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Differential maturation of vesicular glutamate and GABA transporter expression in the mouse auditory forebrain during the first weeks of hearing

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Abstract

Vesicular transporter proteins are an essential component of the presynaptic machinery that regulates neurotransmitter storage and release. They also provide a key point of control for homeostatic signaling pathways that maintain balanced excitation and inhibition following changes in activity levels, including the onset of sensory experience. To advance understanding of their roles in the developing auditory forebrain, we tracked the expression of the vesicular transporters of glutamate (VGluT1, VGluT2) and GABA (VGAT) in primary auditory cortex (A1) and medial geniculate body (MGB) of developing mice (P7, P11, P14, P21, adult) before and after ear canal opening (~P11–P13). RNA sequencing, in situ hybridization, and immunohistochemistry were combined to track changes in transporter expression and document regional patterns of transcript and protein localization. Overall, vesicular transporter expression changed the most between P7 and P21. The expression patterns and maturational trajectories of each marker varied by brain region, cortical layer, and MGB subdivision. VGluT1 expression was highest in A1, moderate in MGB, and increased with age in both regions. *VGluT2* mRNA levels were low in A1 at all ages, but high in MGB, where adult levels were reached by P14. VGluT2 immunoreactivity was prominent in both regions. *VGluT1*⁺ and *VGluT2*⁺ transcripts were co-expressed in MGB and A1 somata, but co-localization of immunoreactive puncta was not detected. In A1, *VGAT* mRNA levels were relatively stable from P7 to adult, while immunoreactivity increased steadily. *VGAT*⁺ transcripts were rare in MGB neurons, whereas VGAT immunoreactivity was robust at all ages. Morphological changes in immunoreactive puncta were found in two regions after ear canal opening. In the ventral MGB, a decrease in VGluT2 puncta density was accompanied by an increase in puncta size. In A1, peri-somatic VGAT and VGluT1 terminals became prominent around the neuronal somata. Overall, the observed changes in gene and protein expression, regional architecture, and morphology relate to—and to some extent may enable—the emergence

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of mature sound-evoked activity patterns. In that regard, the findings of this study expand our understanding of the presynaptic mechanisms that regulate critical period formation associated with experience-dependent refinement of sound processing in auditory forebrain circuits.

Keywords

Glutamate; GABA; Fox-3; Protein; RNA; Development; Sequencing; Cortex; Thalamus; Geniculate; A1; Juvenile; Critical period; Homeostatic plasticity; BDNF; MeCP2

Introduction

In the central nervous system, vesicular transporters are responsible for packaging neurotransmitters into synaptic vesicles and play important roles in the release machinery (Blakely and Edwards 2012; Martin and Krantz 2014; Takamori et al. 2006). Several classes of vesicular transporters have been characterized, each associated with a different neurotransmitter: acetylcholine (VACHT); monoamines (VMAT2); GABA and glycine (VGAT); and glutamate (VGluT1, VGluT2, VGluT3). Activity-dependent regulation of their expression can alter the number of transporters localized to each vesicle, impacting vesicle filling, quantal size, and the amount and probability of neurotransmitter release (Blakely and Edwards 2012; Edwards 2007; Erickson et al. 2006; Fei et al. 2008; Hnasko and Edwards 2012; Omote et al. 2011; Santos et al. 2009; Takamori et al. 2006; Wilson et al. 2005; Wojcik et al. 2004). Accordingly, these transporters contribute to pre- and postsynaptic homeostatic mechanisms that regulate the balance of excitatory and inhibitory neurotransmission (Coleman et al. 2010; De Gois et al. 2005; Lazarevic et al. 2013; Rich and Wenner 2007; Turrigiano 2008; Turrigiano and Nelson 2004; Wilson et al. 2005).

Vesicular transporter expression in the forebrain undergoes substantial changes across development. Prenatally, VGluT1 and VGluT2 mRNA is detected in preplate and marginal zones of mice by E10 (Ina et al. 2007; Schuurmans et al. 2004). Postnatally, changes in VGluT and VGAT expression levels are correlated with the onset of sensory experience (e.g., eye opening), and can be modified by altering sensory experience (Boulland and Chaudhry 2012; Boulland et al. 2004; Chattopadhyaya et al. 2004; De Gois et al. 2005; Liguz-Lecznar and Skangiel-Kramska 2007; Minelli et al. 2003a, b; Nakamura et al. 2005; Takayama and Inoue 2010). Accordingly, these transporters are thought to play an important role in the plastic changes that shape critical period formation (Griffen and Maffei 2014; Hensch 2005; Kotak et al. 2008; Kuhlman et al. 2013; Lefort et al. 2013; Levelt and Hubener 2012; Maffei and Turrigiano 2008; Nahmani and Turrigiano 2014; Wang and Maffei 2014).

Less is known about the maturation of vesicular transporter expression in the developing central auditory system. However, many other developmental milestones for changes in protein expression and neurophysiological responses around the onset of hearing have been established, which provide context for the present studies. The delayed onset of hearing in altricial animals has provided researchers with a convenient window to study activity-dependent changes in basic neuronal response properties (Barkat et al. 2011; Brown and Harrison 2010; Carrasco et al. 2013; Chang et al. 2005; de Villers-Sidani et al. 2007;

Insanally et al. 2010; Kral et al. 2005; Mrcic-Flogel et al. 2003, 2006; Polley et al. 2013; Razak and Fuzessery 2007; Rosen et al. 2010; Sarro et al. 2011; Trujillo et al. 2013) and associated mechanisms (Dorrn et al. 2010; Metherate and Cruikshank 1999; Oswald and Reyes 2008, 2011; Venkataraman and Bartlett 2013, 2014). Critical periods for sound processing are also contained within this window (Barkat et al. 2011; Fitch et al. 2013; Froemke and Jones 2011; Keating and King 2013; Kral et al. 2013; Polley et al. 2013; Sanes and Bao 2009; Sanes and Kotak 2011; Sanes and Woolley 2011; Takesian et al. 2009; Whitton and Polley 2011). As observed in other sensory systems, the vesicular transporters are likely an important component of the synaptic machinery associated with maturation in the auditory forebrain, but their maturational trajectories are yet to be co-registered with established markers of central auditory system development.

Given their potential importance in development and plasticity, and the absence of data on their expression in young animals, the main purpose of the current study was to document the expression of VGluT1, VGluT2, and VGAT in the primary auditory cortex (A1) and medial geniculate body (MGB) of mice at various postnatal ages surrounding the onset of hearing: P7, P11, P14, P21, and adult. We tracked mRNA expression using next-generation RNA sequencing (RNAseq) and in situ hybridization (ISH), which primarily reflects transcript expression in neuronal somata. These assays were complemented by immunohistochemistry (IHC) to index protein expression, which localizes to the axon terminals of neurons that express each gene. The multimodal profiling approach (i.e., RNAseq, ISH, and IHC) enabled the identification of maturational changes in the expression of each transporter and also permitted localization of each gene and protein within single neurons across all layers of A1 and within ventral (MGv) and dorsal (MGd) divisions of the MGB.

Based on known or predicted connectivity, the data in this paper reflect the maturation of several systems of projections: corticocortical (CC) (VGluT1 and VGAT); corticothalamic (CT) (VGluT1); thalamocortical (TC) (VGluT2); tectothalamic (tT) (VGluT2 and VGAT); and thalamorecticular (TRN) (VGAT). In addition, the data support the utility of RNAseq as an efficient means to screen for and identify genes of interest in any brain region, prior to anatomical characterization using ISH or IHC. Altogether, these data add to our understanding of changes in the presynaptic signaling machinery that accompany alterations in sensory experience during postnatal development.

Materials and methods

Tissue acquisition

All procedures were approved by the Animal Care and Use Committee at Massachusetts Eye and Ear Infirmary and followed the guidelines established by the National Institutes of Health for the care and use of laboratory animals. The morning that a new litter of pups was first observed was designated as P0. Brains collected from adult (8–10 weeks) and juvenile (P7, P11, P14, and P21) male and female C57BL/6J mice (Jackson Labs 000664) were used in this study. Animals were given a lethal dose of ketamine and xylazine (200/50 mg/kg, respectively) intraperitoneally and then perfused transcardially with 20–30 ml of perfusion saline (0.1 % NaNO₂ and 0.9 % NaCl in H₂O), followed by 20–30 ml of 4 %

paraformaldehyde dissolved in 0.1 M phosphate-buffered (PB) saline using a medium flow peristaltic pump (Fisher Scientific 13-876-2; 0.4–85.0 ml/min). The brains were sunk in 30 % sucrose dissolved in 0.1 M PB and then stored at –20 °C. Brains were cut frozen in the coronal plane on a sliding microtome at 40 or 50 µm. Sections were stored at 4 °C in 0.1 M PB saline containing 1 % sodium azide. Animals designated for RNAseq (P11 omitted) ($N=6$ per age, total = 24) and multi-fluorescence in situ hybridization ($N=6$) were euthanized in the same manner, but not perfused. Brains from these animals were removed immediately, flash frozen on dry ice, and stored at –80 °C prior to sectioning.

Next-generation sequencing of total RNA

Sample acquisition—For harvesting of RNAseq samples, fresh-frozen brains from 6 animals in each age group (3 male, 3 female) were sectioned at 40–100 µm in the coronal plane (rostral to caudal) on a sliding microtome and viewed through a surgical microscope. The gross anatomical features illustrated in Fig. 1 and described in “Architectonic features of A1 and MGB” were used as a guide to identify areas targeted for sampling (A1, primary auditory cortex; MGB, medial geniculate body). As target regions became visible, they were extracted using a sterile tissue punch or curette of a size appropriate to the brain region. A1 samples were obtained using a 0.5 mm-diameter punch, with the ventral edge beginning approximately 1 mm dorsal to the rhinal fissure. MGB samples were harvested with a curette after using a micro-dissecting scalpel to circumscribe its perimeter. Auditory cortex (AC) samples were centered on A1, but potentially also included some tissue in the adjacent auditory field dorsal to A1. For consistency with the ISH results, all RNAseq samples from the AC were designated as A1, but with the foregoing disclaimer. For the MGB, the microdissection procedure was designed to exclude the lateral geniculate nucleus (LGN) and adjoining nuclei dorsal, medial, and ventral to the MGB (Figs. 1, 2c). The extreme rostral and caudal poles of the MGB were largely excluded from these samples. Punches from homologous areas of both hemispheres were combined in sterile tube containing 400 µl of Trizol, homogenized for 45 s using a mechanized sterile pestle, flash frozen on dry ice, then stored at –80 °C. Samples from the IC were stored, but not further processed for RNAseq.

RNA extraction and sequencing—For each Trizol lysate, 100 µl of reagent grade chloroform (Fisher Scientific, S25248) was added. The samples were centrifuged for 3 min on a desktop centrifuge to fractionate the aqueous and organic layers. After centrifugation, the resulting aqueous layer was carefully removed and transferred to 2.0 ml Sarstedt tubes (Sarstedt, 72.694) which were run on the QIASymphony using the QIASymphony RNA Kit (Qiagen, 931636) and protocol RNA_CT_400_V7 which incorporates DNase treatment. Prior to each run, the desk was UV-irradiated using a programmed cycle. The resulting RNA was eluted to 100 µl of RNase-free water and stored at –80 °C in 2.0 ml Sarstedt tubes until use. Samples were initially quantitated using a Qubit RNA assay. Additional analyses of purity and the quantitation of total RNA were performed using a NanoDrop spectrophotometer (Thermo Scientific) and Agilent RNA 6000 Pico chip (Agilent) according to the manufacturer's protocol using the reagents, chips, and ladder provided in the kit. Quality control data for the 48 samples sequenced are contained in Supplementary Table S1.

RNAseq was performed by the Vanderbilt Technologies for Advanced Genomics core (VANTAGE). Total RNA was isolated with the Aurum Total RNA Mini Kit. All samples were quantified on the QuBit RNA assay. RNA quality was verified using an Agilent Bioanalyzer. RNAseq data were obtained by first using the Ribo-Zero Magnetic Gold Kit (Human/Mouse/Rat) (Epicentre) to perform ribosomal reduction on 1 µg total RNA following the manufacturer's protocol. After ribosomal RNA (rRNA) depletion, samples were then purified using the Agencourt RNAClean XP Kit (Beckman Coulter) according to the Epicentre protocol specifications. After purification, samples were eluted in 11 µl RNase-free water. Next, 1 µl ribosomal depleted samples were run on the Agilent RNA 6000 Pico Chip to confirm rRNA removal. After confirmation of rRNA removal, 8.5 µl of rRNA-depleted sample was input into the Illumina TruSeq Stranded RNA Sample Preparation kit (Illumina) for library preparation. Libraries were multiplexed six per lane and sequenced on the HiSeq 2500 to obtain at least 30 million paired end (2×50 bp) reads per sample.

RNAseq data processing—The RNAseq data went through multiple stages of thorough quality control as recommended by Guo et al. (2013). Raw data and alignment quality control were performed using QC3 (Guo et al. 2014a), and gene quantification quality control was conducted using MultiRankSeq (Guo et al. 2014b). Differential expression analyses between all postnatal ages and brain regions were performed using MultiRankSeq [53], which combines three independent methods for RNAseq analysis: DESeq [57]; edgeR [58]; baySeq [59]. Raw data were aligned with TopHat2 (Kim et al. 2013) against mouse transcript genome mm 10, and read counts per gene were obtained using HTSeq (Anders et al. 2014). Normalized read counts (used in all plots) were obtained by normalizing each gene's read count against the sample's total read count and then multiplying by a constant (1×10^6). Hierarchical clustering analysis and heatmaps were produced using the Heatmap3 (Zhao et al. 2014) package from R. Normalized read counts for *VGluT1* (*SLC17A7*), *VGluT2* (*SLC17A6*), and *VGAT* (*SLC32A1*) were averaged over all samples for each age (P7, P14, P21, adult) and brain region (AC, MGB). Analysis of variance (ANOVA) with Tukey post hoc testing was used to screen for significant differences in expression between ages for each brain area and gene (see Tables 2, 3). Raw sequencing files have been uploaded to the National Center for Biotechnology Information (NCBI) database (accession #SRP053237). Analyses of the complete RNAseq dataset is included in Hackett et al. (2015).

In situ hybridization (ISH)

Single colorimetric ISH assays for *VGluT1*, *VGluT2*, *VGAT*, and the housekeeping gene, *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), were performed in adjacent sections from each brain. This minimized differences between individual animals and permitted within-subject normalization of ISH levels using *GAPDH* as a reference. Multiplex fluorescence ISH (FISH) was performed simultaneously in a separate series of brain sections, permitting visualization of all genes in each tissue section.

Preparation of probes for single colorimetric ISH—Plasmids with inserts of specific sequences to each gene were prepared using the conventional TA-cloning technique. Sequences of primer sets are summarized in Table 1. The sequences were amplified by RT-

PCR from mouse whole brain cDNA (Zyagen, San Diego, CA, USA) and inserted into pCR®II-TOPO plasmid vectors (Invitrogen, Carlsbad, CA, USA). Those plasmids were amplified by transfecting into competent cells (*E. coli*) (Invitrogen) and purified into a 1.0 µg/µl solution. Digoxigenin (DIG)-labeled antisense and sense ribo-probes were prepared from these plasmids using a DIG-dUTP labeling kit (Roche Diagnostics, Indianapolis, IN, USA). RNA Probes were then purified with ProbeQuant G-50 Micro Columns (GE Healthcare Life Science, Pittsburgh, PA, USA) and stored as a 100 µg/ml solution in TE [tris-ethylenediamine-*N,N,N',N'*-tetraacetic acid (EDTA) buffer, pH 8.0].

Colorimetric in situ hybridization (ISH)—Free-floating sections were soaked in 4 % PFA/0.1 M PB (pH 7.4) overnight at 4 °C, permeabilized with 0.3 % Triton-X 100 for 20 min at room temperature, and treated with 1.0 µg/ml proteinase K for 30 min at 37 °C. After acetylation with acetylation buffer (0.13 % triethanolamine, 0.18 % HCl, 0.25 % acetic anhydride) for 10 min at room temperature, the sections were incubated in hybridization buffer [5× standard saline citrate (SSC 150 mM NaCl, 15 mM Na citrate, pH 7.0), 50 % formamide, 2 % blocking reagent (Roche Diagnostics), 0.1 % *N*-lauroylsarcosine (NLS), 0.1 % sodium dodecyl sulfate (SDS), 20 mM maleic acid buffer; pH 7.5] for 60 min at 60 °C and then transferred into the hybridization buffer containing 1.0 µg/ml DIG-labeled riboprobe at 60 °C overnight. Hybridized sections were washed by successive immersion in wash buffer (2× SSC, 50 % formamide, 0.1 % NLS; 60 °C, 20 min, twice), RNase A buffer (10 mM Tris-HCl, 10 mM EDTA, 500 mM NaCl; pH 8.0) containing 20 µg/ml RNase A (37 °C, 30 min), 2× SSC/0.1 % NLS (37 °C, 20 min), and 0.2× SSC/0.1 % NLS (37 °C, 15 min). Hybridization signals were visualized by alkaline phosphatase (AP) immunohistochemical staining using a DIG detection kit (Roche Diagnostics). Sections were mounted onto glass slides, dehydrated through a graded series of increasing ethanol concentrations followed by xylenes, and then coverslipped with Permount. Sense probes detected no signals stronger than background (see Supplementay Fig. S1).

Fluorescence in situ hybridization (FISH)—Tissue blocks (fresh, not fixed) were embedded in OCT compound (Tissue-Tek, Torrance, CA, USA), flash frozen on dry ice, sectioned at 10 µm on a cryostat, and then mounted directly onto Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA, USA). Quadruple fluorescence ISH (FISH) for *GAPDH*, *VGluT1*, *VGluT2*, and *VGAT* was conducted on sections containing A1 and MGB in two adult brains. Custom target and standard control probes were provided by Advanced Cell Diagnostics (ACD, Hayward CA, USA), as described previously (Wang et al. 2012a). In-house comparisons revealed that their assay (RNAscope) was vastly superior to results obtained using DIG-based conjugates. This was attributed to unique signal amplification and background suppression methodology that consistently yielded exceptionally high specificity and low background. Briefly, after a 30-min protease permeabilization step, two independent probes (double Z probe) were hybridized to each target sequence (~20 probe pairs per target molecule). The lower region of each probe is complementary to the target sequence, and the upper region is a 14-base tail sequence. Together, the dual probe construct provides a 28-base binding site for the preamplifiers, which were built up during a three-stage amplification cycle. In the final step, labeled probes containing the fluorescent conjugates were bound to each of the 20 binding sites on each preamplifier. All incubation

steps were performed at 40 °C in a hybridization oven (HybEZ, ACD, Hayward, CA, USA) using the RNAscope Multiplex Fluorescent Reagent Kit, according to the manufacturer's instructions for fresh-frozen brain tissue.

Four types of controls were utilized to evaluate the specificity of the target probes and fluorescence amplification in each channel (Table 1): (1) GAPDH placed in channel 1 as a positive control and reacted along with the target probes in channels 2–4; (2) a three-plex positive control probe containing three highly characterized housekeeping genes (*UBC*, ubiquitin C; *POLR2A*, DNA-directed RNA polymerase II subunit RPB1; *PP1B*, cyclophilin B) in channels 1–3, respectively; (3) a negative control probe (*DAPB*, dihydrodipicolinate reductase), which is a gene from a soil bacterium (*Bacillus subtilis* strain SMY) that has never yielded specific signal in any tissue samples; (4) fluorescence amplification steps in the absence of any positive control or target probes. These controls revealed that all probes were highly specific with no cross-reactivity between any gene or color channel (see Supplementary Fig. S1).

With the exception of Figs. 11 and 12, FISH-reacted sections were counterstained with DAPI (4',6-diamidino-2-phenylindole) to improve identification of layers and subdivisions and provide a focal point for cytoplasmic probe labeling (Figs. 6, 7, 8, 9, 10). As these figures indicate, labeling yield for each target probe was high and readily visible at all magnifications, despite a neuronal density in the 10 µm sections that was roughly 20 % of the 50 µm colorimetric sections.

Antibody selection and immunohistochemistry (IHC)

Vesicular transporter antibody and secondary antibody selection was a lengthy process that began by evaluating the single chromagen staining quality and consistency of several commercially available antibodies against each target protein (for complete listing, see Supplementary Table S2). These assays were performed in parallel in adult mouse and macaque monkey tissue, in support of the present study of mice and prior studies of monkeys (Balaram et al. 2011; Hackett and de la Mothe 2009; Hackett et al. 2014). Antibody specificity was tested by incubating each antibody with a 10× concentration of the control protein provided by the manufacturer, when available. Negative controls, in which the primary antibody was omitted, were used in the testing of all antibodies. Optimal primary antibody concentrations were determined from these tests. Thereafter, antibodies that produced specific staining in single antibody assays were systematically combined in double- and triple-fluorescence assays, with positive and negative controls for both primary and secondary antibodies, to identify combinations that produced strong specific labeling with no cross-reactivity. The antibody combinations used in the present study reflect our judgment of the best combinations for this application (see Table 2). Note that the primary and secondary antibodies listed in Table 2 were included because they all produced reliably good results alone and in combination. To maximize continuity, however, the illustrated figures and analyses were obtained from assays using the primary and secondary antibody combinations indicated by asterisks. VGluT1_{gp} (gp, guinea pig) was always combined with VGAT_{rb} (rb, rabbit), and VGluT1_{rb} was combined with VGluT2_{gp}. These distinctions were added to the text and figure legends where appropriate.

In addition to the vesicular transporters, an additional primary antibody was used in multifuorescence IHC to identify neuronal somata. NeuN (Fox-3) is one of many members of the RNA-binding protein family (Darnell 2013). Although these proteins are mainly involved in the regulation of mRNA, Fox-3/NeuN is widely used as a neuron-specific marker in adult and developing brain across species (Arellano et al. 2012; Fuentes-Santamaria et al. 2013; Hackett et al. 2014; Kim et al. 2009). Antibodies for NeuN produce strong somatic labeling of neurons in most brain areas. In the present study, multifuorescence IHC assays included NeuN in a separate color channel for cytoarchitectonic identification of cortical layers, subcortical nuclei, or particular brain areas.

Multifuorescence immunohistochemistry

Multifuorescence IHC was performed in series of coronal sections (section spacing 1:8). Sections were rinsed for 30 min in 0.01 M PBS-Tx (phosphate-buffered saline, 0.1 % triton), followed by three changes of 0.01 M PBS for 10 min (standard rinse procedure). Nonspecific labeling of myelin by fluorescent secondary antibodies was blocked by incubation in IT-Fx (Life Tech) for 60 min at RT, followed by a standard rinse. Sections were then incubated for 5 min at RT in a single purified glycoprotein blocking solution (Superblock, ScyTek Laboratories, Logan, UT, USA), followed by a single rinse in PBS. Superblock reagent reduces nonspecific antibody binding and was used in lieu of species-specific sera or bovine serum albumin (Buttini et al. 2002; Evans et al. 1996; Turtzo et al. 2014). Sections were then incubated for 48 h in the primary antibody cocktail at 4 °C, rinsed, and then incubated for 3–4 h in the secondary antibody cocktail at RT. All incubations and rinsing steps were performed on a laboratory shaker with constant agitation.

Sections from two animals in each age group were used for quantitative measurements and associated illustrations. In these brains, sections stained for a particular combination of antibodies (VGluT2_{gp} + VGluT1_{rb} + NeuN_{ms} or VGluT1_{gp} + VGAT_{rb} + NeuN_{ms}) were reacted simultaneously for all five age groups in separate well plates under identical conditions (i.e., antibody concentrations, blocking and rinsing steps, solutions, incubation times, etc.). Aliquots of the antibody cocktails and all other solutions were distributed to wells from the same beakers in which they were prepared. These procedures ensured uniformity of conditions across age groups.

ISH image acquisition and analyses

Brightfield images of single colorimetric ISH tissue sections were obtained with a Nikon 80i microscope controlled by Neurolucida 10 software (MBF Bioscience). Fluorescence widefield images and image montages of FISH sections were obtained with a Nikon 90i epifluorescence microscope and Hamamatsu Orca 4.0 CCD camera, controlled by Nikon Elements AR software using 10×, 40×, and 100× objectives. Exposure parameters for brightfield and fluorescence images were maintained at the same levels across all samples to permit comparison of signal intensity measurements between age groups. Images were assembled into figures using Adobe Illustrator CS6 (Adobe Systems, Inc.). 100× images are z-plane stacks collapsed to two dimensions obtained using an Extended Depth of Focus

plugin to the Nikon Elements software. This method was chosen over confocal microscopy because we did not have access to a microscope with an infrared laser.

Estimates of the magnitude of gene expression in colorimetric ISH sections were performed on inverted 10× images from A1, MGB, and IC in both hemispheres that were first converted to 8-bit grayscale using ImageJ software (NIH, nih.gov). For A1, raw grayscale intensity was measured using rectangular selection boxes drawn from the top of layer 2 (L2) to the bottom of L6 (avoiding the subplate layer when present). Raw grayscale values were corrected to minimize differences in tissue staining between samples by subtracting the grayscale intensity of the white matter beneath A1 from the corresponding raw values. For MGB and IC, the same procedures were used, except that selections were hand-drawn using a polygon tool to restrict sampling to targeted subdivisions (MGv, ventral; MGd, dorsal, ICc, central), taking care to exclude section edges, artifacts, and adjoining nuclei. Raw grayscale intensity was corrected against the corpus callosum, where background levels were relatively constant and ISH signals were absent. For all brain regions measured, the background-corrected values for *VGluT1*, *VGluT2*, and *VGAT* were normalized against the background-corrected *GAPDH* values of the same brain regions from the same brain, adapted from a study comparing methods for quantifying ISH images (Lazic 2009). Finally, for each age group, brain area, and gene, normalized grayscale intensity values for each condition were averaged over both hemispheres of all three brains. Note that measurements for *VGAT* in the MGB and *VGluT1* in the IC were entered as null values into tables and plots, since there were typically no labeled cells. Analysis of variance (ANOVA) with Tukey post hoc testing was used to screen for significant differences in expression between ages for each brain area and gene, using $p = 0.05$ as the significance threshold (Tables 3, 5).

Note that although we did not perform RNAseq on the IC, this structure was included in the ISH images and analyses. The rationale for inclusion of the IC data was that *VGluT1* mRNA is not expressed in the IC and therefore not expected to contribute to *VGluT1*-ir terminals in the MGB. In contrast, many *VGluT2*⁺ and *VGAT*⁺ IC neurons do project to the MGB, where their proteins are expressed in terminals (Ito et al. 2009, 2011; Ito and Oliver 2010). At a minimum, demonstration that these genes are expressed in the IC at all ages supports the IHC data and broadens the context for discussion of the circuitry.

IHC image acquisition and analyses

IHC image acquisition—Widefield images and image montages of tissue sections were obtained with a Nikon 90i epifluorescence microscope and Hamamatsu Orca 4.0 CCD camera, controlled by Nikon Elements AR software. Using a 10× objective, composite images of each color channel (red, green, and far red) were acquired sequentially at full resolution. Montages were reconstructed from multiple composite images by the software (Figs. 14, 15, 16, 17; Supplementary Figs. S2–11, S26–27). Each montage comprised several hundred single images of each color channel. Prior to acquisition, exposure times were independently adjusted for each color channel using the RGB histograms to obtain balanced brightness across channels. To avoid over- or underexposure and ensure uniformity of imaging, the color balancing was standardized using adult specimens, where staining density was typically the highest and held constant for the acquisition of images in

the other age groups. Images were assembled in Adobe Illustrator CS6 (Adobe Systems, Inc.).

Confocal images were obtained using an Olympus Fluoview FV1000 laser scanning microscope, using a 60× oil immersion lens (NA—1.42). In A1, image stacks (1024 × 1024 pixels; 0.207 μm/pixel) were obtained at each of seven to eight sequential locations from L1 to the white matter using the mosaic acquisition function. In the MGB, all parameters were identical, except that stacks were obtained from one to two locations near the center of each division. Acquisition parameters were uniform across age groups (Figs. 19, 20, 21, 22, 23; Supplementary Figs. S12–25).

IHC image analyses—The purpose of the image analyses performed was to derive a graphical representation of the changing trajectories in immunoreactivity across layers of cortex and subdivisions of the MGB. Plots of the data pooled over two animals (4 hemispheres) are based on descriptive statistics (means, standard deviations), but statistical comparisons were not performed.

Estimates of the magnitude of immunoreactivity were performed in two different ways, based on separate analyses of 10× widefield and 60× confocal fluorescence images, respectively. Minor differences were observed in density estimates using these two methods, which we attribute to differences in background illumination obtained by the two methods of microscopy.

In the 10× widefield images, measurements were obtained from 8-bit grayscale channels of the composite RGB images using ImageJ software (NIH, nih.gov). The images of each protein marker occupied a different color channel and were measured independently. For cortex, two types of analyses were performed (Fig. 5): (1) grayscale intensity profiles that spanned all layers; and (2) laminar intensity profiles of individual cortical layers or sublayers. First, intensity profiles (e.g., Fig. 5, left panels) were obtained with a line tool oriented perpendicular to the cortical surface extending from the black space above the pia across all layers and into the white matter (Hackett et al. 2001). Tissue edge artifact, created by high-density staining of the pia, was cropped by setting the start of the profile to the mean density of layer 1a for each sample. The stepwise reduction in density at the white matter border marked the bottom of the profile and was cropped at this point. This procedure generated high-resolution profiles of between 1000 and 1500 pixels in length from the top of L1a to the bottom of L6 or the subplate. These raw grayscale profiles were normalized to minimize differences in tissue staining between samples by subtracting the average grayscale intensity of the white matter beneath A1 from the raw density value of each pixel in the profile. The relative grayscale intensities in Fig. 18 represent these normalized profiles averaged over profiles acquired from four hemispheres (two left, two right). The rationale for using adjoining white matter to normalize grayscale intensity was that axons were not labeled by the primary antibodies used, but background staining was present from nonspecific binding of the secondary antibodies, primarily to white matter tracks. Subtraction of the background evened out differences in staining intensity between tissue sections. Alternatively, using other brain areas for normalization confounds interpretation,

because the vesicular transporter proteins are expressed to some degree in most brain areas, and their expression in any or all areas could vary with age.

Second, using a round sampling tool sized to be slightly smaller than the layer of interest, grayscale intensity measurements were taken from 12 samples across the width of A1 in each layer, avoiding its dorsal and ventral borders, and avoiding large blank spaces created by empty blood vessel profiles. These samples were normalized to the average white matter intensity, as for the radial profiles, then averaged to obtain the mean relative grayscale intensity of each layer (Fig. 5, right panels). Layers and sublayers were identified using the NeuN-labeled cells and preserved in the red color channel of each image. This multifuorescence approach enabled greater precision in the identification of layers compared to the matching of adjacent tissue sections stained for different markers. Note that we did not distinguish between L6 and the subplate in the radial profiles illustrated in Fig. 18 and avoided sampling from the subplate in the laminar intensity analyses. Some minor qualitative differences in immunoreactivity between L6 and subplate layers were noted, however, and discussed in the text with reference to higher-magnification images.

In confocal image stacks, immunoreactivity was measured based on the density of immunoreactive (-ir) puncta, adapting the approach of Coleman et al. (2010). RGB confocal image stacks were converted to 8-bit grayscale images at full resolution (1024×1024 pixels), where each color channel was confined to a single file, and each image file contained one confocal slice ($0.74 \mu\text{m/slice}$). For each protein marker, images were thresholded to reduce background and visually separate closely spaced puncta. For each marker, threshold was held constant across samples and set to the upper edge of the histogram for each color channel (typically 25–40 % of maximum intensity). This strict threshold criterion produced the greatest separation between puncta and minimized the counting of particles in which immunoreactivity was low or nonspecific. Estimates of puncta number and density were then calculated from each region of interest (ROI) using the Analyze Particles routine in ImageJ. The thresholded image was filtered to count ovoid puncta between 0.414 and $4.14 \mu\text{m}^2$. For VGluT2 staining of the MGv only, the inclusion range for puncta was 0.414 – $35 \mu\text{m}^2$. The range was expanded to include large VGluT2-ir puncta that were especially prominent in the P21 and adult brain (see “Results”). This expanded range was not used for VGluT1 or VGAT, because these puncta were typically small and inclusion of larger particle sizes would have permitted the counting of aggregates and other artifacts.

From each image stack, puncta counts were obtained from three single confocal slices ($0.74 \mu\text{m/slice}$) selected from planes where immunoreactivity was the most even (typically in the middle third of the stack). For each slice, an ROI was drawn using the polygon selection tool in ImageJ. The ROI was confined to a single cortical layer and restricted to the neuropil. That is, ROIs were drawn in a manner that excluded the empty profiles of blood vessels and somata. This additional step improved the reliability of measurements between slices, as the total area occupied by empty profiles varies between slices and cortical layers and, therefore, can skew the density calculations. To avoid counting the same particles more than once, ROIs were obtained from non-adjacent slices or from different regions in adjacent slices.

The puncta densities (puncta/100 μm^2) in each graph represent the average of three confocal slices.

At the light microscope level, it is difficult to resolve small axon terminals in immunostained material and to distinguish them from small particles that may be nonspecifically stained. Although we chose antibodies that produced the strongest signals with the least nonspecific labeling, some of the labeled particles may not be terminals. In the absence of verification by EM, it is conventional to use the name ‘puncta’ for labeled particles. We adopted this nomenclature, but observed that the levels of nonspecific labeling were both minimal and comparable across samples, which would not bias our results. Previous localization studies increased our confidence and confirmed that the vast majority of VGAT, VGlut1, and VGlut2-ir puncta are axon terminals (Chaudhry et al. 1998; Kaneko et al. 2002; Minelli et al. 2003a, b).

Note also that our impressions concerning differences in the sizes of labeled puncta (below) were limited to qualitative judgments of the confocal images. Those impressions must be validated by other methods, such as EM, as we did not attempt to measure terminal sizes from the confocal images.

Architectonic features of A1 and MGB

The gross anatomical and cytoarchitectonic features used to identify A1 and MGB divisions are illustrated in Fig. 1. Criteria for parcellation were based on reference to online (Allen Brain Atlas, Brain Maps.org) and published atlases (Franklin and Paxinos 2007), and other sources in which architectonic features of the AC and MGB were described (Anderson et al. 2009; Bartlett et al. 2000; Cruikshank et al. 2001; Hackett et al. 2011a; Linke 1999; Linke and Schwegler 2000; Llano and Sherman 2008; Winer et al. 1999). Briefly, in coronal sections, A1 is distinguished from the adjoining areas (dorsal and ventral) by a relatively broad L4, in which cell packing is higher than in L3 and L5 and there is reduced cell density in L5. This feature is visible by Nissl staining, NeuN IHC, *GAPDH*ISH, and *VGlut1* ISH, which permit assessment of the cytoarchitecture. VGlut2-ir density is also higher in the L3b/4 band of A1 compared to surrounding areas. The MGv is larger in size than MGd, and primarily distinguished by higher cell density in MGv. These features are visible in the same histological preparations that permit assessment of the cytoarchitecture. The MGv also contains clusters of VGlut2-ir puncta, compared to more even distribution of smaller puncta in MGd. The medial (MGm) and suprageniculate (Sg) divisions are primarily distinguished by slightly larger neuron size and reduced cell density compared to MGv. Although cytochrome oxidase staining was not formally part of this study, we used this preparation in an earlier study (Hackett et al. 2011a). An image is included for reference in Fig. 1 that shows darker staining in A1 L4 and MGv.

Organization of images

Two sets of photographic images support the findings of the present study. The first set (Figs. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 17, 19, 20, 21, 22, 23) is embedded within the main body of this manuscript. Each is a moderate-resolution version of the key widefield and confocal images. The second set of images is Supplementary (Figs. S1–S28),

and referred to throughout the text. Links to full-resolution versions of all images are contained in Supplementary material 1. Many of these can be viewed through weblinks to high-resolution montages created with the Zoomify™ (Zoomify Inc., Aptos, CA, USA) plugin in Photoshop CS6 (Adobe Systems, Inc.). These images open in a browser window (requires updated Adobe Flash Player). Tools located at the bottom of the Zoomify window permit zoom and move functions, some of which may also be controlled using secondary mouse functions. Note that browser settings may need to be adjusted to allow viewing and that upload/zoom speeds vary.

Results

RNAseq and in situ hybridization

The data obtained for each gene and brain region are described separately in the text and figures below. Figures 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 contain ISH and FISH images for each gene and brain region as a function of postnatal age. Figure 13 contains graphical summaries of the ISH and RNAseq data analyses derived from the corresponding data in Tables 3, 4 and 5. Note that a P11 age group was not available for RNAseq analysis, but the absence of data from this time-point did not alter the conclusions of this study.

Expression of *VGluT1* mRNA—At all ages, *VGluT1* mRNA was expressed in A1 and MGB, but not the IC (Figs. 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13; Tables 3, 4, 5). Expression levels, derived from analyses of RNAseq and ISH assays (Fig. 13), were greater in A1 than MGB, and the spatial expression patterns in the tissue varied by location within each region. In A1, *VGluT1*⁺ cells were densely packed in L2–6. At P7, expression was concentrated in L3b–5, but by P11 there was little visible difference between layers, except L1 where *VGluT1*⁺ cells were rarely observed (Fig. 3). Expression in A1 increased most rapidly between P7 and P14 and then remained stable through adulthood. Overall in the MGB, *VGluT1* levels were lowest at P7. At this age, *VGluT1*⁺ cells were concentrated in the MGd, as very few MGv cells were *VGluT1*⁺. By P11, *VGluT1*⁺ cells were more numerous in the MGv, especially in its medial half. At P14, *VGluT1*⁺ neurons were broadly distributed throughout both divisions. Average expression levels increased significantly between P7 and P14 (ISH) or P21 (RNAseq) and then declined slightly into adulthood.

Although not a focus of the present study, *VGluT1*⁺ transcripts were also present at modest levels in MGM and Sg neurons, but expression in the adjoining nuclei tended to be low or absent (see also the high magnification panels in Fig. 10a). *VGluT1*⁺ cells were relatively sparse in the posterior pole of the MGB at all ages (see Storace et al. 2012).

Expression of *VGluT2* mRNA—In contrast to *VGluT1*, *VGluT2* expression was highest in the MGB and IC (Figs. 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13; Tables 3, 4, 5). In A1, where *VGluT2* levels were relatively low across the age range, expression was slightly, but significantly, elevated at P7 (Figs. 4, 13). This was attributed to a minor concentration of labeled transcripts in L2–3 that rapidly declined after P7. By adulthood, when *VGluT2* transcripts were sparse in L2–3, nominal *VGluT2* expression in A1 was found in some of the larger cell bodies in L5. In the MGB, *VGluT2* expression was already quite strong by P7 in all divisions of the MGB, including MGM and Sg. Expression levels increased significantly

until P14, then declined slightly in a manner similar to *VGluT1*. Expression trajectories and levels were nearly identical for the MGv and MGd. Finally, *VGluT2* expression was robust in all divisions of the IC at all ages (Fig. 4).

Expression of VGAT mRNA—Compared with *VGluT1* and *VGluT2*, the expression of *VGAT* was relatively low at all ages in A1 and MGB (Figs. 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13; Tables 3, 4, 5) (note the change in scale between graphs of *VGAT* and *VGluT1/2*). In A1, *VGAT*⁺ cells were found in all layers at all ages (Fig. 5). Compared to *VGluT1*, the *VGAT*⁺ neurons were much less densely packed, which likely accounts for the relatively low average expression levels (Fig. 13). There were no significant changes in overall expression across the age range, although RNAseq revealed a small, but significant increase in A1 from P7 to P14. *VGAT* mRNA expression in the MGB remained at background levels, occasioned by the rare discovery of a *VGAT*⁺ neuron in the MGd or MGv (e.g., Fig. 10c). However, RNAseq revealed low levels of *VGAT*⁺ transcripts in the MGB. We suspect that this was due to the inclusion of cells from adjacent nuclei (e.g., posterior lateral, PoL; peripeduncular, PPN), where *VGAT*⁺ neurons are abundant. Although not quantified by cell counts, our impression from the density measurements (see Fig. 13) was that *VGAT*⁺-labeled cell density in the IC decreased from P7 to adult in a relatively steady manner. This was similar to A1, where cell density also diminished. In both cases, these qualitative changes were minimized in the grayscale density measures by the normalization.

Transcript Colocalization—Multiplexed FISH assays (Figs. 6, 7, 8, 9, 10, 11, 12) were conducted to confirm anatomical features observed in the single-gene colorimetric ISH assays and to reveal co-localization of transcripts in the same neurons. Figures 6, 7, 8, 9 and 10 show the expression of *GAPDH*, *VGAT*, *VGluT1*, *VGluT2*, and DAPI in adult A1 and MGB at low and high (100×) magnification. High-resolution versions of these images can be viewed using links to their Zoomify™ versions (Supplementary material 1, Supplementary figures). In Figs. 8 and 10, the composite images in panel a are the same as, or derived from, panel a in Figs. 7 and 9, respectively. Transcript labeling for each gene was punctate and intermingled in the cytoplasm in close proximity to the DAPI-labeled nucleus. Some nuclei, possibly glia, lacked significant accumulations of puncta in the surrounding cytoplasm. Puncta associated with different genes were easily distinguishable by color, and specificity of the multiplex FISH assays was exceptionally high. We observed no instances in which labeled puncta were positive for more than one marker. Images of the positive and negative controls further confirm the specificity of both the probes and fluorescent tags (Supplementary Figure 1).

GAPDH (yellow) was present in all neurons and was therefore co-localized in neurons that also contained *VGAT*, *VGluT1*, or *VGluT2* transcripts. Although not quantified, we observed that *GAPDH* expression levels (puncta number) varied between neurons, but no anatomically relevant pattern was evident (e.g., cell type, layer, nuclear division).

VGAT⁺ neurons (green) were located in all layers of cortex at every age, but rarely in MGv, MGd, MGm, or Sg. An example of a rare *VGAT*⁺ neuron in the MGv is illustrated in Fig. 10b, c. The paucity of GABAergic neurons in the MGB is a particular feature of rodents and some bat species (Winer and Larue 1996). In contrast, *VGAT*⁺ neurons were widespread in

the peripeduncular nucleus (PPN) and adjoining posterior group of nuclei that border the MGB ventrally and medially, as well as the superior and inferior colliculus (Figs. 9, 10). These nuclei also contained large numbers of *VGluT2*⁺ neurons. Although highly interspersed in the same brain regions, *VGAT*⁺ transcripts did not co-localize in neurons that contained either *VGluT1* or *VGluT2* transcripts in A1 or MGB at any age.

VGluT1 (red) and *VGluT2* (white) transcripts were frequently co-localized in the same A1 and MGB neurons (Figs. 6, 7, 8, 9, 10, 11, 12). Overall, *VGluT1* transcripts dominated in A1, while expression of *VGluT2* was strongest in the MGB. Most of the *VGluT2*⁺ MGB neurons also expressed *VGluT1*, but *VGluT2*⁺ transcripts in A1 were relatively sparse, especially in the infragranular layers and in adults. In addition, co-expression patterns were age dependent, reflecting changing expression levels during maturation. As observed in the colorimetric assays, *VGluT1* expression in A1 at P7 was relatively low in L2–6, with a minor concentration in L3b–5 (Fig. 11c). *VGluT2* expression in A1 at P7 was concentrated in L2–3, and in some L5 neurons, as also noted in the single ISH assays. Co-localization of both transcripts was therefore more widespread in L2–3 neurons at this age. By P14, *VGluT2* expression in L2–3 declined, and therefore co-localization with *VGluT1* was less pronounced (Fig. 11b). By adulthood, *VGluT2* levels reached a minimum in L2–3, and co-localization with *VGluT1* was only visible at high magnification due to the low number of *VGluT2* transcripts (Figs. 8, 11c). In the MGB, *VGluT2* expression was strong at all ages (Figs. 9, 10, 12). At P7, *VGluT1* mRNA was nearly absent from the MGv and very weak in the MGd, and so co-localization of *VGluT1* and *VGluT2* was limited to a few cells in the MGd at this age (Fig. 12c). By P14, however, *VGluT1* and *VGluT2* co-localization was widespread among MGv and MGd neurons and grew strong through adulthood (Figs. 9, 10, 12a, b).

Immunohistochemistry

In this section, the illustrations, analyses, and descriptions proceed from lower to higher levels of magnification to reveal different aspects of the immunohistochemical expression patterns. Descriptions focus on A1 and MGB, with limited reference to other brain areas for comparison.

Figures 14, 15, 16, 17 are widefield image montages of single coronal sections through A1 and the MGB at each age (Figs. 14, 15: *VGluT2* + *VGluT1* + NeuN; Figs. 16, 17: *VGAT* ? *VGluT1* ? NeuN). The merged color images at the top of each image set are separated into grayscale color channels in the panels below, where each channel corresponds to one protein marker. The left column contains images of the full section. Rectangular insets correspond to images of the MGB and A1 in the middle and right columns, respectively. Image brightness was held constant across these images. Accordingly, signal intensity for some images is quite low, reflecting weak immunore-activity of that marker (e.g., *VGluT1* in Figs. 14, 16 at P7). Image brightness was held constant to maintain the proper impression of relative immunoreactivity between ages and brain regions. Plots of the relative grayscale intensity values are summarized in Fig. 18.

Confocal image stacks, comparing A1 and the MGB at P7 and adult, are depicted in Figs. 19, 20, 21, 22, 23 for each set of markers (A1: Figs. 19, 20; MGB: Figs. 21, 22). Puncta

counts for all ages are graphically summarized below each image set. Figure 23 shows VGluT2-ir puncta in the MGB at high power to show the terminal size at P7 and adult.

VGluT1, VGluT2, and VGAT immunoreactivity: general observations

Immunolabeling of the vesicular transporters was typically punctate and presumed to be contained within putative axon terminals (see Figs. 19, 20, 21, 22, 23; Supp. Figs. S12–25). Labeling was not found within the cytoplasmic domains of neuronal somata or dendrites. Some differences in the size of labeled puncta were evident (but not measured), and in some regions (e.g., MGB) puncta morphology changed with age (details below). We did not find convincing evidence that VGluT2, VGluT1, or VGAT localized to the same puncta within A1 or MGB at any age, suggesting that a given terminal does not normally contain more than one vesicular transporter (at least not at levels detectable by our methods). Because our observations were limited to the light microscope level of inspection, however, we cannot rule out possible co-expression in terminals where expression levels were below the resolution or detection of our approach (e.g., Fig. 26). Generally, the age-related trends visible at low power were also observed at high-power. However, some minor discrepancies are visible, presumably due to differences in illumination (fluorescence excitation) between 10× widefield and 60× confocal.

VGluT2 immunoreactivity in A1 and the MGB

In A1, VGluT2-ir puncta, presumably reflecting mainly TC projections, were present in all layers at all ages, with prominent peaks in L1a and L4 (Figs. 14, 15, 18, 19; Supplementary Figs. S2–6, S12–16). In L5b/6a, a slightly elevated band of VGluT2-ir was visible, but at levels well below L1a and L4, with immunoreactivity reaching a minimum in L6b. The L5b/6a band contained strings of beaded (en passant) VGluT2 puncta (varicosities) that were rare in L6b. The axonal segments joining puncta were not as clearly delineated by the antibody that was used in the final images/analyses, which generated highly punctate labeling of terminal endings (Table 2). However, in preliminary testing of antibodies, the interconnected strings of en passant terminals were clearly visible with one antibody, in particular (Millipore MAB5504). This antibody was excluded from the final assays due to problems associated with reliability in mouse, although it was successfully used in our earlier study of macaque monkey auditory cortex, where the en passant configuration was robust in L5b/6a (Hackett and de la Mothe 2009). Thus, punctate labeling of presumptive TC terminals was observed in varied concentrations in all layers of A1, but in L5b/6a some of the terminals were en passant.

Laminar density profiles in A1 obtained from low-power images were highly overlapping at P7 and P11, and also at P21 and adult, with the greatest increases in terminal density taking place prior to P21, especially in L4 (Fig. 18). Confocal images and puncta counts (Fig. 19) were generally consistent with these trends, indicating that laminar patterns were well established by P7, although immunoreactivity continued to increase through P21. VGluT2-ir terminals were rather uniformly dispersed between cell somata where they likely make contact with dendritic processes (Richardson et al. 2009). Somatic contacts appeared to be rare.

In the MGB, VGluT2-ir puncta, primarily reflecting tT projections, were present in the ventral (MGv) and dorsal (MGd) divisions at all ages (Figs. 14, 15, 18, 20; Supplementary Figs. S2–6, S17–18). Puncta were densely packed in the neuropil of both divisions. In the MGv, VGluT2-ir puncta became concentrated in patch-like formations from P11 onward, producing a lobulated appearance. In contrast to A1, VGluT2-ir decreased in both divisions after P7, with minimal change between P21 and adult (Fig. 20). The reduction was most pronounced in the MGv; so that, by maturity, VGluT2-ir in the MGd was higher than in the MGv. The observations can be explained by reduced numbers of VGluT2-ir puncta, accompanied by an increase in their size (Figs. 20, 23). Qualitatively, the puncta at P7 and P11 were relatively small and densely packed. At P21 and in the adult, most were medium to large in size and were more widely dispersed. A transitional stage was discernible at P14, when small and large puncta were intermixed and weak labeling of distal axons was visible. In the MGd, puncta density also decreased after P7, though puncta size did not appear to change appreciably and puncta counts remained higher than in the MGv. Large VGluT2-ir terminals were rare in the MGd.

VGluT1 immunoreactivity in A1 and the MGB—In A1, VGluT1-ir puncta, primarily reflecting CC projections, were present in all layers of cortex (Figs. 14, 15, 16, 17, 18, 19, 20, 21, 22, 23; Supplementary Figs. S2–16, S19–23).

Compared with VGluT2, laminar density profiles (Fig. 18) lacked prominent peaks, as VGluT1-ir puncta were more uniformly distributed across layers. One exception was in L1; whereas VGluT2 and VGAT were concentrated in L1a and VGluT1-ir puncta were more evenly distributed across L1a and L1b (Figs. 18, 19, 20). A second exception was a relative reduction in L4 immunoreactivity (most prominent after P11). This “negative peak” complemented the positive VGluT2 peak found in L4 (Figs. 18, 19, 20).

Several age-related changes were observed in A1. Overall, VGluT1-ir puncta density increased from P7 to adult, especially prior to P21, but the changes were not uniform across brain regions or cortical layers. Most notable were the changes in L2–4. At P7, immunoreactivity was relatively low in L2/3 and L4, bounded by modest peaks in L1 and L5/6 (Fig. 18). The dip in L2/3 was largely resolved by P14, but the negative peak in L4 persisted through adulthood, becoming more prominent as surrounding layers matured. Another age-related change of interest was an apparent increase in the presence of VGluT1-ir terminals in close proximity to cell somata, most notably in L5–6a (Figs. 19, 20; Supplementary Figs. S12–16, S19–23). In these layers, as the overall numbers of VGluT1-ir terminals increased, the prominence of such terminals also increased. In Figs. 19, 20, note the clustering of VGAT and VGluT1 around somatic profiles in the adult, but not at P7. We found no clear examples of VGluT1 in close apposition to somata at P7 or P11. At P14, as VGluT1 levels were becoming more robust across layers in cortex, VGluT1-ir puncta were in relatively close proximity to a few somata, forming weak outlines. At P21 and in the adult, puncta with a perisomatic arrangement were common and most obvious around L5 pyramidal neurons (see “Discussion” for explanation).

In the MGB, VGluT1-ir puncta, primarily reflecting CT terminals, were evenly distributed in the MGv and MGd, although the density may be slightly higher in MGd (Figs. 14, 15, 16,

17, 18, 21, 22; Supplementary Figs. S2–11, S17–18, S24–25). The expression density was low at P7 and then increased markedly through P14, especially in the MGv, with more gradual increases thereafter. The two VGluT1 antibodies yielded somewhat different appearances, but the same overall trajectories. VGluT1-ir puncta varied somewhat in size (qualitative observation), but were typically small relative to either VGluT2 or VGAT puncta, with no obvious change in either MGB division with age (Figs. 21, 22). We looked for, but only found a few potential examples of larger puncta that might correspond to giant terminals of L5 CT projections. Because it is almost certain that such CT terminals in the MGd are VGluT1-ir, their rarity may reflect a methodological or anatomical peculiarity associated with VGluT1. However, larger VGluT1-ir puncta were visible in the posterior medial nucleus bordering the MGB medially (not shown), suggesting that antibody labeling can delineate at least some changes in terminal size. In any case, the developmental increase in VGluT1-ir puncta density contrasts sharply with the decrease of VGluT2 in MGv and also differed from A1, where the expression of both increased with age. Finally, in both A1 and MGB, we found that maturation of VGluT1 immunoreactivity lagged behind that of VGluT2 and VGAT.

VGAT immunoreactivity in A1 and the MGB—In A1, the distribution of VGAT-ir puncta, reflecting GABAergic interneuron terminals, was relatively even across most layers (Figs. 16, 17, 18, 20; Supp. Figs. S7–11, S19–23), with a couple of exceptions. First, a prominent positive peak was found in L1a at all ages, matching the VGluT2 peak in L1a and contrasting with the even distribution of VGluT1 in L1a and L1b (Fig. 18). Second, at P21 and adulthood, there was also a relatively broad but shallow positive peak centered over L3/4, reflecting a somewhat higher density of puncta in the thalamorecipient layers. VGAT density increased steadily from P7 to adulthood, increasing most rapidly in the superficial layers (L1–3). The prevalence of perisomatic VGAT-ir puncta increased with age in A1 and apparently in tandem with closely apposed VGluT1-ir puncta (Fig. 20). Zooming in on the Supplementary figures at all ages shows more clearly that these terminals were rare at P7 and P11, and started to become more numerous by P14, especially in L5–6a, where perisomatic terminal density was most obvious. At P21, perisomatic labeling in L5–6a was comparable that found in the adult, but below adult levels in L2/3. Thus, it appears that the maturation of this feature continues after P21, along with a gradual increase in VGAT-ir puncta in all layers. The subplate, visible through about P14, contained a slightly higher concentration of VGAT-ir puncta, but these measurements were not included in the quantification of L6 (see Supplementary confocal images).

In the MGB, VGAT-ir puncta, which reflect both tT and TRN terminals, were fairly uniformly distributed in the MGv and MGd, with a somewhat higher density of terminals in the MGd at all ages (Figs. 16, 17, 22; Supp. Figs. S7–11, S24–25). At low magnification (Figs. 16, 17), VGAT-ir puncta in the MGv appeared concentrated in irregular patches, similar to those observed in VGluT2 preparations, and a general increase in the brightness of labeling resulted in an increase in grayscale density over time. The confocal images (Fig. 22; Supp. Figs. S7–11, S24–25) showed that VGAT-ir puncta were present at P7 in both divisions, and at slightly higher levels than for older animals. In the MGv, puncta density decreased modestly from P7 to P11 and then remained fairly stable. In the MGd, puncta

density decreased modestly from P7 to about P14 and then on very slightly through adulthood. Unlike VGluT2 in the MGv, no obvious or widespread changes in terminal morphology were evident that might be correlated with the decrease in VGAT puncta density, although our qualitative impression was that a subpopulation of larger terminals persists through to the adult stage while the numbers of smaller puncta diminish, suggesting some pruning of less developed terminals.

Co-localization of immunoreactive puncta—We looked for, but did not find, any clear instances in which VGluT1-ir and VGluT2-ir puncta were co-localized in presumptive terminals of any layer of A1 or MGB division at any age. Similarly, VGAT-ir puncta were never found to co-localize with either VGluT1 or VGluT2 in any location. We cannot rule out the possibility that very low expression levels were not detectable by our methods. As elaborated below, these results indicate that although *VGluT1* and *VGluT2* mRNA were frequently co-expressed in the same neurons of A1 and MGB, the transporter proteins are not co-localized at detectable levels in the same axon terminals. The results favor the possibility (also beyond the methods used in this study) that if VGluT1 transporter proteins are present in TC axon terminals at significant levels, they are sorted to different axon terminals than VGluT2, in which case they would not be distinguishable from VGluT1-ir CC terminations by immunohistochemistry alone. Similarly, if VGluT2 transporters are normally contained in the axon terminals of any cortical neurons, they are present at relatively low levels, or sorted to different terminals than VGluT1, or both (see “Co-expression of VGluT1 and VGluT2” and “Discussion”).

Correspondence between gene and protein expression

In Fig. 24, the data from Figs. 12 and 16 were replotted to enable comparisons of the RNAseq, ISH, and IHC data. P11 data were omitted from these graphs to facilitate comparisons with RNAseq at this age (P11 samples were not obtained for sequencing). The RNAseq and ISH graphs show a fairly close correspondence in the data obtained by the two mRNA assays. The IHC data were generally comparable, but several interesting differences were observed. In A1, immunoreactivity increased steadily for all three protein markers from P7 through adulthood. In contrast, gene expression increased through P14 for *VGluT1* with a slight decline in adulthood, and remained flat across the age range for *VGluT2* and *VGAT*. In the MGB, *VGluT1* mRNA levels climbed through P14/P21, then declined slightly, but VGluT1-ir grew steadily. *VGluT2* mRNA levels in MGB also peaked at P14, with only a slight decline in adulthood, whereas VGluT2-ir showed a sharp decline from P7 to P21. VGAT-ir also dropped from P7 to P14. As discussed above, these differences partly reflect differences in subcellular localization of mRNA transcripts (somatic) and proteins (terminals), but may also reflect differences in the regulation of transcription or translation during postnatal maturation.

Discussion

In the central auditory pathways, characterization of VGluT1, VGluT2, and VGAT localization and function is ongoing (Barroso-Chinea et al. 2007; Hackett and de la Mothe 2009; Hackett et al. 2011b; Ito et al. 2009, 2011; Ito and Oliver 2010; Nahmani and Erisir

2005; Rovo et al. 2012; Storace et al. 2012). In most structures, expression of at least one of these transporters has been studied in adult animals, but systematic studies of their maturation over the weeks surrounding the onset of hearing are just beginning to emerge.

The main purpose of this study was to characterize postnatal changes in the expression of the vesicular transporters for glutamate (VGluT1, VGluT2) and GABA (VGAT) in A1 and the MGB over the period surrounding the onset of hearing. By profiling the gene (RNAseq, ISH) and protein (IHC) expression patterns, we obtained data from populations of projecting neurons (mRNA transcripts in neuronal somata) and their targets (immunoreactive axon terminations). Accordingly, the combined results reflect maturational features of the corticocortical (CC) corticothalamic (CT), thalamocortical (TC), tectothalamic (tT), and thalamic reticular nucleus (TRN) systems of projections in the auditory forebrain. Overall, significant changes were observed in the expression of all three transporters between P7 and adult, but the rate and direction of change were not uniform between markers or brain regions (summarized in Figs. 24, 25). As discussed below, these differences suggest variable trajectories of maturation between pathways and establish a baseline to which related anatomical features can be compared (e.g., postsynaptic receptors, ion channels, other neuromodulators, specific neuronal subpopulations, etc.). The data also draw attention to the presynaptic machinery related to neurotransmission and its role in activity-dependent homeostatic changes that accompany critical periods of experience-dependent brain development.

Regional expression patterns of VGluT1, VGluT2, and VGAT

Generally, the regional expression patterns of VGluT1 and VGluT2 in the forebrain are complementary, while VGAT is relatively more evenly distributed. This can be instantly appreciated in viewing the low-magnification images in this report (e.g., Figs. 2, 15) and the higher-resolution image libraries that depict immunoreactivity from P7 to adult across the entire brain for all three markers (Supplementary Figures 26, 27). The regional distinctions are most distinct for mRNA. *VGluT1* mRNA is widely expressed by most, if not all, glutamatergic neurons in the neocortex, but is only found in a few subcortical structures—mainly the sensory relay nuclei in the thalamus and the striatum, whereas, *VGluT2* mRNA is widely expressed by glutamatergic neurons in the thalamus and brainstem, but is present at relatively low levels in the cortex (Balaram et al. 2011; Barroso-Chinea et al. 2007; Bryant et al. 2012; De Gois et al. 2005; Fremeau et al. 2001, 2004; Graziano et al. 2008; Hackett et al. 2011b; Herzog 2001; Hur and Zaborszky 2005; Kaneko et al. 2002; Nahmani and Erisir 2005; Rovo et al. 2012). In contrast, VGluT1 and VGluT2 protein expression is more overlapping in both cortex and thalamus, because VGluT1 is contained in CC and CT projections (axon terminals), and VGluT2 is expressed in tT and TC projections. Compared to the glutamate transporters, *VGAT* mRNA is widely expressed by cortical and subcortical neurons. Since most (but not all) of their projections are local, VGAT immunoreactivity also tends to be local; thus there is a much stronger correspondence in the regional expression patterns of VGAT mRNA and protein.

As for the auditory forebrain, the major glutamatergic and GABAergic projection systems in the P7 and adult mouse are summarized in Fig. 25. In these schematics, VGluT1, VGluT2

and VGAT expression in axon terminals (protein) or neuronal somata (mRNA transcripts) are depicted separately. Minor projections and intrinsic connections were omitted for clarity.

In A1, *VGluT1* mRNA appears to be expressed by all glutamatergic neurons. Accordingly, VGluT1-ir is also high, primarily reflecting CC connections among auditory and other cortical areas, but possibly also the terminals of *VGluT1*⁺ subpopulations in the MGB (see Storace et al. 2012 and “Transcript Co-localization”, above). *VGluT2* expression is very low in auditory cortex, although subsets of neurons with detectable transcript levels are present in most areas, with the weakest expression in the infragranular layers (De Gois et al. 2005; Graziano et al. 2008; Hackett et al. 2011b; Ito and Oliver 2010). As observed in the present study, *VGluT2* transcripts in A1 appear to always be contained in neurons that express *VGluT1*, but not all *VGluT1*⁺ neurons express *VGluT2*. Little is known about the neuronal subpopulations that express both genes in cortex, and it remains unclear whether VGluT2 transporter proteins are normally present in any of their axon terminations (but see below). Thus, most of the VGluT2-ir terminals in cortex are presumed to represent TC projections. In A1, these puncta are found in all layers (as depicted in Fig. 25), but form a prominent band centered on L4. *VGAT*⁺ neurons are broadly distributed in all layers of A1, with the fewest number in L1. Most of the VGAT-ir terminals are derived locally, resulting in dense immunoreactivity in most layers, except for L1b (Fig. 25).

In the MGB, nearly all neurons express *VGluT2* mRNA, but a subset also expresses *VGluT1*, at least in rodents (Barroso-Chinea et al. 2007; Ito et al. 2011; Storace et al. 2012). *VGluT1* mRNA appears to be rather sparse in the MGB of adult primates (Hackett et al. 2011b), however, reflecting a possible species difference in the chemoarchitecture of these circuits. VGluT2-ir in the MGB primarily reflects the inputs from *VGluT2*⁺ neurons in the IC, since *VGluT1* transcripts are rare in any division of the IC, as observed in the present and previous studies (Ito et al. 2011; Ito and Oliver 2010). VGluT1-ir in the MGB is also strong, reflecting CT inputs from *VGluT1*⁺ neurons in the infragranular layers of auditory cortex. Since many MGB neurons coexpress *VGluT1* and *VGluT2* mRNA, both transporter proteins could be co-localized to the same terminals or trafficked to different TC terminals. These details (discussed below) remain unknown. Most of the GABAergic inputs to the MGB arise from the thalamic reticular nucleus (TRN) and the IC (Bartlett and Smith 1999; Bartlett et al. 2000; Geis and Borst 2013; Ito et al. 2009, 2011; Mellott et al. 2014a, b; Montero and Scott 1983; Peruzzi et al. 1997; Winer et al. 1996). The TRN neurons receive axon collaterals from both the TC and CT systems of projections, with the exception of the CT axons that originate in L5 (Crabtree 1998; Kimura et al. 2005). In contrast, GABA, GAD, or *VGAT* mRNA expression within MGB neurons is very rare in mice and rats, as observed in the present and previous studies (Ito et al. 2011; Winer and Larue 1996; Yuge et al. 2011). Thus, a network of intrinsic GABAergic projections is absent from the MGB in these species and inhibitory influences on activity are almost entirely external.

Maturation of VGluT1 and VGluT2 in the auditory forebrain

Overall, vesicular glutamate and GABA transporter expression changed significantly between P7 and adulthood (Fig. 25). The greatest changes occurred prior to P21,

encompassing a 2-week period before and after ear canal opening (~P11–P13). Yet, each projection system matured at slightly different rates.

In the IC and MGB, *VGluT2* mRNA expression was well established by P7, with little change thereafter, accompanied by strong VGluT2-ir terminal labeling in the MGB and in L4 of A1. These findings suggest that the potential for VGluT2-mediated transmission in tT and TC projections is well established by P7, in agreement with descriptions of TC transmission prior to the onset of hearing (Barkat et al. 2011). Note also that TC projections to A1 are not limited to L4, but are distributed across most layers in different concentrations. This indicates that TC information reaches neurons in several layers within a narrow window of time. In comparison, *VGluT1* mRNA expression was quite low at P7 in MGB, especially in MGv, and concentrated in L2–3 in A1. VGluT1-ir was also very low in MGB and formed minor peaks in L1a/b and L5/6 of A1. Together, these patterns indicate that VGluT2-mediated tT and TC transmission is established well in advance of VGluT1-mediated CC and CT signaling, which lags behind until after the onset of hearing at P11. These maturational trends correspond well with prior studies of sensory cortex and thalamus in mice and rats (Boulland et al. 2004; De Gois et al. 2005; Liguz-Leczna and Skangiel-Kramska 2007; Minelli et al. 2003b; Nakamura et al. 2005). One interesting difference between modalities is that VGluT1 and VGluT2 expression reaches adult levels by about P14 in S1. This is approximately 1 week faster than in A1 and V1 (Minelli et al. 2003b; Nakamura et al. 2005), suggesting that VGluT1 and VGluT2 expression is linked to the onset of sensation, which can vary between different sensory modalities.

Looking more closely at these maturational trends in A1 and MGB, several observations are of interest, as they may signal important differences in the development of specific pathways.

The first concerns maturation of the CT projections from A1 to MGB. CT inputs to the MGB primarily arise from neurons in L5 and 6 of auditory cortex (Bartlett 2013; Lee 2013). CT neurons in L6 project to the MGv and MGd, ending in small terminals on distal dendrites (Bajo et al. 1995; Bartlett et al. 2000; Llano and Sherman 2008; Ojima 1994; Rouiller and Welker 1991; Smith et al. 2007). In contrast, CT projections from L5 are relatively sparse, have large terminal boutons, and predominantly target the MGd. Neurons in both L5 and 6 are almost exclusively *VGluT1*⁺ at all ages, indicating that CT terminals in the MGB should also be VGluT1-ir. Although *VGluT1* mRNA expression was relatively low at P7 in A1, maturation in L5 was a bit ahead of L6. Further, *VGluT1* mRNA and protein expression was strongly biased toward the MGd at P7, becoming more evenly distributed between the MGv and MGd at later ages. Taken together, the gene and protein expression findings suggest that the L5–MGd CT projections may be active before and mature earlier than the L6 CT projection to the MGv/MGd. These anatomical data support and refine our earlier report that CT axons were biased toward the dorsal subdivision prior to hearing onset but more evenly distributed thereafter (Torii et al. 2013). Note also that over the same period, *VGluT2* mRNA expression was relatively strong in both divisions of the MGB, then VGluT2-ir puncta density decreased, especially after P11, accompanied by an increase in puncta size in MGv (discussed below). Thus, ear canal opening (~P11–13) was followed by distinct patterns of VGluT1 and VGluT2 expression in the MGv and MGd.

Second, the laminar expression patterns of *VGluT1* mRNA and VGluT1-ir in A1 are partially complementary. VGluT1-ir puncta were concentrated in L1 and L5/6 at P7 and gradually increased in the intervening layers over development. In contrast, *VGluT1*⁺ mRNA was most strongly expressed in L3b–5 at P7, but was weaker in L2 and L6. This difference was largely gone by P11, when *VGluT1*⁺ expression became uniform across L2–6. One possible interpretation is that VGluT1-mediated glutamate release by neurons in L3–5 onto targets in L1 and L5/6 matures a bit ahead of the *VGluT1*⁺ projections to other layers prior to the onset of hearing. Alternatively, or additionally, the VGluT1-ir band of terminals in L1 at P7 and later might contain some VGluT1-ir inputs from the MGd (or other nuclei), since modest levels of *VGluT1* mRNA are already present in MGd neurons at P7. These patterns raise intriguing questions about laminar patterns of signaling within A1 before and after hearing onset.

Third, *VGluT2* mRNA levels decreased from P7 to adult in A1, reaching very low levels at maturity. The ISH and FISH assays showed that expression at younger ages was largely concentrated in L2–3. These laminar patterns and their developmental time course are comparable to those observed by De Gois et al. (2005), and also compare well with Allen Brain Atlas data at comparable developmental ages. It is not entirely clear why *VGluT2* levels in L2/3 neurons should be elevated at P7, but the answer may be related to activity-dependent regulation of vesicular transporter expression (discussed below), where the relative levels of VGluT1 and VGluT2 are subject to activity-dependent modification. In addition, the downstream targets of the subpopulations of cells that express *VGluT2* (and are co-localized with *VGluT1*) are also unknown, at present. We are thus led to speculate whether these neurons might have different response properties, and whether the VGluT1 and VGluT2 proteins might be trafficked to the same or different terminals.

Maturation of VGAT expression in the auditory forebrain

Like the glutamatergic system, the GABAergic network in the cortex is in flux for several weeks after birth (Ben-Ari et al. 2004, 2012; Ciceri et al. 2013; Espinosa and Stryker 2012; Gelman and Marin 2010; Griffen and Maffei 2014), resulting in a gradual increase in inhibitory strength (Chattopadhyaya et al. 2004; Li et al. 2012; Maffei and Turrigiano 2008). At the synaptic level, much emphasis has been placed on the documentation of changes in GABA receptor subunit expression, which vary over time by brain region and even cell type, and contribute to the maturation of inhibitory synapses (Ben-Ari et al. 1994, 2012; Bosman et al. 2002; Davis et al. 2000; Fritschy et al. 1994; Heinen et al. 2004; Hensch 2005; Hornung and Fritschy 1996; Huang 2009; Laurie et al. 1992; Maffei and Turrigiano 2008; Mohler 2006; Peden et al. 2008; Takesian et al. 2010).

Overall, the time course of these postsynaptic changes parallels the maturational trajectory of presynaptic VGAT-ir observed in the present and prior studies. Although *VGAT* mRNA levels did not change significantly from P7 to adult in either A1 or MGB, we found that VGAT-ir in A1 increased steadily from P7, leveling off at around P21. The difference in gene and protein expression suggests that translation of VGAT is differentially regulated, perhaps in a manner that reflects the onset of activity. For example, the trajectory of VGAT-ir maturation in A1 closely matches the findings of Boulland and Chaudhry (2012), who

studied the structural and functional properties of VGAT-ir puncta during postnatal development in several brain areas. In the cortex, hippocampus, and thalamus they observed an increase in VGAT expression during the first three post-natal weeks, in tandem with an increase in the expression of markers related to GABA synthesis and neurotransmission (e.g., GAD). In rats Minelli et al. (2003b) found that VGAT levels in S1 increased gradually from P0, reaching 50 % of adult levels between P5 and P10, and adult levels by about P15. The laminar patterns of terminal labeling were comparable to the present study. They also noted that VGAT expression through P5 was slightly ahead of the synaptogenesis of inhibitory contacts, and suggested that at this stage it may contribute to the transition to mature forms of GABA release at synapses. For example, Takayama and Inoue (2010) tracked the expression of the potassium chloride co-transporter 2 (KCC2), GABA, and VGAT proteins in mouse barrel cortex from P0 to adult. KCC2 and VGAT expression followed a similar time course. VGAT puncta were present in L1a at birth, with little change after P5. Puncta appeared in L5/6 after P3, L4 after P5, L2/3 after P7, and were relatively homogeneous across layers by P14. Functionally, the onset of KCC2 expression is correlated with the point at which GABA binding causes hyperpolarization—rather than depolarization—of the postsynaptic cell membrane (Blaesse et al. 2006; Lee et al. 2005; Rivera et al. 2005). Their data suggested that by about P10, GABA is an inhibitory transmitter for most cortical neurons in mice. Compared to S1, then, the time course of VGAT maturation in A1 is delayed. This is likely related to the delayed onset of sensory transmission in the auditory system, which is minimal until after ear canal opening (P11–13). It is not clear whether the shift to inhibition in GABAergic neurons is also delayed in auditory cortex, relative to S1. Pursuing this in future studies would be of interest.

Of additional interest is that the onset of GABA-mediated inhibition in cortex is delayed relative to subcortical nuclei in the thalamus and brainstem (Kullmann and Kandler 2001; Milenkovic et al. 2007; Perreault et al. 2003; Venkataraman and Bartlett 2013; Ziburkus et al. 2003), suggesting that the time course varies between regions. In the MGB, where VGAT-ir terminals originate primarily from the TRN and IC, there was a slight decline in the numbers of VGAT-ir puncta between P7 and P14, but immunoreactivity was otherwise stable after P7. This suggests that the possibility that GABAergic circuitry in the MGB matures ahead of A1. Comparable data on VGAT-ir from other sensory systems are rather limited. Singh et al. (2012) studied GAD67 and VGluT2 expression in the dorsal lateral geniculate nucleus (dLGN) from P3 to adulthood in mice. In their study, GAD67-ir terminal density increased from P7 to P14, but size remained constant. Using an antibody for GABA, Bickford et al. (2010) found little immunoreactivity in the dorsal LGN (dLGN) at P7, but strong labeling by P14. This anatomical change was supported by electrophysiological measurements. These trends match our qualitative impressions of VGAT-ir in LGN from P7 to adult (see Supplementary Figure 27). As for terminal size, there was no obvious maturational change in the MGB, as found in the dLGN (Singh et al. 2012). On average, the size of GABAergic terminals in the MGB were qualitatively smaller than excitatory (VGluT2-ir) terminals from the IC, but there does not appear to be a definitive way to distinguish GABAergic terminal populations from the thalamic reticular nucleus (TRN) and IC based on morphology (Bartlett et al. 2000). Their delineation would be of interest for future anatomical studies, and may serve to support recent neurophysiological studies that

have focused on maturation of excitatory and inhibitory properties in the MGB during postnatal development (Venkataraman and Bartlett 2013, 2014).

Co-expression of VGluT1 and VGluT2

An important observation of the present study was that co-localization of *VGluT1* and *VGluT2* transcripts within the same neurons was common in A1 and MGB of juvenile and adult animals. Throughout the brain, *VGluT1* and *VGluT2* mRNA expression is generally complementary between regions (e.g., cortical versus subcortical); however, co-expression (within a region) and co-localization (within the same cellular compartment, such as somata or terminals) have been reported in some structures in adult animals. Barroso-Chinea et al. (2007) conducted single and dual expression assays of *VGluT1* and *VGluT2* mRNA throughout the thalamus of adult rats. In the MGB and all other sensory relay nuclei (e.g., lateral geniculate nucleus, ventroposterior nucleus), most if not all neurons contained both transcripts. Frequent co-localization was also reported in the MGB (and most other auditory brainstem nuclei) of adult mice and rats by Ito et al. (2011), with the greatest numbers of co-expressing neurons in the MGv (see also Storace et al. 2012).

During postnatal development in rodents, the results of two studies are highly relevant to the present investigation. Danik et al. (2005) studied the co-localization of *VGluT1* and *VGluT2* mRNA in dissociated neurons from the hippocampus, cortex, and cerebellum (Danik et al. 2005). Overall, *VGluT1* mRNA levels increased with age, while *VGluT2* levels decreased. At P14, about 80 % of neurons that expressed *VGluT1* mRNA also expressed *VGluT2*. Co-expression dropped to about 60 % by P60, still reflecting a sizable majority. De Gois et al. (2005) demonstrated widespread co-localization of *VGluT1* and *VGluT2* mRNA in pyramidal neurons and L4 neuronal subtypes in both primary cell cultures and in vivo tissue sections. Somata containing both transcripts increased in numbers through P10 in L2–4 and also L5–6 in the auditory and parietal cortices of rats. *VGluT1* mRNA levels also increased through P14, but then became stable, whereas *VGluT2* mRNA levels decreased through at least P21, but remained at detectable levels. By adulthood, neurons expressing only *VGluT1* mRNA predominated, *VGluT1/2* co-expressing neurons were a significant minority, and cells containing only *VGluT2* were rare. Thus, most of the cells in cortex that expressed *VGluT2*, also expressed *VGluT1*.

Given the widespread co-expression of *VGluT1* and *VGluT2* mRNA, one might predict frequent co-localization of their proteins in axon terminals (e.g., see Fig. 26). However, evidence for this has been inconsistent. Typically, VGluT1 and VGluT2 proteins in adult animals are localized in discrete populations of axon terminals that overlap, but do not co-localize (Bragina et al. 2007). In the present study, we found only rare examples of terminals that were candidates for the co-localization of VGluT1 and VGluT2 proteins in any layer of cortex at any postnatal age. In contrast, in rat primary neocortical cell cultures, De Gois et al. (2005) found that VGluT1 and VGluT2 were co-localized to many (~10 %) terminals between 5 and 21 days in vitro. Similarly, Nakamura et al. (2005) found that VGluT1 and VGluT2 were frequently co-localized in terminals in L4 barrel cortex between P5 and P10, especially in barrel hollows, but rarely in adult brains. Liguz-Lecanar and Skangiel-Kramaska (2007) also noted co-localization in terminals within barrels prior to adulthood (Liguz-

Lecznar and Skangiel-Kramska 2007). These combined results suggest that co-localization might be a unique and transient feature that occurs primarily during the first two postnatal weeks. The reduced levels of *VGluT2* mRNA at later postnatal stages and adulthood may result in lower protein levels and explain why co-localization of *VGluT1* and *VGluT2* transcripts is rarely found in the sensory cortex of older juvenile and mature animals.

The presence of both proteins in neurons or cortical terminals at any stage of development raises intriguing questions about its functional importance and has potentially profound implications for the organization and plasticity of cortical circuitry. First, why should VGluT1 and VGluT2 transporters ever be co-localized, and under what circumstances are expression levels up- or down-regulated? Clues may be found by returning to the study by De Gois et al. (2005), in which expression levels of VGluT1, VGluT2, and VGAT varied dynamically with pharmacological modulation of the local excitatory and inhibitory environment. Treatment of cultures with bicuculline (BIC) or gabazine over 48 h led to decreased expression of *VGluT1* mRNA (in somata) and protein (in terminals), but increases in VGAT and VGluT2. Conversely, application of tetrodotoxin (TTX) increased VGluT1 levels, but decreased VGAT and VGluT2. These findings indicate that prolonged changes in activity levels lead to bidirectional and opposite regulation of VGluT1 and VGluT2/VGAT. The authors suggested that the numbers of VGluT1, VGluT2, or VGAT molecules within a terminal are a critical factor in regulating vesicle filling with glutamate or GABA. Although it has not been determined whether VGluT1 and VGluT2 proteins are located on the same or different populations of synaptic vesicles, the increase in VGluT2 expression after BIC treatment may compensate or substitute for the reduction in VGluT1. Because co-expression occurs in only a subpopulation of terminals, VGluT2 would be selectively increased in a subset of terminals after prolonged activity reduction. This shift in the VGluT1/VGluT2 balance could prevent a reduction in glutamate release and/or increase glutamate release probabilities from these terminals (Edwards 2007; Erickson et al. 2006; Fei et al. 2008; Fremeau et al. 2004; Omote et al. 2011; Takamori et al. 2006; Wilson et al. 2005; Wojcik et al. 2004). Thus, it appears that VGluT1, VGluT2, and VGAT levels can be dynamically altered by the experimental manipulation of activity and also change naturally during development. These results imply that dual expression confers important functionality in both developing and mature animals, and that activity-dependent mechanisms regulate the likelihood of co-expression. For example, overall VGluT2 levels—as well as the probability of co-expression with VGluT1—could be increased under conditions of reduced sensory input occurring either early in development, when cochlear thresholds are still immature, or later in life as a consequence of hearing loss. These presynaptic changes are but a few of the many pre- and postsynaptic homeostatic mechanisms of plasticity that alter the synaptic strength of excitatory and inhibitory circuits, suggesting the need for further study (Coleman et al. 2010; De Gois et al. 2005; Dormn et al. 2010; Lazarevic et al. 2013; Nahmani and Erisir 2005; Sun et al. 2010; Turrigiano 2012; Wang and Maffei 2014; Wilson et al. 2005; Zhang et al. 2011).

Second, what does the apparent absence of significant co-expression of VGluT1 and VGluT2 transporter proteins in axon terminals in normal, mature brains suggest about the organization of TC and CC circuitry in the auditory forebrain? Although *VGluT1* and *VGluT2* transcripts were commonly co-localized in neurons located in supragranular layers

of A1 and principal divisions of the MGB, we did not find evidence that these transporter proteins were co-localized in presumptive terminals of any A1 layer or MGB division at any age. It remains possible that our methods were unable to detect low levels of immunoreactivity in puncta, but widespread or significant co-expression can be ruled out, as the resolution of confocal microscopy is sufficient to reveal co-localization at those levels (Samano et al. 2006). Thus, for A1, the present results (combined with the studies reviewed above) favor the conclusion that if VGluT1 transporter proteins are present in TC axon terminals at significant levels, then VGluT1 and VGluT2 are usually sorted to different axon terminals, in which case they would not be distinguishable from VGluT1-ir CC puncta by immunohistochemistry alone. That is, VGluT1-ir CC and TC terminals could be interspersed in any layer in which TC projections terminate. Similarly, if VGluT2 transporters are normally contained in the axon terminals of any cortical neurons, then they are present at very low levels, or sorted to different terminals than VGluT1, or both. Based on retrograde tracing studies in rat auditory cortex, Storace et al. (2012) found evidence supporting differential expression or co-expression of VGluT1 and VGluT2 transporters in the projections from MGB to different AC areas. Although the present findings were not consistent with significant terminal co-localization in TC projections to A1, the possibility that the transporters are sorted to different terminal populations remains open. Definitive answers may require higher-resolution inspection of terminals in different layers of AC areas that can reliably detect low levels of one or both transporters (e.g., electron microscopy of double-labeled tissue sections).

Several hypothetical configurations are illustrated for consideration in Fig. 26, based on growing evidence that neurons in several brain regions can synthesize and segregate neurotransmitters, their synthesizing enzymes, or associated vesicular transporters to different populations of synaptic vesicles in the same terminals, or sort these molecules to different axon processes (El Mestikawy et al. 2011; Samano et al. 2012; Vaaga et al. 2014). This makes it possible for terminals to co-release (or co-transmit) neurotransmitters onto the same or different targets (Saunders et al. 2015). In addition, the sorting of these molecules is also subject to plasticity (see Samano et al. 2012), which may also contribute to observations of activity-dependent changes in expression levels (De Gois et al. 2005). While presently unknown for TC and CC projections in the auditory forebrain, these findings suggest that some neurons may possess the machinery needed to route transmitters and other molecules to different terminal processes in a dynamic manner.

Although the potential significance of these structural details may not be immediately obvious, they have potentially profound implications for the nature of excitatory neurotransmission in TC and CC circuits. VGluT2 has been associated with a higher probability of glutamate release than VGluT1 in some brain regions (Blakely and Edwards 2012; Hnasko and Edwards 2012; Kaneko and Fujiyama 2002; Santos et al. 2009; Varoqui et al. 2002). In addition, the number of transporters located on each vesicle has an impact on vesicle filling, quantal size, and the amount of neurotransmitter released (Blakely and Edwards 2012; Edwards 2007; Erickson et al. 2006; Fei et al. 2008; Hnasko and Edwards 2012; Omote et al. 2011; Santos et al. 2009; Takamori et al. 2006; Wilson et al. 2005; Wojcik et al. 2004). Given these differences in the synaptic properties associated with VGluT1 and VGluT2, rather different influences could be conferred upon a variety of

postsynaptic targets. For example, if VGluT1 and VGluT2 were segregated to different terminals, their impact on postsynaptic activity in a location would likely be different than if the transporters are co-localized to some or all of those terminals. In addition, since the branches of TC axons end in terminals that are distributed over layers 1–6 in different concentrations, VGluT1 and VGluT2 may also be differentially sorted to (or co-localized within) terminals in a laminar-specific and even cell-specific manner. Such details are currently unknown, but many configurations are possible, each associated with different functional outcomes (see Storace et al. 2012). Further, since transporter expression levels and terminal sorting may be altered through activity-dependent regulation, synaptic properties could shift with changes in the expression of VGluT1 and VGluT2. Thus, while the activity of a TC terminal that contains low levels of VGluT1 and high levels of VGluT2 might not be functionally distinguishable from one in which VGluT1 is entirely absent, the relative levels of either transporter are subject to plastic modifications that could lead to functionally significant changes in that terminal. Clearly, this is fertile ground for further exploration.

Morphological changes in VGluT2-ir puncta in the MGv

One of the more interesting and novel findings of the present study was that the decrease in VGluT2 puncta density after about P11 was accompanied by a substantial increase in puncta size in the MGv, but not MGd. The time course of these morphological changes was closely tied to the onset of hearing, suggesting the involvement of activity-dependent mechanisms. Although this has not been previously described in the MGB, comparable and even more dramatic changes in synaptic morphology associated with the onset of hearing or hearing loss have been identified in the auditory brainstem after the onset of hearing (e.g., endbulb and calyx of Held) (Yu and Goodrich 2014). The collective findings indicate that acoustic stimulation is required for these morphological changes to develop during a critical period of maturation and that these changes are necessary to support the physiological demands in these circuits (i.e., rapid and reliable synaptic transmission at high rates). The molecular mechanisms responsible are still being worked out.

In addition to the auditory brainstem, comparable changes in VGluT2 terminal density and morphology have also been observed in the thalamic relay nuclei of other sensory systems. Nakamura et al. (2005) found that decreased VGluT2 density with age in the ventroposterior nucleus (VP) was concurrent with a visible increase in puncta size. Although the MGB was not specifically mentioned, low-power images of VGluT2-ir (their figures 2 and 8) suggest that immunoreactivity was evenly and densely distributed in the MGB at P7 and then declined thereafter, accompanied by the appearance of clusters of larger terminals by P14. In the dLGN, Bickford et al. (2010) studied axon terminal development of mice at P7, P14, and adult. At P7, the terminals were uniformly small in size and more numerous. By P14 (onset of vision is P12–P14), the terminals were fewer in number and ranged more widely in size from small to large. These patterns were stable through adulthood. Singh et al. (2012) studied GAD67 and VGluT2 expression in the dLGN from P3 to adulthood in mice. Their results were nearly identical to our MGv findings. VGluT2-ir terminal density in the dLGN dropped with age, and terminal size increased. GAD67-ir terminal density also increased, but the size remained constant. In that study, fibroblast growth factor 22 was identified as a

factor contributing to the formation and maturation of the VGluT2 terminals. Although we did not specifically analyze puncta size in LGN and VP, the development of large VGluT2-ir terminals can be clearly seen in our image library that shows immunoreactivity from P7 to adult across the rostral–caudal range of the mouse brain (see Supplementary Figure 26).

These morphological changes are linked to specific activity-dependent synaptic refinements in these systems. Before eye opening around P12, up to 20 retinal ganglion cell (RGC) axons make synaptic contact with each LGN neuron. Within about 2 weeks, the number of contacts declines to between one and three, but these synapses are some 50 times stronger (Chen and Regehr 2000; Jaubert-Miazza et al. 2005). This pruning and synaptic strengthening is inhibited by TTX blockade of retinal activity (Hooks and Chen 2006). In the medial ventroposterior nucleus (VPM), a similar series of events has been observed (Arsenault and Zhang 2006; Wang and Zhang 2008). At P7–P9, about eight lemniscal axons innervate each VPM neuron. This number decreases through P16–P17, when most neurons are innervated by only one or two lemniscal neurons. This ratio is stable thereafter. As in the dLGN, synapse elimination is accompanied by increased strength of the remaining synapses. Sensory deprivation by whisker plucking at this age interferes with synapse elimination and the normal increase in synaptic strength. Comparable anatomical studies have not been conducted in the MGB, but analogous processes are likely to be found. Thus, the available data suggest that the transition from smaller to larger VGluT2-ir terminals is a feature common to the principal thalamic sensory nuclei and is closely tied to the onset of effective sensory stimulation in each system.

Although activity-dependent mechanisms are likely responsible for the morphological changes in MGv, it is not clear why VGluT2-ir terminals in MGd and VGluT1-ir terminals in either division are unaffected. In part, the differences may reflect basic organizational features of the pathways involved. The MGv and MGd have distinct connections, cellular morphology, neurochemical profiles, and neurophysiological response properties (Bartlett 2013; Lee 2013). The MGv primarily receives subcortical inputs from *VGluT2*⁺ neurons in the ipsilateral central nucleus of the inferior colliculus (ICc). These tT terminations range from small to rather large in size (Bartlett and Smith 1999; Bartlett et al. 2000; Pallas and Sur 1994). Subcortical input sources to the MGd are more diffuse and include the dorsal and external (lateral) divisions of the IC, tegmentum, and sagulum. The tT terminations in the MGd are also VGluT2-ir, but tend to be smaller in size, on average, compared to those that target the MGv (Bartlett et al. 2000). Overlaid on the ascending projections to each division are dense CT inputs from *VGluT1*⁺ neurons in cortex, which also exhibit distinct patterns in the MGv and MGd. Neurons in L6 of A1 project to the MGv and MGd, ending in small terminals on distal dendrites (Bajo et al. 1995; Bartlett et al. 2000; Llano and Sherman 2008; Ojima 1994; Rouiller and Welker 1991; Smith et al. 2007). By contrast, layer 5 CT projections are more sparse, feature large terminal boutons, and predominantly target the MGd. Thus, it appears that the morphological changes observed in the MGv are limited to the ICc–MGv projection system, in support of the physiological roles mediated by that neuronal subpopulation.

Delayed formation of perisomatic axon terminals

One additional age-related change of interest in our data was an increase in either the numbers or visibility of VGAT and VGluT1-ir puncta in close proximity to cell somata in L2–6. The clustering of VGAT, and a few VGluT1, puncta around somatic profiles began at around P14, especially in L5/6, and their prevalence increased slowly through P21 into adulthood. We found no clear examples of such terminals in our material at P7 or P11 (Figs. 19, 20; Supp. Figs. S2–3, S7–8, S12–13, S19–20), suggesting that they formed, or became immunoreactive, after the onset of hearing.

Perisomatic GABAergic terminals are an established feature of the inhibitory network in the cortex (DeFelipe 1997; Freund et al. 1983; Kisvarday 1992; Somogyi et al. 1983a, b; Tamas et al. 1997) and typical of fast-spiking parvalbumin expressing basket cells. Thus, it is of interest that this particular type of GABAergic contact does not become prominent in juvenile cortex until after VGAT-ir puncta are already rather abundant in the neuropil (Packer et al. 2013). The developmental trajectory appears to differ between sensory regions. In S1 and hippocampus of mice, perisomatic VGAT contacts form early around P7, with continued maturation through about P21 (Boulland and Chaudhry 2012; Takayama and Inoue 2010). In the primary visual cortex of mice, Chattopadhyaya et al. (2004) found that the numbers of GABAergic perisomatic synapses increased over an extended period of postnatal development that began after eye opening at P14 and continued until about the fifth postnatal week. In addition, the proliferation could be slowed by visual deprivation through about P28, which parallels the onset and closure of the critical period for ocular dominance plasticity (Gordon and Stryker 1996). The anatomical sequence in the visual cortex is more consistent with our findings in A1, as we began to notice perisomatic VGAT-ir terminals only after P11–13, when the ear canals open. Given that proliferation of these terminals does not commence until after the onset of hearing (vision), the process appears to be triggered by experience, and may therefore play some role in the opening and closing of critical periods. GABA-mediated inhibition has a major impact on auditory response properties, such as frequency tuning and temporal precision (Chang et al. 2005; de Villers-Sidani et al. 2008; Dorrn et al. 2010; Tan and Wehr 2009; Tan et al. 2004; Wehr and Zador 2003; Wu et al. 2006, 2008; Zhang et al. 2003), and so it is expected that maturation of these physiological features would be correlated with structural changes of this kind.

In contrast to GABAergic puncta, glutamatergic terminals do not commonly make synaptic contacts with somata or proximal dendrites, although they may be tightly packed around somata in the neuropil (DeFelipe 1997; Freund et al. 1983; Kisvarday 1992; Somogyi et al. 1983a, b; Tamas et al. 1997). We found no examples of perisomatic puncta that were VGluT2-ir, consistent with recent observations in mouse A1, where TC contacts were primarily clustered on proximal dendrites close to the soma, but not in contact with it (Richardson et al. 2009). However, we did find that numerous VGluT1-ir terminals in close proximity to neurons with pyramidal cell profiles had the appearance of perisomatic contacts, sometimes positioned directly next to perisomatic VGAT-ir puncta. Fortunately, this feature has been explored previously and may serve to guide interpretation of our data. Alonso-Nanclares et al. (2004) studied such VGluT1-ir terminals around neurons in L2/3 and L5 in light and electron microscope preparations from the parietal cortex in adult rats.

They found that these terminals were apposed to the cell membrane and frequently adjacent to symmetric (inhibitory) axon terminals that made synaptic contact with the somatic membrane. However, the VGluT1 terminals made asymmetric contacts only with dendritic shafts and spines, and not somata. The functional significance of this unusual arrangement is not known, but the authors suggested that glutamate spillover from the VGluT1 synapse may influence GABAergic activity locally. In any case, the appearance of terminals in contact with or in close proximity to the somatic membrane appears to be a maturational feature that occurs in parallel for both VGluT1 and VGAT, and may contribute to balanced excitation and inhibition in these developing circuits (Dorrn et al. 2010; Sun et al. 2010).

Comments on the precocious maturation of layer 1

Much remains to be learned about the roles of TC and CC inputs to L1, and whether those functions change during postnatal development. Given that VGluT2 and VGAT immunoreactive puncta were prominent in L1a by P7, their impact on activity in A1 prior to the onset of hearing is likely significant. Even VGluT1, which had relatively low expression in L2–4 at P7, formed a significant band of terminals in L1a/b at P7. Although L1 contains few neurons, all GABAergic, it is a prominent site of convergence for the TC, CC, and subcortical projections of neurons from local and distant sources. VGluT2-ir terminals in A1, which are concentrated in L1a, arise from the MGv, but also include inputs from secondary and multisensory thalamic nuclei, such as the MGm, Sg, or posterior lateral/medial nuclei (MGd also projects to L1, but of non-primary areas). VGluT1-ir terminals are presumed to reflect CC inputs, although it is not known whether VGluT1 transporters may be present in TC projections of MGB neurons that express VGluT1 and VGluT2 mRNA (see “Co-expression of VGluT1 and VGluT2”). In their study of A1 in cats, Huang and Winer (2000) noted that some of the thickest TC axons were found after injections of the MGm. Upon reaching L1, these axons (up to 6 μ m in diameter) ran horizontally in L1a over long distances and appeared to come from the large magnocellular neurons unique to the MGm (Mitani et al. 1987; Niimi et al. 1984). These may correspond to the same class of large-diameter axons found in the macaque monkey, which were found to arise from calbindin immunoreactive neurons in the MGm (Hashikawa et al. 1995). Locally, within columns, the axons of subpopulations of *VGluT1*⁺ cells in L2–4 reach L1 (Callaway 2002; Ojima et al. 1991; Wallace et al. 1991b; Wozny and Williams 2011) and blend with convergent CC inputs from both hemispheres. Among the targets of the CC and TC terminations are the apical dendrites of pyramidal and interneuron subpopulations whose somata are located in L2–5 (Code and Winer 1985; Feldmeyer et al. 2005; Lubke and Feldmeyer 2007; Markram 1997; Mitani et al. 1985; Prieto and Winer 1999; Sousa-Pinto 1973; Winer 1984, 1985). The apical dendrites of L2/3 pyramidal neurons are concentrated in L1a, whereas the tufts of L5 neurons are evenly distributed throughout L1 (Smith et al. 2010; Vogt and Peters 1981). Finally, over-lying this dense matrix are VGAT-ir terminals concentrated in L1a, as well as dopaminergic, noradrenergic, cholinergic and serotonergic projections to L1a/b (Avendano et al. 1996; Campbell et al. 1987; Edeline 2003; Mesulam and Geula 1992; Wallace et al. 1991a). Their influences on activity are poorly understood, but likely to be significant.

The impressive blend of inputs to L1 from multiple sources is substantial in scope, even from an early stage of development (Konig et al. 1975), and therefore positioned to modulate

the activity of excitatory and inhibitory neurons from L1–5. As an example, Cruikshank et al. (2012) found that TC inputs to L1 could transmit synaptic signals sufficient to drive interneurons in L1, with associated inhibitory effects on L2/3 pyramidal neurons. The effect of a given TC input to L1, therefore, can have significant impact on activity well beyond L1. In addition, Ma et al. (2013) found that the numbers of different neuronal sub-types increased from P2 to P14 and that the response properties of those neurons evolved substantially over this period. With respect to hearing, it is reasonable to consider how the impact of TC and CC inputs to L1 differs before and after the onset of hearing, as the driving and modulatory influences appear to vary considerably across this period.

Maturation of vesicular transporters and the onset of hearing

Neurophysiological studies in altricial mammals such as mice, rats, and gerbils have shown that many basic response features are adult-like in A1 at the onset of hearing, presumably reflecting intrinsically generated genetic and electrical signals (Gurung and Fritzsche 2004; Tritsch et al. 2010). These include tonotopy (de Villers-Sidani et al. 2007), binaural matching of frequency tuning (Polley et al. 2013), and the laminar and topographic organization of feedforward TC functional response profiles (Barkat et al. 2011). One notable exception can be found in the cortical sensitivity to airborne tone bursts, which increases 100-fold (i.e., 40 dB) between P11 and P14 in accordance with the commensurate maturation of ear canal patency and cochlear biomechanics that also unfold during this brief period (Adise et al. 2014; Mikaelian et al. 1965; Mills and Rubel 1998). However, the dynamic expression levels of VGluT1, VGluT2, and VGAT in conjunction with changes in the intrinsic membrane properties (Metherate and Cruikshank 1999; Oswald and Reyes 2011) and ligand-gated receptor composition (Hsieh et al. 2002; Venkataraman and Bartlett 2013, 2014) in the auditory forebrain between P11 and P14 suggest that the rapid maturation of sound-evoked responses measured in A1 and MGB of intact animals during the first days of hearing may reflect a combination of peripheral and central changes.

Second-order auditory representational features, which are extracted either from the monaural signal over time (e.g., frequency modulation rate or amplitude modulation depth) or from dichotic features (e.g., interaural level difference) mature over a comparatively protracted time course in A1 (Brown and Harrison 2010; Carrasco et al. 2013; Insanally et al. 2010; Polley et al. 2013; Razak and Fuzessery 2007; Rosen et al. 2010; Sarro et al. 2011; Trujillo et al. 2013). Here too, the ongoing maturation of VGluT1, VGluT2, and VGAT considered alongside the delayed development of cortical inhibitory tone (Chang et al. 2005; Dorrn et al. 2010; Oswald and Reyes 2011) and spine protrusion density (Barkat et al. 2011; Schachtele et al. 2011) may provide a biological correlate to support these features. Looking forward, one major challenge for future research will be to understand whether and how changes in these critical biomarkers of auditory forebrain development mechanistically relate to the postnatal maturation of sound features that reach an adult-like state by the end of the second postnatal week (e.g., tonotopy, binaural frequency matching, threshold) versus those that do not reach maturity until the third postnatal week or longer (e.g., frequency modulation direction tuning, amplitude modulation depth encoding, or ipsilateral interaural level difference sensitivity).

Synaptic plasticity, critical periods, and vesicular transporters

The maturation of pre- and postsynaptic signaling machinery supports the progressive refinement of sensory feature representations but also establishes the timing of critical periods during which experience can shape the organization of functional circuits in the sensory cortex (Barkat et al. 2011; Insanally et al. 2009; Polley et al. 2013; Popescu and Polley 2010; Speechley et al. 2007; Takesian and Hensch 2013). Prolonged deprivation of afferent activity can lead to homeostatic adjustments in synaptic strength that serve to balance excitation and inhibition in affected circuits (Maffei et al. 2012). The adjustments are mediated by local structural changes in both pre- and postsynaptic structures, involving modifications at the transcript and protein levels (Batish et al. 2012; Cajigas et al. 2010, 2012; De Gois et al. 2005; Desai et al. 2002; Edwards 2007; Erickson et al. 2006; Holt and Schuman 2013; Lazarevic et al. 2011, 2013; Muller and Davis 2012; Perez-Otano and Ehlers 2005; Turrigiano and Nelson 2004; Vitureira et al. 2012; Wilson et al. 2005).

It is also well established that structural remodeling of axons, spines, and synapses is related to various forms of plasticity in cortical circuits (Bosch et al. 2014; Butz et al. 2009; De Roo et al. 2008; Gogolla et al. 2007; Holtmaat and Svoboda 2009; Yamahachi et al. 2009; Zito et al. 2009). The gross changes in axon terminal density and size that we observed in A1 and the MGB from P7 to adulthood could provide a small window into the numerous structural changes taking place at the synaptic level during this period. As an example, Coleman et al. (2010) studied modifications of VGluT2-ir thalamocortical synapses in L4 of the mouse visual cortex (V1). Synapse density and size were reduced after brief monocular deprivation lasting 3 days, but recovered by 7 days. The synaptic changes peaked at 3 days, corresponding to maximal depression of responses to the deprived eye (Blais et al. 2008). In contrast, monocular inactivation with TTX for 17–22 h had no effect on VGluT2-ir synapses. These results suggest that the diminished retinal activity in the deprived animals had a different impact on TC synapses compared to the absence of retinal activity in the TTX group, and that the structural changes varied with time (Nahmani and Erisir 2005).

Although presynaptic mechanisms are more often associated with short-term synaptic dynamics (Blackman et al. 2013; Fioravante and Regehr 2011), the Coleman study also draws attention to the possible involvement of presynaptic structures in longer-term homeostatic changes. In the presynaptic realm, there is tremendous heterogeneity in the proteins that regulate neurotransmitter release probability, which is an important determinant of synaptic strength (Cajigas et al. 2012; Lazarevic et al. 2013; Turrigiano 2012). Some of the mechanisms involved contribute to balancing glutamatergic and GABAergic signaling at the synapse (Burrone et al. 2002; Dickman et al. 2012; Thiagarajan et al. 2005; Tokuoka and Goda 2008; Wierenga et al. 2006). A variety of evidence indicates that the vesicular transporter proteins may be involved in scaling presynaptic GABA or glutamate storage and release through alterations in their levels on vesicles, as discussed earlier (De Gois et al. 2005; Erickson et al. 2006; Wilson et al. 2005; Wojcik et al. 2004).

With respect to mechanisms of plasticity, these results are intriguing since they imply that the different types of vesicular transport proteins are regulated independently, even in response to identical changes in activity. These differences, in turn, may reflect specific interactions with other presynaptic proteins associated with neurotransmitter release.

Bragina et al. (2007, 2010), for example, studied co-expression of VGluT1, VGluT2, and VGAT with several protein families involved in vesicle fusion and exocytosis, trafficking, and neurotransmitter release (e.g., synapsin, synaptophysin, synaptosomal-associated protein, synaptogyrin, vesicle-associated membrane protein, and syntaxin) (Bragina et al. 2007, 2010). The degree to which each of these proteins co-localized with VGluT1, VGluT2, and VGAT was distinctive. Thus, while there is significant heterogeneity in the molecular machinery that governs neurotransmitter release presynaptically, there is also specificity. Further, to the extent that the co-expression of these proteins is constant, the expression of VGluT1, VGluT2, and VGAT could be a useful index of associated structural modifications that occur during development, aging, and pathology.

Correspondence between gene and protein expression assays

In the present study, *VGluT1*, *VGluT2*, and *VGAT* mRNA levels derived from ISH and RNAseq assays were comparable across brain areas and developmental ages. In some instances (e.g., VGluT2 in MGv, VGAT in A1), however, gene and protein expression trajectories were not always well correlated. One factor that could account for these differences is that the vesicular transporter proteins and transcripts are typically localized in separate neuronal compartments and often different brain regions (e.g., cortex vs. thalamus). As summarized in Figs. 25 and 26, the mRNA transcripts of these genes are largely confined to the somatic cytoplasm, whereas their proteins are trafficked to presynaptic axon terminals, which may be local or distant from the soma (Chaudhry et al. 1998; Kaneko et al. 2002; Melone et al. 2005; Minelli et al. 2003a, b). Accordingly, neurons synthesizing *VGluT1* mRNA predominate in cortical areas, and the transporter protein can be found in the axon terminals of their CC, CT, and Ct targets (Rovo et al. 2012). *VGluT2*⁺ neurons are concentrated in subcortical brain areas, and the transporter protein is expressed in their thalamocortical (TC), tectothalamic (tT), and subcortical projections. *VGAT* mRNA appears to be expressed by all GABAergic neurons in cortical and subcortical regions, whereas expression of the protein in axon terminals may reflect both local and distant projections (Chaudhry et al. 1998; Dumoulin et al. 1999; Frahm et al. 2006; Henny and Jones 2006; Ito et al. 2011; Minelli et al. 2003a; Wang et al. 2009). Thus, differential localization of mRNA transcripts and their corresponding proteins is one reason why gene and protein expression levels may not be correlated within a given brain region and must be factored into the interpretation, as above.

Secondly, differing trajectories for gene and protein expression suggest that transcription and translation are regulated differently during postnatal development and in a manner that is also region specific. Numerous recent studies have demonstrated rather convincingly that gene and protein expression levels are only modestly correlated, implying that indices of mRNA abundance do not reliably predict protein concentrations (Ghazalpour et al. 2011; Nie et al. 2007; Park et al. 2009; Vogel and Marcotte 2012). Although methodological factors have been noted, these poor correlations are linked to fundamental differences in the regulation of transcription and translation for many genes. In the case of VGluT1 and VGluT2, for example, their transcription and translation can be differentially regulated by other proteins (e.g., BDNF and MeCP2) in a level-dependent manner that varies with postnatal age (Melo et al. 2013; Nguyen et al. 2012) (see Supplementary Fig. S28). In

addition, epigenetic factors and noncoding RNA can also regulate gene and protein expression differentially through multiple mechanisms that may change during development (Earls et al. 2014; Elramah et al. 2014). This variability underscores the importance of a multimodal approach to profiling gene and protein expression in neural circuits to account for such differences and more accurately describe their structural elements.

Species differences in vesicular transporter expression

In comparing the results of the present study to our prior studies of the auditory and visual systems in primates (Balaram et al. 2011; Balaram et al. 2013; Hackett et al. 2011b), we found significant differences in the expression of both *VGluT1* and *VGluT2*. In sensory cortex of the adult monkey, *VGluT2* mRNA levels are present at moderate levels in most pyramidal neurons in L2–4, punctuated by strong expression in larger pyramidal neurons of L3b. Expression in L5/6 is very weak in primates. In adult rodents, *VGluT2* levels are relatively low across layers. In the MGB and LGN, *VGluT1* mRNA is strongly expressed in mice, but not in primates. Conversely, *VGAT* is expressed in MGB of primates, but not rodents. We have noticed species differences for several other genes in preliminary studies, in agreement with a growing number of reports (Mashiko et al. 2012; Nehme et al. 2012; Van der Zee and Keijser 2011; Watakabe 2009; Zeng et al. 2012). Detailed documentation of the differences in gene expression between model species is clearly needed, but in its absence we must be vigilant to consider the potential for differences in the interpretation gene and protein profiling results.

Summary and future directions

The data contained in this study establish a broad baseline for further study of the changes in excitatory and inhibitory circuitry that occur during postnatal development in the auditory forebrain. The data also draw attention to the presynaptic machinery involved in neurotransmission, as well as homeostatic mechanisms that operate in development, aging, and hearing loss. Building on this foundation, future studies could reveal additional features that are likely to impact auditory-related activity during maturation.

First, given that gene and protein expression levels may be differentially regulated in both developing and adult animals, it will be important to identify and characterize the factors involved. Among these factors are the non-coding regulatory sequences (e.g., micro-RNA, long non-coding RNA) associated with the expression of each of these genes, as they would expand our understanding of the factors that govern changes in their expression during postnatal development before and after the onset of hearing (Elramah et al. 2014; Gao 2010; Olde Loohuis et al. 2012; Schratt 2009). Our current models of developmental plasticity and activity-dependent alterations in sensory processing lack these details, but would certainly be improved by their inclusion.

Second, in studies of developing sensory cortex, much emphasis has been placed on the maturation of pre- and postsynaptic glutamatergic (Barth and Malenka 2001; Blundon et al. 2011; Chun et al. 2013; Jiang et al. 2006; Lu et al. 2001; Walz et al. 2010; Wang et al. 2012b; Yanagisawa et al. 2004) and GABA receptors (Ben-Ari et al. 1994, 2012; Bosman et al. 2002; Davis et al. 2000; Fritschy et al. 1994; Heinen et al. 2004; Hensch 2005; Hornung

and Fritschy 1996; Huang 2009; Laurie et al. 1992; Maffei and Turrigiano 2008; Mohler 2006; Peden et al. 2008; Takesian et al. 2010). Changes in the amount and subunit composition of ligand-gated receptors are thought to be causally related to the timing of developmental windows that govern the translation of afferent activity patterns into long-term modification of synapses and representational maps (Kotak et al. 2013; Takesian et al. 2010; Venkataraman and Bartlett 2013). However, the developmental trajectories of all receptor types have not been fully documented in the auditory forebrain. The present findings indicate that their developmental trajectories will vary by brain region, cortical layer, and even cell type (see also Hackett et al. 2015). In addition, an important complement to the glutamatergic and GABAergic networks are the neuromodulatory systems (cholinergic, noradrenergic, and monoaminergic), whose projections converge in A1 and MGB from various sources. Integration of these patterns would provide a useful synthesis of the changing relationships between excitatory, inhibitory, and modulatory systems in adult and developing animals.

A third line of inquiry concerns the apparent absence of VGluT1 and VGluT2 co-localization in TC and CC terminals from P7 to adult. The two main possibilities are: (1) VGluT1 and VGluT2 are expressed at nominal levels in TC and CC projections, respectively (or not at all); or (2) VGluT1 and VGluT2 are sorted to different axon terminals (Fig. 26). Many configurations are possible. Given their importance for understanding normal patterns of excitatory neurotransmission in auditory cortex, a thorough understanding of these relationships should be pursued. A closely related subject is that glutamate (and GABA) transporter expression and trafficking are subject to plastic modification and may therefore be linked to homeostatic adjustments at the synaptic level. It would be of interest to document whether and how expression (or co-expression) and trafficking may be altered, and whether critical period windows are modified by plastic changes in the magnitude and sites of expression.

A fourth topic concerns the morphological changes in axon terminals that accompanied the onset of hearing. In A1, perisomatic VGAT and VGluT1 terminals became prominent after the onset of hearing. What functionality is conferred to cortical pyramidal cells as these terminals coalesce around the action potential initiation zone? What is the nature of the interaction between the VGAT terminals that form somatic synapses and the closely apposed VGluT1 terminals that do not? In MGB, we observed changes in the numbers and size of VGluT2 terminals during the period of pruning and morphological change between P7 and adulthood. Does the increase in VGluT2 terminal size in MGv follow a decrease in the number of terminals that synapse with MGv neurons, as in the VPM and LGN? What are the molecular mechanisms involved, and how might these events be impacted by alterations in auditory input? In addition, why did terminal size remain unchanged in the MGd and how might that impact the activity of MGd neurons as a function of age?

Fifth, the laminar patterns of VGluT1 maturation in A1 are of some interest, as expression in the middle layers (L2–4) lagged behind L1 and L5/6. The VGluT1 projections to each layer may reflect several different input sources, including local and distant cortical areas, inter-hemispheric projections, and even thalamic sources. Inputs to the middle layers also include subplate neurons, which are largely depleted from primary sensory areas by the onset of

hearing (Kanold 2004; Kanold and Luhmann 2010; Viswanathan et al. 2012). Presumably, these are VGluT1-ir but protein expression levels in the middle layers are very low before P14. A related question concerns the nature of the relationship between TC inputs and CC inputs as they mature. TC inputs to L1a and L4 are established well before hearing onset, but the CC projections develop later. How do those differential trajectories impact activity in A1?

Finally, the present study demonstrates that a multi-modal sequencing-driven approach to neurochemical profiling is both efficient and highly advantageous. On the front end, RNA sequencing is a powerful tool that can be used to reveal *all* of the genes that are highly expressed in a brain region or cell population of interest, or those that may be changing with time, experience, or other manipulation (see Hackett et al. 2015). Sequencing also provides a means to explore relationships among a set of functionally related genes, as well as make testable predictions about their regulation by other coding and non-coding genes or proteins. In that sense, the present study supports the viability and utility of a sequencing-driven approach to transcriptomic and proteomic profiling of neural circuitry and architecture. Upon identification of target genes from the sequencing data, targeted ISH and IHC can be subsequently employed to reveal their spatial expression patterns in intact tissue sections, where natural anatomical features are preserved. Multiplexed ISH and IHC using fluorescent probes permit detailed evaluation of co-expression and co-localization of several genes and proteins in cells or regions of interest. Documentation of the subpopulations that express one or more genes (or proteins) is an important endeavor in the neurochemical profiling of neuronal circuitry in auditory pathways, with implications for functional studies where genetic specificity is important (e.g., optogenetics). Together, this type of deep structural profiling provides a unique window into the functional subsystems that comprise a given pathway and can foster improved hypotheses about function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

A1	Primary auditory cortex, area 1
AC	Auditory cortex
AuD	Auditory cortex, dorsal area
AuV	Auditory cortex, ventral area
CC	Corticocortical connections

CT	Corticothalamic connections
Ct	Corticotectal connections
D	Medial geniculate body, dorsal division
DAPI	4',6-Diamidino-2-phenylindole
DC	Inferior colliculus, dorsal cortex
dLGN	Lateral geniculate nucleus, dorsal division
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
IC	Inferior colliculus
ICc	Inferior colliculus, central nucleus
IHC	Immunohistochemistry
ISH	In situ hybridization
Hip	Hippocampus
LC	Inferior colliculus, lateral cortex
M	Medial geniculate body, medial division
MGB	Medial geniculate body
MGd	Medial geniculate body, dorsal division
MGm	Medial geniculate body, medial division
MGv	Medial geniculate body, ventral division
NeuN	Neuron-specific RNA-binding protein, Fox-3
PAG	Periaqueductal gray
PP	Thalamus, peripeduncular nucleus
rf	Rhinal fissure
Rt	Reticular nucleus
RT	Reticulothalamic connections
SC	Superior colliculus
Sg	Thalamus, suprageniculate nucleus
TC	Thalamocortical connections
TRN	Thalamic reticular nucleus
tT	Tectothalamic connections
TeA	Temporal cortex area A
V	Medial geniculate body, ventral division
VGluT1	Vesicular glutamate transporter 1

VGluT2	Vesicular glutamate transporter 2
VGAT	Vesicular GABA/glycine transporter

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Fig. 1.

Architectonic delineation of A1 and MGB. Coronal sections at the level of A1 and MGB. **a** Nissl stain at low magnification showing A1 and adjoining areas. **b** Cytochrome oxidase stain at the same level as **a**. *Asterisk* denotes darker staining in L4. **c** Nissl stain at higher magnification showing laminar details of A1 and adjoining areas. **d** Nissl stain showing cytoarchitectonic details of MGB. *A1* primary auditory cortex area 1, *AuD* dorsal auditory cortex, *AuV* ventral auditory cortex, *TeA* temporal cortex area A, *V* ventral division of MGB, *D* dorsal of MGB, *M* magnocellular/medial division of MGB, *PP* peripeduncular, *Sg* supragenicular nucleus. *Roman numerals* denote cortical layers. *Scale bars a–c* 500 μ m, **d** 250 μ m

Fig. 2.

In situ hybridization and sample acquisition. **a** Single colorimetric ISH of *VGluT1*, *VGluT2*, *VGAT*, and *GAPDH* in adjacent sections from the same brain. **b** Triple FISH of a section from a different brain showing co-expression of *VGluT1* (red), *VGluT2* (white), and *VGAT* (green). **c** Photograph of a frozen brain during harvesting of samples from A1 and MGB for sequencing. The location of A1 within the AC is shown, along with a sketch of the 0.5 mm punch used to obtain samples. Note that the size and shape of the punch compresses tissue outside of the punched volume. The left MGB has been circumscribed prior to extraction. *Scale bars 1 mm all panels. A1* primary auditory cortex area 1, *AC* auditory cortex, *MGB* medial geniculate body

Fig. 3. Single colorimetric ISH assay for *VGlut1* in AC centered on A1 (*left column*), MGB (*middle column*), and IC (*right column*) at P7, P11, P14, P21, and adult. Coronal sections. *Roman numerals* indicate layers (*sp* subplate). *Scale bars* 250 μ m in *all panels*. *d* dorsal division of MGB, *v* ventral division of MGB, *DC* dorsal cortex, *IC* inferior colliculus, *ICc* central nucleus, *LC* lateral cortex, *PAG* periaqueductal gray

Fig. 4.

Single colorimetric ISH assay for *VGlut2* ISH in AC centered on A1 (*left column*), MGB (*middle column*), and IC (*right column*) at P7, P11, P14, P21, and adult. Coronal sections. *Roman numerals* indicate layers (*sp* subplate). *Scale bars* 250 μ m in *all panels*. *d* dorsal division of MGB, *v* ventral division of MGB, *DC* dorsal cortex, *IC* inferior colliculus, *ICc* central nucleus, *LC* lateral cortex, *PAG* periaqueductal gray

Fig. 5. Single colorimetric ISH assay for *VGATISH* in AC centered on A1 (*left column*), MGB (*middle column*), and IC (*right column*) at P7, P11, P14, P21, and adult. Coronal sections. *Roman numerals* indicate layers (*sp* subplate). *Scale bars* 250 μ m in *all panels*. *d* dorsal division of MGB, *v* ventral division of MGB, *DC* dorsal cortex, *IC* inferior colliculus, *ICc* central nucleus, *LC* lateral cortex, *PAG* periaqueductal gray

Fig. 6. Triple FISH assay in coronal sections of adult mouse at the level of A1 and MGB. The low-magnification image montages obtained at 10× show combined (a) and single channel expression (b–f) for *VGAT*, *VGluT1*, and *VGluT2* mRNA, counterstained by DAPI. *SC* superior colliculus, *Hip* hippocampus, *MG* medial geniculate body. Scale bars 500 μm in all panels

Fig. 7.

Quadruple FISH assay in a coronal section of adult mouse showing A1 and surrounding areas of auditory cortex. The image montages obtained at 40× in **a–f** show combined or single channel expression for *VGAT*, *GAPDH*, *VGlut1*, and *VGlut2* mRNA, counterstained by DAPI. *Scale bars* 500 μm in *all panels*. *A1* primary auditory cortex area 1, *AuD* dorsal auditory cortex, *AuV* ventral auditory cortex, *rf* rhinal fissure, *TeA* temporal cortex area A

Fig. 8. Quadruple FISH assay in a coronal section of adult mouse centered on A1 (from Fig. 7). **a** The image montages obtained at 40 $\times$ in **a** show combined expression for *VGAT*, *GAPDH*, *VGluT1*, and *VGluT2* mRNA, counterstained by DAPI. *Subpanels 1–8* show 100 $\times$ image stacks taken at sites in different layers of A1, as indicated in the composite image (*left*). **b–g** Higher-resolution examples of transcript labeling from subpanel 4 shown separately for each gene. *Scale bars a* 250 μ m, all other *panels* 20 μ m

Fig. 9.

Quadruple FISH assay in a coronal section of adult mouse showing MGB and surrounding regions. The image montages obtained at 40× in **a–f** show combined or single channel expression for *VGAT*, *GAPDH*, *VGluT1*, and *VGluT2* mRNA, counterstained by DAPI. *Scale bars* 500 μm in *all panels*. *D* dorsal division of MGB, *Hip* hippocampus, *M* medial division of MGB, *PPN* peripeduncular nucleus, *V* ventral division of MGB, *SC* superior colliculus, *Sg* supragenulate

Fig. 10.

Quadruple FISH assay in a coronal section of adult mouse MGB (from Fig. 9). **a** The image montages obtained at 40 $\times$ show combined expression for *VGAT*, *GAPDH*, *VGluT1*, and *VGluT2* mRNA, counterstained by DAPI. *Subpanels (right)* show 100 $\times$ image stacks from MGB divisions, adjoining nuclei, and the central nucleus of the inferior colliculus (ICc). **b–g** Higher-resolution examples of transcript labeling from MGv shown separately for each gene. *Scale bars a* 250 μ m, *all other panels* 20 μ m. *D and MGd* dorsal division of MGB, *Hip* hippocampus, *ICc* central nucleus of inferior colliculus, *M and MGm* medial division of

MGB, *PPN* peripeduncular nucleus, *V* ventral division of MGB, *SC* superior colliculus, *Sg* suprageniculate

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Fig. 11.

Triple FISH assays in the mouse auditory cortex at P7, P14 and adult. *Panels* show combined or single channel expression for *VGAT* (green), *VGluT2* (white), and *VGluT1* (red). **a** Adult AC centered on A1. *Row 1* low-magnification images (10×) of each gene shown separately and all three combined (Merge). *Row 2* higher magnification (40×) of L1–3. Note low levels of *VGluT2* in all layers. **b, c** Same as **a**, but at P14 and P7. Note elevated expression of *VGluT2* in L2–3 at P7 and co-localization with *VGluT1*. *Scale bars: top*

panels 10×, 200 μm; *other panels* 40×, 100 μm. *A1* primary auditory cortex area 1, *Hip* hippocampus

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Fig. 12.

Triple FISH assays in the mouse MGB at P7, P14 and adult. *Panels* show combined or single channel expression for *VGAT* (green), *VGlut2* (white), and *VGlut1* (red). **a** Adult MGB. *Row 1* low-magnification images (10×) of each gene shown separately and all three combined (Merge). Note absence of *VGAT* in MGB. *Row 2* 40× images in the MGv (v) to show co-localization of *VGlut1* and *VGlut2*. **b** Same as **a**, but at P14. Note co-localization of *VGlut1* and *VGlut2* at this age. **c** Same as in **b** and **c**, but at P7. Note very low levels of *VGlut1* expression in the MGd shown at 40× in the *bottom panels* (*VGlut1* absent in MGv)

at this age and co-localization with *VGluT2*. *Scale bars: top panels* 10×, 200 µm; *other panels* 40×, 100 µm. *D* dorsal division of MGB, *Hip* hippocampus, *M* medial division of MGB, *PPN* peripeduncular nucleus, *V* ventral division of MGB

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Fig. 13.

Graphical summaries of gene expression obtained by analyses of RNAseq and ISH measurements. *Top* Mean normalized read counts from RNAseq for *VGluT1*, *VGluT2*, and *VGAT* in A1 (blue lines) and MGB (red lines). *Bottom* Mean relative optical density of ISH for the same genes in A1 (blue), MGv (red), and MGd (green). In all plots, error bars denote standard deviation. For each data series, significant differences in expression levels between each age and all others ($p < 0.05$) are listed in Tables 4 (RNAseq) and 5 (ISH). Note that RNAseq samples contained both MGv and MGd (i.e., MGB). A1 primary auditory cortex area 1, ICc inferior colliculus central nucleus, MGB medial geniculate body, MGd dorsal division of MGB, MGv ventral division of MGB

Fig. 14.

Coronal section at the level of A1 and MGB showing immunoreactivity for VGluT2, VGluT1_{rb}, and NeuN at P7. *Top* the merged RGB images show VGluT2 (*green*), VGluT1 (*red*), and NeuN (*blue*) reactivity in the same tissue section. The *left column* contains an image of the full section. *Rectangular insets* correspond to midpower images of MGB and A1 in the *middle* and *right columns*. *Below* the RGB composites in panels below the *top row* are separated into *grayscale color* channels, where each channel corresponds to one protein

marker. *Scale bars: left column 1 mm; middle and right column 500 μ m. d dorsal division of MGB, v ventral division of MGB*

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Fig. 15.

Coronal section at the level of A1 and MGB showing immunoreactivity for VGluT2, VGluT1_{rb}, and NeuN in adult. *Top* the merged RGB images show VGluT2 (*green*), VGluT1 (*red*), and NeuN (*blue*) reactivity in the same tissue section. The *left column* contains an image of the full section. *Rectangular insets* correspond to midpower images of MGB and A1 in the *middle* and *right columns*. *Below* the RGB composites in *panels below the top row* are separated into *grayscale color* channels, where each channel corresponds to one protein

marker. *Scale bars: left column* 1 mm; *middle and right column* 500 μ m. *d* dorsal division of MGB, *v* ventral division of MGB

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Fig. 16.

Coronal section at the level of A1 and MGB showing immunoreactivity for VGAT, VGlut1_{gp}, and NeuN at P7. *Top* the merged RGB images show VGAT (green), VGlut1 (red), and NeuN (blue) reactivity in the same tissue section. The *left column* contains an image of the full section. *Rectangular insets* correspond to midpower images of MGB and A1 in the *middle and right columns*. *Below* The RGB composites in panels *below the top row* are separated into *grayscale color channels*, where each channel corresponds to one

protein marker. *Scale bars: left column 1 mm; middle and right column 500 μ m. d dorsal division of MGB, v ventral division of MGB*

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Fig. 17.

Coronal section at the level of A1 and MGB showing immunoreactivity for VGAT, VGlut1_{gp}, and NeuN in adult. *Top* the merged RGB images show VGAT (*green*), VGlut1 (*red*), and NeuN (*blue*) reactivity in the same tissue section. The *left column* contains an image of the full section. *Rectangular insets* correspond to midpower images of MGB and A1 in the *middle* and *right columns*. Below the RGB composites in panels below the *top row* are separated into *grayscale color* channels, where each channel corresponds to one protein

marker. *Scale bars: left column* 1 mm; middle and right column 500 μ m. *d* dorsal division of MGB, *v* ventral division of MGB

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Fig. 18.

Relative grayscale intensity measurements of VGLUT1_{gp}, VGLUT2, and VGAT immunoreactivity in A1. *Left* laminar intensity profiles from L1a to L6 at each age. *Right* mean relative grayscale intensity measurements (and standard deviations) of immunoreactivity by layer or sublayer (layers 1a–6)

Fig. 19.

Confocal images and puncta counts for VGluT2, VGluT1_{rb}, and NeuN in A1 at P7 and adult. *Top* single confocal slices from each layer of A1, showing immunoreactivity for VGluT2 and VGluT1. *Bottom* average puncta density of each protein by layer and age group. Scale bar 20 μm (links to Zoomify™ images for all ages contained in Supp. Figs. S12–S16)

Fig. 20.

Confocal images and puncta counts for VGAT, VGluT1_{gp}, and NeuN in A1 at P7 and adult. *Top* single confocal slices from each layer of A1, showing immunoreactivity for VGluT2 and VGluT1. *Bottom* average puncta density of each protein by layer and age group. *Scale bar* 20 μm (links to ZoomifyTM images for all ages contained in Supp. Figs. S19–S23)

Fig. 21. Confocal images and puncta counts for VGluT2, VGluT1_{rb}, and NeuN in MGB at P7 and adult. *Top* single confocal slices from each layer of A1, showing immunoreactivity for VGluT2 and VGluT1. *Bottom* average puncta density of each protein by layer and age group. *Scale bar* 20 μm . *MGv* ventral division of MGB, *MGd* dorsal division of MGB (links to Zoomify™ images for all ages contained in Supp. Figs. S17–S18)

Fig. 22. Confocal images and puncta counts for VGAT, VGlut1_{gp}, and NeuN in MGB at P7 and adult. *Top* single confocal slices from each layer of A1, showing immunoreactivity for VGlut2 and VGlut1. *Bottom* average puncta density of each protein by layer and age group. *Scale bar* 20 μm . *MGv* ventral division of MGB, *MGd* dorsal division of MGB (links to Zoomify™ images for all ages contained in Supp. Figs. S24–S25)

Fig. 23.

High-magnification confocal maximum intensity projection from five consecutive slices to show details of VGlut2 (*green*) and VGAT (*red*) puncta labeling in MGv and MGd at P7 and adult. *Scale* 20 μ m. *MGv* ventral division of MGB, *MGd* dorsal division of MGB

Fig. 24.

Graphical summaries of VGluT1, VGluT2, and VGAT expression in A1 and MGB obtained by analyses of RNAseq, ISH, and IHC assays. The data from Figs. 13, 19, 20, 21 and 22 were replotted to facilitate comparison between conditions and methods as a function of brain region and age. *Top* Primary auditory cortex (A1) at P7, P14, P21, and adult. *Bottom* medial geniculate body (MGB) at the same ages. Other conventions as in Fig. 13. Note the general correspondence between RNAseq and ISH data and differences for some IHC conditions (e.g., VGluT2 in MGB)

Fig. 25.

Summary of vesicular transporter gene and protein expression in A1, MGB, and IC at P7 and adult for VGluT1, VGluT2, and VGAT. Schematic drawings illustrate relative expression of each marker in cortical layers of A1, MGD (d), MGv (v), Rt, and IC (ICc and adjacent divisions). Gene expression levels of neuronal somata (*triangles, circles, ovals*) in each structure are indicated by *grayscale shading* (*black* highest expression). Protein expression levels are indicated by grayscale shading by cortical layer or subcortical region. For IC and Rt, relative immunoreactivity at P7 and adult were based on qualitative impressions (see Supplementary Figures 26 and 27). Major connections between structures are indicated by arrows. *Solid arrows* corticocortical (CC), thalamocortical (TC), and tectothalamic (tT) projections. *Dashed arrows* corticothalamic (CT), corticotectal (Ct), and reticulothalamic (RT) projections. *Brackets* from L1 to L6 indicate that TC projections are found in all layers. For VGluT2, *grayscale shading* density by layer is correlated with TC terminal density. *Question marks* (?) denote connections for which the vesicular transporter involved is not known or is speculative (i.e., VGluT1 TC; VGluT2 CC). *Asterisk* in the VGAT panel denotes that CC* projections of VGAT are primarily local (some long-range projections exist)

Fig. 26.

Schematic diagrams depicting possible variations in the trafficking and expression of VGLUT1 and VGLUT2 transporters in TC (MGB to AC) and CC (AC to OTHER) projections. In all examples, *VGLUT1* and *VGLUT2* mRNA is co-expressed in somata, but relative levels differ by brain region. **a** Only VGLUT2 is expressed on vesicles in TC axon terminals, and VGLUT1 is expressed on vesicles in all CC terminals. **b** VGLUT1 and VGLUT2 are sorted to different terminals. **c** VGLUT1 and VGLUT2 are co-expressed in TC and CC axon terminals; **d** VGLUT2 or VGLUT1 is expressed alone in some terminals, but co-expressed in others. Note that many additional configurations are possible. Sorting may vary by cortical layer or target cell type and may be modified by sensory experience or other manipulations. Note also that variations in trafficking may apply to other molecules expressed in these neurons (for further discussion, see “Co-expression of VGLUT1 and VGLUT2”). *MGB* medial geniculate body, *AC* auditory cortex

Table 1

Details of probes designed for in situ hybridization

Gene	Forward primer	Reverse primer	Accession no.	Position	Product length
Colorimetric probes					
<i>VGLUT1</i> (SLC17A7)	cttctacctgctcctcatctct	acacttctcctcgctcatct	NM_182993	972–1545	574
<i>VGLUT2</i> (SLC17A6)	catgggtcaacaacagcactatc	ctctccaatgctcctctctatg	XM_006540602	298–871	574
<i>VGAT</i> (SLC32A1)	taagaacctcaaggccgtgtccaa	cacataaatggccatgagcagcgt	NM_009508	1255–1832	578
<i>GAPDH</i>	tgctgagtatgtcgtggagtct	ggctccagggtttcttactcctt	NM_001289726	359–1107	749
Gene	Channel (color)	Catalog number	Accession no.	Position	
RNAscope multiplex fluorescence probes					
<i>GAPDH</i>	1 (FITC)	314091	NM_008084.2	21–935	
<i>VGLUT1</i> (SLC17A7)	2 (Cy3)	416631-C2	NM_182993.2	464–1415	
<i>VGLUT2</i> (SLC17A6)	3 (Alexa 647)	319171-C3	NM_080853.3	1986–2998	
<i>VGAT</i> (SLC32A1)	4 (Alexa 750)	319191-C4	NM_009508.2	894–2037	
3-Plex positive control probe					
<i>UBC</i>	1	320881	NM_019639.4	34–860	
<i>POLR2A</i>	2		NM_009089.2	2802–3678	
<i>PPIB</i>	3		NM_011149.2	98–856	
3-Plex negative control probe					
<i>DAPB</i>	1–3	320871	EF191515	414–862	

Table 2

Primary and secondary antibodies used

Antibody	Species	Supplier	Part number	Dilution	References
Primary antibodies					
VGAT*	rb	Synaptic Systems	131002	1:1000	Dudanova et al. (2007), Panzanelli et al. (2007)
VGluT1*	rb	Synaptic Systems	135303	1:1000	Herzog et al. (2006)
VGluT1*		Synaptic Systems	135304	1:2500–5000	Michalski et al. (2013), Siembab et al. (2010), Wouterlood et al. (2012)
VGluT1	rb	MABTech	VGT1-3	1:2000	Raju et al. (2006), Villalba and Smith (2011), Wojcik et al. (2004)
VGluT2	rb	Synaptic Systems	135403	1:2000	Gomez-Nieto and Rubio (2009), Herzog et al. (2006), Persson et al. (2006), Sergeeva and Jansen (2009), Toyoshima et al. (2009), Zhou et al. (2007)
VGluT2*	gp	Synaptic Systems	135404	1:2000	Michalski et al. (2013), Mikhaylova et al. (2014), Perederiy et al. (2013)
Fox3/NeuN*	ms	Covance	SIG39860	1:2000	Wimmer et al. (2010)
Control proteins					
VGluT1	CP	Millipore	AG208	10×	–
VGluT1	CP	Synaptic Systems	135-3P	10×	–
VGluT2	CP	Synaptic Systems	135-4P	10×	–

Antibody	Species	Supplier	Part number	Dilution	Primary antibody combination
Secondary antibodies					
Alexa 488	G-ms	Lifetech	A21200	1:500	
Alexa 488	Ch-ms	Lifetech	A21200	1:500	
Alexa 488*	Ch-rb	Lifetech	A21442	1:500	VGAT 131002
Alexa 594*	Ch-ms	Lifetech	A21201	1:500	Fox3 SIG39860
Alexa 594	G-ms	Lifetech	A11032	1:500	
Alexa 568	G-ms	Lifetech	A11004	1:500	
Alexa 647	G-gp	Lifetech	A21450	1:500	VGluT2 135403; VGluT1 135304
Alexa 647	G-rb	Lifetech	A21200	1:500	VGluT1 135303
Alexa 750*	G-ms	Lifetech	A21037	1:500	Fox3 SIG39860

ms mouse, *rb* rabbit, *gp* guinea pig, *G/g* goat, *Ch* chicken, *H* horse, *CP* control protein

Table 3

Analysis of variance (ANOVA) comparing differences in expression levels across age (P7 to adult) by brain region and gene for RNAseq (mean normalized read counts) and ISH (normalized grayscale intensity) assays

	VGAT	VGluT1	VGluT2
RNASeq			
A1	$F(3,20) = 10.437, p < 0.001$	$F(3,20) = 102.848, p < 0.001$	$F(3,20) = 23.083, p < 0.001$
MGB	$F(3,18) = 3.169, p = 0.050$	$F(3,18) = 15.062, p < 0.001$	$F(3,18) = 3.714, p = 0.031$
ISH			
A1	$F(4,25) = 0.766, p = 0.557$	$F(4,25) = 28.169, p < 0.001$	$F(4,25) = 7.206, p = 0.001$
IC	$F(4,25) = 1.905, p = 0.141$	$F(4,25) = \text{n/a}$	$F(4,25) = 14.960, p < 0.001$
MGd	$F(4,25) = \text{n/a}$	$F(4,25) = 10.343, p < 0.001$	$F(4,25) = 5.211, p = 0.003$
MGv	$F(4,25) = \text{n/a}$	$F(4,25) = 16.043, p < 0.001$	$F(4,25) = 7.054, p = 0.001$

$F(x,y) = z$, where x is degrees of freedom for between-groups comparison, y for within groups, and z is the F statistic. n/a denotes that no measurements were made due to absence of reactivity

Table 4

Summary statistics for RNAseq sample comparisons

	VGluT1		VGluT2		VGAT	
	A1	MGB	A1	MGB	A1	MGB
Adult (Ad)						
Mean	93.00	21.30	2.82	46.25	2.67	3.01
SD	7.45	6.81	0.27	5.29	0.22	0.87
$p < 0.05$	P7	P7	P7	None	P7	None
P21						
Mean	105.61	30.69	2.85	43.77	2.75	3.88
SD	14.03	11.72	0.25	5.63	0.35	1.34
$p < 0.05$	P7	P7, P14	P7	None	P7	None
P14						
Mean	99.59	17.91	3.23	56.38	2.81	5.01
SD	6.61	4.15	0.28	10.06	0.61	1.69
$p < 0.05$	P7	P7, P21	P7	None	P7	None
P7						
Mean	27.91	2.35	5.37	44.14	1.70	6.02
SD	2.88	0.87	1.16	5.56	0.30	1.75
$p < 0.05$	All	All	All	None	All	None

For each gene, the mean normalized read count and standard deviation (SD) are listed by postnatal age and brain region. For each condition, comparisons with all other age groups that reached significance are listed by age (i.e., P7, P14, P21, Ad, or All). Significance determined by Tukey post hoc comparisons ($p < 0.05$)

n/a no measurements available, *none* no significant differences with any other age group

Table 5

Summary statistics for ISH sample comparisons

	VGluT1				VGluT2				VGAT			
	AI	MGv	MGd	IC	AI	MGv	MGd	IC	AI	MGv	MGd	IC
Adult (Ad)												
Mean	0.91	0.39	0.35	0.0	0.02	0.58	0.63	0.48	0.21	0.00	0.00	0.26
SD	0.15	0.11	0.10	0.0	0.03	0.11	0.12	0.13	0.08	0.00	0.00	0.02
$p < 0.05$	P7	P7	P7	n/a	P7	P14	None	P11/14/21	None	n/a	n/a	None
P21												
Mean	0.99	0.47	0.34	0.0	0.04	0.67	0.65	0.74	0.19	0.00	0.00	0.30
SD	0.05	0.08	0.07	0.0	0.03	0.08	0.10	0.10	0.03	0.00	0.00	0.06
$p < 0.05$	P7	P7/11	P7	n/a	P7	P7	None	P7/Ad	None	n/a	n/a	None
P14												
Mean	0.95	0.51	0.40	0.0	0.04	0.74	0.77	0.77	0.18	0.00	0.00	0.26
SD	0.03	0.10	0.06	0.0	0.02	0.10	0.09	0.04	0.04	0.00	0.00	0.06
$p < 0.05$	P7	P7/11	P7/11	n/a	P7	P7/Ad	P7	P7/Ad	None	n/a	n/a	None
P11												
Mean	0.87	0.30	0.24	0.0	0.04	0.62	0.63	0.72	0.18	0.00	0.00	0.32
SD	0.05	0.11	0.08	0.0	0.02	0.03	0.03	0.05	0.01	0.00	0.00	0.08
$p < 0.05$	P7	P7/14/21	P14	n/a	P7	None	None	P7/Ad	None	n/a	n/a	None
P7												
Mean	0.56	0.13	0.16	0.0	0.09	0.49	0.53	0.56	0.19	0.00	0.00	0.35
SD	0.06	0.07	0.05	0.0	0.02	0.09	0.08	0.06	0.02	0.00	0.00	0.11
$p < 0.05$	All	All	P14/21/Ad	n/a	All	P14/21	P14	P11/14/21	None	n/a	n/a	None

For each gene, the mean normalized read count and standard deviation (SD) is listed as a function of postnatal age and brain region. For each condition, comparisons with all other age groups that reached significance are listed by age (i.e., P7, P11, P14, P21, Ad, or All). Significance determined by Tukey post hoc comparisons ($p < 0.05$)

n/a no measurements available, none no significant differences with any other age group