Published in final edited form as:

J Comp Neurol. 2011 February 1; 519(2): 316–340. doi:10.1002/cne.22521.

Expression of Glutamate and Inhibitory Amino Acid Vesicular Transporters in the Rodent Auditory Brainstem

Tetsufumi Ito^{1,2}, Deborah C. Bishop², and Douglas L. Oliver²

¹Department of Anatomy, Faculty of Medical Sciences, University of Fukui, Fukui, Japan

²Department of Neuroscience, University of Connecticut Health Center, Farmington, CT, 06030-3401 USA

Abstract

Glutamate is the main excitatory neurotransmitter in the auditory system, but associations between glutamatergic neuronal populations and the distribution of their synaptic terminations have been difficult. Different subsets of glutamatergic terminals employ one of three vesicular glutamate transporters (VGLUT) to load synaptic vesicles. Recently, VGLUT1 and VGLUT2 terminals were found to have different patterns of organization in the inferior colliculus suggesting that there are different types of glutamatergic neurons in the brainstem auditory system with projections to the colliculus. To positively identify VGLUT-expressing neurons as well as inhibitory neurons in the auditory brainstem, we used in situ hybridization to identify the mRNA for VGLUT1, VGLUT2, and VIAAT (the vesicular inhibitory amino acid transporter used by GABAergic and glycinergic terminals). Similar expression patterns were found in subsets of glutamatergic and inhibitory neurons in the auditory brainstem and thalamus of adult rats and mice. Four patterns of gene expression were seen in individual neurons. 1) VGLUT2 expressed alone was the prevalent pattern. 2) VGLUT1 co-expressed with VGLUT2 was seen in scattered neurons in most nuclei but was common in the medial geniculate body and ventral cochlear nucleus. 3) VGLUT1 expressed alone was found only in granule cells. 4) VIAAT expression was common in most nuclei but dominated in some. These data show that the expression of the VGLUT1/2 and VIAAT genes can identify different subsets of auditory neurons. This may facilitate the identification of different components in auditory circuits.

Keywords

GABA; glycine; in situ hybridization; VGAT; VGLUT; mouse; rat

Introduction

As in other brain regions, glutamate is the prevalent excitatory neurotransmitter at synapses in the auditory system (e.g. Altschuler and Shore, 2010; Fitzgerald and Sanes, 1999; Fujino et al., 1997; Isaacson and Walmsley, 1995). Nevertheless, the identification of glutamatergic neurons and terminals is often made indirectly. For example, terminals are identified as excitatory (and therefore, presumably glutamatergic) if they contain round synaptic vesicles (Oliver, 1987). Cell bodies are considered as excitatory if they do not contain molecules associated with inhibitory neurotransmitters (Saint Marie et al., 1997). The positive identification of cell bodies of glutamatergic neurons has been more problematic: Since

glutamate is an essential amino acid present in all cells, immunoreactivity for the amino acid is not a useful way to identify neurons that release glutamate from synaptic vesicles.

The identification of the vesicular glutamate transporters (VGLUT) was a major breakthrough in the search for molecular markers for glutamatergic neurons. Three genes were identified; VGLUT1 (Bellocchio et al., 2000; Ni et al., 1994), VGLUT2 (Freneau et al., 2001; Fujiyama et al., 2001; Herzog et al., 2001), and VGLUT3 (Freneau et al., 2002; Gras et al., 2002; Schafer et al., 2002; Takamori et al., 2002). When cultured GABAergic neurons were made to express one of the genes for VGLUT, they released synaptic glutamate (Takamori et al., 2000; 2001), so the expression of VGLUT is sufficient for the glutamatergic phenotype. In general, the expression of the three VGLUT genes is complementary (Fujiyama et al., 2001; Gras et al., 2002; Kaneko and Fujiyama, 2002; Kaneko et al., 2002): Expression of VGLUT1 is strong in the cerebral cortex, while that of VGLUT2 is strong in the thalamus. Although the expression of VGLUT1 and VGLUT2 is mainly segregated (Kaneko and Fujiyama, 2002), colocalization within some neurons is also reported (Billups, 2005; Blaesse et al., 2005; Ito et al., 2009; Nakamura et al., 2007). Neurons expressing VGLUT3 are different since they often co-express GABA (Hioki et al., 2004), acetylcholine, or serotonin (Gras et al., 2002). Although immature neurons in the medial nucleus of the trapezoid body (MNTB) express VGLUT3 (Gillespie et al., 2005), VGLUT3 immunoreactivity is almost absent in the adult superior olivary complex (SOC) (Blaesse et al., 2005) and inferior colliculus (IC) (Ito et al., 2009). Therefore, a “typical” auditory glutamatergic neuron may express VGLUT1 and/or VGLUT2. Thus, the identification of the expression pattern of the two VGLUT molecules not only reveals the distribution of all glutamatergic neurons, but it also reveals subgroups of glutamatergic neurons with different combinations of VGLUT expression. Since these molecules are abundant in synaptic vesicles but sparse in the somata, the *in situ* labeling of the mRNA for these molecules is an excellent means to identify the cell bodies of these glutamatergic cells.

In this study, we examined the expression of VGLUT1 and VGLUT2 mRNAs in the auditory brainstem of rats and mice. For comparison, we also examined the expression of the vesicular inhibitory amino acid transporter (VIAAT; also called VGAT) to identify inhibitory neurons since it is expressed in both GABAergic and glycinergic neurons (Chaudhry et al., 1998; Wang et al., 2009). The data demonstrate the distribution of all neurons using the amino acid neurotransmitters in the auditory brainstem, including the cochlear nuclei complex (CNC), SOC, nuclei of the lateral lemniscus (NLL), IC, and also in the medial geniculate body (MGB). The presence of VGLUT proteins has been shown in axonal terminals in many of these auditory nuclei (Altschuler et al., 2008; Billups, 2005; Blaesse et al., 2005; Ito et al., 2009; Zhou et al., 2007), and our data provide information about the origin of these terminals.

Material and methods

Subjects

Six adult Long-Evans rats and 10 Swiss-Webster mice were used for this study. All experiments were conducted in accordance with the institutional guidelines of the University of Connecticut Health Center and the NIH guidelines for the care and use of laboratory animals. All efforts were made to minimize the number of animals used and their suffering.

Rats were deeply anesthetized with ketamine (97.5 mg/kg *i.m.*) and xylazine (2.4 mg/kg *i.m.*). Mice were deeply anesthetized with a mixture of ketamine (100mg/kg *i.p.*), xylazine (20mg/kg *i.p.*), and acepromazine (3mg/kg *i.p.*). The animals were perfused transcardially with normal saline followed by 4% paraformaldehyde diluted in 0.1M phosphate buffer (pH 7.4; PB). After cryoprotection in diethylpyrocarbonate- (DEPC) treated 30% sucrose in PB

for 2 days, serial coronal sections were cut at a thickness of 40 μm (for rats) or 30 μm (for mice) with a freezing microtome. Every 5th section was stained with Cresyl Violet. Auditory nuclei were identified by Nissl cytoarchitecture, and in accordance with Mugnaini et al. (1980) for the CNC, Schofield and Cant (1991) for the SOC, Saint Marie et al. (1997) for the NLL, Loftus et al. (2008) for the IC, and Clerici and Coleman (1990) for the MGB.

Bright field in situ hybridization histochemistry

Both digoxigenin (DIG)- and fluorescein (FL)-labeled sense and antisense riboprobes were made from the cDNAs of mouse VGLUT1 (nucleotides of 152–1085, GenBank accession number NM_182993.2), VGLUT2 (nucleotides of 848–2044, GenBank accession number NM_080853.2), and VIAAT (nucleotides of 620–1599, GenBank accession number NM_031782). Sequences of VGLUT1, VGLUT2, and VIAAT probes have very high similarities with the corresponding regions of rat cDNAs (96%, 94%, and 97%, respectively). On the other hand, similarities of these riboprobes with other members of the family of molecules are low: The mouse VGLUT1 riboprobe shares only 74% and 71% identity with the corresponding regions of the rat VGLUT2 and VGLUT3 mRNA; and the mouse VGLUT2 riboprobe has only 74% and 76% homology with the rat VGLUT1 and VGLUT3 mRNA. The mouse VGLUT1 riboprobe shares only 75% and 71% homology with the mouse VGLUT2 and VGLUT3 mRNA; and the mouse VGLUT2 riboprobe is only 74% and 75% homologous with the mouse VGLUT1 and VGLUT3 mRNA. Sections reacted with sense riboprobes exhibited no signal at all.

The procedure for non-radioactive in situ hybridization (ISH) was described previously (Ito et al., 2008; Ito et al., 2007; Liang et al., 2000). Free-floating sections consisting of a series of every fifth section from each animal were washed in 0.1 M PB (pH 7.0) for 5 minutes twice, immersed in PB containing 0.3% Triton X-100, and washed in 0.1 M PB. Then, sections were acetylated for 10 minutes at room temperature with 0.003% acetic acid anhydride, 1.3% (v/v) triethanolamine, and 6.5% (w/v) HCl diluted in DEPC-treated water. After being washed in 0.1M PB twice, the sections were incubated for 1 hour at 70°C in a prehybridization buffer containing 50% (v/v) formamide, 5×SSC buffer (a 5× concentration of SSC buffer containing 16.65 mM sodium chloride and 16.65 mM sodium citrate buffer, pH 7.0), 2% blocking reagents (Roche Diagnostics, Mannheim, Germany), 0.1% N-lauroylsarcosine (NLS), and 0.1% sodium dodecyl sulfate. Then, the sections were hybridized with 1 $\mu\text{g}/\text{ml}$ DIG-labeled sense or antisense RNA probe for VGLUT1, VGLUT2, or VIAAT in freshly prepared prehybridization buffer for 20 hours at 70°C. After two washes in 2×SSC, 50% formamide, and 0.1% NLS for 20 minutes at 70°C, the sections were incubated with RNase A (20 $\mu\text{g}/\text{ml}$; Sigma-Aldrich, St. Louis, MO) in 0.01 M Tris-HCl (pH 8.0), 1 mM ethylenediaminetetraacetic acid, 0.5 M NaCl for 30 minutes at 37°C. The sections were washed in 2×SSC with 0.1% NLS for 20 minutes twice at 37°C, and in 0.2×SSC with 0.1% NLS for 20 minutes twice at 37°C. These sections were incubated with 1% blocking reagent (Roche) diluted in 0.1 M Tris-HCl (pH 7.5) and 0.15 M NaCl (TS7.5) for 1 hour at room temperature, and then they were incubated with alkaline phosphatase-conjugated sheep anti-DIG antibody Fab fragment (1:2000; Roche) in 1% blocking reagent (Roche) diluted in TS7.5 at room temperature overnight. The bound phosphatase was visualized by a reaction with nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (Roche) for 4 hours at 37°C in 0.1 M Tris-HCl (pH 9.5), 0.15 M NaCl, and 10 mM MgCl_2 . Sections were mounted on glass slides, dehydrated, cleared with Histoclear (National Diagnostics, Atlanta, GA), and coverslipped.

Double fluorescent ISH histochemistry for VGLUT1 and VGLUT2

A series of every fifth section from three rats and three mice was used for double fluorescent ISH (FISH) for VGLUT1 and VGLUT2. A FL-labeled riboprobe for VGLUT1 and a DIG-

labeled riboprobe for VGLUT2 were diluted in hybridization buffer at a final concentration of 1 µg/ml. The hybridization temperature was set at 70°C. After the incubation and washing described above, these sections were incubated with alkaline phosphatase-conjugated sheep anti-FL Fab fragment (1:2000; Roche) and peroxidase-conjugated sheep anti-DIG antibody Fab fragment (1:2000; Roche) in 1% blocking reagent (Roche) diluted in TS7.5 at room temperature overnight. The sections were washed three times, and the bound peroxidase was reacted with dinitrophenol-tyramide signal amplification (Perkin-Elmer, Waltham, MA). After three more washes, the sections were incubated with AlexaFluor 488-conjugated rabbit anti-dinitrophenol (1:250; Invitrogen, Carlsbad, CA) in 1% blocking reagent diluted in TS7.5. To visualize bound alkaline phosphatase by fluorescent microscopy, sections were developed with 0.005% (w/v) 4-chloro-2-methylbenzenediazonium hemi-zinc chloride (Fast Red TR, Roche), 1% (v/v) 2-hydroxy-3-naphtoic acid-2'-phenylanilide phosphate (HNPP, Roche) diluted in 0.1 M Tris-HCl (pH8.0), 0.15 M NaCl, 10 mM MgCl₂, for 30 minutes at room temperature. The sections were mounted on glass slides with CC/Mount (DBS, Pleasanton, CA).

Image analysis

Fluorescent images were acquired with a laser scanning confocal microscope (510 META, Carl Zeiss Microimaging, Göttingen, Germany) or a structural illuminated microscope (Axiovert microscope with Apotome, Zeiss). For the laser scanning confocal microscope, AlexaFluor 488 was excited by a 488 nm Ar laser, and the emitted fluorescence was filtered with a 500–530 nm band-pass filter. Fast Red/ HNPP was excited by a 543 nm He-Ne laser, and the emitted fluorescence was filtered with a 565–615 nm band-pass filter. The images of each dye were taken sequentially to avoid bleed-through artifact. For the structural illuminated microscope, filter sets for FITC and rhodamine were used to visualize AlexaFluor 488 and Fast Red/ HNPP, respectively. Minimal adjustments of the levels were made in Photoshop CS3 (Adobe Systems, San Jose, CA).

To plot cells positive for VGLUT1 and VGLUT2, we collected images of all main auditory nuclei (for a total of 90 images) in three rats and three mice. The images were assembled into a single montage for each section with Illustrator CS3 (Adobe), and the montages were analyzed in Stereo Investigator (MBF Bioscience, Inc., Williston, VT). For each structure (i.e. CNC, NLL, MGB, and SOC), at least three equally spaced montages were used for the analysis. Auditory nuclei were first identified in Nissl-stained neighboring sections. We then examined those nuclei that had VGLUT gene expression in the bright-field specimens. Within a single nucleus, a sampling frame was set, and we counted cells positive for only VGLUT1, only VGLUT2, or both. We compared the bright-field ISH and fluorescent ISH specimens and counted neurons as positive only when the same pattern of labeling was seen in both specimen types. This reduced false positive counts. Counts are given as percentage of neurons expressing these molecules. Granule cells, which have small diameter (Table 1) and express only VGLUT1 (see Results; Figs. 3a4–c4 and 5a3–c3), were not included for counting.

Results

General CNS distribution of VGLUT1, VGLUT2, and VIAAT expression

We examined the overall distribution of expression of VGLUT1, VGLUT2, and VIAAT in the CNS to investigate the specificity of our probes. The expression pattern was very similar for the rat (Fig. 1, a–c) and mouse (Fig. 1, d–f). VGLUT1 was strongly expressed in the olfactory tract, cerebral cortex, cerebellar granule cells, medial habenula, pontine nucleus, and hippocampus (Fig. 1a, d). Lightly stained neurons were found in some thalamic nuclei (Fig. 1a2, d2). In contrast, VGLUT2 expression was prevalent in all thalamic nuclei (except

for the reticular nucleus), hypothalamus, superior and inferior colliculi, deep cerebellar nuclei, and many brainstem nuclei (Fig. 1b, e). Weak expression of VGLUT2 was found in the hippocampus, restricted neocortical areas, and the amygdala. The intensity of VGLUT2 staining was stronger in the amygdala and olfactory cortex than the neocortex. This expression pattern of VGLUT1 and VGLUT2 was virtually identical to that in previous studies using different riboprobes (Herzog et al., 2001; Hisano, 2003; Ni et al., 1995).

VIAAT-expressing neurons were found in many brain regions that also expressed one of the VGLUT markers (Fig. 1c, f). However, the density of VIAAT-expressing neurons was especially high in the reticular thalamic nucleus, ventromedial hypothalamus, central amygdaloid nucleus, caudate putamen, olfactory tubercle, and it was scarce in most thalamic nuclei. This expression pattern in our material is very similar to a previous study in which authors used a different riboprobe to examine the gene expression of VIAAT in comparison to fluorescent protein expressed under the VIAAT promoter (Wang et al., 2009). These general patterns of gene expression show that our riboprobes recognize only the appropriate structures in the CNS and are consistent with the notion that our riboprobes have a high degree of specificity.

Cochlear nucleus

VGLUT1 and 2 are co-expressed in the anteroventral and posteroventral cochlear nuclei—(AVCN, PVCN) in both rat (Fig. 2, a1–d1, a2–d2) and mouse (Fig. 4, a1–d1, a2–d2). In rat, VGLUT1- and VGLUT2-expressing neurons are densely packed (Fig. 2a–b), while VIAAT-expressing neurons, presumably D-stellate cells (Doucet et al., 1999), are sparsely distributed (Fig. 2c). Double FISH for VGLUT1 and VGLUT2 revealed that stained neurons in the ventral cochlear nucleus (VCN) expressed both genes (Figs. 3, a1–c1, a2–c2; Table 2). Cochlear root neurons (CRNs, Merchan et al., 1988) also express both VGLUT1 and VGLUT2 (Fig. 3, a3–c3). An almost identical pattern is seen in the mouse with co-expression of both VGLUT1 and 2 in almost all AVCN and PVCN neurons (Figs. 5, a1–c1, a2–c2; Table 3).

Granule cells express only VGLUT1 in both rat and mouse—In the granule cell layer (GrC in Figs. 2 and 4), glutamatergic cells express only VGLUT1, similar to cerebellar granule cells (Ni et al., 1994). VIAAT-expressing neurons were sparse in the GrC. The dense cluster of GrC VGLUT1-expressing neurons extends to layer 2 of the dorsal cochlear nucleus (DCN) (Fig. 2a2; Fig. 4a2). In the DCN, small VGLUT1-expressing cells are mainly distributed in layer 2 but are found in other layers as well (Fig. 2a2, a3; Fig. 4a2). We measured the perimeter of granule cells in the CNC as well as cells expressing both VGLUT1 and VGLUT2 in the CNC in double FISH specimens. The average perimeter of neurons expressing only VGLUT1 (Fig. 3 a4–c4; Fig. 5a3–c3) is roughly half that of the cells expressing VGLUT2 in both rat and mouse (Table 1).

Fusiform cells in the DCN express only VGLUT2 in both rat and mouse—In contrast to the co-expression seen in AVCN and PVCN, the large cells in layer 2 and the deeper layer of DCN, presumably fusiform cells and giant cells respectively, express only VGLUT2 (Fig. 2b3; Fig. 4b2). VIAAT-expressing cells are distributed in all layers of the DCN and represented a distinct population with a distribution and packing density different from the fusiform and granule cells (Fig. 2c3; 4c2). Double FISH sections reveal a complete lack of co-expression for VGLUT1 and 2 (Figs. 3a4–c4; 5a3–c3; Tables 2, 3).

Expression of VGLUT2 and VIAAT is complementary in the SOC

In our study, we subdivided the SOC into 9 nuclei based on Nissl cytoarchitecture in the rat (Fig. 6d). The pattern is almost identical in the mouse (Fig. 8d). The main nuclei are the

lateral and medial superior olives (LSO, MSO) and the medial nucleus of the trapezoid body (MNTB). Surrounding these are the periolivary nuclei, starting dorsally and moving counter clockwise: the dorsal periolivary (DPO), lateroventral periolivary (LVPO), the medioventral periolivary (MVPO), the ventromedial periolivary (VMPO), and the superior paraolivary (SPO) nuclei. The most rostral is the rostral periolivary (RPO) nucleus.

The SPO, MNTB, and MVPO express only VIAAT in both rat (Fig. 6c1–c3) and mouse (Fig. 8c1–c3), and they show a complete absence of VGLUT2 (Figs. 6b1–b3; 8b1–b3) or VGLUT1 expression (Figs. 6a1–a3; 8a1–a3). Thus, they are different from the other SOC nuclei and were not included in the analysis of VGLUT co-expression in the SOC.

The LSO is found at caudal levels of SOC in both rat (Figs. 6a3–d3) and mouse (Fig. 8a3–d3). Neurons expressing VGLUT2 or VIAAT are equally common and fusiform-shaped. VIAAT neurons are located at the periphery of the nucleus in the mouse but are scattered throughout in the rat. VGLUT1 message is found in few neurons. In rats, double FISH shows that only 4% of VGLUT2-expressing LSO cells co-express VGLUT1 (Fig. 7a4–c4; Table 2). In mice, glutamatergic cells express almost exclusively VGLUT2 (Fig. 9a4–c4; Table 3), and VGLUT1 co-expression is absent.

The MSO is found in the center of the SOC. The nucleus is more easily distinguished in the rat (Figs. 6a2–d2, 6a3–d3) than in the mouse (Fig. 8a2–d2, 8a3–d3). VIAAT-expression is almost absent (Fig. 6c2–c3), while VGLUT2-expressing cells are densely packed (Fig. 6b2–b3). Many VGLUT2-expressing neurons have spindle-shaped cell bodies. In rats, VGLUT1-expressing cells are fewer than VGLUT2 cells (Fig. 6a2), and VGLUT1 is almost absent in mice (Fig. 8a2). Double FISH reveals that all VGLUT1-expressing cells co-express VGLUT2 in rats, and these represent 19% of glutamatergic neurons (Fig. 7a2–c2; Table 2).

The DPO is located in the dorsolateral part of the SOC above the LSO (rat: Fig. 6a2–d2, a3–d3; mouse: Fig. 8a2–d2, a3–d3). Higher cell density differentiates the DPO from non-SOC regions (Fig. 6d3). Bright-field microscopy shows that all three molecules are expressed in the DPO of both species. The VGLUT2-expressing cells are more prevalent than the VGLUT1-expressing cells. Double FISH reveals that the majority of glutamatergic cells express VGLUT2 alone, but 20% of VGLUT neurons co-express VGLUT1 and VGLUT2 in rat (Fig. 7a3–c3; Tables 2). A similar pattern is seen in the mouse with 13% of VGLUT neurons expressing both VGLUTs (Fig. 9a3–c3; Table 3).

The LVPO is located ventral and lateral to the LSO and MSO (rat: Figs 6a2–d2, a3–d3; mouse: Figs. 8a1–d1, 8a2–d2, 8a3–d3). All three molecules are expressed in the LVPO with VIAAT being most common and VGLUT1 being least common (rat: Fig. 6a2–c2, a3–c3; mouse: Fig. 8). There are few VGLUT1-expressing cells. VIAAT-expressing cells are closer to the lateral surface than VGLUT2 cells, although there is no clear boundary. Double FISH shows that all glutamatergic cells express VGLUT2, but only 8–12% of VGLUT2 cells co-express VGLUT1 in both rat and mouse (Figs. 7a5–c5, 9a5–c5; Tables 2 and 3).

The VMPO is ventromedial to the MSO (Figs. 6a2–d2, 6a3–d3; 8a2–d2, 8a3–d3). It contains both glutamatergic and inhibitory cells. Cell density was lower in the VMPO than in the MSO. VGLUT1-expressing cells are found in rat (Fig. 6a2, a3) but not in mice (Fig. 8a2, a3). Double FISH reveals that almost all VGLUT1-expressing cells co-express VGLUT2, and the majority of VGLUT2-expressing neurons co-express VGLUT1 in rats (Fig. 7a6–c6; Table 2). In mice, glutamatergic cells express VGLUT2 exclusively (Fig. 9a6–c6; Table 3) similar to the MSO and LSO.

The RPO is located in the most rostral part of the SOC and is encircled by VNLL, SPO and MVPO (Figs. 6a1–d1; 8a1–d1). All 3 molecules are expressed in these nuclei, although

there are fewer VGLUT1-expressing cells. A dense cluster of VGLUT2-expressing cells differentiates the RPO from the encircling nuclei that largely express VIAAT (Figs 6b1–c1; 8b1–c1). Over 60% of VGLUT2-expressing neurons co-express VGLUT1 in the rat (Fig 7a1–c1, 9a1–c1; Table 2), but only 5% of neurons show co-expression in mice (Table 3).

Dorsal and ventral NLL are inhibitory but intermediate NLL is glutamatergic

In the dorsal NLL (DNLL) of both rat and mouse, dense clusters of VIAAT-expressing neurons are found, while neurons expressing VGLUT are few (Figs. 10a1–d1; 11a1–d1). In the intermediate NLL (INLL), all three molecules are expressed with VGLUT2 as the most frequent and the VIAAT-expressing cells the least frequent (Figs. 10a1–d1; 11a11–d1). Small and large VGLUT1-expressing neurons appear to be distributed differently: Small neurons are mainly distributed in the lateral and caudal part of the INLL, and larger neurons are distributed throughout the nucleus. Double FISH reveals that the small neurons express only VGLUT1 (a green neuron in Fig. 17a1–c1), while the larger neurons co-express VGLUT1 and VGLUT2 (arrows in Fig. 15a1–c1; Table 2 and 3).

In the ventral NLL (VNLL) ventral to the INLL (Figs. 10a2–d2, 11a2–d2), the VIAAT-expressing cells are densely packed, while VGLUT-expressing ones are much fewer. The VGLUT2-expressing cells outnumber the VGLUT1-ones. As in the INLL, two kinds of VGLUT1-expressing neurons are found: Small neurons are mainly distributed in the lateral and caudal part of the VNLL and larger neurons are distributed throughout the nucleus. Double FISH reveals that the small neurons are VGLUT2-negative (not shown), while larger neurons mainly express both VGLUT1 and VGLUT2 (Table 2 and 3). About 3–5% of VGLUT2-expressing neurons co-express VGLUT1 (Tables 2 and 3).

We measured the perimeter of cells expressing only VGLUT1 or only VGLUT2 in double FISH specimens from the NLL (Table 1). Cells expressing only VGLUT1 form a clearly separated population of small neurons (perimeter < 45 μ m). These cells are most likely granule cells as they are distributed along the lateral surface of the lateral lemniscus, forming a continuous band with the granule cells of the CNC, medial cerebellar peduncle, and the cerebellum.

VGLUT1 is not expressed in the IC

In the IC, there are numerous VGLUT2-expressing cells (Figs. 12b and 13b) and no VGLUT1-expressing cells (Figs. 12a and 13a). Fewer VIAAT-expressing neurons are seen, but they are still numerous (Figs. 12c and 13c). It is possible to identify the dorsal and lateral cortex and central nucleus (DC, LC, and ICC, respectively) in ISH sections for VGLUT2 and VIAAT. For example, the dense packing of VGLUT2 cells in the ICC forms a relatively sharp border with the lower density in the LC. In layer 2 of the LC, dense clusters of VIAAT-expressing cells are found in both rats and mice; and these correspond to the GABA modules described in previous studies (Chernock et al., 2004; Ito et al., 2009).

Most cells in ventral MGB express both VGLUT1 and 2

In the MGB, VIAAT-expressing cells are rare (Figs. 14c and 16c). VGLUT2 is expressed in all parts of the MGB (Figs. 14b and 16b). VGLUT1-expressing cells are mainly limited to the ventral division (MGV) and the adjacent part of the dorsal division (MGD). There appears to be a rostro-caudal gradient in the MGV with a higher density of VGLUT1-expressing cells in the rostral MGV (Figs. 14a1–3 and 16a1–3). A reverse of this gradient is seen for VGLUT2 where the higher density is in the middle to caudal thirds (Figs. 14b1–3 and 16b1–3). There is also a dorso-ventral gradient of VGLUT1 expression in the MGD with the highest density of VGLUT1 seen ventrally (Figs. 14a and 16a). The medial and supragenulate nuclei (MGM and SG), have few VGLUT1-expressing cells, and in the

caudodorsal nucleus (CD), the expression of VGLUT1 is almost absent (Figs. 14a3 and 16a3). The VGLUT1 signal is the strongest in the MGv amongst all MGB subdivisions; however, the double FISH demonstrates that vast majority of VGLUT1-expressing neurons co-express VGLUT2 (Tables 2 and 3). In contrast, the incidence of co-expression of VGLUT1 in VGLUT2-expressing neurons varies among subnuclei; it is the highest (80–85%) in the MGv (Figs. 15a2–c2, 17a2–c2); lower in the MGD (Figs. 15a3–c3, 17a3–c3), MGM (Figs. 15a4–c4, 17a4–c4), and SG (Figs. 15a5–c5, 17a5–c5); and absent in the CD (Tables 2 and 3). Incidence of co-expression in the MGD and MGM is higher in mice than rats (Tables 2 and 3).

Discussion

In this study, we demonstrated the distribution of the presumed excitatory (glutamatergic) and inhibitory (GABAergic and glycinergic) cells in the auditory brainstem and thalamus (Fig. 18). Glutamatergic cells can be divided into subpopulations in terms of VGLUT expression. VGLUT2 was expressed in most glutamatergic cells in the auditory brainstem except for the granule cells that expressed only VGLUT1. Subpopulations of VGLUT2-expressing neurons co-expressed VGLUT1, and this pattern was most obvious in the MGv and VCN. On the other hand, IC glutamatergic cells and DCN fusiform cells expressed only VGLUT2.

Species difference

There was no obvious qualitative difference in the distribution of VIAAT-expressing cells in the rat and mouse. The expression of VGLUT2 as the prevalent transporter was also very consistent between species. However, there are fewer VGLUT1-expressing neurons in the SOC of the mouse compared to the rat. Accordingly, the number of SOC neurons that expressed both molecules was reduced in the mouse. Since there is a tendency for the staining intensity of VGLUT1 to be weaker than that of VGLUT2 in the SOC and NLL, there may be a limit in the detection of the VGLUT1 mRNA. Nevertheless, it is reasonable to conclude that expression of VGLUT1 mRNA is weaker in the mouse SOC than rat. In contrast, co-expression of VGLUT1 and VGLUT2 is more frequent in the mouse MGD and MGM than in the rat.

Subdivisions of the SOC determined by expression of vesicular transporters

The nuclei of the SOC can be divided into 3 groups; a glutamatergic nucleus (MSO), nuclei with a mixture of both glutamatergic and inhibitory neurons (LSO, DPO, RPO, VMPO, and LVPO), and nuclei with only inhibitory neurons (MNTB, SPO, and MVPO). The inhibitory nuclei are located medially; the nuclei with a mixture of excitatory and inhibitory neurons are located laterally, and the purely excitatory nucleus (MSO) is located in the middle. Interestingly, the medial, inhibitory nuclei are driven by inputs mostly from the contralateral VCN (Schwartz, 1992). In contrast, the lateral, mixed function nuclei are driven mostly by inputs from the ipsilateral VCN. The purely glutamatergic MSO is driven by both sides. Both medial and central nuclei project to the ipsilateral IC (except for the MNTB), while the lateral nuclei project bilaterally to the IC (Adams, 1983). In the LSO, glycinergic cells project ipsilaterally and glycine-negative, presumably glutamatergic cells project contralaterally (Saint Marie et al., 1989). Our preliminary data suggest that the other lateral nuclei have a similar projection pattern. Finally, the descending projections of SOC neurons also differ between medial and lateral regions: Lateral nuclei (LSO, LVPO, DPO, and RPO) project to ipsilateral VCN and cochlea, while MVPO neurons project bilaterally (Adams, 1983; Horvath et al., 2000). Therefore, the topology, gene expression, and connectivity are closely related in the SOC.

Granule cells in the lateral lemniscus

This may be the first report of presumed granule cells in the NLL. Granule cells are easily identified in the CNC and cerebellum by their high density and small size. On the other hand, the granule-like cells in the NLL may have escaped previous notice because the small VGLUT1-expressing cells in the surface of the NLL are sparsely distributed and few in number. Bajo and colleagues (1993) reported that small cells were distributed throughout the DNLL; however, those cells are not likely to be granule cells since VGLUT expression in the DNLL is rare.

Comparison with the previous studies

Previous studies of the glutamate transporters have focused on the presence of VGLUT proteins in axonal terminals in the auditory pathway (Altschuler et al., 2008; Billups, 2005; Blaesse et al., 2005; Ito et al., 2009; Zhou et al., 2007). In the cochlear nucleus, the terminals of the auditory nerve contain VGLUT1 but not VGLUT2 (Zhou et al., 2007). The dense VGLUT1-immunoreactive terminals found in layer 1 of the DCN (Zhou et al., 2007) are likely to be the terminals of granule cells. In contrast, the dense VGLUT2-immunoreactive terminals in the shell region of the CNC may originate from the IC since this region receives IC efferents (Caicedo and Herbert, 1993).

In the SOC, terminals colocalize VGLUT1 and VGLUT2 proteins (Blaesse et al., 2005) and are likely to originate from bushy cells, octopus cells, and T-stellate cells in the VCN (Schofield, 1995; Tolbert et al., 1982; Warr, 1966). In the MVPO, the prevalent VGLUT2-positive/ VGLUT1-negative terminals (Billups, 2005) may be from the IC since the MVPO receives dense IC inputs (Caicedo and Herbert, 1993).

In the IC, terminals colocalizing VGLUT1 and VGLUT2 proteins (Ito et al., 2009) are likely to originate mainly from T-stellate cells in the VCN (Oertel et al., 1990; Oliver, 1987). Terminals that contain only VGLUT2 in the IC may arise from local neurons in the IC or from the DCN fusiform cells, the SOC, or the INLL as these nuclei project to the IC (Alibardi, 1998; Gonzalez-Hernandez et al., 1996; Oliver, 1985; Oliver et al., 1995; Saint Marie and Baker, 1990; Whitley and Henkel, 1984). Some of these terminals in the IC may contribute to the dense VGLUT2 axosomatic synapses on the large tectothalamic GABAergic neurons (Ito et al., 2009). Consequently, the DCN, SOC, or INLL inputs to IC may be associated with feed-forward inhibition to the thalamus through the tectothalamic GABAergic neurons. Terminals that contain only VGLUT1 in the IC are most likely from the layer 5 pyramidal neurons in the auditory cortex (Winer et al., 1998). Such neocortical neurons express VGLUT1 but not VGLUT2 (Hisano, 2003).

In the MGB, the mRNA expression of VGLUT1 and VGLUT2 was shown by Barroso-Chinea et al. (2007). They concluded that VGLUT2 was expressed in the entire MGB, while VGLUT1 was expressed only in the MGv. However, they provided no details on colocalization and no quantitative analysis. The present results differ - VGLUT1 signal was the strongest in the MGv, but it did extend to the adjacent subdivisions. The present results also show that neurons in the MGv express both genes. The discrepancy between the present results and the previous study might arise from different methods that have different levels of sensitivity.

Functional significance

Our present data show that auditory neurons use different combinations of VGLUT molecules. The nuclei of the auditory pathway appear to differ in the types of glutamatergic neurons and the prevalence of inhibitory neurons (expressing VIAAT). Although the properties of the VGLUT molecules are very similar (Schafer et al., 2002; Varoqui et al.,

2002), the VGLUT1 vesicles may have more pathways for recycling and may recycle faster under prolonged, high-frequency stimulation (Voglmaier et al., 2006). For example, VGLUT2 synapses in hippocampal neurons depress more rapidly than VGLUT1 synapses after high-frequency stimulation; moreover, the VGLUT2 synapses recover more slowly (Fremeau et al., 2004). This suggests that the high expression levels of VGLUT1 in the auditory nerve and VCN may be useful for high-frequency firing and phase-locking. In contrast, the prevalent expression of VGLUT2 in the SOC, NLL, and IC suggest that rapid vesicle recycling may be less important for those structures. Interestingly, the VGLUT1-expression pattern returns in the MGv and auditory cortex where phase locking is not commonly expected, and it raises the possibility that VGLUT1 may be associated fast spiking neurons or other synaptic functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge Drs. Tetsuo Yamamori and Akiya Watakabe (National Institute for Basic Biology, Japan) for their gift of the VGLUT1 and VGLUT2 riboprobes, and Dr. Richard Altschuler for reading the manuscript.

Grant support

This work was supported by grants from NIH DC00189 (DLO), the Health Center Research Advisory Committee of the University of Connecticut Health Center (DLO), and The Uehara Memorial Research Scholarship (TI).

Abbreviation list

Cochlear Nuclei Complex (CNC)

AVCN	anteroventral cochlear nucleus
PVCN	posteroventral cochlear nucleus
VCN	ventral cochlear nucleus
DCN	dorsal cochlear nucleus
CRN	cochlear root neurons

Superior Olivary Complex (SOC)

DPO	dorsal periolivary nucleus
LSO	lateral superior olive
LVPO	lateroventral periolivary nucleus
MSO	medial superior olive
MNTB	medial nucleus of the trapezoid body
MVPO	medioventral periolivary nucleus
RPO	rostral periolivary nucleus
SPO	superior paraolivary nucleus
VMPO	ventromedial periolivary nucleus

Nuclei of the Lateral Lemniscus (NLL)

DNLL	dorsal nucleus of the lateral lemniscus
INLL	intermediate nucleus of the lateral lemniscus
VNLL	ventral nucleus of the lateral lemniscus
PBG	parabigeminal nucleus
Inferior Colliculus (IC)	
ICC	central nucleus of the IC
DC	dorsal cortex of the IC
LC	lateral cortex of the IC
Medial Geniculate Body (MGB)	
MGD	dorsal division of the MGB
MGV	ventral division of the MGB
CD	caudodorsal division of the MGB
MGM	medial division of the MGB
SG	supragenicular division of the MGB
PP	peripeduncular nucleus
PIL	posterior intralaminar thalamic nucleus
LP	lateroposterior thalamic nucleus
other	
ISH	in situ hybridization
FISH	fluorescent ISH
FL	fluorescein
DIG	digoxigenin
VGLUT	vesicular glutamate transporter
VIAAT	vesicular inhibitory amino acid transporter

Literature cited

- Adams JC. Cytology of periolivary cells and the organization of their projections in the cat. *J Comp Neurol.* 1983; 215(3):275–289. [PubMed: 6304156]
- Alibardi L. Ultrastructural and immunocytochemical characterization of neurons in the rat ventral cochlear nucleus projecting to the inferior colliculus. *Ann Anat.* 1998; 180(5):415–426. [PubMed: 9795692]
- Altschuler, RA.; Shore, SE. Central auditory neurotransmitters. In: Rees, A.; Palmer, AR., editors. *The Auditory Brain.* 1 ed. Oxford: Oxford University Press; 2010. p. 65-92.
- Altschuler RA, Tong L, Holt AG, Oliver DL. Immunolocalization of vesicular glutamate transporters 1 and 2 in the rat inferior colliculus. *Neuroscience.* 2008; 154(1):226–232. [PubMed: 18436385]
- Bajo VM, Merchan MA, Lopez DE, Rouiller EM. Neuronal morphology and efferent projections of the dorsal nucleus of the lateral lemniscus in the rat. *J Comp Neurol.* 1993; 334(2):241–262. [PubMed: 8366195]

- Barroso-Chinea P, Castle M, Aymerich MS, Perez-Manso M, Erro E, Tunon T, Lanciego JL. Expression of the mRNAs encoding for the vesicular glutamate transporters 1 and 2 in the rat thalamus. *J Comp Neurol*. 2007; 501(5):703–715. [PubMed: 17299752]
- Bellocchio EE, Reimer RJ, Fremeau RT Jr, Edwards RH. Uptake of glutamate into synaptic vesicles by an inorganic phosphate transporter. *Science*. 2000; 289(5481):957–960. [PubMed: 10938000]
- Billups B. Colocalization of vesicular glutamate transporters in the rat superior olivary complex. *Neurosci Lett*. 2005; 382(1–2):66–70. [PubMed: 15911123]
- Blaesse P, Ehrhardt S, Friauf E, Nothwang HG. Developmental pattern of three vesicular glutamate transporters in the rat superior olivary complex. *Cell Tissue Res*. 2005; 320(1):33–50. [PubMed: 15714284]
- Caicedo A, Herbert H. Topography of descending projections from the inferior colliculus to auditory brainstem nuclei in the rat. *J Comp Neurol*. 1993; 328(3):377–392. [PubMed: 7680052]
- Chaudhry FA, Reimer RJ, Bellocchio EE, Danbolt NC, Osen KK, Edwards RH, Storm-Mathisen J. The vesicular GABA transporter, VGAT, localizes to synaptic vesicles in sets of glycinergic as well as GABAergic neurons. *J Neurosci*. 1998; 18(23):9733–9750. [PubMed: 9822734]
- Chernock ML, Larue DT, Winer JA. A periodic network of neurochemical modules in the inferior colliculus. *Hear Res*. 2004; 188(1–2):12–20. [PubMed: 14759566]
- Clerici WJ, Coleman JR. Anatomy of the rat medial geniculate body: I. Cytoarchitecture, myeloarchitecture, and neocortical connectivity. *J Comp Neurol*. 1990; 297(1):14–31. [PubMed: 2376630]
- Doucet JR, Ross AT, Gillespie MB, Ryugo DK. Glycine immunoreactivity of multipolar neurons in the ventral cochlear nucleus which project to the dorsal cochlear nucleus. *J Comp Neurol*. 1999; 408(4):515–531. [PubMed: 10340502]
- Fitzgerald KK, Sanes DH. Serotonergic modulation of synapses in the developing gerbil lateral superior olive. *J Neurophysiol*. 1999; 81(6):2743–2752. [PubMed: 10368394]
- Fremeau RT Jr, Burman J, Qureshi T, Tran CH, Proctor J, Johnson J, Zhang H, Sulzer D, Copenhagen DR, Storm-Mathisen J, Reimer RJ, Chaudhry FA, Edwards RH. The identification of vesicular glutamate transporter 3 suggests novel modes of signaling by glutamate. *Proc Natl Acad Sci U S A*. 2002; 99(22):14488–14493. [PubMed: 12388773]
- Fremeau RT Jr, Kam K, Qureshi T, Johnson J, Copenhagen DR, Storm-Mathisen J, Chaudhry FA, Nicoll RA, Edwards RH. Vesicular glutamate transporters 1 and 2 target to functionally distinct synaptic release sites. *Science*. 2004; 304(5678):1815–1819. [PubMed: 15118123]
- Fremeau RT Jr, Troyer MD, Pahner I, Nygaard GO, Tran CH, Reimer RJ, Bellocchio EE, Fortin D, Storm-Mathisen J, Edwards RH. The expression of vesicular glutamate transporters defines two classes of excitatory synapse. *Neuron*. 2001; 31(2):247–260. [PubMed: 11502256]
- Fujino K, Koyano K, Ohmori H. Lateral and medial olivocochlear neurons have distinct electrophysiological properties in the rat brain slice. *J Neurophysiol*. 1997; 77(5):2788–2804. [PubMed: 9163393]
- Fujiyama F, Furuta T, Kaneko T. Immunocytochemical localization of candidates for vesicular glutamate transporters in the rat cerebral cortex. *J Comp Neurol*. 2001; 435(3):379–387. [PubMed: 11406819]
- Gillespie DC, Kim G, Kandler K. Inhibitory synapses in the developing auditory system are glutamatergic. *Nat Neurosci*. 2005; 8(3):332–338. [PubMed: 15746915]
- Gonzalez-Hernandez T, Mantolan-Sarmiento B, Gonzalez-Gonzalez B, Perez-Gonzalez H. Sources of GABAergic input to the inferior colliculus of the rat. *J Comp Neurol*. 1996; 372(2):309–326. [PubMed: 8863133]
- Gras C, Herzog E, Bellenchi GC, Bernard V, Ravassard P, Pohl M, Gasnier B, Giros B, El Mestikawy S. A third vesicular glutamate transporter expressed by cholinergic and serotonergic neurons. *J Neurosci*. 2002; 22(13):5442–5451. [PubMed: 12097496]
- Herzog E, Bellenchi GC, Gras C, Bernard V, Ravassard P, Bedet C, Gasnier B, Giros B, El Mestikawy S. The existence of a second vesicular glutamate transporter specifies subpopulations of glutamatergic neurons. *J Neurosci*. 2001; 21(22):RC181. [PubMed: 11698619]

- Hioki H, Fujiyama F, Nakamura K, Wu SX, Matsuda W, Kaneko T. Chemically specific circuit composed of vesicular glutamate transporter 3- and preprotachykinin B-producing interneurons in the rat neocortex. *Cereb Cortex*. 2004; 14(11):1266–1275. [PubMed: 15142960]
- Hisano S. Vesicular glutamate transporters in the brain. *Anat Sci Int*. 2003; 78(4):191–204. [PubMed: 14686474]
- Horvath M, Kraus KS, Illing RB. Olivocochlear neurons sending axon collaterals into the ventral cochlear nucleus of the rat. *J Comp Neurol*. 2000; 422(1):95–105. [PubMed: 10842220]
- Isaacson JS, Walmsley B. Receptors underlying excitatory synaptic transmission in slices of the rat anteroventral cochlear nucleus. *J Neurophysiol*. 1995; 73(3):964–973. [PubMed: 7608781]
- Ito T, Bishop DC, Oliver DL. Two classes of GABAergic neurons in the inferior colliculus. *J Neurosci*. 2009
- Ito T, Hioki H, Nakamura K, Kaneko T, Iino S, Nojyo Y. Some gamma-motoneurons contain gamma-aminobutyric acid in the rat cervical spinal cord. *Brain Res*. 2008; 1201:78–87. [PubMed: 18294622]
- Ito T, Hioki H, Nakamura K, Tanaka Y, Nakade H, Kaneko T, Iino S, Nojyo Y. Gamma-aminobutyric acid-containing sympathetic preganglionic neurons in rat thoracic spinal cord send their axons to the superior cervical ganglion. *J Comp Neurol*. 2007; 502(1):113–125. [PubMed: 17335042]
- Kaneko T, Fujiyama F. Complementary distribution of vesicular glutamate transporters in the central nervous system. *Neurosci Res*. 2002; 42(4):243–250. [PubMed: 11985876]
- Kaneko T, Fujiyama F, Hioki H. Immunohistochemical localization of candidates for vesicular glutamate transporters in the rat brain. *J Comp Neurol*. 2002; 444(1):39–62. [PubMed: 11835181]
- Liang F, Hatanaka Y, Saito H, Yamamori T, Hashikawa T. Differential expression of gamma-aminobutyric acid type B receptor-1a and -1b mRNA variants in GABA and non-GABAergic neurons of the rat brain. *J Comp Neurol*. 2000; 416(4):475–495. [PubMed: 10660879]
- Loftus WC, Malmierca MS, Bishop DC, Oliver DL. The cytoarchitecture of the inferior colliculus revisited: a common organization of the lateral cortex in rat and cat. *Neuroscience*. 2008; 154(1):196–205. [PubMed: 18313229]
- Merchan MA, Collia F, Lopez DE, Saldana E. Morphology of cochlear root neurons in the rat. *J Neurocytol*. 1988; 17(5):711–725. [PubMed: 2463341]
- Mugnaini E, Warr WB, Osen KK. Distribution and light microscopic features of granule cells in the cochlear nuclei of cat, rat, and mouse. *J Comp Neurol*. 1980; 191(4):581–606. [PubMed: 6158528]
- Nakamura K, Watakabe A, Hioki H, Fujiyama F, Tanaka Y, Yamamori T, Kaneko T. Transiently increased colocalization of vesicular glutamate transporters 1 and 2 at single axon terminals during postnatal development of mouse neocortex: a quantitative analysis with correlation coefficient. *Eur J Neurosci*. 2007; 26(11):3054–3067. [PubMed: 18028110]
- Ni B, Rostock PR Jr, Nadi NS, Paul SM. Cloning and expression of a cDNA encoding a brain-specific Na(+)-dependent inorganic phosphate cotransporter. *Proc Natl Acad Sci U S A*. 1994; 91(12):5607–5611. [PubMed: 8202535]
- Ni B, Wu X, Yan GM, Wang J, Paul SM. Regional expression and cellular localization of the Na(+)-dependent inorganic phosphate cotransporter of rat brain. *J Neurosci*. 1995; 15(8):5789–5799. [PubMed: 7643219]
- Oertel D, Wu SH, Garb MW, Dizack C. Morphology and physiology of cells in slice preparations of the posteroventral cochlear nucleus of mice. *J Comp Neurol*. 1990; 295(1):136–154. [PubMed: 2341631]
- Oliver DL. Quantitative analyses of axonal endings in the central nucleus of the inferior colliculus and distribution of 3H-labeling after injections in the dorsal cochlear nucleus. *J Comp Neurol*. 1985; 237(3):343–359. [PubMed: 4044892]
- Oliver DL. Projections to the inferior colliculus from the anteroventral cochlear nucleus in the cat: possible substrates for binaural interaction. *J Comp Neurol*. 1987; 264(1):24–46. [PubMed: 2445792]
- Oliver DL, Beckius GE, Shneiderman A. Axonal projections from the lateral and medial superior olive to the inferior colliculus of the cat: a study using electron microscopic autoradiography. *J Comp Neurol*. 1995; 360(1):17–32. [PubMed: 7499562]

- Saint Marie RL, Baker RA. Neurotransmitter-specific uptake and retrograde transport of [3H]glycine from the inferior colliculus by ipsilateral projections of the superior olivary complex and nuclei of the lateral lemniscus. *Brain Res.* 1990; 524(2):244–253. [PubMed: 1705464]
- Saint Marie RL, Ostapoff EM, Morest DK, Wenthold RJ. Glycine-immunoreactive projection of the cat lateral superior olive: possible role in midbrain ear dominance. *J Comp Neurol.* 1989; 279(3): 382–396. [PubMed: 2918077]
- Saint Marie RL, Shneiderman A, Stanforth DA. Patterns of gamma-aminobutyric acid and glycine immunoreactivities reflect structural and functional differences of the cat lateral lemniscal nuclei. *J Comp Neurol.* 1997; 389(2):264–276. [PubMed: 9416921]
- Schafer MK, Varoqui H, Defamie N, Weihe E, Erickson JD. Molecular cloning and functional identification of mouse vesicular glutamate transporter 3 and its expression in subsets of novel excitatory neurons. *J Biol Chem.* 2002; 277(52):50734–50748. [PubMed: 12384506]
- Schofield BR. Projections from the cochlear nucleus to the superior paraolivary nucleus in guinea pigs. *J Comp Neurol.* 1995; 360(1):135–149. [PubMed: 7499559]
- Schofield BR, Cant NB. Organization of the superior olivary complex in the guinea pig. I. Cytoarchitecture, cytochrome oxidase histochemistry, and dendritic morphology. *J Comp Neurol.* 1991; 314(4):645–670. [PubMed: 1726174]
- Schwartz, IR. The superior olivary complex and lateral lemniscal nuclei. In: Webster, DB.; Popper, AN.; Fay, RR., editors. *The Mammalian Auditory Pathway: Neuroanatomy.* New York: Springer-Verlag; 1992. p. 117-167.
- Takamori S, Malherbe P, Broger C, Jahn R. Molecular cloning and functional characterization of human vesicular glutamate transporter 3. *EMBO Rep.* 2002; 3(8):798–803. [PubMed: 12151341]
- Takamori S, Rhee JS, Rosenmund C, Jahn R. Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. *Nature.* 2000; 407(6801):189–194. [PubMed: 11001057]
- Takamori S, Rhee JS, Rosenmund C, Jahn R. Identification of differentiation-associated brain-specific phosphate transporter as a second vesicular glutamate transporter (VGLUT2). *J Neurosci.* 2001; 21(22):RC182. [PubMed: 11698620]
- Tolbert LP, Morest DK, Yurgelun-Todd DA. The neuronal architecture of the anteroventral cochlear nucleus of the cat in the region of the cochlear nerve root: horseradish peroxidase labelling of identified cell types. *Neuroscience.* 1982; 7(12):3031–3052. [PubMed: 6298659]
- Varoqui H, Schafer MK, Zhu H, Weihe E, Erickson JD. Identification of the differentiation-associated Na⁺/PI transporter as a novel vesicular glutamate transporter expressed in a distinct set of glutamatergic synapses. *J Neurosci.* 2002; 22(1):142–155. [PubMed: 11756497]
- Voglmaier SM, Kam K, Yang H, Fortin DL, Hua Z, Nicoll RA, Edwards RH. Distinct endocytic pathways control the rate and extent of synaptic vesicle protein recycling. *Neuron.* 2006; 51(1): 71–84. [PubMed: 16815333]
- Wang Y, Kakizaki T, Sakagami H, Saito K, Ebihara S, Kato M, Hirabayashi M, Saito Y, Furuya N, Yanagawa Y. Fluorescent labeling of both GABAergic and glycinergic neurons in vesicular GABA transporter (VGAT)-venus transgenic mouse. *Neuroscience.* 2009; 164(3):1031–1043. [PubMed: 19766173]
- Warr WB. Fiber degeneration following lesions in the anterior ventral cochlear nucleus of the cat. *Exp Neurol.* 1966; 14(4):453–474. [PubMed: 4378200]
- Whitley JM, Henkel CK. Topographical organization of the inferior collicular projection and other connections of the ventral nucleus of the lateral lemniscus in the cat. *J Comp Neurol.* 1984; 229(2):257–270. [PubMed: 6501602]
- Winer JA, Larue DT, Diehl JJ, Hefti BJ. Auditory cortical projections to the cat inferior colliculus. *J Comp Neurol.* 1998; 400(2):147–174. [PubMed: 9766397]
- Zhou J, Nannapaneni N, Shore S. Vesicular glutamate transporters 1 and 2 are differentially associated with auditory nerve and spinal trigeminal inputs to the cochlear nucleus. *J Comp Neurol.* 2007; 500(4):777–787. [PubMed: 17154258]

Figure 1.

Brightfield micrographs of in situ hybridization for VGLUT1 (**a, d**), VGLUT2 (**b, e**), and VIAAT (**c, f**) in the rat (**a–c**) and mouse (**d–f**) brain. Both coronal sections of the forebrain (**a1–f1**) and sagittal sections of whole brain (**a2–f2**) are shown. Abbreviations: LOT, nucleus of lateral olfactory tract; Pn, pontine nucleus; MD, mediodorsal thalamic nucleus; Th, thalamus; SC, superior colliculus; VMH, ventromedial hypothalamic nucleus; Ce, central amygdaloid nucleus; CPu, caudate putamen; MHb, medial habenular nucleus; dC, deep cerebellar nuclei; Tu, olfactory tubercle; Rt, reticular thalamic nucleus. Scale bar = 2 mm.

Figure 2. Expression of VGLUT1 (**a**), VGLUT2 (**b**), VIAAT (**c**), and Nissl stain (**d**) in the rat CNS. Sections at the AVCN level (**a1–d1**). Sections at the PVCN and DCN level (**a2–d2**). Sections at the DCN level (**a3–d3**). Scale bar = 500 μ m.

Figure 3.

Colocalization and segregation of VGLUT1 (a) and VGLUT2 (b) in the rat CNC. Pseudocolor merged images are shown in (c). Neurons co-expressing VGLUT1 and VGLUT2 are indicated by arrows. In the AVCN (1), PVCN (2), and CRN (3), most stained neurons express both molecules. In the DCN (4), small cells, presumably granule cells, expressed only VGLUT1 whereas large cells, presumably fusiform cells, expressed only VGLUT2. Scale bar = 50 μ m. The magenta version of this figure is Figure S1.

Figure 4. Expression of VGLUT1 (**a**), VGLUT2 (**b**), VIAAT (**c**), and Nissl stain (**d**) in the mouse CNC. Sections at the AVCN level (**a1–d1**). Sections at the PVCN and DCN level (**a2–d2**). Scale bar = 250 μ m.

Figure 5. Colocalization and segregation of VGLUT1 (a) and VGLUT2 (b) in the mouse CNC. Pseudocolor merged images are shown in (c). Neurons co-expressing VGLUT1 and VGLUT2 are indicated by arrows. In the AVCN (1) and PVCN (2), most stained neurons express both molecules. In the DCN (3), small cells, presumably granule cells, expressed only VGLUT1; whereas large cells, presumably fusiform cells, expressed only VGLUT2. Scale bar = 50 μ m. The magenta version of this figure is Figure S2.

Figure 6.

Expression of VGLUT1 (**a**), VGLUT2 (**b**), VIAAT (**c**), and Nissl stain (**d**) in the rat SOC. Sections at the RPO level (**a1–d1**). Sections at the MSO level (**a2–d2**). Sections at the LSO level (**a3–d3**). Scale bar = 500 μ m.

Figure 7.

Colocalization of VGLUT1 (**a**) and VGLUT2 (**b**) in the rat SOC. Pseudocolor merged images are shown in (**c**). Neurons co-expressing VGLUT1 and VGLUT2 are indicated by arrows. In the RPO (**1**), the majority of stained neurons express both VGLUT1 and VGLUT2. In the MSO (**2**) and VMPO (**6**), colocalization of both molecules was frequently found. In the DPO (**3**), LSO (**4**), and LVPO (**5**), neurons expressing both molecules were fewer. In the all SOC nuclei, neurons expressing only VGLUT1 are rare. Note: small green cells in **a**₅ are red blood cells (artifact caused by endogenous peroxidase). Scale bar = 50 µm. The magenta version of this figure is Figure S3.

Figure 8. Expression of VGLUT1 (a), VGLUT2 (b), VIAAT (c), and Nissl stain (d) in the mouse SOC. Sections at the RPO level (a1–d1). Sections at the MSO level (a2–d2). Sections at the LSO level (a3–d3). Note the fewer VGLUT1-expressing cells, and smaller MSO and MNTB than rat. Scale bar = 500 μ m.

Figure 9.

Colocalization of VGLUT1 (a) and VGLUT2 (b) in the mouse SOC. Pseudocolor merged images are shown in (c). Neurons co-expressing VGLUT1 and VGLUT2 are indicated by arrows. In the RPO (1), DPO (3), and LVPO (5), few neurons expressed both molecules. In the other nuclei, the colocalization is rare. Scale bar = 50 μ m. The magenta version of this figure is Figure S4.

Figure 10. Expression of VGLUT1 (a), VGLUT2 (b), VIAAT (c), and Nissl stain (d) in the rat NLL. Sections at the DNLL and INLL level (a1–d1). Sections at the VNLL level (a2–d2). Note the complementary expression of VGLUT2 and VIAAT. Scale bar = 1 mm.

Figure 11. Expression of VGLUT1 (a), VGLUT2 (b), VIAAT (c), and Nissl stain (d) in the mouse NLL. Sections at the DNLL and INLL level (1). Sections at the VNLL level (2). PBG, parabigeminal nucleus. Scale bars = 250 μ m (1) and 500 μ m (2).

Figure 12. Expression of VGLUT1 (a), VGLUT2 (b), VIAAT (c), and Nissl stain (d) in the rat IC. Note the almost complete absence of VGLUT1 expression except in the mesencephalic trigeminal nucleus (arrow). Scale bar = 1 mm.

Figure 13. Expression of VGLUT1 (**a**), VGLUT2 (**b**), VIAAT (**c**), and Nissl stain (**d**) in the mouse IC. Note the almost complete absence of VGLUT1 expression except in the mesencephalic trigeminal nucleus (arrow). Scale bar = 500 μ m.

Figure 14.

Expression of VGLUT1 (**a**), VGLUT2 (**b**), VIAAT (**c**), and Nissl stain (**d**) in the rat MGB. Rostral level (**a1–d1**). Intermediate level (**a2–d2**). Caudal level (**a3–d3**). PP, peripeduncular nucleus; PIL, posterior intralaminar thalamic nucleus; LP, lateroposterior thalamic nucleus. Scale bar = 1 mm.

Figure 15.

Colocalization and segregation of VGLUT1 (**a**) and VGLUT2 (**b**) in the rat INLL (**1**) and MGB (**2–5**). Pseudocolor merged images are shown in (**c**). Neurons co-expressing VGLUT1 and VGLUT2 are indicated by arrows. In the MGV (**2**), most stained neurons express both molecules. In the MGD (**3**), fewer neurons co-express both. In the MGM (**4**) and SG (**5**), VGLUT1 expression is negligible. No neurons expressing only VGLUT1 were found. Scale bar = 50 μ m. The magenta version of this figure is Figure S5.

Figure 16. Expression of VGLUT1 (**a**), VGLUT2 (**b**), VIAAT (**c**), and Nissl stain (**d**) in the mouse MGB. Rostral level (**a1–d1**). Intermediate level (**a2–d2**). Caudal level (**a3–d3**). PP, peripeduncular nucleus; PIL, posterior intralaminar thalamic nucleus. Scale bar = 500 μ m.

Figure 17.

Colocalization and segregation of VGLUT1 (a) and VGLUT2 (b) in the mouse INLL (1) and MGB (2–5). Pseudocolor merged images are shown in (c). Neurons co-expressing VGLUT1 and VGLUT2 are indicated by arrows. In the MGCV (2), most stained neurons express both molecules. In the MGD (3), fewer neurons co-express both. In the MGM (4) and SG (5), expression of VGLUT1 is rare. Scale bar = 50 μ m. The magenta version of this figure is Figure S6.

Figure 18.

Summary of VGLUT and VIAAT expression in auditory nuclei of mouse (a) and rat SOC (b). Color filled in each nucleus indicates expression pattern of VGLUT and VIAAT. Red: Nuclei in which most neurons are glutamatergic and more than 20% of glutamate neurons co-express VGLUT1 and VGLUT2. Orange: Nuclei in which most neurons are glutamatergic and more than 80% of glutamate neurons express only VGLUT2. Green: Nuclei in which substantial number of VIAAT and VGLUT neurons is present and more than 80% of glutamate neurons co-express VGLUT1 and VGLUT2. Yellow: Nuclei in which substantial number of VIAAT and VGLUT neurons is present and 20–80% of glutamate neurons co-express VGLUT1 and VGLUT2. Light blue: Nuclei in which substantial number of VIAAT and VGLUT neurons is present and more than 80% of glutamate neurons express only VGLUT2. Dark blue: Nuclei in which most neurons express VIAAT. Both species show the same pattern except for some nuclei in the SOC. Note: Granule cells that express only VGLUT1 are found in nuclei with asterisk (*), and were excluded from categorization.

Table 1

Perimeters (in $\mu\text{m} \pm \text{S.D.}$) of VGLUT1-positive/ VGLUT2-negative cells and VGLUT2-positive cells in the CNC and NLL.

rat				
nucleus	VGLUT1+/VGLUT2-	(N)	VGLUT2+*	(N)
CNC	$32.6 \pm 4.5^{**}$	(89)	66.7 ± 10.1	(57)
NLL	$33.1 \pm 3.9^{**}$	(74)	59.8 ± 8.7	(42)
mouse				
nucleus	VGLUT1+/VGLUT2-	(N)	VGLUT2+*	(N)
CNC	32.2 ± 4.6	(82)	60.3 ± 7.9	(62)
NLL	27.5 ± 5.4	(31)	55.2 ± 5.9	(23)

* CNC cells chosen were also VGLUT1+, while NLL cells chosen were VGLUT1-

** not significant by Tukey's multiple comparison

Table 2

Quantitative analysis of colocalization of VGLUT1 and VGLUT2 in the rat auditory nuclei.

nucleus	V1 +	percentage V2+	V1+/V2+	N of cells
AVCN	0.0 ± 0.0	4.9 ± 7.6	95.1 ± 7.6	1041
CNC				
PVCN	0.0 ± 0.0	4.3 ± 2.1	95.7 ± 2.1	144
DCN*	0.0 ± 0.0	98.5 ± 2.6	1.5 ± 2.6	121
DPO	4.6 ± 5.5	75.4 ± 12.6	20.0 ± 17.5	80
LSO	0.0 ± 0.0	96.1 ± 5.0	3.9 ± 5.0	263
LVPO	0.0 ± 0.0	91.3 ± 6.4	8.7 ± 6.4	77
MSO	0.0 ± 0.0	80.9 ± 7.3	19.1 ± 7.3	138
RPO	0.0 ± 0.0	38.1 ± 6.7	61.9 ± 6.7	74
VMPO	1.0 ± 1.7	30.3 ± 11.6	68.8 ± 10.4	81
NLL				
INLL*	0.0 ± 0.0	95.1 ± 1.9	4.9 ± 1.9	367
VNLL*	0.0 ± 0.0	89.1 ± 9.4	10.9 ± 9.4	93
MGD	0.0 ± 0.0	69.8 ± 6.6	30.2 ± 6.6	595
CD	0.0 ± 0.0	99.6 ± 0.6	0.4 ± 0.6	326
MGB				
MGM	0.0 ± 0.0	91.9 ± 2.7	8.1 ± 2.7	576
MGV	0.1 ± 0.2	13.0 ± 8.3	86.9 ± 8.2	1075
SG	0.0 ± 0.0	95.5 ± 3.8	4.5 ± 3.8	450

* granule cells (VGLUT1+/VGLUT2-) are eliminated from the analysis.

Table 3

Quantitative analysis of colocalization of VGLUT1 and VGLUT2 in the mouse auditory nuclei.

nucleus	V1 +	percentage V2+	V1+/V2+	N of cells
AVCN	5.1 ± 6.4	3.0 ± 2.9	91.9 ± 6.8	767
CNC				
PVCN	2.7 ± 2.8	4.9 ± 4.6	92.4 ± 2.6	230
DCN*	0.0 ± 0.0	98.0 ± 2.0	2.0 ± 2.0	153
Soc				
DPO	1.2 ± 2.1	85.5 ± 22.1	13.3 ± 23.1	60
LSO	0.6 ± 1.0	99.5 ± 1.0	0.0 ± 0.0	213
LYPO	0.0 ± 0.0	88.0 ± 20.7	12.0 ± 20.7	98
MSO	0.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0	102
RPO	1.0 ± 1.7	94.1 ± 7.8	4.9 ± 6.1	84
VMPO	0.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0	33
NLL				
INLL*	2.3 ± 3.9	94.2 ± 7.7	3.6 ± 3.9	436
VNLL*	4.2 ± 7.2	86.4 ± 11.8	9.4 ± 10.1	56
MGB				
MGD	0.8 ± 0.9	35.2 ± 19.6	64.0 ± 19.8	915
CD	0.0 ± 0.0	97.5 ± 0.7	2.5 ± 0.7	396
MGM	0.3 ± 0.6	84.1 ± 4.2	15.6 ± 4.3	420
MGV	1.1 ± 1.4	18.1 ± 12.6	80.8 ± 12.0	1586
SG	0.3 ± 0.5	90.1 ± 8.2	9.6 ± 7.7	377

* granule cells (VGLUT1+/VGLUT2-) are eliminated from the analysis.