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VGLUT1 and VGLUT2 mRNA expression in the primate auditory pathway

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Abstract

The vesicular glutamate transporters (VGLUTs) regulate storage and release of glutamate in the brain. In adult animals, the VGLUT1 and VGLUT2 isoforms are widely expressed and differentially distributed, suggesting that neural circuits exhibit distinct modes of glutamate regulation. Studies in rodents suggest that *VGLUT1* and *VGLUT2* mRNA expression patterns are partly complementary, with *VGLUT1* expressed at higher levels in cortex and *VGLUT2* prominent subcortically, but with overlapping distributions in some nuclei. In primates, *VGLUT* gene expression has not been previously studied in any part of the brain. The purposes of the present study were to document the regional expression of *VGLUT1* and *VGLUT2* mRNA in the auditory pathway through A1 in cortex, and to determine whether their distributions are comparable to rodents. *In situ* hybridization with antisense riboprobes revealed that *VGLUT2* was strongly expressed by neurons in the cerebellum and most major auditory nuclei, including the dorsal and ventral cochlear nuclei, medial and lateral superior olivary nuclei, central nucleus of the inferior colliculus, sagulum, and all divisions of the medial geniculate. *VGLUT1* was densely expressed in the hippocampus and ventral cochlear nuclei, and at reduced levels in other auditory nuclei. In auditory cortex, neurons expressing *VGLUT1* were widely distributed in layers II – VI of the core, belt and parabelt regions. *VGLUT2* was most strongly expressed by neurons in layers IIIb and IV, weakly by neurons in layers II – IIIa, and at very low levels in layers V – VI. The findings indicate that *VGLUT2* is strongly expressed by neurons at all levels of the subcortical auditory pathway, and by neurons in the middle layers of cortex, whereas *VGLUT1* is strongly expressed by most if not all glutamatergic neurons in auditory cortex and at variable levels among auditory subcortical nuclei. These patterns imply that VGLUT2 is the main vesicular glutamate transporter in subcortical and thalamocortical (TC) circuits, whereas VGLUT1 is dominant in cortico-cortical

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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 and goal to be reached. As a friend and mentor, Jeff was patient and true. He encouraged me to continue on in the field 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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(CC) and cortico-thalamic (CT) systems of projections. The results also suggest that *VGLUT* mRNA expression patterns in primates are similar to rodents, and establishes a baseline for detailed studies of these transporters in selected circuits of the auditory system.

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; Fremeau et al., 2004b; Fremeau et al., 2001; Gras et al., 2002; Herzog et al., 2001; Kaneko et al., 2002a; Kaneko et al., 2002b; Takamori, 2006; Takamori et al., 2000; Takamori et al., 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 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 et al., 2002a; Mohrmann et al., 2008; Santos et al., 2009; Varoqui et al., 2002). For example, in the cortex of adult animals, including 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., 2002b). The concentration of VGLUT2-ir in layer IV terminals suggests that these inputs are thalamic in origin (Hur et al., 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 ventroposterior nuclei (Barroso-Chinea et al., 2008; Barroso-Chinea et al., 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 et al., 2008; Nair-Roberts et al., 2008; Stornetta et al., 2002; Wang et al., 2009), whereas *VGLUT1* is strong in only a few (e.g., vestibular and cochlear nuclei, lateral reticular, external cuneate). The transcripts of 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 gleaning 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

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circuits that are largely segregated, and therefore likely to subserve different functional roles (Altschuler et al., 2008; Ito et al., 2009; Kaneko et al., 2002b; 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 were comparable to patterns in rats and mice (Kaneko et al., 2002b; Zhou et al., 2007). In macaque auditory cortex, the regional and laminar distribution of VGLUT2-ir terminals was related to established architectonic markers of the core, belt and parabelt regions (Hackett et al., 2009). Across laminae, VGLUT2-ir terminals were concentrated in the thalamorecipient layers (IIIb, IV), coextensive with elevated expression of parvalbumin (PV), acetylcholinesterase (AChE) and cytochrome oxidase (CO). A lesser band of immunoreactive terminals was located in layer VI. Regionally, VGLUT2-ir was highest in the primary, or core, region, intermediate in the surrounding belt areas, and very sparse in the parabelt, matching systematic reductions in the expression of AChE and CO along the core-belt-parabelt hierarchy (Hackett, 2010). The conspicuous concentration of VGLUT2-ir in the core, and significant reductions in the belt and parabelt suggested that subcortically, *VGLUT2* mRNA might be preferentially expressed by neurons in the primary (lemniscal) pathway, which includes the ventral division of the medial geniculate complex (MGv), consistent with findings in rodents (Barroso-Chinea et al., 2007; Herzog et al., 2001). Although it would be convenient if *VGLUT1* mRNA were preferentially expressed by neurons in non-primary auditory structures, the rodent data suggest that *VGLUT1* mRNA will be expressed at reduced levels in the subcortical nuclei of primates and strong in cortical neurons.

To address these questions and better understand the distributions of neurons expressing *VGLUT1* and *VGLUT2* in the auditory pathway of primates, we employed *in situ* hybridization to study mRNA expression in owl monkeys from the cochlear nucleus to A1. Accordingly, it follows that VGLUT2 is the glutamate transporter utilized in the subcortical and thalamocortical (TC) auditory pathways, while VGLUT1 is prominent in corticocortical (CC) and corticothalamic (CT) circuits. A secondary purpose of this study was to provide much needed data on basic architectonic features of the primate auditory brainstem nuclei, which remain poorly studied. The combined results indicated that the distributions of *VGLUT1* and *VGLUT2* mRNA 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.

Methods

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.

Tissue preparation for histology

Animals were deeply anesthetized with a lethal dose of sodium pentobarbital (80mg/kg) and perfused transcardially with 0.9% saline in 0.1M phosphate buffer (PB) followed by 4% paraformaldehyde (PFA) in 0.1M PB. The brain was removed and postfixed for 3–6 hours in

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4% PFA in 0.1M PB. The thalamus and brainstem were separated from the cerebral hemispheres, blocked, and cryoprotected in 30% sucrose in 0.1M PB for 3–5 days at 4°C. The block was cut into 40um frozen coronal sections on a sliding microtome and sections were stored at −20°C in cryoprotectant solution (30% glycerol, 30% ethylene glycol, 40% 0.1M phosphate-buffered saline). Brain sections were saved in 12 series, with alternating series of sections processed for Nissl using thionin, cytochrome oxidase (CO) (Wong-Riley, 1979), *in situ* hybridization for vesiclular 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 DH20, Invitrogen Corp, Carlsbad, CA) to show laminar details.

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-dUTP 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 ccgctacattatcgccatca and cgatgggcacgatgatggtc respectively, which targeted position 204-1093 of human *VGLUT1* (AB032436). The forward and reverse primers for *VGLUT2* were gccatygtggacatggtcaa (y indicates c or t) and atractccaccatagtggac (r 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; Takahata et al., 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 anyhydride in 0.9% triethanolamine and 0.12% hydrochloric acid, the sections were incubated in hybridization buffer (pH 7.5) containing 5X standard saline citrate (SSC; 150mM sodium chloride, 15 mM sodium citrate, pH 7.0), 50% formamide (FA), 2% blocking reagent (Roche Diagnostics), 0.1% N-lauroylsarcosine (NLS), 0.1% sodium dodecyl sulphate (SDS), 20mM maleic acid buffer, and 1ug/ml of the appropriate DIG-labeled riboprobe. Sections were hybridized overnight at 60°C and washed twice by successive immersion in 2X SSC, 50% FA, 0.1% NLS for 20 minutes at 60°C. Nonspecific mRNA was removed with 20ug/ml RNase A in RNase A buffer (10 mM Tris-HCl, 10 mM ethylenediamine-N,N,N′,N′-tetraacetic acid (EDTA), 500 mM NaCl; pH 8.0) for 15 minutes at 37°C and sections were washed again in 2X SSC, 0.1% NLS, followed by 0.2X 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.

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

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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.

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). This layer could be divided into two domains based on CO expression (Fig. 3D). 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. 3J). In contrast, *VGLUT1* mRNA was rather weakly expressed in this zone and elsewhere in the DCN, with expression at levels just above background (Fig. 3G). In the deepest third layer, was a thin line of cells that expressed *VGLUT2* mRNA.

In the anteroventral (AVCN) and posteroventral (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 Discussion).

Superior Olivary Complex (SOC)

The SOC of the owl monkey contained nuclei identified herein as the medial (MSO), lateral (LSO), medial (MNTB) and ventral (VNTB) nuclei of the trapezoid body, as well as a several periolivary 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) periolivary nuclei. In the MSO, the central line of principal cells stained darkly for Nissl substance and CO (Fig. 4A, B). CO reactivity was dense within soma and proximal dendrites (Fig. 4B). Cells in the central column densely expressed *VGLUT2* mRNA (Fig. 4D, F). *VGLUT1* mRNA was also expressed in these cells, but at greatly reduced levels compared to *VGLUT2* in these cells and also to *VGLUT1* in the AVCN and PVCN (Fig. 4C, E).

Based on cell distribution patterns in the Nissl preparations (Fig. 4A), it appears that *VGLUT1* and *VGLUT2* mRNA were also expressed in a subpopulation of neurons in the LSO (Fig. 4C, D). The arrows denote a location where the labeled neurons expressing *VGLUT2* mRNA were contained in a cluster in the LSO along the lateral boundary of the MSO. *VGLUT1* expression was rather weak in these neurons.

In the MNTB region, the somata of the principal neurons expressed CO, but only a subpopulation of neurons in the presumptive VNTB or other periolivary nuclei expressed

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CO (Fig. 4B). Scattered neurons in these periolivary nuclei expressed *VGLUT1* or *VGLUT2* mRNA. Generally, mRNA expression in these neurons was weaker compared to the MSO and LSO neurons.

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 et al., 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 et al., 2010). In contrast, numerous cells in the INLL and nearby sagulum strongly expressed *VGLUT2*, and frequently *VGLUT1* at reduced levels.

Inferior Colliculus

On the basis of Nissl and CO staining (Cant et al., 2006; Morest et al., 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). We 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.

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., 2006b;Morel et al., 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 (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), supragenicular (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,

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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 varies between divisions. Note that *VGLUT1* and *VGLUT2* mRNA were also contained neurons of the LGN, but *VGLUT1* levels were greater than in the MGC (data not shown).

Auditory Cortex (Area A1)

In the auditory cortex (Fig. 8), *VGLUT1* mRNA was strongly expressed by neurons from layers II to 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.

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 coexpressed 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;Herzog et al., 2001;Hioki et al., 2003;Hisano et al., 2002).

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; Barroso-Chinea et al., 2007; Fremeau et al., 2004a; Fremeau et al., 2001; Graziano et al., 2008; Herzog et al., 2001; Ito et al., 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)

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pathways and layers IIIb – 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 cortico-cortical (CC) and cortico-thalamic (CT) systems of projections.

Yet, the overlapping 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 et al., 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 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 *VGLUT*2 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 differences in neuronal firing rates in some brain regions (see Fremeau et al 2001 see Fremeau et al 2004). 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 the 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 (2008) 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.

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

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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 et al., 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.

VGLUT1 and VGLUT2 expression in the auditory brainstem and thalamus

In the cochlear nuclei, *VGLUT1* and *VGLUT2* mRNA was 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* coexpression in a majority of glutamatergic neurons in the ventral nuclei is likely, as found in the rat (Ito et al., 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, 2007; Moore, 1987; Moore, 2000; Moore et al., 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 et al., 1986; Cant et al., 2003; Suneja et al., 1995), and IC (Cant et al., 2003; Ito et al., 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 et al., 2010), as well as earlier observations that most neurons in the DNLL and VNLL are GABAergic or glycinergic (Adams et al., 1984; Aoki et al., 1988; Moore et al., 1987; Roberts et al., 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 fluoribunda* 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

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the IC in cats, injections of retrograde tracers in a corresponding dorsolateral zone labeled neurons clustered in the ipsilateral dorsal (low-frequency) MSO (93%) and dorsolateral LSO (7%) (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 et al., 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 et al., 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 et al., 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 et al., 1992), and similar to the organizational schemes adopted in other monkeys (de la Mothe et al., 2006b; 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 et al., 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).

VGLUT1 and VGLUT2 expression in auditory cortex

VGLUT1 and *VGLUT2* gene expression in A1 showed a striking pattern in this study. *VGLUT1* mRNA was expressed by the majority of pyramidal neurons in layers II – VI. In contrast, *VGLUT2* mRNA was most strongly expressed by neurons in layers IIIb, weaker in layers II, IIIa and IV, and absent or at very low levels in layers V and VI. This differs from the typical pattern of VGLUT2 protein expression in auditory cortex, which is concentrated in presumptive TC terminals in layer IIIb, IV, and VI (Hackett et al., 2009). Of additional interest was that *VGLUT1* and *VGLUT2* mRNA expression did not appear to vary significantly between the core, belt, and parabelt regions of auditory cortex. This contrasts with the gradients typically observed with other functional markers (see Hackett, 2010). Overall, these expression patterns are very similar to those found in the sensory cortices of rats (Fremeau et al., 2001; Graziano et al., 2008; Ito et al., 2010), and have a number of important implications with respect to the organization of auditory pathways.

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First, we can conclude that VGLUT1 is the main glutamate transporter used in the CC projections of auditory cortex neurons, and that VGLUT2 is utilized in a subset of those, perhaps favoring feedforward projections to higher areas and lateral type connections to adjoining areas (Fitzpatrick et al., 1980; Galaburda et al., 1983; Hackett, 2010). Evidence of *VGLUT1 – VGLUT2* coexpression suggests that some neurons in the middle layers express both transporters (De Gois et al., 2005).

Second, the apparent absence of *VGLUT2* mRNA in layers V and VI neurons suggests that VGLUT1 is the principal or only glutamate transporter utilized by the descending corticothalamic and corticotectal (CT) projections to MGC and IC (de la Mothe et al., 2006b; FitzPatrick et al., 1978; Luethke et al., 1989; Winer et al., 2002). A similar conclusion was reached by Ito and Oliver (2010), based on the absence of *VGLUT2* expression in layer V neurons labeled by tracer injections in the IC. If it turns out that cortico-thalamo-cortical (CTC) circuits contribute to driven activity in higher-order areas of the auditory cortex (e.g., belt, parabelt), as recently reported in the somatosensory system (Theyel et al 2010), then the present findings indicate that VGLUT1 would mediate the descending CT projections, whereas mainly VGLUT2 (and possibly VGLUT1) would be utilized in the ascending TC projections. The very low levels of VGLUT2-ir terminals in the parabelt (Hackett et al., 2009), however, suggest that a VGLUT2-mediated CTC circuit would be unlikely to drive activity in that region of auditory cortex, but could perhaps facilitate the actions of feedforward projections to the parabelt from lower areas in the belt region.

Third, the CT projections, which arise from neurons that primarily or exclusively express *VGLUT1*, differ from the system of descending projections with neurons of origin in the thalamus or upper brainstem, because *VGLUT2* is the dominant transporter in most nuclei, and *VGLUT1* is more selectively or more weakly expressed (Barroso-Chinea et al., 2007). In the IC, for example, the LN and DC receive descending projections from the MGC and AC, and so there would be an overlapping distribution of terminals utilizing the VGLUT1 and/or VGLUT2 transporters. Studies combining anterograde tract tracing and immunohistochemistry for the VGLUTs and various recipient cell types will be needed to sort out these details.

Conclusions and future directions

In this study, *VGLUT1* and *VGLUT2* gene expression were 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

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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.

Research Highlights

- Vesicular glutamate transporter 1 and 2 mRNA are expressed in partly overlapping circuits in the auditory pathway of primates.
- Vesicular glutamate transporter 1 is most strongly expressed in auditory cortex and ventral cochlear nuclei.
- Vesicular glutamate transporter 1 expression is dominant in the subcortical nuclei.
- Patterns of vesicular glutamate transporter 1 and 2 mRNA expression are comparable to rodents.

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List of Abbreviations

A1	Auditory area 1
AVCN	Anteroventral cochlear nucleus
CB	Calbindin
CN	Cochlear nucleus
CO	Cytochrome oxidase
DC	Dorsal cortex of the inferior colliculus
DCN	Dorsal cochlear nucleus
GC	Substantia griseum centralis
IC	Inferior colliculus
ICc	Inferior colliculus, central nucleus
LGN	Lateral geniculate nucleus, thalamus
Lim	Limitans nucleus, thalamus
LN	Lateral nucleus of the inferior colliculus
LSO	Lateral superior olivary nucleus
MGad	Medial geniculate complex, anterodorsal division
MGC	Medial geniculate complex
MGm	Medial geniculate complex, magnocellular division
MGpd	Medial geniculate complex, posterodorsal division
MGv	Medial geniculate complex, ventral division

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MNTB	Medial nucleus of the trapezoid body
MSO	Medial superior olivary nucleus
PL	Lateral pulvinar nucleus, thalamus
PM	Medial pulvinar nucleus, thalamus
Po	Posterior nucleus, thalamus
PVCN	Posteroventral cochlear nucleus
Sg	Suprageniculate nucleus, thalamus
SOC	Superior olivary complex
VGLUT	Vesicular glutamate transporter
VNTB	Ventral nucleus of the trapezoid body

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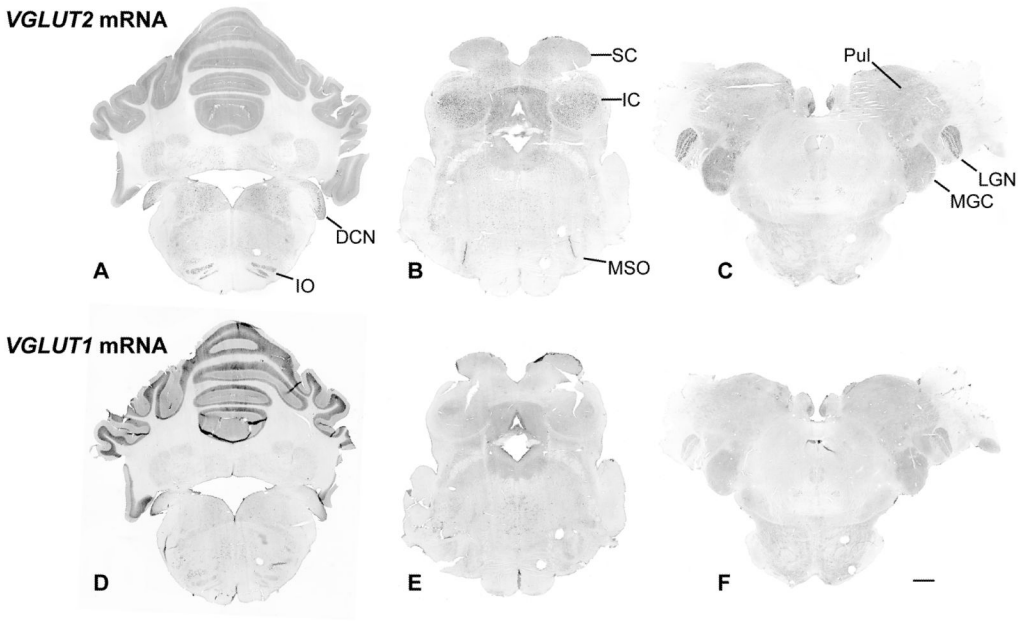

Fig. 1.

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 are illustrated in adjacent sections to show their respective distribution patterns. Scale bar = 1mm.

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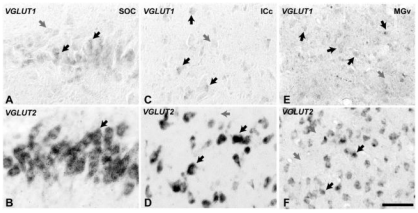

Fig. 2.

High magnification images of cells containing *VGLUT1* and *VGLUT2* mRNA. *VGLUT1* signals (A, C, E) were found at reduced levels in these subcortical auditory structures, and its subcellular distribution was partial compared to *VGLUT2* (B, D, F). Medial superior olivary nucleus (A, B); central nucleus of the inferior colliculus (C, D); ventral division of the medial geniculate (E, F). Scale bar = 50 um.

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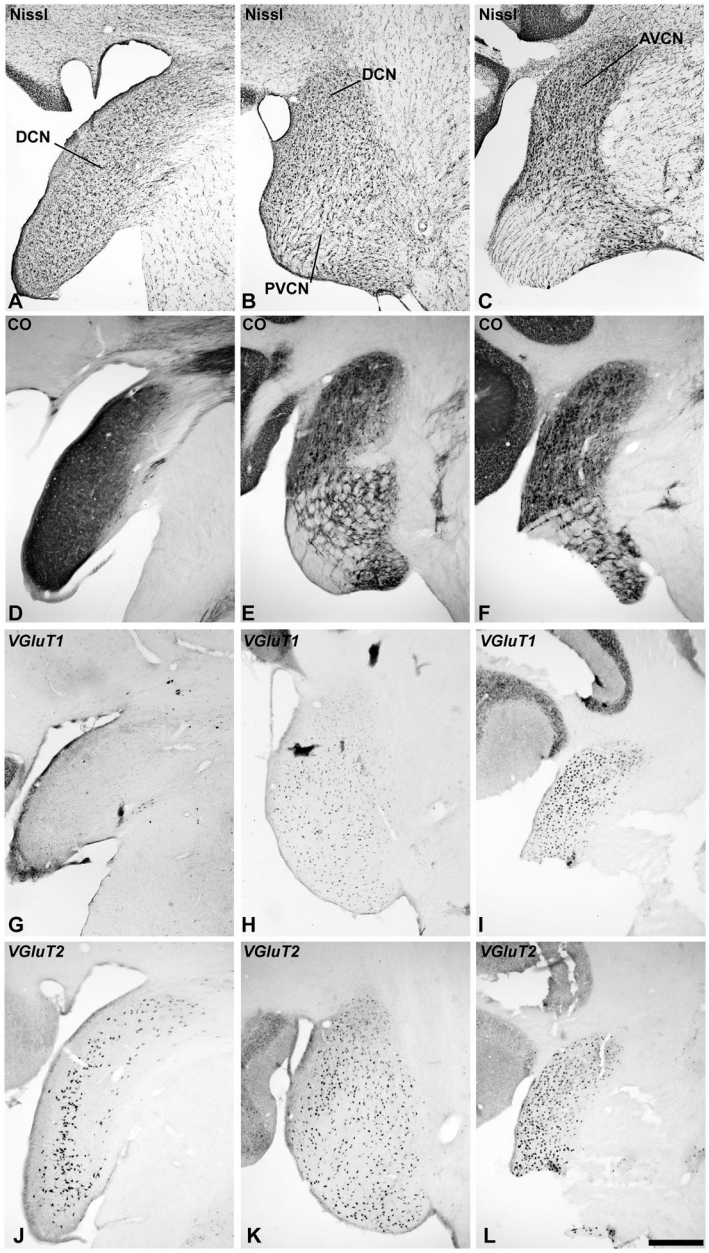

Fig. 3.

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 um.

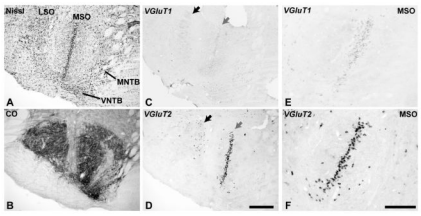

Fig. 4.

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 um.

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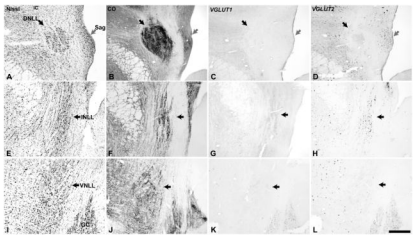

Fig. 5.

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 um.

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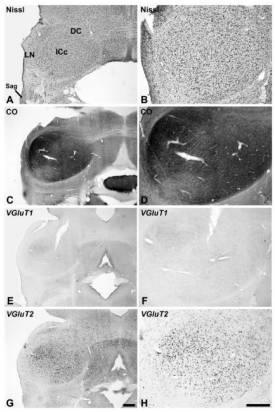

Fig. 6.

VGLUT mRNA expression in the inferior colliculus (IC). The general locations of the central (ICc), lateral (LN), dorsal cortex (DC) divisions are indicted in panel A. Sections processed for Nissl (A, B), CO (C, D), *VGLUT1* mRNA (E, F) and *VGLUT2* mRNA (G, H) are illustrated at two different magnifications to show detail. Scale bars = 500 um.

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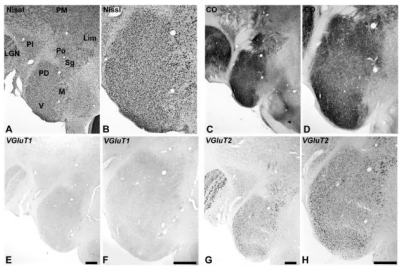

Fig. 7.

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 um.

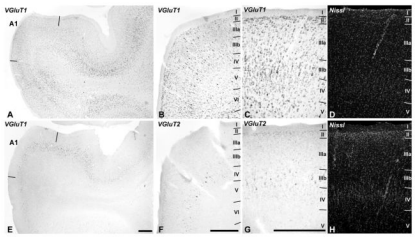

Fig. 8.

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 um.

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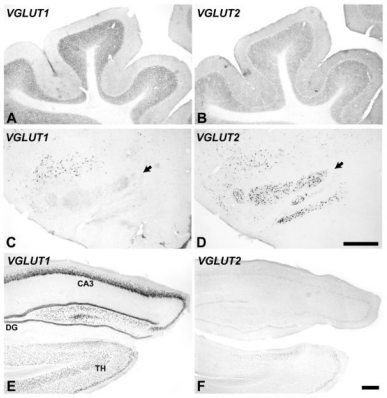

Fig. 9.

VGLUT mRNA expression in the cerebellum (A, B), inferior olivary complex (C, D black arrows), and hippocampus (E, F). *VGLUT1* mRNA (left panels). *VGLUT2* mRNA (right panels). Scale bars = 500 um.

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