Published in final edited form as:

J Comp Neurol. 2016 April 15; 524(6): 1292–1306. doi:10.1002/cne.23907.

THE ULTRASTRUCTURE OF GENICULOCORTICAL SYNAPTIC CONNECTIONS IN THE TREE SHREW STRIATE CORTEX

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Abstract

To determine whether thalamocortical synaptic circuits differ across cortical areas, we examined the ultrastructure of geniculocortical terminals in the tree shrew striate cortex in order to directly compare the characteristics of these terminals to that of pulvinocortical terminals (examined previously in the temporal cortex of the same species, Chomsung et al. *Cerebral Cortex* 2010). Tree shrews are considered to represent a prototype of early prosimian primates, but are unique in that sublaminae of striate cortex layer IV respond preferentially to light onset (IVa) or offset (IVb). We examined geniculocortical inputs to these two sublayers labeled by tracer or virus injections, or an antibody against the type 2 vesicular glutamate antibody (vGLUT2). We found that layer IV geniculocortical terminals, as well as their postsynaptic targets, were significantly larger than pulvinocortical terminals and their postsynaptic targets. In addition, we found that 9–10% of geniculocortical terminals in each sublamina contacted GABAergic interneurons, whereas pulvinocortical terminals were not found to contact any interneurons. Moreover, we found that the majority of geniculocortical terminals in both IVa and IVb contained dendritic protrusions, while pulvinocortical terminals do not contain these structures. Finally, we found that synaptopodin, a protein uniquely associated with the spine apparatus, and telencephalin (TLCN, or Intercellular Adhesion Molecule type 5, ICAM5), a protein associated with maturation of dendritic spines, are largely excluded from geniculocortical recipient layers of the striate cortex. Together, our results suggest major differences in the synaptic organization of thalamocortical pathways in striate and extrastriate areas.

Graphical Abstract

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Conflict of interest statement

The authors have no known conflicts of interest that could inappropriately influence this work.

Role of authors

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: DF and MB. Acquisition of data: DF, RQ, SM, WD, ASS and MEB. Analysis and interpretation of data: DF and MEB. Drafting of the manuscript: DF, MEB, and HMP. Critical revision of the manuscript for important intellectual content: DF, HMP, and MEB. Statistical analysis: DF and MEB. Obtained funding: MEB and HMP. Administrative, technical, and material support: MEB and ASS. Study supervision: MEB.

Injection of adenoassociated virus in layer 1 of the dorsal lateral geniculate nucleus induced the expression of the fluorescent protein TdTomato (purple) in layer IVa of the tree shrew striate cortex. The section was additionally stained with an antibody against the type 2 vesicular glutamate transporter (vGLUT2, green). Scale = 20 (μm). Cortical layers IIIc, IVb and V are also indicated.

Keywords

geniculocortical; pulvinocortical; spiny stellate; pyramidal; type 2 vesicular glutamate transporter; synaptopodin; telencephalin; GABA; RRID:AB_10015246; RRID:AB_1587626; RRID:AB_477652; RRID:AB_11202657; RRID:nif-0000-30467

Introduction

Although now classified in the order Scandentia, tree shrews are considered to represent a prototype of early prosimian primates, and the tree shrew visual system exhibits many of the characteristics of the primate visual system. These features include a 6-layered dorsal lateral geniculate nucleus (dLGN; Jain et al. 1994; Holdefer and Norton 1995), a large pulvinar nucleus (Luppino et al. 1988; Lyon et al. 2003; Chomsung et al. 2008), and a highly laminated striate cortex (Fitzpatrick 1996; Balaram and Kaas 2014). However, the tree shrew striate cortex is unique in that layer IV is divided into sublayers that respond preferentially to light onset or offset (Norton et al. 1985; Kretz et al. 1986; Fitzpatrick 1996; Van Hooser et al. 2013).

Striate cortex layer IVa receives input from a matched pair of dLGN layers 1 and 2, which receive input from ON-center ganglion cells in the contralateral and ipsilateral retina respectively. Layer IVb receives input from a matched pair of dLGN layers 4 and 5, which receive input from contralateral and ipsilateral OFF-center ganglion cells respectively. The remaining two dLGN layers form an unmatched pair that receives contralateral retinal input.

Layer 3 contains a roughly equal mixture of ON-center and OFF-center cells that project primarily to striate layer IIIb, but also display a secondary projection to the center of layer IV. Layer 6 contains ON-OFF center cells that project primarily to striate layer IIIc with some branches terminating in lower layer IVb (Raczkowski and Fitzpatrick 1990; Usrey et al. 1992; Holdefer and Norton 1995; Usrey and Fitzpatrick 1996).

Thus, in the tree shrew, geniculocortical projection patterns maintain the segregation of parallel retinogeniculate channels. Furthermore, the dendrites of spiny stellate cells are restricted to the sublayers IVa or IVb (Muly and Fitzpatrick 1992), suggesting that, at least within layer IV, thalamocortical synapses are organized to limit the mixing of visual signals. In contrast, our previous studies of the tree shrew pulvinar suggested that the projections of this nucleus may be organized to integrate signals across multiple brain regions. The projections from the pulvinar nucleus innervate two different areas of the temporal cortex, where the terminals are distributed across layers I–IV (Chomsung et al., 2010). Furthermore, the pulvinar nucleus additionally projects to the striatum and amygdala (Day-Brown et al., 2010).

These differences in thalamic projection patterns prompted us to ask whether individual geniculocortical and pulvinocortical terminals also exhibit functional distinctions. Specifically, we wished to determine whether the ultrastructure of thalamocortical synapses differs across cortical areas, an issue that has not previously been examined directly in any species. In the present study, we examined the ultrastructure of geniculocortical terminals in layers IVa and IVb of the tree shrew striate cortex in order to directly compare the characteristics of these terminals to that of pulvinocortical terminals which we examined previously in the temporal cortex of the same species (Chomsung et al. 2010).

This comparison revealed a number of differences in the ultrastructure of dendrites postsynaptic to geniculocortical and pulvinocortical terminals. We therefore investigated the distribution of synaptopodin, an actin-binding protein that is uniquely associated with a structure known as the spine apparatus (Mundel et al. 1997; Deller et al. 2003), and telencephalin (TLCN, also known as Intercellular Adhesion Molecule type 5, ICAM5), a protein proposed to regulate the maturation of dendritic spines in the telencephalon (Yoshihara et al. 2009). We found that these two proteins are largely excluded from geniculocortical recipient layers of the striate cortex. Together our present and previous results highlight major differences in the synaptic organization of thalamocortical pathways in striate versus extrastriate areas of the cortex.

Materials and Methods

A total of 5 adult tree shrews (*Tupaia belangeri*) were used for the current study. All the methods used were approved by the University of Louisville Animal Care and Use Committee and conform to the National Institutes of Health guidelines. One tree shrew received an injection of biotinylated dextran amine (BDA) in dLGN layers 5 and 6 which labeled, by anterograde transport, geniculocortical terminals in the layer IVb of the primary visual cortex (V1). One tree shrew received an injection of an adeno-associated virus (AAV) in dLGN layer 1 which induced the expression of the fluorescent protein TdTomato in

geniculocortical terminals in V1 layer IVa. Tissue from 3 additional tree shrews was used for the immunocytochemical labeling of the type 2 vesicular glutamate transporter (vGLUT2), synaptopodin and/or telencephalin. Data generated in the current study was compared to our previous electron microscopic results from 4 tree shrews that received injections of BDA in the pulvinar nucleus to label pulvinocortical terminals by anterograde transport (Chomsung et al. 2010).

AAV and BDA injections

Tree shrews were initially anesthetized with intramuscular injections of ketamine (100mg/kg) and xylazine (6.7mg/kg), and to maintain surgical anesthesia, supplemental doses of ketamine and xylazine were administered throughout the surgical procedure. The tree shrews were placed in a stereotaxic apparatus and prepared for sterile surgery. A small area of the skull overlying the dLGN was removed and the dura reflected. For the BDA injection, a glass pipette (3 μ m tip diameter) filled with a solution of 5% BDA (3000 MW; Molecular Probes, Eugene, OR) in saline was lowered vertically into the dLGN and BDA was ejected iontophoretically using 2 μ A positive current for 30 minutes. For the AAV injection, the virus (carrying a vector for channelrhodopsin 1 and 2 fused to TdTomato, as described in by Jurgens, Bell, McQuiston, & Guido, 2012) was loaded into an oil-filled nanofil syringe with an attached 34 gauge needle. The needle was lowered vertically into the dLGN and 100 nl of virus was pressure injected at a rate of 30nl/min via UMP-4 UltraMicroPump (World Precision Instruments, Sarasota, FL).

Perfusion and sectioning

One week following the BDA injection and 10 days following the AAV injection, the injected tree shrews were given a lethal dose of ketamine (600 mg/kg) and xylazine (130 mg/kg) and perfused with Tyrode solution, followed by a fixative of 2% paraformaldehyde and 2% glutaraldehyde in 0.1M phosphate buffer, pH 7.4 (PB). Tree shrews used for immunocytochemistry were similarly anesthetized and perfused with either 2% paraformaldehyde and 2% glutaraldehyde in PB or 4% paraformaldehyde in PB. Following the perfusion, the brains were removed from the skull, and 50 μ m thick coronal sections were cut using a vibratome.

Immunohistochemistry for confocal microscopy

Selected sections that contained TdTomato-labeled geniculocortical terminals were incubated for 15 minutes in a 1% sodium borohydride in PB. After rinsing in PB, the sections were then incubated in a solution of 10% normal goat serum (NGS) and 0.1% Triton X-100 in phosphate buffered saline (PBS; 0.01M PB and 0.9% NaCl) for 30 minutes, and then transferred to a 1:10,000 dilution of an antibody against the type 2 vesicular glutamate transporter (vGLUT2, made in guinea pig, Chemicon Temecula, CA) in 1% NGS in PBS overnight at 4°C. The following day the sections were rinsed in PB and incubated for 1 hour in a 1:100 dilution of a goat-anti-guinea-pig antibody conjugated to AlexoFluor-488 (Invitrogen). After washing the sections in PB, they were mounted on slides, coverslipped using ProLong® Gold antifade agent (Invitrogen) and viewed with a confocal microscope (Olympus Fluoview).

Sections from uninjected animals were incubated with 10% NGS in PBS for 30 minutes and then transferred to a 1:10,000 dilution of the vGLUT2 antibody and a 1:20 dilution of a mouse-anti-synaptopodin antibody (Fitzgerald, Acton, MA) or a 1:5,000 dilution of the rabbit-anti-telencephalin antibody (kindly provided by Dr. Yoshihara; Yoshihara et al. 1994) in 1% NGS/PBS overnight at 4°C. The next day the sections were incubated for 1 hour in a solution containing a 1:100 dilution of biotinylated goat-anti-guinea pig antibody and a 1:100 dilution of a goat-anti-mouse or a goat-anti-rabbit antibody conjugated to Alexafluor 488 (Invitrogen) in 1% NGS/PBS. After washing in PB, the sections were then incubated for 1 hour in a 1:100 dilution of avidin conjugated to Alexafluor 546 (Invitrogen). After washing in PB, the sections were mounted and coverslipped for confocal viewing as described above.

Histochemistry for electron microscopy

To reveal BDA, sections were incubated in a 1:100 dilution of avidin and biotinylated horseradish peroxidase (ABC; Vector Laboratories, Burlingame, CA) in PBS overnight at 4°C. The following day the sections were rinsed in PB and reacted with nickel-intensified 3,3'-diaminobenzidine (DAB) for 5 minutes, washed in PB and mounted on slides for light microscope examination or prepared for electron microscopy as described below. To reveal TdTomato- or vGLUT2-labeled terminals for electron microscopy, sections were pre-incubated with 10% NGS in PBS for 30 minutes and then transferred to a 1:1000 dilution of a rabbit anti-DsRed antibody (Clontech Laboratories, Mountainview, CA), or a 1:15,000 dilution of the vGLUT2 antibody in 1% NGS/PBS overnight at 4°C. The next day the sections were incubated for 1 hour in a solution containing a 1:100 dilution of either biotinylated goat-anti-rabbit or biotinylated goat-anti-guinea pig antibodies (Vector). After rinsing in PB, the sections were subsequently incubated in ABC for 1 hour, and reacted with DAB as described above.

Antibody characterization

The primary antibodies used in this study are listed in Table 1. All DsRed antibody binding was confined to cells and terminals that contained TdTomato (as determined by their fluorescence under green epifluorescent illumination). No staining was detected in sections that did not contain TdTomato.

Preabsorption of the vGLUT2 antiserum with immunogen peptide eliminates all immunostaining (manufacturer's product information). The vGLUT2 antibody has previously been demonstrated to stain geniculocortical terminals in the ferret (Nahmani and Erisir 2005) and mouse (Coleman et al. 2010). This was accomplished by staining sections containing geniculocortical terminals labeled by anterograde transport with the vGLUT2 antibody and using confocal microscopy to establish colocalization. In addition, vGLUT2 did not colocalize with corticocortical axons. In tree shrew striate cortex, the vGLUT2 antibody labels a dense band of terminals in striate cortex layers IV and III, the major target of geniculocortical terminals, and also stains geniculocortical terminals labeled by anterograde transport, as demonstrated with confocal microscopy.

The GABA antibody shows positive binding with GABA and GABA-keyhole limpet hemocyanin, but not bovine serum albumin (BSA), in dot blot assays (manufacturer's product information). In tree shrew tissue, the GABA antibody stains a subset of neurons in the dorsal thalamus and cortex (Chomsung et al. 2008, 2010). This labeling pattern is consistent with other GABAergic markers used in a variety of species (Bickford et al. 1999).

Staining with the synaptopodin antibody was found in the tree shrew cortex and striatum, where spine apparatus are found, but was absent in the thalamus and brainstem, where spine apparatus are absent. This is consistent with previous studies (Mundel et al. 1997; Deller et al. 2002). The telencephalin antibody recognizes 130 kDa telencephalin (also known as intercellular adhesion molecule 5, or ICAM-5) by immunoblot (Hino et al. 1997). Staining with the telencephalin antibody was also confined to the telencephalon of the tree shrew brain, as previously reported in other species (Imamura et al. 1990; Kelly et al. 2014a). Staining with the telencephalin antibody is absent in ICAM-5 knock-out mice (Kelly et al. 2014a).

Electron microscopy

To prepare tissue for electron microscopy, selected sections that contained DAB-labeled terminals were postfixed in 2% osmium tetroxide, dehydrated in an ethyl alcohol series and embedded in Durcupan resin between sheets of Aclar plastic. Using a light microscope, the embedded sections were examined to select areas of interest. Selected areas were mounted on resin blocks and ultrathin sections (70–80 nm) were cut using a diamond knife. To ensure that we did not photograph the same synapse in more than one section, a one in five series of ultrathin sections was collected on Formvar-coated nickel slot grids and every fourth collected section was stained to reveal the presence of GABA, as previously described (Chomsung et al. 2010). Briefly, a GABA antibody made in rabbit (Sigma Chemical Company, Saint Louis, MO) was used at a concentration of 1:2000 and was subsequently tagged with a 1:25 dilution of a goat-anti-rabbit antibody conjugated to 15 nm gold particles (Amersham, Arlington Heights, IL). After air drying the sections were stained with a 10% solution of uranyl acetate in methanol for 30 minutes and examined with a Phillips CM 10 electron microscope equipped with a digital camera (SIA-12C). Each examined section was separated from the other examined sections by a minimum of 840 nm.

Ultrastructural data analysis

A total of 303 thin sections were examined. In each section, electron microscopic images of all DAB-labeled terminals that were involved in synaptic contacts were captured at a magnification of 18000X. Ultrastructural features were noted, and the number of gold particles overlying each DAB-labeled presynaptic profile, and each postsynaptic profile was counted. Using Maxim DL © 5 software, the areas of the DAB-labeled presynaptic profiles and the profiles postsynaptic to them were measured, and the gold density overlying each was calculated. The postsynaptic profiles were considered to be GABAergic if the overlying gold particle density was at least 2 times the density found overlying the DAB-labeled presynaptic terminals. For statistical analysis of size differences, an independent t-test was used. Throughout the text, all area measurements are expressed as mean $\pm$ standard deviation.

Confocal image analysis

To quantify the colocalization of TdTomato and vGLUT2, we used ImageJ software (RRID: nif-000-30467).

Results

Distribution and morphology of geniculocortical terminals

Figures 1 and 2 illustrate the location of BDA and AAV injections in the dLGN and the resulting distribution of terminals in the striate cortex labeled by these injections. The BDA injection was limited to the most lateral regions of the dLGN (layers 5 and 6, Figure 1A). The AAV injection resulted in TdTomato expression that was primarily limited to the most medial regions of the dLGN (layer 1, Figure 2A). Confirming previous reports (Raczkowski and Fitzpatrick 1990), the BDA injection labeled geniculocortical terminals in layers IVb and III of V1 (Figure 1B), while the virus injection labeled terminals in layer IVa of V1 (Figure 2B). In both layers IVa and IVb, labeled geniculocortical terminals formed dense horizontally-oriented bands.

Geniculocortical terminals contain vGLUT2

To investigate whether tree shrew geniculocortical terminals contain vGLUT2, we incubated sections that contained TdTomato-labeled geniculocortical terminals with an anti-vGLUT2 antibody and tagged this antibody with Alexafluor 488. Examination of this tissue with a confocal microscope revealed that many TdTomato-labeled geniculocortical terminals were labeled with the vGLUT2 antibody (white areas in Figure 2E). To quantify the colocalization of TdTomato and vGLUT2, we calculated the percentage of TdTomato-labeled axons (purple + white) that contained vGLUT2 (white). We only analyzed areas that included both TdTomato and vGLUT2 labeling to account for limitation in the vGLUT2 antibody penetration. Three images of a 0.5 μm scan thickness were analyzed. Using this method, we calculated that $7.9 \pm 2.5\%$ of the TdTomato-labeled axons contain vGLUT2. It should be noted that labeling with the vGLUT2 antibody is restricted to synaptic terminals, and is absent from portions of axons that do not contain vesicles (Nahmani and Erisir 2005; Coleman et al. 2010; Wei et al. 2011). In the same images, we found that $25.5 \pm 8.6\%$ of the vGLUT2-labeled terminals (green + white) contained TdTomato (white). This suggests that our virus injections labeled only a portion of the geniculocortical terminals in layer IVa.

Ultrastructural comparison of layer IVa and layer IVb geniculocortical terminals

We examined the synaptic targets of 100 geniculocortical terminals in layer IVa labeled with TdTomato and 103 geniculocortical terminals in layer IVb labeled with BDA. There was no significant difference ($p = 0.15$) in the size of geniculocortical terminals labeled with TdTomato in layer IVa (mean area $0.93 \pm 0.54 \mu\text{m}^2$) or with BDA in layer IVb (mean area $0.86 \pm 0.53 \mu\text{m}^2$). Representative electron micrographs are illustrated in Figures 2F–L (layer IVa terminals) and 1C–K (layer IVb terminals).

The geniculocortical terminals labeled by either method formed asymmetric synaptic contacts (arrows in Figures 1 and 2) with spines and small dendrites of similar sizes (mean area of profiles postsynaptic to TdTomato-labeled terminals in layer IVa was 0.36 ± 0.2

μm^2 , mean area of profiles postsynaptic to BDA-labeled terminals in layer IVb was $0.44 \pm 0.25 \mu\text{m}^2$, $p < 0.01$). The majority of these synaptic contacts were classified as simple, i.e. the terminal and postsynaptic profile were connected by a synaptic cleft with one continuous postsynaptic density (72% of the layer IVa geniculocortical terminals made simple contacts and 63% of the layer IVb geniculocortical terminals made simple contacts). The remaining synapses were classified as perforated (two separate postsynaptic densities were identified in the postsynaptic profile; 25% of layer IVa geniculocortical synapses, 28% of layer IVb geniculocortical synapses) or multiple (more than two separate postsynaptic densities identified; 3% of layer IVa geniculocortical synapses, 9% of layer IVb geniculocortical synapses).

Erisir and Dreusicke, 2005, noted that geniculocortical terminals in the ferret contain small protrusions, which extend from postsynaptic dendrites and are embedded inside the presynaptic terminal bouton. We also noted this feature in tree shrew geniculocortical terminals (green profiles within the terminals in Figure 1C–K and 2F–H, J–L). We found that 58% of layer IVa and 58% of layer IVb geniculocortical terminals contained dendritic protrusions.

The examined sections were stained for GABA as described in the methods section to evaluate whether geniculocortical terminals contact GABAergic interneurons in layer IVa or IVb. The density of gold particles overlying pre- and postsynaptic profiles was calculated to classify profiles as GABAergic or nonGABAergic. Presuming that thalamocortical terminals are non-GABAergic (Freund et al. 1989; Shostak et al. 2003; Nahmani and Erisir 2005; Coleman et al. 2010; Chomsung et al. 2010), the gold particle density overlying presynaptic profiles was considered to represent background staining. Postsynaptic profiles were considered to be GABA-positive if they displayed a gold particle density at least two times greater than that of the adjacent presynaptic profile (e.g. Figure 1F, 1G, 2H). According to this criterion, the majority of profiles postsynaptic to labeled geniculocortical terminals were non-GABAergic (91% of profiles postsynaptic to labeled terminals in layer IVa and 90% of profiles postsynaptic to labeled terminals in layer IVb).

Ultrastructure of vGLUT2-labeled terminals in V1 layer IV

We examined the ultrastructure of layer IV terminals labeled with the vGLUT2 antibody in tissue from an additional animal ($n = 100$ synaptic contacts, examples shown in Figure 2M–O). As reported previously (Nahmani and Erisir 2005; Chomsung et al. 2008), when revealed with a DAB reaction, the vGLUT2 antibody identifies synaptic terminals; the DAB reaction product is distributed throughout profiles that contain vesicles, but is largely absent from profiles that do not contain identifiable vesicles.

Terminals labeled with the vGLUT2 antibody exhibited the same ultrastructural features displayed by the terminals labeled with either TdTomato or BDA (described above). Terminals labeled with the vGLUT2 antibody contained dendritic protrusions (46%; examples illustrated in Figure 2N and O) and primarily contacted nonGABAergic profiles (94%). However, we found significant ($p < 0.001$) differences between the size of vGLUT2-labeled terminals in layers IV ($1.6 \pm 0.69 \mu\text{m}^2$), TdTomato-labeled terminals in layer IVa ($0.93 \pm 0.54 \mu\text{m}^2$), BDA-labeled terminals in layer IVb ($0.86 \pm 0.53 \mu\text{m}^2$), and their

postsynaptic targets (vGLUT2 targets: $0.5 \pm 0.3 \mu\text{m}^2$, TdTomato targets: $0.36 \pm 0.2 \mu\text{m}^2$, BDA targets: $0.44 \pm 0.25 \mu\text{m}^2$).

Comparison of the synaptic targets of geniculocortical terminals and pulvinocortical terminals

We compared the features of the geniculocortical synapses examined in this study (labeled by TdTomato or BDA, $n = 203$) to 444 pulvinocortical synapses examined in a previous study from our laboratory (Chomsung et al, 2010). Geniculocortical terminals (mean area $0.9 \pm 0.53 \mu\text{m}^2$) were found to be nearly twice the size of pulvinocortical terminals ($0.51 \pm 0.28 \mu\text{m}^2$), and this difference was significant ($p < .001$). The postsynaptic targets contacted by geniculocortical terminals were also significantly ($p < .001$) larger (mean area $0.4 \pm 0.23 \mu\text{m}^2$) than those contacted by pulvinocortical terminals ($0.28 \pm 0.11 \mu\text{m}^2$). Geniculocortical and pulvinocortical synapses also differed in their innervation of GABAergic interneurons: 9.5% of geniculocortical terminals contacted GABAergic profiles, but none of the pulvinocortical terminals contacted GABAergic profiles. Pulvinar synapses as a group were more complex (51% simple, 35% perforated, 14% multiple) when compared to geniculocortical terminals (71% simple, 28% perforated and 4% multiple). Finally, while we observed dendritic protrusions embedded within the majority of geniculocortical terminals (58%), no pulvinocortical terminals contained dendritic protrusions. This suggests that the finger-like extension of dendrites into presynaptic terminals may be a feature specifically associated with spiny stellate cells.

Lamellar bodies, synaptopodin and telencephalin distribution

We carefully examined profiles postsynaptic to geniculocortical terminals to determine if any contained a spine apparatus. While most profiles postsynaptic to geniculocortical terminals contained membranous structures, with our single section analysis it was difficult to accurately quantify the prevalence of spine apparatus. When we could clearly identify organized membranous stacks (Figure 3, indicated by the arrows), they were located within larger dendrites that also contained mitochondria (Figure 3, m). An electron dense material could be seen between these membranous stacks, as previously described for lamellar bodies postsynaptic to geniculocortical terminals in the macaque (Freund et al. 1989).

To determine the distribution of spine apparatus in the tree shrew striate cortex, we stained tissue for the actin-binding protein synaptopodin, which has been found to be uniquely associated with spine apparatus (Mundel et al. 1997; Deller et al. 2003) or telencephalin (intercellular adhesion molecule 5; ICAM5), a protein associated with dendritic protrusions (Kelly et al. 2014). In each case the tissue was additionally stained for vGLUT2 to identify the location of geniculocortical terminals. As illustrated in Figure 4A–C, synaptopodin staining was densest in regions that contained the least vGLUT2 labeling (layers I, II, IIIa, IIIc, V and VI) and synaptopodin staining was lightest in regions that contained the greatest density of vGLUT2 labeled terminals (layers IVa, IVb, and IIIb, which receive the greatest density of input from the dLGN, Raczkowski and Fitzpatrick 1990). Telencephalin staining was also most densely distributed in layers that do not receive geniculocortical input, including infragranular layers V and VI (Figure 4D–F; as previously described in cat striate cortex (Imamura et al. 1990)).

Discussion

Comparison with previous studies of geniculocortical terminals

The distribution of geniculocortical terminals labeled by our tracer and adenovirus injections confirm the findings of previous studies of the tree shrew (reviewed by (Fitzpatrick 1996). We found that an injection in layer 1 of the dLGN labeled terminals in sublayer IVa of V1 and an injection in layer 5 and 6 of the dLGN labeled terminals in layer IVb and III of V1. The labeled geniculocortical axons formed arbors that were oriented parallel to the cortical surface, similar to the orientation of the dendrites of spiny stellate cells (Muly and Fitzpatrick 1992). The ultrastructure of the labeled geniculocortical terminals, as well as terminals labeled with an antibody against vGLUT2, was similar to previous descriptions of geniculocortical terminals in layer IV of rodents, carnivores and primates (Garey and Powell 1971; Winfield et al. 1982; Freund et al. 1985, 1989; LeVay 1986; Dehay et al. 1991; Erisir and Dreusicke 2005; Nahmani and Erisir 2005; Coleman et al. 2010; da Costa and Martin 2011). Most previous studies have described geniculocortical terminals as large terminals that primarily contact dendritic spines. Our measurements of layer IV geniculocortical terminals in the tree shrew (mean area layer IVa $0.93 \pm 0.54 \mu\text{m}^2$ and layer IVb $0.86 \pm 0.53 \mu\text{m}^2$) fall within the range reported for carnivores (ferret $0.74 \pm 0.04 \mu\text{m}^2$, Nahmani and Erisir 2005) and primates (owl monkey parvocellular $1.29 \pm 0.54 \mu\text{m}^2$ and magnocellular $1.40 \pm 0.65 \mu\text{m}^2$, Shostak et al. 2003), but are well above the sizes reported for geniculocortical terminals in rodents (0.37 ± 0.02 , Owe et al. 2013).

Our results also are comparable to previous studies that have examined the percentage of contacts on GABAergic and nonGABAergic dendrites. Erisir and Dreusicke (2005) found that 9% of geniculocortical terminals in the adult ferret contact GABAergic dendrites, nearly identical to the percentage of contacts on GABAergic dendrites revealed in our study (9.5%). Similar percentages of GABAergic profiles postsynaptic to layer IV geniculocortical terminals have been demonstrated in the macaque (4.5–9.5%, (Freund et al. 1989) and owl monkey (4.6–13.3%, Shostak et al. 2003).

We also confirmed that geniculocortical terminals in the tree shrew contain vGLUT2, as has been demonstrated in rodents (Coleman et al. 2010), carnivores (Nahmani and Erisir 2005) and primates (Marion et al. 2013). Not all geniculocortical axons were double-labeled with the vGLUT2 antibody, but this can be attributed to limitations in antibody penetration as well as the fact that vGLUT2 is limited to regions of axons that contain synaptic vesicles. Although a previous study found no significant difference in the size of geniculocortical terminals labeled by tracer injections or vGLUT2 immunocytochemistry (Nahmani and Erisir 2005), we found that layer IV terminals labeled by the vGLUT2 antibody were larger than virus- and tracer-labeled terminals in either layer IVa or IVb. This could be due to the fact that we sampled tracer-labeled geniculocortical terminals and vGLUT2-labeled terminals in tissue obtained from separate animals. Alternatively, vGLUT2 may be more densely distributed in larger terminals which contain greater numbers of vesicles. Therefore our sampling of immunocytochemically-stained terminals may have been biased toward larger profiles.

What is the functional significance of dendritic protrusions?

As previously reported in V1 of the ferret (Erisir and Dreusicke 2005; Nahmani and Erisir 2005), we found that small dendritic protrusions were contained within the majority of layer IV geniculocortical terminals in the tree shrew. Dendritic protrusions are also evident in previous studies of geniculocortical terminals in the cat (Garey and Powell 1971) and studies of corticocortical terminals in the macaque middle temporal area (MT or V5) that originate from V1 or V2 (Anderson et al. 1998; Anderson and Martin 2002). However, dendritic protrusions are not found in all geniculocortical terminals, or in all studies of these terminals. The presence or absence of dendritic protrusions could be accounted for by differences in tracer labeling techniques. However, we noted that dendritic protrusions were present even when terminals were labeled using the immunocytochemical detection of vGLUT2 in tissue from animals that had not been injected with any tracers (also seen by Nahmani and Erisir 2005). Interestingly, dendritic protrusions have not been identified in geniculocortical terminals in studies in which the animals were anesthetized for long periods of time prior to perfusion (e.g. Freund et al. 1989, in which axons were labeled via intracellular injection of horseradish peroxidase following physiological recording). Thus, an alternative explanation for differences observed in the morphology of dendrites postsynaptic to geniculocortical terminals is that dendritic protrusions exhibit activity-dependent properties. Because monocular lid suture and dark rearing paradigms have been shown to cause alterations in the number of spines on the dendrites of spiny stellate cells (Lund et al. 1991), it is possible that V1 activity levels may elicit changes in the dendritic protrusions that presumably arise from these cells.

Dendritic protrusions are also seen in large terminals of the dorsal thalamus, such as retinogeniculate terminals. As for the protrusions within geniculocortical terminals, dendritic protrusions in retinogeniculate terminals can be the site of synaptic contacts (Bickford et al. 2010, 2015; Budisantoso et al. 2012; Hammer et al. 2014). Both retinogeniculate and geniculocortical terminals are associated with a high probability of neurotransmitter release and frequency-dependent synaptic depression (Chen et al. 2002; Boudreau and Ferster 2005; Swadlow et al. 2005; Budisantoso et al. 2012). Thus the increased surface area for synaptic contact that protrusions provide could strengthen and/or alter the dynamics of synaptic signal transfer.

Do spiny stellate cells contain spine apparati?—Spine apparati are composed of stacks of smooth endoplasmic reticulum and are found in most mature (mushroom-shaped) dendritic spines of hippocampal pyramidal cells (Segal et al. 2010; Vlachos 2012). This organelle, together with its uniquely associated actin-binding protein synaptopodin, is thought to participate in the long-term potentiation of synapses (Deller et al. 2003, 2007; Jedlicka et al. 2008, 2009). In the neocortex, spines do not appear to fall into discrete categories (Arellano et al. 2007) and the presence of spine apparati has yet to be correlated with specific cell types, synapses, or spine shapes.

In our single-section analysis, it was difficult to identify spine apparati in profiles postsynaptic to geniculocortical terminals. When we did identify structures similar to spine apparati, they were located within dendritic profiles that also contained mitochondria. In a

previous study of geniculocortical terminal synaptic targets (Freund et al. 1989), similar structures were defined as lamellar bodies rather than spine apparati because reconstructions carried out in this same study indicated that the spines do not contain mitochondria. As reported by Freund et al (1989), we did not observe any structures that could be identified as spine apparati or lamellar bodies within GABAergic profiles postsynaptic to geniculocortical terminals.

To determine the distribution of spine apparati in the tree shrew V1, we stained tissue with antibodies against synaptopodin, which has been found to be specifically associated with spine apparati and is an essential component of spine plasticity in the hippocampus (Mundel et al. 1997; Deller et al. 2003). We also incubated the same tissue sections in the vGLUT2 antibody to identify the location of geniculocortical terminals. We found an inverse relationship between synaptopodin staining and vGLUT2 staining, indicating that spine apparati are least abundant in regions innervated by geniculocortical terminals. This corresponds to previous studies in mice, where synaptopodin protein was found to be most densely distributed in the supragranular layers, and synaptopodin mRNA was found in cells in layers I–III and V but largely excluded from layer IV cells (Deller et al. 2002).

Telencephalin (TLCN, or ICAM5) is a protein present within dendritic protrusions in the developing mouse visual cortex (Kelly et al. 2013, 2014). The expression of TLCN has been shown to slow filopodia to spine transitions (Matsuno et al. 2006), and its deletion accelerates these transitions (Barkat et al. 2011). In the adult tree shrew V1, we found that distribution of TLCN was very similar to that of synaptopodin, i.e. the densest TLCN staining was found in regions that did not contain vGLUT2-stained geniculocortical terminals. A similar pattern was previously described in cat striate cortex (Imamura et al. 1990). In mice it has been demonstrated that the regulatory element that directs TLCN to neurons of the telencephalon is expressed by subsets of neocortical pyramidal neurons in layers I–III and VI (Mitsui et al. 2007). Together, these staining patterns suggest the spines of spiny stellate cells could exhibit structural features that are distinct from those of pyramidal cell spines. Future studies will be needed to determine whether lamellar bodies and spine apparati are distinct or identical structures.

Comparison of geniculocortical terminals in the ON and OFF sublamina

The presence of parallel retino-geniculo-cortical pathways has prompted a number of comparisons of their synaptic organization at the cortical level. Freund et al., (1989) concluded that geniculocortical axons originating from the magnocellular and parvocellular layers of the macaque dLGN use similar synaptic strategies at the first stage of cortical information transfer. In contrast, Shostak et al (2003) found a number of differences in the synaptic arrangements of magnocellular and parvocellular terminals in the striate cortex of the owl monkey.

Since magnocellular-like and parvocellular-like pathways have not been described in the tree shrew, we investigated potential differences in the synaptic organization of geniculocortical terminals related to the organization of the ON and OFF sublamina of the tree shrew visual cortex. To this end, we compared geniculocortical terminals in layer IVa and IVb labeled following a virus injection in dLGN layer 1 (labeling terminals in V1 layer

IVa) or a BDA injection in dLGN layers 5 and 6 (labeling terminals in V1 layers IVb and III). Although it has been shown that cells in the tree shrew dLGN layer 1 are larger than cells in dLGN layer 5 (Brauer et al. 1981), we found no significant differences in the size of terminals originating from these layers. In addition, we found that each set of terminals contacted similar numbers of GABAergic profiles (9% of postsynaptic profiles in layer IVa and 10% in layer IVb). Finally, we found that 58% of both populations of terminals, contained dendritic protrusions. Therefore we found no obvious ultrastructural differences in the synaptic arrangements of geniculocortical terminals that would differentially influence their responses to thalamic input.

This finding is relevant to differences in the processing of luminance increments and luminance decrements. The observation that humans are better at detecting luminance decrements compared to luminance increments dates back to Blackwell (1946), and has been confirmed and extended in a variety of studies since then (reviewed in Yeh et al. 2009). Recording from single neurons in macaque V1, Yeh et al. (2009) discovered that whereas OFF-cells (black-dominant responses) and ON-cells (white dominant responses) were roughly equivalent in number in layer IV, OFF-cells significantly outnumbered ON-cells in layers II/III. This result has also been documented in tree shrews where responses to luminance decrements were faster and larger than those to luminance increments (Veit et al. 2011). This difference was present in layer IV, but magnified in the supragranular layers. Unfortunately, our findings shed no light on the mechanism for this difference in ON-cell and OFF-cell responses.

Comparison of geniculocortical and pulvinocortical terminals in the tree shrew

Comparison of data collected in the present study to that collected in our previous study of pulvinocortical connections in the temporal cortex of the tree shrew (Chomsung et al. 2010), revealed a number of differences. First, the distribution of these two types of terminals is quite distinct. Whereas geniculocortical terminals form dense horizontally-oriented bands in V1, pulvinocortical terminals form columns of terminals that extend from layer I–IV in the temporal cortex. Second, geniculocortical terminals and their postsynaptic profiles are significantly larger when compared to pulvinocortical terminals and their postsynaptic profiles. Third, whereas pulvinocortical terminals do not contact any GABAergic profiles, 9.5% of geniculocortical terminals contact GABAergic profiles. Finally, we did not observe any dendritic protrusion within pulvinocortical terminals, but found protrusions in 58% of geniculocortical terminals. Thus, our two studies clearly indicate that the synaptic organization of thalamocortical synapses is quite different in the striate and extrastriate cortex of the tree shrew. The unique ultrastructural features of layer IV geniculocortical terminals may be due to the fact that they innervate spiny stellate cells. In support of this, the size of koniocellular geniculocortical terminals in the owl monkey ($0.51 \pm 0.36 \mu\text{m}^2$; Shostak et al. 2003), which presumably innervate pyramidal cells in the more superficial layers of V1, is nearly identical to that of pulvinocortical terminals in the tree shrew ($0.51 \pm 0.28 \mu\text{m}^2$; (Chomsung et al. 2010). On the other hand, corticocortical terminals in area MT have been found to be quite large (Anderson et al. 1998; Anderson and Martin 2002), and spiny stellate cells have not been identified in this cortical region.

Thalamocortical microcircuits in the tree shrew—In summary, our results suggest that parallel pathways from the ON and OFF sublayers of the dLGN innervate identical elements within layer IVa and IVb of the tree shrew striate cortex, supporting the concept of canonical microcircuits that are repeated throughout the neocortex (Douglas and Martin 2007). On the other hand, comparison of the results of the current study to those of our previous study of pulvinocortical terminals in the tree shrew (Chomsung et al. 2010) indicates that unique thalamocortical microcircuits are found within the extrastriate (homotypical, Mountcastle 1997) cortex. As recently reviewed by Harris and Shepherd (2015), thalamic nuclei can be subdivided based on a number of criteria, including their projections patterns to primary and secondary cortical areas, as well as subcortical regions such as the striatum and amygdala (Day-Brown et al. 2010). Our results suggest that another key distinction between thalamic nuclei may be whether their major synaptic targets are spiny stellate cells or pyramidal cells. As schematically summarized in Figure 5, our results reveal potential differences in the ultrastructure of the spines of these two cell types that could influence signal transfer across the thalamocortical synapse.

As discussed above, our results suggest that geniculocortical synapses on spiny stellate cells are highly secure. In contrast, pulvinocortical are about half the size of geniculocortical terminals, and contact small dendritic spines that do extend protrusions (Chomsung et al. 2010). Since spiny stellate cells have not been identified in extrastriate cortical areas, pulvinocortical terminals most likely contact spines distributed across the dendritic arbors of pyramidal neurons in layers I through IV. In fact, our preliminary data in mice indicates that pulvinocortical terminals target pyramidal cells that project to the striatum and amygdala (Zhou et al 2014), implying that thalamocortical circuits are highly dependent on the cortical area innervated. Therefore, continued comparisons of the cortical circuits innervated by the thalamus may be particularly important in furthering our understanding of neocortical function.

Acknowledgments

Funding: This work was supported by the National Institutes of Health, grant numbers R01EY016155 and R21EY021016

The authors thank Phillip S. Maire and the University of Louisville veterinary staff for maintenance of the tree shrew colony and assistance with surgical procedures, and Dr. Yoshihiro Yoshihara (Laboratory for Neurobiology of Synapse, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan) for his generous contribution of the telencephalin antibody.

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Figure 1.

A) Coronal section showing an injection of biotinylated dextran amine (BDA) in layers 5 and 6 of the dorsal lateral geniculate (dLGN). B) Terminals labeled following the injection illustrated in A are primarily located in layer IVb of the striate cortex. C–K) BDA-labeled geniculocortical terminals in layer IVb form synaptic contacts (white arrows) with GABAergic (red, F, G) and nonGABAergic (green, C–E, H–K) dendrites. Dendritic protrusions (green, C–J) can be seen inside the geniculocortical terminals. Scales A = 250 μ m, B = 50 μ m F = 0.5 μ m and applies to panels C–K. Pul, pulvinar, OT, optic tract.

Figure 2.

A) Coronal section of an injection of adenoassociated virus (AAV) in layer 1 of the dorsal lateral geniculate nucleus induced the expression of the fluorescent protein TdTomato (purple). The section was additionally stained with an antibody against the type 2 vesicular glutamate transporter (vGLUT2, green). B) TdTomato-labeled terminals (purple) labeled following the injection illustrated in A are primarily located in layer IVa of striate cortex. Section also stained for vGLUT2 (green). C)–E) Confocal images (1 μ m optical section) of TdTomato-labeled geniculocortical terminals (C, purple) in tissue stained for vGLUT2 (D, green). Overlapping areas (E, white) indicate that geniculocortical terminals contain vGLUT2. F–L) AAV-labeled geniculocortical terminals in layer IVa form synaptic contacts (white arrows) with GABAergic (red, H) and nonGABAergic (green, F, D, I–L) dendrites. Dendritic protrusions (green, F–H, J, K) can be seen inside the geniculocortical terminals. M–O) vGLUT2-labeled terminals in layer IV form synaptic contacts (white arrows) with GABAergic (not shown) and nonGABAergic (green, M–O) dendrites. Dendritic protrusions

(green, N, O) can be seen inside the geniculocortical terminals. Scales: B = 20 μm and also applies to A, E = 10 μm and also applies to C and D, O = 0.5 μm and applies to F–O.

Figure 3. Lamellar bodies (indicated by the arrows) are occasionally found in dendrites postsynaptic to geniculocortical terminals (A–D). These dendrites also contain mitochondria (m). Scale = 0.25 μ m and applies to all panels.

Figure 4.

Confocal images of tree shrew visual cortex stained with antibodies against the type 2 vesicular glutamate transporter (vGLUT2, purple, A and D) and either synaptopodin (green, B) or telencephalin (green, E). Overlap of synaptopodin and vGLUT2 is shown in panel C and overlap of telencephalin and vGLUT2 is shown in panel F. Both synaptopodin and telecephalin are largely excluded from lamina innervated by geniculocortical terminals. Scale in panel C = 100 μ m and applies to all panels.

Figure 5.

A schematic summary of the organization of geniculocortical and pulvinocortical pathways in the tree shrew. The six layers of the dorsal lateral geniculate nucleus (dLGN) project to different sublamina of the striate cortex (modified from Fitzpatrick 1996). In layers IVa and IVb, horizontally oriented geniculocortical axons align with the restricted dendritic arbors of spiny stellate cells (Muly and Fitzpatrick 1992). In contrast, projections from the dorsal (Pd) and central (Pc) pulvinar innervate layers I–IV of the temporal cortex, aligning with the apical dendrites of pyramidal neurons (Chomsung et al. 2010). The insets summarize the ultrastructure of geniculocortical terminals (in striate cortex layers IVa and IVb) and pulvinocortical terminals in the temporal cortex (black, with white vesicles and light gray synaptic densities). Geniculocortical terminals are larger than pulvinocortical terminals and contact postsynaptic dendrites with dendritic protrusions (DP). Geniculocortical terminals also contact interneurons (9.5% of synapses) while pulvinocortical terminals do not contact interneurons. Spine apparatus (SA) or lamellar bodies (LB) can occasionally be identified in dendrites postsynaptic to pulvinocortical and geniculocortical terminals respectively.

Table 1

Primary antibodies used in this study

Antigen	Description of Immunogen	Source, Host species, Cat.#, RRID	Concentration used
DsRed	DsRed Express, a variant of <i>Discosoma</i> sp. red fluorescent protein	Clontech Laboratories, rabbit polyclonal, Cat.#632496, RRID: AB_10015246	0.5µg/ml
VGLUT2	GST-tagged peptide corresponding To the C-terminal of rat VGLUT2	Chemicon (Millipore), guinea pig polyclonal, Cat.# AB2251, RRID:AB_1587626	0.07µg/ml to 0.3µg/ml
GABA	GABA conjugated to bovine serum albumin using glutaraldehyde	Sigma-Aldrich, rabbit polyclonal, Cat.# A2052, RRID:AB_477652	0.25µg/ml
Telencephalin	carboxy terminal 17 amino acids of mouse telencephalin	Gift from Dr. Yoshihara	1:5000 dilution
Synaptopodin	Isolated rat kidney glomeruli	Fitzgerald Industries International, mouse monoclonal, Cat.# 10R-2373, RRID:AB_11202657	cell culture supernatant, diluted 1:20