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Distributions of vesicular glutamate transporters 1 and 2 in the visual system of tree shrews (*Tupaia belangeri*)¹

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Abstract

Vesicular glutamate transporter (VGLUT) proteins regulate the storage and release of glutamate from synapses of excitatory neurons. Two isoforms, VGLUT1 and VGLUT2, are found in most glutamatergic projections across the mammalian visual system, and appear to differentially identify subsets of excitatory projections between visual structures. To expand current knowledge on the distribution of VGLUT isoforms in highly visual mammals, we examined the mRNA and protein expression patterns of VGLUT1 and VGLUT2 in the lateral geniculate nucleus (LGN), superior colliculus, pulvinar complex, and primary visual cortex (V1) in tree shrews (*Tupaia belangeri*), which are closely related to primates but classified as a separate order (Scandentia). We found that VGLUT1 was distributed in intrinsic and corticothalamic connections, whereas VGLUT2 was predominantly distributed in subcortical and thalamocortical connections. VGLUT1 and VGLUT2 were coexpressed in the LGN and in the pulvinar complex, as well as in restricted layers of V1, suggesting a greater heterogeneity in the range of efferent glutamatergic projections from these structures. These findings provide further evidence that VGLUT1 and VGLUT2 identify distinct populations of excitatory neurons in visual brain structures across mammals. Observed variations in individual projections may highlight the evolution of these connections through the mammalian lineage.

Keywords

VGLUT; lateral geniculate nucleus; pulvinar; superior colliculus; V1; NeuroLucida; nif-0000-10294

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Introduction

Vesicular glutamate transporters (VGLUTs) are a common feature of glutamatergic synaptic terminals in the mammalian central nervous system (CNS) (Ni et al., 1995; Bellocchio et al., 1998; Aihara et al., 2000; Bellocchio et al., 2000; Herzog et al., 2001; Fremeau et al., 2004b; Blakely and Edwards, 2012). Two isoforms, VGLUT1 and VGLUT2, are utilized in the majority of glutamatergic neurons in the CNS (Fremeau et al., 2001; Herzog et al., 2001; Kaneko et al., 2002), and may confer distinct presynaptic properties to their host neurons (Bellocchio et al., 1998; Takamori et al., 2000; Herzog et al., 2001; Kaneko and Fujiyama, 2002; Boulland et al., 2004; Fremeau et al., 2004a; b; Ito et al., 2011; Weston et al., 2011; Rovó et al., 2012; Storace et al., 2012; Balaram et al., 2013). In sensory pathways, VGLUT1 and VGLUT2 are largely restricted to discrete subsets of glutamatergic projections that also appear to be functionally segregated (Takamori et al., 2000; Kaneko and Fujiyama, 2002; Li et al., 2003; Todd et al., 2003; Hur and Zaborszky, 2005; Persson et al., 2006; Scherrer et al., 2010; Hackett et al., 2011; Balaram et al., 2013). In general, VGLUT2 is present in the terminals of feedforward or driving projections, which significantly alter or 'drive' neural activity in their postsynaptic targets (Sherman and Guillery, 1996; 1998; Sherman, 2005). For example, retinal projections to the lateral geniculate nucleus (LGN) are commonly identified as driving projections (Sherman and Guillery, 1996) and predominantly utilize VGLUT2 (Kaneko et al., 2002; Fujiyama et al., 2003; Nahmani and Erisir, 2005; Islam and Atoji, 2009; Balaram et al., 2011b). In contrast, VGLUT1 is often present in feedback or modulatory projections, which are described as projections that variably alter postsynaptic activity in their target cells (Sherman and Guillery, 1998; 2006; Sherman, 2007; 2012). Projections from visual cortex to the LGN are commonly identified as modulatory projections (Sherman and Guillery, 1996) and primarily utilize VGLUT1 in their terminations within the LGN (Bellocchio et al., 1998; Kaneko et al., 2002; Fujiyama et al., 2003; Balaram et al., 2011a; 2013). Both proteins are occasionally found in nontraditional driving and modulatory synapses (Wei et al., 2011; Rovó et al., 2012; Marion et al., 2013), but in general, VGLUT1 and VGLUT2 appear to be restricted to distinct glutamatergic projections in the CNS.

The use of a VGLUT isoform within a given projection is demonstrated by correlation of *VGLUT* mRNA labeling in neuronal cell bodies with VGLUT protein label in their respective terminals. This method appears to identify similar types of VGLUT1-positive and VGLUT2-positive projections between brain regions (Herzog et al., 2001; Kaneko and Fujiyama, 2002; Fremeau et al., 2004b; Graziano et al., 2008; Balaram et al., 2013), but the mRNA and protein distribution patterns of either VGLUT isoform may vary between species to some extent. For example, *VGLUT2* mRNA is only expressed in a subset of layers in primary visual cortex (V1) of rodents (Hisano et al., 2000; Fremeau et al., 2004b) but is found throughout the superficial layers of V1 in primates (Balaram et al., 2013). Species-specific variations in VGLUT1 and VGLUT2 distributions may simply reflect changes in the intracellular processes of glutamatergic neurons within a particular projection. Alternatively, the functional contributions of individual sensory projections may vary between species and VGLUT1 and VGLUT2 are still correlated with the presynaptic characteristics of those projections. In this case, differences in the relative distributions of

VGLUT1 and VGLUT2 in a known sensory projection could reflect alterations in the functional roles of that pathway within the larger sensory network. For the same example, the widespread distribution of *VGLUT2* mRNA in the superficial layers of V1 in primates may highlight driving projections from V1 to other visual areas, which could differ from similar projections in the superficial layers of V1 in rodents that lack *VGLUT2* mRNA.

In order to expand current knowledge on VGLUT distributions within mammalian visual projections, we chose to examine the relative distributions of VGLUT1 and VGLUT2 mRNA and protein in the visual system of tree shrews. Tree shrews provide a model visual system that contains features found in both rodents and primates, as well as specialized features that differ from those in other mammalian species (see Lund et al, 1985 for review). They are grouped in the Euarchontoglires clade between rodents and primates, but are more closely related to primates (Huchon et al., 2002). Tree shrews are small, quadruped mammals, similar in size to rodents, but maintain a diurnal lifestyle, similar to most primates. Tree shrews possess many of the same visual structures found in rodents and primates – the superior colliculus, lateral geniculate nucleus, lateral posterior or pulvinar nucleus, and primary and secondary visual cortical areas for example – but each structure and its associated pathways is slightly different in tree shrews compared to other species (Clark, 1925; 1929; 1942; Chomsung et al., 2008; Wong and Kaas, 2009; Chomsung et al., 2010). The tree shrew retina is cone-dominated (Samorajski et al., 1966), rather than rod-dominated as in rodents or primates (Glickstein, 1969; Wässle, 2004). The superior colliculus and lateral geniculate nucleus in tree shrews are both large and distinctly laminated, similar to primates, but both structures contain projections that differ from those found in rodent or primate species (Abplanalp, 1970; Harting et al., 1973b; Casagrande and Harting, 1975; Albano et al., 1979; Conway and Schiller, 1983; Conley et al., 1984). The pulvinar complex in tree shrews anatomically resembles the lateral posterior nucleus of rodents (Diamond et al., 1970; Harting et al., 1973a), but contains similar subdivisions and connections as the pulvinar complex of primates (Lyon et al., 2003a; b). Additionally, visual cortical areas such as V1 and V2 in tree shrews are located at similar cortical positions as V1 and V2 in highly visual rodents such as squirrels (Wong and Kaas, 2008; 2009), but their connections and lamination patterns more closely resemble those of V1 and V2 in primates (Clark, 1925; Snyder and Diamond, 1968; Lund et al., 1985). Lastly, V1 in tree shrews contains a unique pattern of segregation of geniculate inputs in layer 4 (Casagrande and Harting, 1975; Hubel, 1975; Humphrey et al., 1977; Norton et al., 1985), which relates to the larger segregation of parallel pathways across most projections in the tree shrew visual system (Lund et al., 1985). Thus, if VGLUT1 and VGLUT2 distinguish between subsets of glutamatergic projections in visual circuits, regardless of the species in question, then their relative distributions across visual structures in the tree shrew may reflect the functional properties of their host projections. These distributions should also be comparable, to some extent, to known VGLUT distributions in the visual systems of both rodents and primates as well.

Materials and Methods

Four adult tree shrews were used to examine the distribution of VGLUT1 and VGLUT2 mRNA and protein across subcortical and cortical visual structures. Three animals were

male, aged 8–9 months and weighing 120–135g, and one animal was female, aged 20 months and weighing 123g, but no age or sex-related differences were noted in this study. An additional three adult tree shrews were used to correlate the distributions of VGLUT2 protein across coronal, sagittal, and horizontal planes of section. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Louisville, and followed the guidelines published by the National Institutes of Health.

Tissue acquisition and histology

Each animal received a lethal intraperitoneal dose of sodium pentobarbital (250mg/kg; Beuthanasia-D, Merck Animal Health, Summit NJ) and placed in a dark enclosure for 3–5 minutes. When areflexive, the animal was transcardially perfused with sterile 0.1M phosphate-buffered saline (PBS; 0.9% NaCl) followed by sterile 4% paraformaldehyde (PFA) in 0.1M PBS. After fixation, the brains were removed from the skulls, rinsed briefly in sterile PBS, and postfixed overnight in 4% PFA. The following day, brains were blocked, bisected through the corpus callosum, placed in sterile cryoprotectant (30% sucrose in 0.1M phosphate buffer), and stored at 4°C prior to histology. Each block was cut into 40µm coronal cryosections using a sliding microtome, and separated into 8 series for further study. One series from every block was processed for cytochrome oxidase (CO; Wong-Riley, 1979) to reveal boundaries of visual structures.

Immunohistochemistry (IHC)

Reagents and concentrations for all immunohistochemistry preparations are listed in Table 1. One series from every block was stained for neuronal cell bodies using a commercial antibody against neuronal nuclei (NeuN) to reveal areal and laminar divisions of cortical and subcortical visual structures. A second series was stained for VGLUT2 protein and a third series was stained for VGLUT1 protein, both using commercial antibodies, to identify the distribution of both proteins in synaptic terminals across visual structures. All IHC reactions were carried out as previously described (Balaram et al., 2013).

Antibody characterization

Both VGLUT antibodies have been extensively characterized in previous studies (VGLUT2: AB_262186, VGLUT1: AB_887876, in JCN antibody database) and were specifically tested against primate and rodent tissue for related projects (Balaram et al., 2013 and unpublished results). Rabbit anti-VGLUT1 from Synaptic Systems recognizes a large fragment of the VGLUT1 protein and labels a single band at 65kDa in western blot preparations (manufacturer's information and Balaram et al, 2013). Mouse anti-VGLUT2 from Millipore accurately recognizes VGLUT2 protein in primates (Balaram et al., 2011b; Rovó et al., 2012; Balaram et al., 2013; Garcia-Marin et al., 2013), as well as squirrels and tree shrews (Wong and Kaas, 2008; 2009) and labels a single band at 56kDa in western blot preparations (manufacturer's information and Baldwin et al, 2012; Balaram et al, 2013).

In situ hybridization

Custom RNA probes against *VGLUT1* and *VGLUT2* were generated through cDNA libraries obtained from tree shrew liver and brain tissue using previously described techniques

(Tochitani et al, 2001). Forward and reverse primers for *VGLUT1* were *cttctacctgctcctcatctct* and *acacttctctctgctcatct* respectively, and targeted nucleotides 972–1545 of mouse *VGLUT1* (NM_182993). Forward and reverse primers for *VGLUT2* were *ggcaagggtcatcaaggagaa* and *gcacaagaatgccagctaaag* respectively, and targeted nucleotides 735–1125 of human *VGLUT2* (NM_020346). BLAST comparisons of each probe showed ~90–95% sequence homology with primate *VGLUT1* and *VGLUT2* and ~88–92% sequence homology with rodent *VGLUT1* and *VGLUT2*. However, comparisons of both sequences to each other showed no overlap, confirming the specificity of each probe to its target sequence. One series from each block was labeled for *VGLUT1* mRNA and a second series from each block was labeled for *VGLUT2* mRNA, to identify the cell bodies of neurons using each *VGLUT* isoform. ISH procedures were carried out as previously described (Balaram et al., 2013).

Image acquisition and analysis

Sections containing relevant visual structures were identified in CO or NeuN preparations and photographed using an MBF CX9000 camera mounted on an E80i microscope with NeuroLucida software (RRID:nif-0000-10294). Adjacent sections stained for *VGLUT1* and *VGLUT2* mRNA and protein were then photographed at low magnification, and digitally montaged at high magnification to preserve details of cell bodies and processes. Boundaries of individual layers and nuclei within each structure were determined by superimposing images of *VGLUT*-labeled sections on images of adjacent CO- or NeuN-labeled sections and marking boundaries revealed by CO and NeuN staining on the *VGLUT*-labeled images. All images were cropped and equally adjusted for brightness and contrast, but were otherwise unaltered. Images were then qualitatively analyzed for labeling intensity and cell density of *VGLUT1* and *VGLUT2* positive neurons and terminals, and then correlated to known projections in the tree shrew visual system to identify *VGLUT1*- or *VGLUT2*-positive pathways between visual structures.

Results

Lateral geniculate nucleus

The lateral geniculate nucleus (LGN) in tree shrews consists of six distinct layers, numbered 1 through 6 from medial to lateral (Glickstein, 1967; Laemle, 1968; Harting et al., 1973a; Casagrande and Harting, 1975; Hubel, 1975; Conway and Schiller, 1983; Conley et al., 1984; Lund et al., 1985) (Figures 1 and 2). CO and NeuN stained sections clearly revealed all six LGN layers (Fig. 1A, B). As previously described (Wong-Riley and Norton, 1988), LGN layers 1, 2, 4, and 5 showed dense CO reactivity, layers 3 and 6 showed moderate CO reactivity, and the interlaminar zones showed weaker reactivity compared to the surrounding LGN layers. In NeuN sections, NeuN-positive cell bodies were largely restricted to individual LGN layers, although fewer labeled cell bodies were present overall, compared to previous descriptions of Nissl-stained sections in the tree shrew LGN (Brunso-Bechtold and Casagrande, 1982; Conway and Schiller, 1983; Conley et al., 1984). However, other visual structures presented here showed nearly identical staining in NeuN or Nissl preparations (data not shown).

Diffuse labeling of VGLUT1 protein was present throughout the LGN (Figs. 1C, 2A), with only slightly denser labeling in layers 3 and 6 compared to layers 1, 2, 4 and 5. The interlaminar zones between LGN layers all labeled darkly for VGLUT1 protein, indicating greater densities of VGLUT1-positive terminals in these regions compared to the LGN layers. In contrast to VGLUT1, dense labeling of VGLUT2 protein was largely restricted to LGN layers 1 through 5, and far weaker VGLUT2 labeling was seen in layer 6, as well as the interlaminar zones (Fig. 1D, 2B). Layers 3 and 4 also appeared to merge at the dorsal and ventral LGN boundaries in all VGLUT2-labeled sections, but a clear difference in labeling density as well as a weakly labeled interlaminar zone separated these two layers in each case. Similarly, the ventral portion of layer 6 appeared continuous with the interlaminar zone between layers 4 and 5, and showed similarly diffuse VGLUT2 labeling as the remaining interlaminar zones. Laminar boundaries for layers 1, 2, and 5 were clearly visible in VGLUT2-labeled sections, but were less apparent for layers 3, 4, and 6.

In sections hybridized for *VGLUT1* mRNA (Fig. 1E, 2C), scattered populations of weakly labeled cells were present throughout the LGN, but were concentrated in different locations depending on the layer. Layers 1 and 2 both showed even distributions of neurons that weakly expressed *VGLUT1* mRNA, with no apparent intralaminar differences. Layer 3 contained weakly expressing *VGLUT1*-positive cells concentrated through the center of the layer. Layer 4, in contrast, contained two strips of cells along its dorsal borders that moderately expressed *VGLUT1* mRNA with hardly any *VGLUT1*-positive cells in between them. Towards the medial and ventral portions of layer 4, cells that expressed *VGLUT1* mRNA were more evenly distributed through the layer (Figs. 2C). Layer 5 contained similar distributions of cells as layers 1 and 2. Lastly, cells in layer 6 weakly expressed *VGLUT1* mRNA and were primarily located in the dorsal half of the layer, with far fewer cells in ventral locations throughout the LGN. Very few cells in the interlaminar zones expressed *VGLUT1* mRNA.

Sections hybridized for *VGLUT2* mRNA (Fig. 1F, 2D) showed dense distributions of *VGLUT2*-positive (*VGLUT2*+) neurons throughout the LGN. Layers 1 and 2 both contained even distributions of neurons that densely expressed *VGLUT2* mRNA. Neurons in layer 3 labeled more moderately for *VGLUT2* mRNA and were clustered together in the center of the layer compared to the medial and lateral edges. Layer 4 contained two narrow strips of neurons, which densely expressed *VGLUT2* mRNA and were packed in line with each other, along its lateral and medial boundaries, with more diffusely spread *VGLUT2*+ cells between the two strips. The lateral strip of *VGLUT2*-positive cells contained the densest *VGLUT2* expression of all geniculate cells and was easily visible at the border of layers 4 and 5 throughout the LGN (Fig. 2D). Layer 5 contained a similar distribution of neurons as the center of layer 4 that all densely expressed *VGLUT2* mRNA. Layer 6, in contrast, only contained a few scattered cells that were smaller and more moderately labeled for *VGLUT2* mRNA compared to cells in the other LGN layers. Lastly, all the interlaminar zones of the tree shrew LGN contained mixed distributions of neurons that moderately labeled for *VGLUT2* mRNA.

Superior Colliculus

The superior colliculus (SC) in tree shrews is large and distinctly laminated (Campbell et al., 1967; Glickstein, 1967; Laemle, 1968), compared to that of similarly sized mammals (May, 2006), with segregated visual and visuomotor functions at different laminar depths. Retinal and cortical projections largely target the superficial layers of the SC (Figures 3 and 4). Retinal projections terminate primarily in the zonal layer (SZ) and upper and lower superficial gray layers (uSGS and lSGS, respectively), and secondarily in the optic layer (SO) (Campbell et al., 1967; Laemle, 1968). Striate cortex projections to the SC terminate densely along the boundary between the upper and lower superficial gray layers and more diffusely through the remaining superficial SC layers (Harting and Noback, 1971). CO stained sections through the SC (Fig. 3A) identified an additional separation within the upper SGS, where the ventral division (USGS_v) labeled more densely for CO than the dorsal division (USGS_d) in all cases. These divisions are visible, but not defined, in previous descriptions of CO (Wong-Riley and Norton, 1988) and Nissl (Snyder and Diamond, 1968; Albano et al., 1979) sections through the tree shrew SC. In comparison to the upper SGS, the lower SGS and SO both labeled moderately for CO. NeuN stained sections through the SC (Fig. 3B) clearly identified the SZ and SO as cell sparse layers flanking the SGS. A clear separation was not visible between the upper and lower sublayers within the SGS, but the upper SGS was differentiable from the lower SGS by smaller, more densely packed neurons. Cells in the upper SGS primarily project to the LGN, while cells in the lower SGS project to subdivisions of the pulvinar complex (Albano et al., 1979; Luppino et al., 1988; Chomsung et al., 2008).

Both VGLUT1 and VGLUT2 protein (Fig. 3C–D, 4A–B) were differentially distributed in the five laminar divisions of the SC. The SZ labeled weakly for VGLUT1 and densely for VGLUT2. The upper SGS could be divided into dorsal and ventral divisions with complementary staining patterns; the dorsal division stained weakly for VGLUT1 but densely for VGLUT2, while the ventral division stained densely for VGLUT1 and weakly for VGLUT2 (best seen in Figs 3C, D). The lower SGS stained moderately for VGLUT1 and weakly for VGLUT2, and the SO showed even labeling of both VGLUTs surrounding the fiber pathways traversing this layer.

In mRNA preparations, no *VGLUT1* mRNA was visible anywhere in the SC (Fig. 3E, 4C). A few darkly labeled neurons were present along the boundary of the periaqueductal gray (Fig. 3E), but no neurons in the visually responsive SC layers expressed *VGLUT1* mRNA. *VGLUT2* mRNA however, was differentially expressed across the superficial SC (Fig. 3F, 4D). The SZ did not express *VGLUT2* since almost no cells are present in this layer of the SC. The upper SGS showed scattered populations of small neurons that weakly expressed *VGLUT2* mRNA, most of them concentrated in the dorsal division and far fewer present in the ventral division. The lower SGS contained dense populations of larger neurons that densely expressed *VGLUT2* mRNA, while the SO contained only a few scattered neurons that expressed *VGLUT2* mRNA. Thus, all VGLUT preparations identified the SZ, two tiers of the upper SGS, the lower SGS, and the SO in the tree shrew SC.

Pulvinar complex

The pulvinar complex in tree shrews can be divided into three distinct nuclei that differentially label for VGLUT1 and VGLUT2 mRNA and protein (Figs. 5 and 6) (Chomsung et al., 2008; Wei et al., 2011). All three pulvinar divisions have been identified previously, based on connectional and architectonic evidence (Lyon et al., 2003a; b; Chomsung et al., 2008; 2010). The dorsal pulvinar (Pd) lies dorsomedial to the central pulvinar (Pc), while the ventral pulvinar (Pv) lies ventral to Pc. Most of Pc and Pv lie lateral to the brachium of the superior colliculus, but parts of both nuclei are often seen medial to the brachium as well. All divisions of the pulvinar complex showed relatively even distributions of CO and NeuN (Fig 5A, B); a few patches of darker CO staining were scattered through the central nucleus, and neurons here appeared slightly more clustered than cells in Pd or Pv, but overall, both stains did not conclusively differentiate pulvinar divisions in tree shrews. Similar results for CO and Nissl stained sections of the tree shrew pulvinar have been previously reported (Lyon et al., 2003a).

VGLUT1 and VGLUT2 protein distributions were less distinct in the pulvinar complex compared to the LGN or SC, but some differences between nuclei were still noticeable in both preparations (Figs 5C–D, 6A–B). First, although VGLUT1 immunoreactivity was evenly distributed across most of the pulvinar, the region between Pd and Pc showed irregular patches of slightly darker labeling for VGLUT1 (white asterisk, Fig 5C) and most of Pc showed uniformly denser VGLUT1 labeling compared to Pd and Pv (Figs 6A–C). Similar to previous descriptions (Chomsung et al., 2008; Wei et al., 2011), in VGLUT2 preparations, Pd showed dense, punctate labeling for VGLUT2 while Pv showed almost none, especially in the region of Pv medial to the brachium of the colliculus, which lacks SC input and receives dense input from striate cortex instead. Pc showed irregular distributions of moderately labeled VGLUT2 (Fig 5D, 6D–F). A small region between the dorsal borders of Pd and Pc (white asterisk, Fig. 5D) showed much sparser VGLUT2 terminal labeling, but the few VGLUT2-positive terminals present in this region were clustered together in tight groups compared to VGLUT2-positive terminals in the surrounding regions of Pd and Pc. This pattern of VGLUT2 labeling was remarkably similar to that seen in the medial inferior pulvinar of monkeys (Balaram et al., 2013) and the whole pale-stained region did not continue across the brachium of the SC.

Cells in all three divisions of the pulvinar expressed both *VGLUT1* and *VGLUT2* mRNA (Figs 5E–F, 6C–D). *VGLUT1* mRNA was expressed at low levels in all three nuclei, with Pd and Pv containing slightly fewer *VGLUT1*-expressing neurons compared to Pc (Fig. 5E, 6G–I)). In contrast, *VGLUT2* mRNA was expressed at high levels in all three nuclei, and cells in Pd showed denser *VGLUT2* labeling compared to cells in Pc and Pv (Fig. 5F, 6J–L). Distinct boundaries between nuclei were not visible in *VGLUT* mRNA preparations, but both *VGLUT1* and *VGLUT2* mRNA distributions distinguished the dorsal pulvinar from the central and ventral pulvinar nuclei based on the density of *VGLUT* expression within positively stained cells.

Primary visual cortex (V1)

Primary visual cortex, or V1, occupies a large expanse of caudomedial neocortex and is easily identified by the distinct stratification of its neocortical layers, compared to those of other cortical areas, in the tree shrew brain (Figures 7 and 8) (Snyder and Diamond, 1968; Kaas et al., 1972; Lund et al., 1985; Wong and Kaas, 2009). Layer 1, the dorsal most laminar division below the pial surface, largely consists of dense neuropil while layer 2, immediately ventral to layer 1, consists of small, densely packed neurons arranged in a thin band above layer 3. Layer 3 occupies most of the supragranular depth of V1, and can be divided into three distinct sublayers based on variations in neuronal size and density, termed 3A, 3B, and 3C, from dorsal to ventral in V1. Layer 4 of V1 is remarkably distinct in tree shrews, and consists of two densely populated granular layers, 4A and 4B, separated by a distinct cell-sparse cleft. Two thin rows of neurons immediately adjacent to either side of the cleft receive monocular input from the contralateral eye, while the remaining neurons throughout 4A and 4B receive binocular input instead (Fitzpatrick, 1996). Layer 5 is sparsely populated with medium and large cells, while layer 6 displays an upper tier of closely arranged large and medium cells and a lower tier of small cells that diffuse into the white matter below V1. These layer boundaries follow Hässler's (1967) scheme for laminar designations in V1 across mammals, rather than Brodmann's (1909) laminar scheme, since Hässler's scheme is more appropriate for comparisons of V1 functions across rodents, tree shrews, and primates (Brodmann, 1909; Hässler, 1967; Harting et al., 1973a; Hubel, 1975; Casagrande and Kaas, 1994). NeuN stained sections through V1 identified the same layers and sublayers described above (Fig. 7B), and revealed sharper interlaminar boundaries compared to those seen in Nissl stains of tree shrew V1 (see Lund et al, 1985 for example). A distinct subdivision of layer 5 was also visible, consisting of a thin band of weakly labeled cells below the ventral boundary of layer 4 by and above the larger, more densely stained neurons in the ventral portion of layer 5, known as 5B. This superficial portion of layer 5, termed 5A in primates, appears to be a common feature of highly visual mammals (Balaram and Kaas, 2014). Layer 6 could also be differentiated into two divisions, a dorsal layer 6A and a ventral layer 6B, based on the size and packing density of neurons in this layer. CO stained sections of V1 also showed similar results to those previously described (Fig 7A) (Wong-Riley and Norton, 1988; Wong and Kaas, 2009). Layer 4 showed the densest CO reactivity, with slightly denser reactivity at the dorsal and ventral boundaries compared to the middle of the layer, followed by moderate CO reactivity in 6A, 5A, and parts of 3C. The remaining layers showed evenly weak CO reactivity throughout V1.

Labeling for VGLUT1 and VGLUT2 protein in V1 of tree shrews revealed dense distributions of both proteins, with remarkably distinct laminar profiles for each isoform. (Figs 7C–D, 8A–B). VGLUT1 was abundantly expressed in all V1 layers, including layer 1, but each layer showed slight changes in staining density throughout the area (Fig. 7C, 8A). The superficial extent of V1, from layer 1 to layer 3C, displayed a gradual decrease in VGLUT1 label, which successively distinguished each layer at its dorsal and ventral boundaries. Both divisions of layer 4, 4A and 4B, showed significantly less VGLUT1 label than any other layer, clearly distinguishing them in the middle of V1. However, the cell-sparse cleft between 4A and 4B contained high levels of VGLUT1 (best seen in fig. 7C). Both subdivisions of layer 5 and layer 6A displayed moderate VGLUT1 reactivity, and

appeared continuous through the ventral depth of V1. Layer 6B however, labeled weakly for VGLUT1 and appeared as a light band above the white matter below V1. In contrast to the evenly dense VGLUT1 label, VGLUT2 labeling in V1 was highly restricted (Fig. 7D, 8B). The greatest density of VGLUT2 label in V1 was located in layer 4, which was clearly visible as a dark band throughout V1. Layers 4A and 4B, as well as the cell-sparse cleft, contained punctate distributions of densely labeled VGLUT2-positive terminals, with slightly denser labeling along the ventral boundary of 4B compared to the rest of layer 4. Similarly dense VGLUT2 label was seen in layer 3B, which contained a thin band of VGLUT2-labeled terminals that periodically clustered and extended up through the superficial layers to layer 1 (fig. 8B). These extended VGLUT2-positive terminations were more frequent in the monocular zone of V1 (best seen in fig. 7D). The remaining superficial V1 layers contained low levels of VGLUT2 protein in the neuropil as well. The deep layers of V1 contained sparse patches of VGLUT2-positive terminations in layer 5B, and an even band of moderately labeled VGLUT2 terminals in 6A, but little to no VGLUT2 label in layers 5A or 6B.

VGLUT1 mRNA distributions in V1 followed similar patterns compared to VGLUT1 protein distributions (Figs. 7E, 8C). *VGLUT1* mRNA was densely expressed in all V1 layers, excluding layer 1, but each layer showed slight variations in the density of *VGLUT1*-positive neurons. The superficial V1 layers showed a gradual decrease in *VGLUT1* mRNA expression from dorsal to ventral, such that layer 2 contained large cells that strongly expressed *VGLUT1* mRNA and layer 3C contained small cells with little or no *VGLUT1* mRNA expression. Most cells in layer 4 expressed moderate levels of *VGLUT1* mRNA as well. Interestingly, two thin bands of neurons with slightly elevated *VGLUT1* expression were clearly visible along either side of the cell sparse cleft in V1 (best seen in fig. 7E). Lastly, layers 5A and 6B contained small cells with moderate or weak *VGLUT1* mRNA expression while layers 5B and 6A contained much larger cells with denser *VGLUT1* mRNA expression. Cells in 6A were more condensed compared to cells in 5B, which identified the border between layers 5 and 6 in V1. *VGLUT2* mRNA was almost entirely absent in V1. A few weakly positive neurons were found scattered along the ventral border of 5B, and several more were present in layer 6B, but the vast majority of neurons in V1 did not express *VGLUT2* mRNA.

Discussion

The present study aimed to characterize the distribution of VGLUT1 and VGLUT2 in the visual system of tree shrews, in order to determine whether VGLUT isoforms are differentially restricted to functional classes of glutamatergic projections. A summary of these findings is presented in figure 9. The results demonstrate that VGLUT1 and VGLUT2 are separately utilized in most glutamatergic projections through visual structures in tree shrews, but the overlapping distribution of both VGLUTs in some structures suggests a more heterogeneous use of VGLUT isoforms in the transmission of visual information. The distributions of each VGLUT isoform in the tree shrew visual system are largely consistent with VGLUT distributions in rodent (Bellocchio et al., 1998; Aihara et al., 2000; Hisano et al., 2000; Kaneko and Fujiyama, 2002; Kaneko et al., 2002) and primate (Wong and Kaas, 2010; Balaram et al., 2011a; b, 2013; Baldwin et al., 2013) visual systems as well, indicating

that VGLUT1 and VGLUT2 characterize distinct types of glutamatergic projections. When compared with the functional attributes of these projections, it becomes evident that VGLUT1 and VGLUT2 are generally characteristic of modulatory and driving glutamatergic neurons in most visual structures.

VGLUT1- and VGLUT2-positive projections in the tree shrew visual system

In every visual structure examined, VGLUT2 was restricted to a discrete subset of glutamatergic pathways (figure 9). The dense VGLUT2 labeling seen in retinorecipient layers of the LGN and SC (Campbell et al., 1967; Laemle, 1968) indicate that retinal ganglion cell projections to both structures utilize VGLUT2. LGN projections to layer 4 of V1 (Diamond et al., 1970; Harting et al., 1973a; Casagrande and Harting, 1975; Conley et al., 1984; Fitzpatrick, 1996) also predominantly use VGLUT2, as revealed by robust *VGLUT2* mRNA expression in all LGN layers, as well as dense VGLUT2 terminal labeling in layer 4 of V1. The band of VGLUT2-positive terminations in layer 3B, as well as the regular pattern of extended VGLUT2-positive terminations through layer 1, likely arises from LGN layers 3 and 6 (Hubel, 1975; Carey et al., 1979; Conley et al., 1984; Lund et al., 1985) and may alternate with modules of intrinsic connections in V1 (Rockland et al., 1982). Some of these superficial projections may arise from cells expressing *VGLUT2* mRNA in the ventral pulvinar as well (Carey et al., 1979; Lund et al., 1985; Lyon et al., 2003b). The relevance of sublaminar differences in *VGLUT2* expression in the LGN, particularly the densely labeled *VGLUT2*-positive neurons along the boundaries of LGN layer 4, is unclear since corresponding changes in VGLUT2 terminal labeling in layer 4 of V1 were not apparent. However, the dense band of *VGLUT2*-positive neurons along the lateral boundary of LGN layer 4 may also be associated with the interlaminar zone between LGN layers 4 and 5, which projects to layer 1 of V1 instead (Carey et al., 1979), and these cells could contribute to the periodic VGLUT2 terminal labeling seen in that layer of V1.

Tectal projections to the LGN and pulvinar in tree shrews predominantly utilize VGLUT2 as well. Projections from the upper SGS of the SC target layers 3 and 6 of the LGN as well as the interlaminar zone between LGN layers 4 and 5 (Harting et al., 1973b; Albano et al., 1979; Fitzpatrick et al., 1980; Diamond et al., 1991), and small, moderately labeled cells expressing *VGLUT2* mRNA in the upper SGS correspond to the moderately labeled VGLUT2-positive terminals distributed in these LGN regions. All three tectorecipient LGN layers target the superficial layers of V1 (Carey et al., 1979; Conley et al., 1984) and also predominantly use VGLUT2, as discussed above. Projections from the lower SGS of the SC target the dorsal and central divisions of the pulvinar (Harting et al., 1973b; Casagrande and Harting, 1975; Albano et al., 1979; Luppino et al., 1988; Chomsung et al., 2008), and the dense expression of *VGLUT2* mRNA by cells in this layer matches the dense VGLUT2 terminal labeling seen across the dorsal pulvinar and corresponding parts of the central pulvinar as well. Individual tectopulvinar terminals have been found to contain VGLUT2 (Wei et al., 2011), and the ultrastructure of VGLUT2-positive terminals in the tree shrew pulvinar nucleus is identical to that of tectopulvinar terminals (Chomsung et al., 2008). Tectopulvinar terminals in tree shrews do not exhibit classic driving or modulatory characteristics (Wei et al., 2011), and may release other neurotransmitters in addition to glutamate (Stepniewska et al., 2000; Masterson et al., 2010), but the presence of VGLUT2

in this projection is consistent with VGLUT2-positive tectopulvinar projections in other visual mammals as well (Baldwin et al., 2011; Balaram et al., 2011b; Baldwin et al., 2013).

Pulvinar projections to cortical visual areas are rather complex in tree shrews (Harting et al., 1972; 1973a; Carey et al., 1979). Retrograde tracing techniques suggest two major pathways (Luppino et al., 1988; Lyon et al., 2003b; Chomsung et al., 2008). One pathway from the Pc and Pv projects to occipital visual areas, and another pathway from the Pd and Pc projects to temporal visual areas (Luppino et al., 1988; Lyon et al., 2003b). More recent anterograde tracing techniques (Chomsung et al., 2010) have shown that Pd and Pc each project to two different regions of the temporal cortex. The dense *VGLUT2* mRNA seen in all divisions of the pulvinar implies that pathways to both the striate and temporal cortex predominantly utilize VGLUT2 in their synaptic terminations, but the weaker expression of *VGLUT1* mRNA in many pulvinar cells suggests that both VGLUT isoforms are utilized to different extents in individual pulvinar projections to cortical areas. Lastly, the scattered distributions of *VGLUT2* mRNA expressing neurons in the deep layers of V1 may project to the ventral pulvinar, as well as layers 3 and 6 of the LGN (Casseday et al., 1979; Usrey and Fitzpatrick, 1996), and contribute to more diffusely labeled VGLUT2 terminations in these locations.

VGLUT1 also appears to be restricted to a subset of glutamatergic projections in the tree shrew visual system. The dense expression of *VGLUT1* mRNA across all layers of V1 suggests that most intrinsic V1 projections exclusively utilize VGLUT1. The same is likely true for V1 projections to other cortical visual areas, given the exclusive *VGLUT1* mRNA expression in the superficial layers of V1. Feedback projections from V1 to the SC primarily arise in layer 5 (Casseday et al., 1979; Lund et al., 1985), and the strong expression of *VGLUT1* mRNA by neurons in this layer match the dense VGLUT1 terminal labeling in the corticorecipient layers of the SC (Huerta et al., 1985). Similarly, V1 projections to the LGN primarily arise from layer 6A, and neurons in this layer strongly expressed VGLUT1 mRNA while the corticorecipient interlaminar zones of the LGN contain dense distributions of VGLUT1-positive terminals. The ventral pulvinar also receives projections from layer 5 and 6B of V1 (Usrey and Fitzpatrick, 1996), both of which also express *VGLUT1* mRNA and likely contribute to the dark VGLUT1 terminal labeling across this pulvinar division. Although corticopulvinar terminals are larger than those of other corticothalamic projections, such as cortical terminations in the LGN, corticopulvinar projections in other species appear to utilize VGLUT1 in their terminations as well (Rovó et al., 2012).

Some projections in the tree shrew visual system appear to employ both VGLUT1 and VGLUT2. Weak *VGLUT1* mRNA expression alongside strong *VGLUT2* mRNA expression in neurons of the LGN and pulvinar complex implies that these projections utilize both isoforms in their target synapses. Individual neurons have been found to express both *VGLUT1* and *VGLUT2* mRNA (Barroso-Chinea et al., 2007; 2008), but both proteins do not colocalize to single synapses, suggesting that each transcript is differentially regulated within single neurons (Herzog et al., 2001; De Gois et al., 2005; Rovó et al., 2012). Thus, the LGN and pulvinar may contain overlapping or segregated distributions of VGLUT-positive projections. In the LGN for example, neurons expressing *VGLUT1* mRNA may contribute to the diffuse VGLUT1 terminal label in the geniculorecipient layers of V1, or they may project intrinsically (Hajdu et al., 1982) and contribute to the moderate VGLUT1

terminal labeling within each LGN layer. Similarly, given the variety of efferent projections from each division of the pulvinar nucleus (Lyon et al., 2003b), weakly expressing *VGLUT1*-positive neurons in the pulvinar that project to the cortex, striatum, or amygdala, may be coincident with *VGLUT2*-positive projections, or they could represent distinct projections with different targets in cortical or subcortical areas. In cortex, layers 5B and 6B both contained neurons that weakly expressed *VGLUT2* mRNA alongside many more neurons that densely expressed *VGLUT1* mRNA, and both layers specifically target the ventral pulvinar and layers 3 and 6 of the LGN (Casseday et al., 1979; Usrey and Fitzpatrick, 1996). It is likely that neurons in these V1 layers are related to the fine *VGLUT2*-positive terminations seen in the recipient locations of both thalamic structures, but whether these terminations are distinct from *VGLUT1*-positive terminations in the same locations is unknown. The diffuse and even nature of *VGLUT1* terminal labeling in every visual structure did not allow us to differentiate between individual *VGLUT1*-positive projections, but further studies involving colocalized labeling and tracing methods will shed light on the segregation of *VGLUT1*- and *VGLUT2*-positive projections from individual visual structures. Overall, *VGLUT2* is predominantly found in ascending projections such as retinogeniculate, retinotectal, and tectopulvinar connections while *VGLUT1* is predominantly distributed in descending projections such as corticopulvinar and corticogeniculate connections, as well as intrinsic connections in the thalamus and cortex. Both *VGLUT1* and *VGLUT2* appear to be dually expressed in thalamocortical projections and some corticothalamic projections as well, but further experiments are needed to fully demonstrate their colocalization and identify their respective contributions to synaptic transmission within a single glutamatergic projection.

Functional correlates of *VGLUT1*- or *VGLUT2*-positive projections in the tree shrew: comparisons with *VGLUT* distributions in other species

Recent studies on the distribution of *VGLUT1* and *VGLUT2* in mammalian brains have converged on the hypothesis that *VGLUT1* and *VGLUT2* characterize functionally distinct types of glutamatergic projections (Herzog et al., 2001; Varoqui et al., 2002; Fremeau et al., 2004a; Weston et al., 2011) and some studies (Rovó et al., 2012; Balaram et al., 2013) have correlated *VGLUT* distributions with previously defined driving and modulatory projections (Sherman and Guillery, 1996; 1998; Sherman, 2005), although direct evidence is still scant. Modulatory and driving projections (Sherman and Guillery, 1996) have not been specifically documented in the tree shrew visual system, but comparisons of *VGLUT1*- or *VGLUT2*-positive projections in the tree shrew with known *VGLUT1*/modulatory or *VGLUT2*/driving projections in other species gave us some insight on the functional attributes of these distributions in visual structures.

The use of *VGLUT2* in retinal projections to the SC and LGN in tree shrews is identical to retinal projections in other mammals (Fujiyama et al., 2003; Land et al., 2004; Gong et al., 2006; Islam and Atoji, 2009; Balaram et al., 2011b; 2013), and retinal projections are known to drive their postsynaptic targets (Sherman and Guillery, 1996; 1998; Sherman, 2005). Similarly, all projections from the LGN to V1 across rodents, primates, and carnivores are *VGLUT2*-dominant, consistent with the driving nature of geniculostriate projections (Sherman and Guillery, 1996; Sherman, 2005). However, rodents and primates are known to

exhibit low levels of *VGLUT1* mRNA in the LGN (Fremeau et al., 2001; Fujiyama et al., 2001; Herzog et al., 2001; Balaram et al., 2011a; b; 2013), perhaps in the same neurons that express *VGLUT2* mRNA (Herzog et al., 2001), which blurs the segregation of VGLUT1 and VGLUT2 between modulatory and driving projections. Additionally, VGLUT2-positive tectopulvinar projections have been previously identified in tree shrews (Chomsung et al., 2008; Wei et al., 2011) rodents (Hisano et al., 2000) and primates (Balaram et al., 2011b; Baldwin et al., 2013), but tectopulvinar projections appear to represent a separate category of terminations that have distinct features from classic driving (i.e. retinogeniculate) or modulatory (i.e. corticogeniculate) projections (Chomsung et al., 2008; Masterson et al., 2009; 2010; Wei et al., 2011). VGLUT2-positive tectogeniculate projections are present in tree shrews, and likely exist in prosimian primates (Balaram et al., 2011b), which closely resemble early primate species (Kaas, 2013), although similar results have not been documented in anthropoid primates (Balaram et al., 2013). In rodents, tectogeniculate projections likely utilize VGLUT2, since neurons expressing *VGLUT2* mRNA are found across the upper SGS in rats (Fremeau et al., 2001) and squirrels (unpublished results), and further experiments will determine if tectogeniculate terminations can be characterized as classic driving or modulatory projections (Sherman and Guillery, 1996; 1998; Sherman, 2005; Sherman and Guillery, 2006; 2011).

The predominant use of VGLUT1 in infragranular projections from V1 to the thalamus and midbrain is consistent across rodents and primates as well as tree shrews (Ni et al., 1995; Bellocchio et al., 1998; Fujiyama et al., 2001; Kaneko et al., 2002; Balaram et al., 2011a; 2013). Most visual cortical projections to subcortical regions are classified as modulatory in nature (Sherman and Guillery, 1996; Sherman, 2005), but subsets of corticopulvinar projections have driver-like qualities across sensory systems (Sherman, 2007; 2012). The nearly exclusive expression of *VGLUT1* mRNA in the deep layers of V1, where both driving and modulatory corticopulvinar projections originate, further confounds the association of VGLUT1 and VGLUT2 with modulatory and driving projections. *VGLUT2* mRNA was present in some infragranular V1 neurons in tree shrews, and has been described in other rodent cortical areas (Hisano et al., 2000). These *VGLUT2*-positive neurons could contribute to cortical driving projections from V1 but further experiments are necessary to identify the location and function of their terminations in other brain regions. Experiments in galagos (Balaram et al., 2011a; Marion et al., 2013) suggest that presumed driving projections from layer 5 of V1 to the pulvinar complex utilize VGLUT1 while presumed driving projections from the pulvinar to V2 utilize VGLUT2; thus, within cortico-thalamo-cortical circuits, there may be no strict correlation between VGLUT isoforms and their effects on their postsynaptic targets.

The dual expression of VGLUT1 and VGLUT2 in some projections may contribute to mechanisms of homeostatic plasticity within a glutamatergic circuit (De Gois et al., 2005; Nakamura et al., 2005; Anne and Gasnier, 2014), and since most neurons release more than one neurotransmitter from individual synapses (Hnasko and Edwards, 2012), the co-expression of VGLUT1 and VGLUT2 with other neurotransmitter transporters may contribute to a wide range of signal transmission, perhaps between distinct terminations from an individual projection (Herzog et al., 2001). Nevertheless, the results presented here

provide further evidence that VGLUT1 and VGLUT2 are largely segregated within distinct glutamatergic pathways. While the classification of glutamatergic projections as solely driving or modulatory is still debatable (Groh et al., 2008 for example), the use of VGLUT1 and VGLUT2 content to define specific pathways can contribute to further testing of this concept in sensory circuits (Sherman, 2012; Petrof and Sherman, 2013).

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The distribution patterns of VGLUT1 and VGLUT2 in the tree shrew visual system indicate that VGLUT2 predominates in driving projections while VGLUT1 predominates in modulatory projections, and both are coexpressed in some thalamocortical and corticothalamic projections. This suggests that VGLUTs identify functionally distinct types of glutamatergic connections between visual structures.

Figure 1.

Coronal sections through the tree shrew lateral geniculate nucleus (LGN) stained for (A) cytochrome oxidase (CO), (B) neuronal nuclear antigen (NeuN), (C) VGLUT1 protein, (D) VGLUT2 protein, (E) VGLUT1 mRNA, and (F) VGLUT2 mRNA. LGN layers are adapted from Glickstein, 1967. Midline is to the left, scale bar is 1mm.

Figure 2.

High magnification images of panels C–F in figure 1 highlight laminar differences in (A) VGLUT1 and (B) VGLUT2 protein, and (C) *VGLUT1* and (D) *VGLUT2* mRNA through the tree shrew LGN. Layer conventions as in figure 1. Midline is to the left, scale bar is 100 μ m.

Figure 3.

Coronal sections through the tree shrew superior colliculus (SC) stained for (A) CO, (B) NeuN, (C) VGLUT1 protein, (D) VGLUT2 protein, (E) *VGLUT1* mRNA, and (F) *VGLUT2* mRNA. SC layers are adapted from May, 2006. Midline is to the right, scale bar is 500um. Abbreviations: SZ – zonal layer, uSGSd – dorsal division of the upper superficial gray layer, uSGSv – ventral division of the upper superficial gray layer, LSGS – lower superficial gray layer, SO – optic layer.

Figure 4.

High magnification images of panels C-F in figure 3 show variations in (A) VGLUT1 and (B) VGLUT2 protein and (C) *VGLUT1* and (D) *VGLUT2* mRNA distributions between layers of the SC. Layer conventions as in figure 3. Scale bar is 50um. Abbreviations: SZ – zonal layer, uSGSd – dorsal division of the upper superficial gray layer, uSGSv – ventral division of the upper superficial gray layer, LSGS – lower superficial gray layer, SO – optic layer.

Figure 5. Coronal sections through the tree shrew pulvinar complex stained for (A) CO, (B) NeuN, (C) VGLUT1 protein, (D) VGLUT2 protein, (E) *VGLUT1* mRNA, and (F) *VGLUT2* mRNA. Individual nuclei are demarcated after Lyon et al., 2003, white asterisks indicate regions of interest within pulvinar divisions (see text). Midline is to the right, scale bar is 500um. Abbreviations: Pd – dorsal pulvinar, Pc – central pulvinar, Pv-ventral pulvinar.

Figure 6.

High magnification images of individual pulvinar divisions from panels C-F in figure 5 reveal differences in (A–C) VGLUT1 and (D–F) VGLUT2 protein, and (G–I) *VGLUT1* and (J–L) *VGLUT2* mRNA labeling across these nuclei. Scale bar is 50µm. Abbreviations: Pd – dorsal pulvinar, Pc – central pulvinar, Pv-ventral pulvinar.

Figure 7.

Coronal sections through primary visual cortex (V1) in tree shrews stained for (A) CO, (B) NeuN, (C) VGLUT1 protein, (D) VGLUT2 protein, (E) VGLUT1 mRNA, and (F) VGLUT2 mRNA. Individual V1 layers are named following the convention of Hässler (1967) and are shown on the left of each section. Solid lines are boundaries between V1 layers, dotted lines are sublamina divisions. Hemispheric midline is to the right, scale bar is 500um.

Figure 8. High magnification images of panels C-F in figure 7 identify sublaminar differences in (A) VGLUT1 and (B) VGLUT2 protein and (C) *VGLUT1* and (D) *VGLUT2* mRNA distributions in V1. Laminar conventions as in figure 7. Scale bar is 100 μ m.

Figure 9.

Summary of VGLUT1-positive and VGLUT2-positive projections in the tree shrew visual system. VGLUT1 projections are shown in blue, VGLUT2 projections are shown in red, and dual VGLUT1/VGLUT2 projections are shown in alternating red and blue. Circles denote the origin of individual projections while arrowheads indicate the terminations of each projection. Abbreviations: LGN – lateral geniculate nucleus, SC – superior colliculus, SZ – zonal layer, uSGSd – dorsal division of the upper superficial gray layer, uSGSv – ventral division of the upper superficial gray layer, LSGS – lower superficial gray layer, SO – optic

layer, Pd – dorsal pulvinar, Pc – central pulvinar, Pv-ventral pulvinar, V1 – primary visual cortex.

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Table 1

Antibodies used to label VGLUT1 and VGLUT2 protein in visual brain structures

Antigen	Immunogen	Source, Host Species, Cat. #, RRID	Concentration
Vesicular Glutamate Transporter 1 (VGLUT1)	Strep-Tag [®] fusion protein of rat VGLUT1	Synaptic Systems, rabbit polyclonal, 135 303, AB_887876	0.2ug/ml
Vesicular Glutamate Transporter 2 (VGLUT2)	Recombinant protein from rat VGLUT2	Millipore, mouse monoclonal, MAB5504, AB_2187552	0.2ug/ml
Biotinylated horse anti-mouse IgG	Mouse IgG	Vector Labs, mouse polyclonal, BA-2000, AB_2313581	3ug/ml
Biotinylated goat anti-rabbit IgG	Rabbit IgG	Vector Labs, rabbit polyclonal, BA-1000, AB_2313606	3ug/ml