we can conclude that VGLUT2 is the main vesicular glutamate transporter in subcortical and thalamocortical (TC) circuits, whereas VGLUT1 is dominant in corticocortical (CC) and corticothalamic (CT) systems of projections.

Yet, the nonoverlapping distributions of neurons expressing VGLUT1 and VGLUT2 in the primary nuclei and middle layers of cortex suggest that both transporters are used in some circuits. In early postnatal development in rats, the co-expression of VGLUT1 and VGLUT2 transcripts occurs in the majority of neurons in the cortex, cerebellum and hippocampus, then decrease in numbers over time (Danik et al., 2005; De Gois et al., 2005). In adult animals, co-expression is found at variable levels in several structures, including the hippocampus, thalamus, brainstem and cortex (Danik et al., 2005; De Gois et al., 2005; Herzog et al., 2001; Ito and Oliver, 2010). Of particular relevance to the present study is a recent study of the auditory brainstem nuclei of the rat by Ito and Oliver (2010). They not only found that VGLUT2 expression was prominent in nuclei containing glutamatergic neurons, but also found that many of these neurons also co-expressed VGLUT1. In thalamus, co-expression has been observed in the primary sensory and association nuclei, but is limited to VGLUT2 in the midline and intralaminar nuclei (Barroso-Chinea et al., 2007). In cortex, most of the neurons in layer IV that express VGLUT2 mRNA also express VGLUT1 mRNA (De Gois et al., 2005). The functional significance of VGLUT co-expression is less clear, as relatively little is known about the ways in which VGLUT isoforms differ with respect to glutamate regulation, and how those factors may impact the systems of projections in which they are expressed. Among the more general observations is that VGLUT1 and VGLUT2 are associated with different release probabilities, but apparently not with differences in neuronal firing rates in some brain regions (see Fremeau et al., 2001, 2004a, 2004b). Hisano et al. (2002) suggested that the VGLUTs may transport glutamate independently according to different kinetic properties, or perhaps are segregated into two different populations of synaptic vesicles that selectively transport glutamate. Whether these are properties that characterize all circuits remains to be determined, but given the known differences in their distributions throughout the brain, it does seem likely that glutamate is differentially regulated in circuits using these transporters. Therefore, we would expect their impact on neuronal activity to reflect those differences.

It is of special interest in this context that VGLUT gene and protein expression are developmentally regulated (Blaesse et al., 2005; Danik et al., 2005; De Gois et al., 2005; Gillespie et al., 2005) and also modulated by activity (De Gois et al., 2005). Changes in levels of expression and co-expression at selected synapses may alter synaptic transmission and play a role in plasticity. The VGLUTs are critical for establishment of normal hearing (Seal et al., 2008), and not surprisingly, their expression is modified by hearing loss. For example, Zeng et al. (2009) found that VGLUT1-ir is downregulated in the magnocellular (deep layers) of the DCN after unilateral deafening, whereas VGLUT2-ir was upregulated in the outer layers, which receive significant nonauditory inputs, suggesting compensatory enhancement of those inputs. Thus, given their roles in signaling, in general, and dynamic changes during development and various forms of synaptic plasticity, it is important that we establish more precisely the patterns of VGLUT expression and co-expression throughout the brain.

4.1. Implications for organization of the primate auditory pathways

Historically, the organization of the auditory pathways has emphasized and depended on studies conducted in species other than primates. Relatively few studies have focused on human or nonhuman primates, especially in the more caudal portions of the brainstem. The present study is one of very few in which the architectonic features of the entire pathway have been considered in a primate. As such, it may serve as a useful reference. Overall, our findings revealed no gross differences in organization of the subcortical pathways between that of primates and other species, especially as compared to the auditory cortex, where differences between species are more pronounced (or more obvious) (Hackett, 2010; Winer, 1992; Winer and Lee, 2007). Otherwise, the main contribution of this study was to document VGLUT1 and VGLUT2 mRNA expression in the auditory pathway and provide a baseline for future studies. Some of these results are briefly discussed below.

4.2. VGLUT1 and VGLUT2 expression in the auditory brainstem and thalamus

In the cochlear nuclei, VGLUT1 and VGLUT2 mRNA were densely expressed in principal neurons of the AVCN and PVCN, suggesting that ascending projections from these divisions of the CN utilize either or both transporters. In the DCN, VGLUT2 mRNA was prominent in what appears to correspond to the fusiform layer, but VGLUT1 expression was very weak overall. These findings corroborate findings in mice (Graziano et al., 2008; Lein et al., 2007). Since we did not perform dual ISH in these experiments, we could not assess whether the transporters were colocalized in the same neurons. However, comparison of the distributions of VGLUT and Nissl stained cells suggests that VGLUT1 and VGLUT2 co-expression in a majority of glutamatergic neurons in the ventral nuclei is likely, as found in the rat (Ito and Oliver, 2010).

In the SOC, little is known about the architectonic organization or connections of the principal and especially periolivary nuclei in primates compared to some other species. The present results are in agreement with previous studies, however, in demonstrating that the SOC of the owl monkey has a well developed MSO and LSO, although the LSO does not appear to be as large as in other mammals, such as cats, and its internal organization not as elaborate (Bazwinsky et al., 2005; Hilbig et al., 2007; Kulesza and Randy, 2007; Moore, 1987, 2000; Moore and Moore, 1971). Given that the principal cells of the AVCN and PVCN expressed VGLUT1 and/or VGLUT2 mRNA, it can be assumed that some of their projections contribute to the glutamatergic terminations on neurons in the MSO and LSO (Blaesse et al., 2005; Cant and Casseday, 1986; Cant and Benson, 2003; Suneha et al., 1995), and IC (Cant and Benson, 2003; Ito and Oliver, 2010; Oliver, 1987). By comparison, the prominent expression of VGLUT2 by MSO and LSO neurons indicates that their targets mainly utilize the VGLUT2 transporter.

In the lateral lemniscus, VGLUT2 expression was primarily limited to a population of neurons in the INLL. The DNLL and VNLL
contained few, if any, neurons that expressed either VGLUT1 or VGLUT2. This is consistent with findings in the rat, where very few neurons were found to express the mRNA of either transporter (Ito and Oliver, 2010), as well as earlier observations that most neurons in the DNLL and VNLL are GABAergic or glycinegic (Adams and Mugnaini, 1984; Aoki et al., 1988; Moore and Moore, 1987; Roberts and Ribak, 1987; Winer et al., 1995).

In the inferior colliculus, we found that architectonic organization was rather similar to other species. Of particular interest was the structural heterogeneity within the central nucleus (ICc), which is well known from both anatomical and physiological work in other species (Cant, 2005; Oliver, 2005). For example, VGLUT2 mRNA and CO expression were strongest in the central nucleus (ICc) overall, but especially in the dorsolateral quadrant of the ICc, which is an ITD-sensitive low best frequency domain. By comparison, VGLUT1 mRNA was weakly expressed by neurons in the ICc, but distributed in a similar manner as VGLUT2. This gradient is complementary to that observed in the rhesus monkey IC for Wisteria floribunda agglutinin binding, which was more intense in dorsally and medially (Hilbig et al., 2007). Given these gradients, it may be worth noting that in a recent study of the IC in cats, injections of retrograde tracers in a corresponding dorsolateral zone revealed double-labeled neurons clustered in the ventral IC (fovea) (Loftus et al., 2010). It was suggested that these projections represented two sources of excitatory input to this portion of the IC, and that ITD-sensitive MSO inputs contributed to binaural properties of neurons in this sector. This is consistent with recent anatomical work in the gerbil (Cant and Benson, 2006). Given that neurons in the MSO and LSO most strongly expressed VGLUT2 mRNA, it is therefore likely that most or all excitatory SOc projections to the IC mainly express the VGLUT2 protein, while weaker VGLUT1 co-expression appears to characterize a subset of these projections (Ito and Oliver, 2010). Of related interest are recent studies of the IC in rats, where dual fluorescence immunohistochemistry was used to examine VGLUT1 and VGLUT2 protein expression in relation to cells labeled by specific markers (Altschuler et al., 2008; Ito et al., 2009). Although both proteins were densely expressed in the IC, their colocalization in terminals tended to be infrequent. Altschuler et al. (2008) reported that VGLUT1 and VGLUT2 immunoreactivity was mainly expressed in different terminals throughout the IC. VGLUT2 terminals made somatic and dendritic contacts, and outnumbered those reactive for VGLUT1, which primarily contacted dendrites. The findings by Ito et al. (2009) were comparable in those respects, adding that distinct patterns of VGLUT2 terminations characterized two types of GABAergic neurons in the IC: a larger-celled population with VGLUT2 axosomatic contacts, and a smaller-celled population without somatic contacts. Taken together, the collective findings are consistent with the established view that projections to the IC contribute distinct information to functional zones in the IC, which are passed on to other structures (e.g., MGC) (Cant and Benson, 2007), but also indicate that glutamate is differentially regulated by VGLUT1 and VGLUT2 in these circuits.

The architectonic features of the owl monkey medial geniculate complex were consistent with a previous study of this species (Morel and Kaas, 1992), and similar to the organizational schemes adopted in other monkeys (de la Mothe et al., 2006; Hackett et al., 2007; Jones, 2003; Molinari et al., 1995). VGLUT1 and VGLUT2 were expressed in all divisions of the MGC, but their patterns varied. As observed in mice and rats (Barroso-Chinea et al., 2007; Fremeau et al., 2001; Lein et al., 2007), VGLUT2 mRNA was strongly expressed by neurons in all of its subdivisions. VGLUT1 was also expressed across all divisions, but was much weaker overall. The broad distribution of VGLUT1 and VGLUT2 mRNA in neurons of all MGC divisions indicates that both transporters are used by TC projections to core, belt and parabelt fields of auditory cortex (Hackett and de la Mothe, 2009), and may even be co-expressed by the majority of glutamatergic MGC neurons. In that case, we can expect that the majority of axon terminals in the thalamorecipient layers of auditory cortex will express both VGLUT1 and VGLUT2 proteins, as observed in somatosensory cortex of mice (Graziano et al., 2008).
4.4. Conclusions and future directions

In this study, VGLUT1 and VGLUT2 gene expression was surveyed in the auditory pathway of the owl monkey. Overall, it can be concluded that VGLUT1 and VGLUT2 mRNA are widely expressed throughout the auditory pathway. Upon closer inspection of their distributions, however, and from consideration of other studies, it appears that the VGLUT1 and VGLUT2 proteins are utilized by distinct systems of projections in most structures, and overlapping in others. VGLUT2 is most strongly expressed in the subcortical pathways, especially in the primary nuclei. VGLUT1 is more weakly and variably expressed in neurons of most subcortical nuclei with the exception of the ventral cochlear nuclei, and strongly expressed by most pyramidal neurons in cortex. Thus, as indexed by VGLUT expression, excitatory neurotransmission in the ascending and descending auditory pathways is mediated by at least two systems, but in a manner that varies between nuclei and neuronal subpopulations.

The present findings and those of related studies reviewed above suggest that much could be learned about normal and impaired auditory function by conducting studies that document and expand our understanding of VGLUT expression and regulation in auditory circuits. Among the studies that could be pursued include 1) co-labeling of cells and terminals in tracer studies of specific projections/circuits; 2) co-expression of VGLUT1, VGLUT2, VGLUT3 mRNA with markers of specific cell types to reveal neurochemically-distinct populations of neurons; 3) physiological characterization of neurons that exhibit different VGLUT expression profiles; 4) tracking up- or down-regulation of VGLUTs in development or after specific environmental manipulations.

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Comments about Dr. Jeffrey Winer

This manuscript is dedicated to the memory of Dr. Jeffrey Winer, a brilliant and creative scientist and friend, whose contributions to the auditory community will never be forgotten. As an anatomist, he had no equal. The attention to detail in the beautiful work that he so carefully prepared is unmatched. It has inspired me from the first paper I read and shall continue to serve as an example to follow in times of great doubt, and gave advice and support to see it through. I shall always be grateful for his life and friendship.

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