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Morphological and neurochemical comparisons between pulvinar and V1 projections to V2

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Abstract

The flow of visual information is clear at the earliest stages: the retina provides the driving (main signature) activity for the lateral geniculate nucleus (LGN), which, in turn, drives the primary visual cortex (V1). These driving pathways can be distinguished anatomically from other modulatory pathways that innervate LGN and V1. The path of visual information after V1, however, is less clear. There are two primary feedforward projections to the secondary visual cortex (V2), one from the lateral/inferior pulvinar and the other from V1. Because both lateral/inferior pulvinar and V2 can't be driven visually following V1 removal, either or both of these inputs to V2 could be drivers. Retinogeniculate and geniculocortical projections are privileged over modulatory projections by their layer of termination, bouton size, and the presence of vesicular glutamate transporter 2 (Vglut2) or parvalbumin (PV). It has been suggested that such properties might also distinguish drivers from modulators in extrastriate cortex. We tested this hypothesis by comparing lateral pulvinar-to-V2 and V1-to-V2 projections with LGN-to-V1 projections. We found that V1 and lateral pulvinar projections to V2 are similar in that they target the same layers and lack PV. Projections from pulvinar to V2, however, bear a greater similarity to projections from LGN to V1 because of their larger bouton sizes (measured at the same location in V2) and positive staining for Vglut2. These data lend support to the hypothesis that the pulvinar could act as a driver for V2.

Keywords

pulvinar; V2; bouton area

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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: RM, VAC. Acquisition of data: RM, KL, GP, YJ, VAC. Analysis and interpretation of data: RM, GP, VAC. Drafting of the manuscript: RM, GP, VAC. Critical revision of the manuscript for important intellectual content: RM, KL, GP, VAC. Statistical analysis: RM, KL, GP, VAC. Obtained funding: VAC. Study supervision: VAC.

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INTRODUCTION

A fundamental problem in neuroscience is to define the flow of information from the sensory periphery through the brain. For primary sensory nuclei of the thalamus, such as the lateral geniculate nucleus (LGN), the “feedforward” direction of information flow is clear: signals are sent to the LGN of the thalamus which, in turn, are combined to activate cells in primary visual cortex (V1). This is despite the fact that both LGN and V1 receive numerous other connections from many brain areas both subcortical and cortical (Casagrande and Kaas, 1994; Casagrande and Norton, 1991). The question is “who drives and who modulates?” a question that is more complicated for thalamic nuclei and cortical areas which are not directly connected to the periphery. The primate pulvinar and the secondary visual area (V2) are exemplars of this problem. The visual pulvinar receives its drive from V1 (Bender, 1983), the lateral and inferior portions of the visual pulvinar and V1 send outputs to V2, (Casagrande and Kaas, 1994; Kaas and Lyon, 2007 for review). As seen in Figure 1A there are two routes by which feedforward information can travel from V1 to V2. The traditional view is that feedforward information flows from LGN to V1 and from there to V2 and other extrastriate areas in a hierarchical manner (Felleman and Van Essen, 1991; Goodale and Milner, 1992; Mishkin and Ungerleider, 1982). One alternative view is that for each cortical area, the main drive comes from the thalamus with cortico-cortical connections providing a modulatory input (Sherman and Guillery, 1998; Sherman and Guillery, 2002; Sherman, 2007). How can one decide between these alternatives? Rockland and Pandya (1979) argued that feedforward (potential driving connections) in cortex are distinct from feedback (modulatory connections) based on the cells of origin and the layers of termination of their axons. Their data showed that feedforward visual system connections are found within the middle layers of cortex (layers III and IV) while feedback connections are found in the supragranular layers, especially layer I, and in infragranular layers. These anatomical distinctions were subsequently used, in part, by Felleman and Van Essen (1991) to construct the now well-known dorsal and ventral hierarchies of cortical visual areas in macaque monkeys highlighted in many neuroscience textbooks. These cortical hierarchies continue to be referenced to each other based on the original criterion of laminar terminations of their inputs and outputs. At the level of the LGN and V1, however, driving connections have been distinguished from modulatory connections based on a number of additional anatomical criteria. Examples include the sizes, positions and arrangements of boutons and the protein content of these boutons (for review see Sherman and Guillery, 1998; Sherman and Guillery, 2002; Sherman, 2007).

Thus, the primary goal of this study was to examine and compare the two key inputs to V2 that could serve as drivers for this area, input from the lateral pulvinar (hereafter referred to simply as pulvinar) and input from V1 (see Symonds and Kaas, 1978; Wong et al., 2009). Prior studies have shown that inputs from both sources end in layer IV of V2 in macaque, bush baby and other primates consistent with the criterion for feedforward connections (Adams et al., 2000; Collins et al., 2001; Cragg, 1969; Cusick and Kaas, 1988; Felleman and Van Essen, 1991; Gattass et al., 1981; Kaske et al., 1991; Kennedy and Bullier, 1985; Kuypers et al., 1965; Lund et al., 1981; Mettler, 1935; O'Brien et al., 2001; Preuss et al., 1993; Rockland and Pandya, 1979; Shipp, 2003; Sincich and Horton, 2002; Tigges et al., 1973; Wong et al., 2009; Zeki, 1969). Hence, both inputs are possible drivers. Additionally, studies have shown that cells in primate V2 lose responsiveness following removal of V1 (Schiller and Malpeli, 1977), so V2 could be either driven directly by V1 or indirectly via the pulvinar (or both). Since LGN input to V1 is a known driver, we used the characteristics of this pathway for comparison, focusing here on axon distribution, bouton size, and the presence or absence of the vesicular glutamate transporter number 2 (Vglut2) and the calcium binding protein, parvalbumin (PV). The latter proteins are strongly expressed in the

LGN axons within layer IV of V1 (Blumcke et al., 1991; DeFelipe and Jones, 1991; Nahmani and Erisir, 2005).

METHODS

Overview

Ten bush babies (*Otolemur garnettii*) of both sexes, also known as “greater galagos”, were used in this study. Animal ages ranged from 6 months to 6 years with the median age being between 1 and 2 years. Four animals were optically imaged, 5 animals received injections in V1, 4 animals received injections in the LGN and all animals received injections in the pulvinar. In total 11 hemispheres were examined. All of the animals were cared for according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and according to the guidelines of the Vanderbilt University Institutional Animal Care and Use Committee under an approved protocol.

Anesthesia, Surgery, and Recovery

All surgical procedures were carried out under aseptic conditions and all survival procedures were performed under sterile conditions. Prior to surgery, anesthesia was induced with 2-4% isoflurane in O₂. During this period animals were given intramuscular Dexamethasone to control for brain swelling, (1mg/kg), Glycopyrrolate (Robinul) to reduce salivation and fluid accumulation (0.015mg/kg) and Naxcel as an antibiotic (2.2 mg/kg). Two venous catheters were inserted, the pupils were dilated with 2% cyclopentolate (Cyclogyl) and/or 1% atropine drops and contact lenses were placed in the eyes to protect the corneas. The heart rate and temperature were monitored continuously and the temperature was regulated using a water circulating heating pad and heat packs. After intubation animals were mounted in a stereotaxic apparatus and connected to CO₂ and heart monitors and baseline measures of ECG, heart rate and CO₂ were taken. Once the animal was stable under anesthesia, a midline incision was made and the skull exposed. In the initial surgery a craniotomy of ~10 mm in diameter was performed over either pulvinar or LGN and in some cases another craniotomy was made over the primary visual cortex (V1). After each craniotomy, the dura was cut and retracted. At the end of each surgery, artificial dura (Tecoflex®) and a protective cap of thermoplastic and dental cement were placed over the craniotomies. In subsequent surgeries the brain was accessed by removal of the protective cap and the artificial dura.

Before an experiment was performed, the animal was switched to an anesthesia consisting of 75% N₂O along with Propofol delivered at ~10 mg/kg/h if given during surgery, which was reduced during the experiments to 4-7 mg/kg/h. During the transition between anesthetic methods the animal was maintained temporarily on Propofol and isoflurane at the levels previously described. After the animal's physiological state was stable under Propofol/N₂O anesthesia, neuromuscular blockade was induced with IV injection of 1mg/kg of Vencuronium bromide and maintained throughout the experiments with Vencuronium bromide (0.6 mg/kg/h) in a 5% dextrose lactated Ringers solution. Animals were artificially ventilated with a mixture of 75% N₂O, 23.5% O₂, and 1.5% CO₂ that was delivered at a rate of 35-40 breaths/min in a volume sufficient to maintain the peak expired CO₂ at 3.5-4%. Contact lenses with sufficient power were used to bring the animal to focus at a viewing distance of 28.5 cm or 57 cm. Retinal landmarks including the optic disks and *areae centralii* were plotted using back reflection of the blood vessel pattern from the tapetum at the beginning of each experiment. At this juncture one of two experiments could be performed, either optical imaging or visual evoked potential mapping and tracer injections. Both experimental procedures are described below.

At the end of each tracer injection or optical imaging session, animals were weaned from the ventilator and anesthetic after infusion of the paralytic agent was stopped and were watched carefully until they were capable of eating and drinking on their own. These animals also were given the analgesic buprenorphine (Buprenex 0.01 mg/kg) and the antibiotic, ceftiofur sodium (2.2 mg/kg) postoperatively. In some cases additional terminal experiments for a separate set of studies were performed before the animals were perfused.

Optical imaging

Our imaging equipment and procedures have been previously described (Khaytin et al., 2008; Xu et al., 2005; Xu et al., 2004). After a V1 craniotomy was performed and the dura removed and replaced with Tecoflex®, the opening was covered with 1% agarose in saline, and sealed with a glass cover-slip. Cortical reflectance changes following stimulus presentation were imaged using an Optical Imager 2001 system (Optical Imaging Inc., Mountainside, NJ). Visual stimuli were presented on a 21 inch monitor (Sony FD Trinitron, F400, Sony, Tokyo, Japan) at 120Hz (non-interlaced mode, background luminance = 30 cd/m²) using the VSG 2/5 stimulus generator (Cambridge Research Systems, Rochester, UK) synchronized with the imaging system. A macroscope consisting of two front-to-front tandem Nikon lenses (50 mm/50 mm or 50 mm/135 mm) magnified the cortex for the CCD camera. The image was focused at about 500-600 µm below the cortical surface and the diaphragm was closed by one or two f-stops. The light source was filtered at 540 nm to acquire a reference image of the surface vasculature and 611 nm to collect functional data. Raw images for each trial were normalized by the image acquired during a blank screen condition at the start of the trial (Crair et al., 1997). Visual topography of V1 was determined by use of retinotopically restricted rectangular windows, disks and rings of different sizes containing 100% contrast square-wave gratings (0.5 c/deg fundamental spatial frequency) presented at 2 orthogonal orientations and drifted at 2 Hz. Analysis was performed using WinMix (Optical Imaging Inc.).

Visuotopic mapping and tracer injections

Animals received tracer injections in the LGN, pulvinar and/or V1. The basic connections for LGN, pulvinar, V1 and V2 in bush baby are shown in Figure 1B. All tracer injection experiments began by initially visuotopically mapping the location of the lateral geniculate nucleus (LGN) using tungsten microelectrodes (~1 MΩ, FHC) and stimulating with spots or bars of light on a tangent screen. LGN single and multi unit receptive fields were hand mapped and these data were used to estimate the relative stereotactic location of the area of interest (either in LGN or pulvinar) where the next penetration would be placed. In all but two of the hemispheres additional locations were mapped until a location was found that represented central vision. After a desirable location was found the electrode was retracted and an injectrode (made in house using 2-4 MΩ tungsten microelectrodes joined with a glass pipette with a tip diameter of 15-30 µm fitted with a plunger) was inserted at the location of the previous penetration. The injectrode was used to confirm the visuotopic location using either multiunit or single unit recording. This location was then injected with tracer. There were three hemispheres where this procedure was not followed. In the first case after the initial LGN visuotopic mapping penetration the pulvinar and LGN were injected without further electrophysiological mapping. In the second case the stereotaxic coordinates from one hemisphere that had been mapped were used to guide injectrode penetrations and injections in the opposite hemisphere. In the last case two injectrode penetrations were made into the LGN, one guided by electrophysiological mapping and a second guided stereotactically and using the same injectrode as the first. All injections made in the thalamus were pressure injections and in all cases silicone oil was initially pulled into the tip of the injectrode to prevent leakage of the tracer during the penetration. However, if the injectrode was used to make multiple injections the silicone oil was not replenished between injections. Injections

generally were 300 nl of tracer (although in two cases 400 or 450 nl was used) and 100-200 nl of silicone oil injected slowly over a period of ~20-30 min. The tracers used in the thalamic injections were biotinylated dextran (BDA), dextran conjugated to Alexa-fluor 488 (dex 488) and dextran conjugated to Alexa-fluor 568 (dex 568). The dextran portion of each of these tracers had a molecular mass of 10,000 u and all were purchased from Invitrogen. All dextran based tracers were used at 10% in 0.01 M phosphate buffer (PB pH 7.35-7.45).

Following injection of the thalamus, some animals received one or more injections into V1. In 4 cases these injections were guided by prior visuotopic mapping using optical imaging, while in one case the injections were guided stereotactically. Cases received between 1-7 injections in 1, 3 or 4 penetrations. Both pressure and iontophoretic injections were placed in V1 containing dex 568 or the plant lectin, *Phaseolus vulgaris* leucoagglutinin (PHAL, Vector), respectively. Iontophoretic injections consisted of 7 or 15 minutes of 7 μ A current alternating at 5 sec on, 5 sec off. Pressure injections consisted of 100 to 200 nl of tracer. The majority of injections were placed between 300 and 500 μ m from the cortical surface, though some injections were confirmed to be as deep as 900 μ m or extended just below the surface.

Perfusion, cutting and processing

Two to four weeks after injections were placed, the animals were administered a lethal dose of sodium pentobarbital and perfused transcardially with a saline rinse followed by a fixative consisting of 3% paraformaldehyde, 0.1% glutaraldehyde and 0.2% picric acid (saturated solution v/v) in 0.1M PB. The brain was blocked in the coronal plane at a known AP coordinate with the head in a stereotaxic apparatus, and following blocking, the brain was removed. The brain was then cryoprotected, allowing the tissue to equilibrate overnight in 30% sucrose in 0.1M PB at room temperature. The brain was then frozen on dry ice and stored until use at -70°C. All cases were cut in the coronal plane using a sliding microtome. In all except 3 cases, sections were 40 μ m thick, in the others the sections were 30 or 52 μ m. In several cases some of the cut sections were processed immediately (see below); in all cases some or all sections were re-frozen in a solution of 30% glycerol in 0.1 M Tris buffered saline (TBS pH 7.35-7.45), or 30 % ethylene glycol and 30% sucrose in 0.1 M in TBS, and stored at either -70°C or -20°C respectively until processed. The processing steps resulting in fluorescent sections were typically done using TBS.

Immunohistochemistry

Two buffers were repeatedly used in the immunohistochemical processes: blocking buffer (bb), consisting of 3% normal donkey serum, 2% cold water fish gelatin, 0.1% Triton X-100 and 0.1% sodium azide in TBS, and antibody buffer (ab), consisting of 0.5% cold water fish gelatin, 0.2% Triton X-100 and 0.1% sodium azide in TBS. To visualize an antigen of interest (Vglut2, PV, or PHAL) the sections were rinsed 3 times, placed in bb for 1 hour, then placed in primary diluted with ab for 12-48 hrs (see table 1 for dilution). The sections were then rinsed 3 times in ab and placed in secondary antibody (see table 2) diluted using ab for 2 hours. The sections were then rinsed one time in ab and two times in TBS before mounting. Sections were stored in the dark at 4°C. Occasionally for ease of dilution, the Alexa-fluor secondary antibodies were used at 1:500; no difference was seen in the resulting staining and staining at the typical concentration. Primary absent controls showed a uniform lack of staining.

Antibody characterization

The mouse monoclonal anti-parvalbumin antibody is specific and does not react with other members of the EF-hand family (technical information provided by Sigma-Aldrich). As in

all other species examined PV morphologically identifies specific classes of cells in bush baby thalamus and cortex (Johnson and Casagrande, 1995; Wong and Kaas, 2010).

Staining using anti-PHAL (table 1) and the procedure above failed to result in stained processes in control sections lacking PHAL indicating primary antibody specificity.

In western blots of bush baby cerebellum and V1 tissue, the mouse monoclonal anti-Vglut2 recognizes a single band at 56kDa, the expected molecular weight (technical information provided by Millipore; Balaram, 2012 personal communication). This antibody shows that this protein is distributed in a similar way in the cortex of tree shrew, bush baby and macaque monkey suggesting that the same protein is being recognized across these distantly related species (Balaram et al., 2011; Hackett and de la Mothe, 2009; Wong et al., 2009; Wong and Kaas, 2010).

For all combinations of primary and secondary antibodies used, omission of the primary antibody resulted in a complete lack of staining indicating specificity of the secondary antibodies.

Visualization of dextran based tracers and cytochrome oxidase (CO)

Dex 568 and dex 488 could be visualized after cutting without additional processing. To visualize BDA, sections were placed in 1:400 or 1:500 Streptavidin Alexa-fluor 488 or Streptavidin Alexa-fluor 568 (Invitrogen) for two hours then rinsed three times in TBS, mounted and coverslipped with Vectashield (Vector Laboratories Inc.). CO staining was done using methods described previously (Boyd and Matsubara, 1996).

Identification and reconstructions of injection and projection sites

Cases were considered successful if injections were localized to the areas of interest and injections produced projections dense enough to clearly reveal what cortical layer the projection targeted. In all successful cases an initial set of sections (every 3rd section in 2 cases, or every 6th section for all other cases) was developed for the tracers injected into that case. Then these sections were inspected in series for the injection sites and their extent, and the locations and extents of axon terminations, centers of most dense projection and regions where axonal projections in V2 from pulvinar and V1 came into close proximity or overlapped. To determine the laminar and nuclear (thalamus) locations of the fluorescent injection and projection sites, a series of adjacent sections were reacted to visualize CO and this set used to confirm areal borders and to align bright field images of CO sections with fluorescent micrographs of injection and projection sites (using Photoshop) so that placement could be confirmed.

Selection of sections analyzed

After an initial survey of sections was taken (see above), sections (either from the initial series of sections or from sections reacted subsequently) were chosen for further analysis using confocal microscopy at high power (63X of 1.4 numerical aperture). Sections chosen were for one of two types of analysis, either co-localization analysis (to determine if projections to V1 or V2 co-localized with parvalbumin or Vglut2), or bouton size analysis (to determine the size of boutons on projections to V1 or V2). Sections selected for co-localization analysis of Vglut2 or parvalbumin with a projection of either V1-V2, pulvinar-V2 or LGN-V1 had good staining in expected areas (layer IV of V1 for Vglut2 and many stained cells and processes through the cortex for parvalbumin) and sufficient density of the axonal projections of interest so that multiple axons in layer IV could be sampled. These samples were chosen from at least two cases for each projection type (V1-V2, pulvinar to V2 or LGN to V1). Except in two cases, sections were chosen for bouton size analysis

because they had low background staining and contained the densest center of the projection foci. In the remaining two cases, sections were chosen for bouton size analysis because they had low background staining and contained an area where projections from pulvinar and V1 came into close proximity to each other ($< 40 \mu\text{m}$), and had a significant numbers of boutons.

Microscopy and Image analysis

Images were collected using bright field, fluorescent and confocal microscopy. A Zeiss LSM 510 confocal microscope with a motorized stage was used to montage a large portion of the sections so that areal and layer data could later be confirmed by alignment with an adjacent CO section. When data pertaining to Vglut2 or parvalbumin were collected, location within the cortical layers was determined by eye at the time of image acquisition from low power fluorescent micrographs of the area of interest. Images for bouton analysis were aligned with a montaged image of the fluorescent section and with a CO section so that a more exact estimation of the layers could be made. Alignment of sections was performed in Photoshop using the vasculature and the outline of the edge of the sections. Levels were adjusted in all photomicrographs subsequently displayed.

Bouton size analysis

High power confocal stacks destined for bouton size analysis were, in most cases, taken from the center of projection foci or in two cases from areas where projections from pulvinar and V1 came into close proximity to each other ($< 40 \mu\text{m}$). In three cases the stack collected contained many more boutons than needed for analysis (as determined by eye), and in these cases analysis was limited to a cube centrally located in the stack, drawn by eye, and containing $\sim 50\%$ of the volume of the stack. Once high power stacks had been obtained, they were converted to grey scale and given to a naive observer who was instructed to pick out boutons. Boutons were defined as enlargements on axon shafts or terminals that were larger than the local average width of the axon and did not occur at points where the axon branched. Once the observer had selected the boutons for analysis, the stack was transferred into Metamorph (Molecular Devices). To measure the area of a given bouton inside Metamorph, first the brightest point within the bouton was found, then working within that plane of the stack, the image was thresholded at half the intensity level of the brightest point and the software was used to automatically circle the thresholded area and record the result. This process was repeated until all the boutons in a stack or designated sub-region were measured. This process is the areal analog to measuring full width at half maximum. If a bouton had a maximum intensity at or above the saturation point of the intensity range, that bouton was excluded from analysis. Such exclusions, however, happened infrequently because during the collection of the high power stacks destined for bouton size analysis, care was taken to minimize the number of pixels that were at saturated intensity within the axons.

During analysis the data were double checked to make sure that no areas were measured that exceeded the limit of the microscope's resolution. The theoretical limit of lateral resolution was calculated using equation 1 where r is the lateral resolution and λ is the wavelength of the light measured and $N.A.$ is the numerical aperture of the objective (see <http://www.olympusconfocal.com/theory/resolutionintro.html> for more information).

$$r = 0.4 \lambda / N.A. \quad \text{Equation 1}$$

In our case λ is either 488 nm or 547 nm and $N.A.$ is 1.4. The lateral resolution was then treated at the radius of a circle and the area of the resulting area of the circle was calculated and compared against the data. No datum was smaller than the area of the circle for the wavelength of the light measured.

In practice the resolution limit of a microscope should be determined experimentally. The management at the Vanderbilt Cell Imaging Shared Resources Core has measured the full width at half maximum resolution in the systems we used to be approximately 300 nm. When extrapolated into two dimensions a radius of 150 nm yields a circle with an area of approximately $0.07 \mu\text{m}^2$. No data measured were equal to or smaller than this area.

Co-localization analysis

To quantify the portion of Vglut2 or PV containing boutons in a population, 2 stacks for each projection type (LGN to V1, V1 to V2, pulvinar to V2) containing the greatest density of projections from two separate cases were chosen from the volume of data that had already been collected. In stacks used to quantify PV only the first 10 (3.41 μm) optical sections beginning at the tissue surface were used for the analysis. This was done because of PV signal drop off deeper in the tissue. Then each bouton within each stack was ranked on a scale of certainty of overlap with 3 categories: clearly co-localizing, clearly not co-localizing, and uncertain degree of co-localization.

Statistics

The bouton areas for each projection type (LGN to V1, V1 to V2, pulvinar to V2) in each case were considered as a single sample. Each sample was tested to see if it violated the assumption of being normally distributed using Lilliefors test (Matlab on line documentation, MathWorks Inc.). Only 2 of the 11 samples were not normally distributed ($p < 0.05$), hence we have assumed for statistical comparison that the data are normally distributed but provide both means and medians for the 2 samples that were not normally distributed. In sample pairs where a t-test was to be performed, a two sample F-test was first administered. If the F-test was significant ($p < 0.05$) then the samples were subsequently compared using a Student's t-test; otherwise the samples were compared using Welch's t-test. A uniform α level of 0.05 was adopted except in the case of multiple t-tests performed after an ANOVA in which case the α level (0.05) was corrected using the Bonferroni method where the α level is divided by the number of possible comparisons (in this specific case 6) before it was compared with the p-value obtained. The variability of all samples is reported as standard errors of the mean (SE).

RESULTS

Thalamic injections

Nine successful pulvinar injections were made in 6 cases. Reconstructions of these injections showed that the sizes of these injections varied between 350 and 1100 μm in diameter and tended to be wider in the dorsal/ventral than in the other two dimensions. In each penetration, either one or two injections were made in the Pulvinar. In the cases where a second injection was made, it was always in the same penetration as the first. Figure 2 shows a typical single injection in the lateral pulvinar. Injections were always located at or above the level of the brachium of the superior colliculus, which has traditionally been used to architectonically subdivide the lateral or superior pulvinar from the inferior pulvinar in bushy (Diamond et al., 1992; Glendenning et al., 1975; Kaas and Lyon, 2007; Raczkowski and Diamond, 1980; Raczkowski and Diamond, 1981; Stepniewska, 2004; Symonds and Kaas, 1978; Wong et al., 2009). Pulvinar neural responses measured at the tip of the injectrode prior to the injection showed that the ventral injection was made at a visuotopic location within 20 deg of *area centralis* (AC). Within the injected part of pulvinar, there are two retinotopic maps (Li et al., 2011), one dorsal and one ventral, which are joined at their central vision representation at the vertical meridian. All of the injections were made at or dorsal to the most central point in the penetration. Histological analysis of the injections suggested that the center of the injection site tended to fall slightly dorsal to

the end of the injectrode track, likely indicating a tendency for the tracer to pool in the space above the tip. Therefore, although involvement of the ventral map cannot be excluded, the bulk of our projections come from the dorsal map within PL. In all cases selected for further analyses (see Methods), a large number of brightly labeled cells (see Figure 2) were seen in the pulvinar, with a significant number of clearly labeled axons leaving the pulvinar. Furthermore, in all of these cases, only a few scattered cells along the injection track were seen to have taken up the label and none of these appeared to have labeled axons.

Successful BDA injections into the LGN were placed at 8, 12-15 and ~20° eccentricities in 2 cases. Two injections involved all layers (see Figure 2), while one involved the medial aspect of M layers alone. All injections resulted in a large number of brightly labeled LGN cells (see Figure 2) with significant numbers of axons leaving the LGN.

V1 injection sites

Nineteen injections were made in V1 in 5 cases. Complete reconstructions of these fluorescent injections and comparison with adjacent sections stained for CO showed that they were 120-400 μm in size and were centered in different layers of V1 mainly in layer III and were always confined to V1 based on the density of CO staining in layer IV and the presence of CO blobs in layer IIIB. Injections into V1 were all placed within the area representing 10° of AC as evidenced by both the OI data used to place the injection (Figure 3A) and by comparison with past mapping studies of V1 in the bush baby (DeBruyn et al., 1993; Xu et al., 2007; Xu et al., 2005). An example of a superficial injection is shown in Figure 3B. These injections resulted in dense labeling in V2. One or two injections were placed per penetration. Most injections were centered on the superficial layer as shown in Figure 3B although, when multiple injections were made in the same penetration, deep layer involvement was common.

Pulvinar projections to V2

Projections from PL were seen in the temporal, parietal and occipital cortices (data not shown). The bulk of these projections were to cortical layers III and IV except in area V1 where there were sparse projections to layers III and IV and dense projections to layer I. For this study, we focused on PL projections to area V2. Projections outside of area V2 will be considered in future studies. V2 received strong projections from the pulvinar, and like other areas of the extrastriate cortex, the bulk of the projections running to V2 terminated in layers III and IV (see Figure 4A), with the area of greatest density in layer IV. Projections from the pulvinar tended to be organized in a columnar manner, particularly in layer III where they were almost always oriented perpendicular to the laminar structure. In addition, patchiness was observed in the projections from the pulvinar to the cortex, with one or two injections leading to 3-5 patches of projections. Curiously, the distinctness of the patches varied from case to case, and no correlation between the distinctness of the patches and the placement of the injections in the pulvinar was observed. In areas where the projections from the pulvinar were densest, a layer I projection also was observed. The layer I projection tended to emanate from the top of the densest parts of the projections in the layers below and then spread out, running parallel to the laminar structure of the cortex within layer I.

V1 projections to V2

Projections from V1 were observed in V2 as well as in an area anterior to V2 assumed to be V3 (see Fan et al., 2012 for review). In both areas, the bulk of the projections terminated in layers III and IV (see Figure 4B), with the area of greatest density in layer IV. In this respect, the projections from V1 to V2 were similar to those from pulvinar to V2, however, two subtle differences were observed at low power. First, pulvinar projections to layer III of V2 tended to extend in a more radial fashion than V1 projections. Second, no V1 projections

were observed in layer I of V2 whereas pulvinar projections to the same layer were repeatedly observed. This latter difference could be due to the fact that individual injections in V1 were smaller than those in the pulvinar, yielding a less dense overall projection pattern.

LGN projections to V1

Figure 4C shows dense layer IV projections from the LGN to V1 as has been widely reported (see Casagrande and Kaas, 1994 for review). We observed these projections in both layer IV α and layer IV β indicating the involvement of both M and P pathways (Florence and Casagrande, 1987). Sparse projections of small caliber axons also were observed in layers III and I confirming involvement of the K layers.

Quantitative comparisons of projections to V2

Figure 5A shows projections from V1 (green) and pulvinar (magenta) in close proximity within the same flattened confocal stack taken from layer IV of V2. Both projections have similar fine morphology with many *en passant* (beads on a string) boutons and occasional stalk-like boutons. As seen in Figure 5A, axons and boutons tended to be larger in projections coming from the pulvinar. Figure 5B shows a quantification of bouton sizes for pulvinar and V1 in three cases. In case 09-02, pulvinar boutons (n=24) had a mean area of $0.52 \mu\text{m}^2$ (± 0.04 SE) and V1 boutons (n=52) had a mean area of $0.39 \mu\text{m}^2$ (± 0.03 SE) and a median of $0.33 \mu\text{m}^2$. In case 09-07, pulvinar boutons (n=36) had a mean area of $0.46 \mu\text{m}^2$ (± 0.03 SE) and V1 (n=8) boutons had a mean area of $0.21 \mu\text{m}^2$ (± 0.03 SE). In case 09-08, pulvinar boutons (n=27) had a mean area of $0.44 \mu\text{m}^2$ (± 0.03 SE) and V1 boutons had a mean area of $0.34 \mu\text{m}^2$ (± 0.03 SE). In each case, the difference between the size of V1 boutons and pulvinar boutons was found to be significant ($p < 0.05$) using Student's t-test in cases 09-02 and 09-08 and using Welch's t-test in case 09-07 (because samples were shown to have unequal variances, see Methods). Boutons measured in cases 09-07 and 09-08 were taken from locations where the axons from V1 and pulvinar innervated the same region of V2 (in areas such as the one pictured in Figure 5A). Therefore, the difference in bouton size is not the result of the two projections to V2 representing different eccentricities. The boutons in case 09-02, however, were taken from non-overlapping projection centers located ~3mm apart thus indicating that the size differences are not specific to the cortical locations where projections overlap.

Quantitative comparisons of projections from LGN to V1

In order to compare our findings in V2 to known thalamocortical driving projections, we examined the projections from the LGN to layer IV of V1. Figure 6A shows large *en passant* boutons in layer IV α (IVC α of Brodmann, 1909) of V1 resulting from a BDA injection in the LGN. Bouton areas in layer IV α (M cell projections) and layer IV β (P cell projections) were quantified and compared to bouton areas of pulvinar and V1 projections to V2 (shown in Figure 5B). As Figure 6B depicts, M boutons in V1 (n = 110 from 2 cases) were found to have a mean area of $0.70 \mu\text{m}^2$ (± 0.03 SE) while P boutons in V1 (n = 41 from 1 case) were found to have a mean area of $0.52 \mu\text{m}^2$ (± 0.03 SE) and a median of $0.52 \mu\text{m}^2$. We compared these results with the findings of bouton areas in V2 depicted in Figure 5B; as seen in Figure 6B. When collapsed across three cases, pulvinar boutons (n=87) had a mean area of $0.47 \mu\text{m}^2$ (± 0.02 SE) and V1 boutons (n=88) had a mean area of $0.36 \mu\text{m}^2$ (± 0.02 SE). A one-way ANOVA run on the M LGN, P LGN, pulvinar and V1 bouton areas described above found significant variation ($F(3, 322) = 40.13, P = 0$). Student's t-tests with Bonferroni correction show that both the LGN projections to V1 and the pulvinar projections to V2 have larger boutons than the V1 projections to V2 ($p < 0.05$); further, the boutons on M LGN projections to V1 are larger than boutons on both the P LGN projections to V1 and the pulvinar projections to V2 ($p < 0.05$). The sizes of boutons on P LGN projections to V1 and

pulvinar projections to V2, however, were not significantly different (Welch's t-test $P > 0.05$).

Control for tracer type

In the cases where boutons were quantified, the majority (14 of 16) of injections in V1 were made with PHAL while all injections in the thalamus were made with dextran based tracers. To control for the possibility that tracer type might affect the measurements of bouton size, we examined a case where both dex 568 and PHAL were injected into V1 in the same area. As seen in Figure 7A, the resulting projections in V2 share the same fine morphology using either tracer type. Boutons on these projections were quantified and the results are shown in Figure 7B. The boutons on dex 568 projections ($n=23$) had a mean area of $0.28 \mu\text{m}^2$ (± 0.02 SE) while boutons on PHAL projections ($n=9$) had a mean area of $0.30 \mu\text{m}^2$ (± 0.02 SE). A Student's t-test showed no difference between these two populations ($p > 0.05$).

Co-localization of parvalbumin in thalamic axons

Parvalbumin has been found to co-localize with known driving axons from LGN to V1 layer IV (Blumcke et al., 1991; DeFelipe and Jones, 1991; Jones and Hendry, 1989; van Brederode et al., 1991). At low power, PV staining revealed brightly labeled cells and processes in both V1 and V2, likely due to the presence of GABAergic interneurons known to express parvalbumin (Johnson and Casagrande, 1995). These cells and processes were particularly dense in layers II, III and IV in the bushy baby. Examination at high power showed that PV co-localized with projections from the LGN to V1 in layer IV α and IV β (parts of both sub-layers are shown in Figures 8A, B, C, D). Interestingly, some geniculov1 processes did not contain any PV and many of those that did contain PV appeared to contain less PV (i.e. were more dimly labeled) compared to surrounding non-geniculofugal processes (presumably belonging to GABAergic interneurons). In V2, the axons from V1 (Figures 9A, B, C, D) and those from pulvinar (Figures 10A, B, C, D) rarely co-localized with PV. In order to compare the amount of co-localization seen in each projection type (LGN-V1, V1-V2 and pul-V2), we chose one confocal stack from 2 cases (see Methods) and determined for each of the boutons in a stack whether that bouton co-localized with PV. Although in V1 LGN axons and boutons co-localized with PV, this analysis focused on boutons because these structures are larger and more readily resolved. Of the boutons examined on LGN to V1 projections, 78% ($n=119$) clearly co-localized with PV while 2% ($n=3$) clearly did not co-localize and a remaining 20% ($n=31$) of boutons examined had an ambiguous degree of overlap. These results contrast with the results of the same analysis conducted on boutons on pulvinar to V2 projections where 1% ($n=1$) clearly co-localized with PV while 69% ($n=49$) clearly did not co-localize and a remaining 30% ($n=21$) of boutons examined had an ambiguous degree of overlap. Similar to the pulvinar to V2 projections, in the V1 to V2 projections, 5% ($n=5$) clearly co-localized with PV, while 73% ($n=73$) clearly did not co-localize and a remaining 22% ($n=21$) of boutons examined had an ambiguous degree of overlap.

Co-localization of Vglut2 in thalamic boutons

When viewed at low power, Vglut2 was seen to brightly label puncta in V1 layer IV with all other parts of V1 and V2 appearing dim in comparison. In V2, however, it was clear that layers III and IV showed increased staining compared to the other layers although this staining as mentioned, was weaker than that seen in V1 layer IV. No labeled cell bodies were observed in the cortex in any layer. Examination at high power showed that Vglut2 expressed as discrete puncta in both V1 and V2 (Figure 11 B, E, and H). In V1, Vglut2 (Figure 11B) was seen to co-localize (Figure 11C) with boutons on projections from the LGN in layer IV α (Figures 9A and C) and IV β (not shown). In V2, boutons on axons from V1 (Figures 9D and F) rarely co-localized (Figure 11F) with Vglut2 (Figures 11E and F),

while boutons on axons from the pulvinar (Figures 11G and I) frequently co-localized (Figure 11I) with Vglut2 (Figures 11H and I). In order to compare the amount of co-localization seen in each projection type (LGN-V1, V1-V2 and pul-V2), we chose one stack from each of 2 cases (see Methods) and determined for each of the boutons in a stack whether that bouton co-localized with Vglut2. Of the boutons examined on LGN to V1 projections, 70% (n=119) clearly co-localized with Vglut2 while 12% (n=20) clearly did not co-localize and a remaining 18% (n=31) of boutons examined had an ambiguous degree of overlap. These results are similar to the results of the same analysis conducted on boutons on pulvinar to V2 projections where 60% (n=176) clearly co-localized with Vglut2 while 20% (n=58) clearly did not co-localize and a remaining 20% (n=59) of boutons examined had an ambiguous degree of overlap. Of the boutons on V1 to V2 projections, only 6% (n=3) clearly co-localized with Vglut2, while 73% (n=39) clearly did not co-localize and a remaining 20% (n=11) of boutons examined had an ambiguous degree of overlap.

DISCUSSION

Our primary goal for this study was to examine and anatomically compare the two main inputs to bushy baby visual area V2 that could serve as drivers for this area, namely, inputs from the pulvinar and inputs from V1. Since the LGN input to V1 is a known driver, we compared its key characteristics to the two potential driving pathways to V2. Our main findings were as follows: 1) both V1 and pulvinar pathways to V2 had some characteristics of driver inputs such as their relative density and terminal patterns in layer IV of V2 and 2) both V1 and pulvinar pathways to V2 were distinct from LGN to V1 layer IV inputs in several ways (see Figure 12). Both pulvinar and V1 inputs to layer IV of V2 showed irregular patches and extended densely into layer III. Also, unlike LGN projections to layer IV of V1 that contain the calcium binding protein parvalbumin, neither pulvinar nor V1 axons in V2 contained this protein. Pulvinar axons, however, were more like LGN axons in two ways: their boutons were as large as the majority of LGN boutons, namely P boutons, and, like LGN boutons in V1, the majority of pulvinar boutons in V2 contained the glutamate transporter Vglut2. By contrast, V1 boutons were significantly smaller than either pulvinar boutons in V2 or LGN boutons in V1 and V1 to V2 boutons rarely showed any evidence of Vglut2.

Below we consider the significance of these results in light of findings of others and in light of the Sherman and Guillery (1998) hypothesis that higher order thalamic nuclei could act as drivers of extrastriate cortex.

Laminar organization of inputs to V2

Others have shown in simian and prosimian primates that the primary inputs to V2 are from V1 and pulvinar and that these inputs end principally in V2 layer IV and lower layer III (Adams et al., 2000; Collins et al., 2001; Cragg, 1969; Cusick and Kaas, 1988; Felleman and Van Essen, 1991; Gattass et al., 1981; Kaske et al., 1991; Kennedy and Bullier, 1985; Kuypers et al., 1965; Lund et al., 1981; O'Brien et al., 2001; Preuss et al., 1993; Rockland and Pandya, 1979; Shipp, 2003; Sincich and Horton, 2002; Tigges et al., 1973; Wong et al., 2009; Zeki, 1969; for reviews see Casagrande and Kaas, 1994; Kaas and Lyon, 2007). Additionally, bushy baby V2, like V2 in other primates gets a variety of inputs from higher order cortical visual areas including MT, V3, the dorsal medial area (DM/V3a) and the dorsal lateral visual area (DL/V4) among others as well as other subcortical sources (Casagrande and Kaas, 1994; Collins et al., 2001). The latter inputs, however, do not uniquely target layer IV and tend to terminate above and below layer IV (see Felleman and Van Essen, 1991 for review). Most of these inputs are far less dense in their distribution than either input to V2 from pulvinar or from V1 (Sincich and Horton, 2002). V2 of bushy baby also shows a similar second order visuotopic organization to that seen in simian primates

(Rosa et al., 1997; Xu et al., 2007; Xu et al., 2005). One difference that has been noted between prosimian bush babies and simian primates is in the compartmental organization in V2 which does not appear to exhibit clear thick and thin stripes as defined by CO or areas of weak orientation selectivity as seen in simian primates (Collins et al., 2001; Condo and Casagrande, 1990; 2007; Xu et al., 2005). Nevertheless, studies of the cortical connections of V2 in bush baby have reported clear evidence of a compartmental organization in V2 based on retrograde labeling following tracer injections into a variety of visual areas (Boyd and Casagrande, 1999; Collins et al., 2001). In our material we found clear regions of overlap between boutons from pulvinar and V1 in layer IV of V2 but also regions without overlap. No differences were noted between regions where these inputs overlapped and where they did not but we cannot rule out the possibility that one or the other input dominates functionally in specific compartments of V2 especially since our data show a patchy pattern of input to V2 from either pathway. Previous studies in macaque and squirrel monkey have demonstrated that pulvinar projects most densely to the CO dark stripes (both thick and thin) with very little input between these zones (Adams et al., 2000; Curcio and Harting, 1978; Levitt et al., 1995; Livingstone and Hubel, 1982; Ogren and Hendrickson, 1977; Sincich and Horton, 2002; Wong-Riley, 1977; Wong-Riley and Carroll, 1984) and data in several simians have document functional differences between V2 compartments although not a property that unifies the CO thick and thin stripes (see Roe and Ts'o, 1995 for review). Therefore, it remains possible that both V1 and pulvinar drive V2 but that they dominate in different compartments for functional reasons that remain to be elucidated.

Bouton Size

Increased bouton size has been correlated with an increased amount of release machinery associated with transmitters and is presumed to cause a greater post synaptic effect (Pierce and Lewin, 1994). Further, in comparisons between retino-geniculate and cortico-geniculate inputs, larger boutons have been found on the driving retino-geniculate afferents (see Sherman and Guillery, 1996 for review) while corticogeniculate afferents clearly act as modulators of the main message. Prior work in macaque monkey already had suggested that lateral pulvinar axons in V2 had larger boutons than boutons on axons from V1 in V2 (Rockland et al., 1999). These qualitative observations, however, were never quantified and came from different cases where injections were not matched for visuotopic eccentricity. Therefore, in the latter study it was never certain whether the differences seen could also reflect known differences related to visuotopy in V1 and V2 (Tootell et al., 1988; Vanduffel et al., 2002; Xu et al., 2007). Controlling for these factors, we quantitatively showed that boutons on pulvinar axons in V2 in bush baby are significantly larger than boutons on axons from V1, confirming and extending the earlier observations in macaque monkeys. When we compared the boutons found in V2 to those present on geniculo-cortical projections, we found that boutons on axons from pulvinar were similar in size to boutons on P LGN axons in layer IV of V1, although both populations were significantly smaller in size than boutons found on M axons in layer IV of V1. Our confocal finding that the M axons have larger boutons than those found on P axons also confirmed prior ultrastructural measurements made in the macaque and cat (Freund et al., 1989; Freund et al., 1985). Our findings also show that boutons on LGN to V1 projections are larger than those on V1 to V2 projections as would be suggested by comparing the work of Anderson and Martin (2009) to the work of Freund et al. (1989).

Neurochemical Content of Boutons

Parvalbumin has been shown to co-localize with geniculo-cortical projections (Blumcke et al., 1991; DeFelipe and Jones, 1991; Jones and Hendry, 1989; van Brederode et al., 1991). Our data show that, as in macaque monkeys, parvalbumin is located in the M and P LGN axons in layer IV of bush baby. Parvalbumin also is found in axons from relay cells in the

medial geniculate nucleus projecting to A1 and in axons from cells in the ventrobasal nucleus projecting to S1 (Jones and Hendry, 1989; Wong and Kaas, 2010). Correlative studies using single labeling of axons and PV also have suggested that PV could occur in axons between thalamus and motor cortex (Melchitzky et al., 1999). Most of this protein, however, is found in the cell bodies and processes of *interneurons* in V1 and other cortical areas both in bush babies and other primate species (see Johnson and Casagrande, 1995). Also, interestingly, the calcium binding content of thalamic cells is not necessarily reflected in their axon content since koniocellular LGN relay cells contain calbindin and project to the CO blobs of V1, but the CO blobs clearly contain the highest amount of parvalbumin while the interblobs contain higher amounts of calbindin (Blumcke et al., 1991; Johnson and Casagrande, 1995). In both of the latter cases the density differences of these calcium binding proteins is reflected in the processes of cortical interneurons, not direct projections from thalamus. Therefore, the presence of the calcium binding proteins parvalbumin and calbindin in excitatory thalamic relay cells does not always correlate with the presence of this protein in their axons nor its cortical distribution in interneurons (see also Negyessy and Goldman-Rakic, 2005). Our data provide no evidence of parvalbumin in the axons from pulvinar to V2, nor evidence of parvalbumin in the axons from V1 to V2. Since virtually all parvalbumin positive cells in V1 are GABAergic interneurons and not excitatory relay cells, the latter result is not surprising. On the other hand parvalbumin is present in some neurons of the lateral pulvinar in both bush babies and other primates (Gutierrez et al., 2000; Imura and Rockland, 2007; Jones and Hendry, 1989). We can't, of course, say if these particular cells send axons to V2 but it is highly likely that they were involved in our thalamic injections. So why is parvalbumin uniquely present in thalamic axons to primary sensory cortex layer IV? Parvalbumin has been associated with cells known to have a relatively high firing rate in cortex (Kawaguchi and Kubota, 1993). No data is available directly for thalamic relay cells providing cell by cell correlations between parvalbumin and firing rate but P and M LGN cells in bush baby have higher firing rates than K cells (Norton and Casagrande, 1982) perhaps requiring more effective calcium buffering in their axons as a result.

Vglut2 is one of three vesicular glutamate transporters, the others being Vglut1 and Vglut3 (see Fremeau et al., 2004 for review). In the visual system Vglut2 has been found to co-localize with retino-geniculate and geniculo-cortical afferents in the rat and ferret, respectively (Land et al., 2004; Nahmani and Erisir, 2005) while Vglut1 is found mainly in connections from cortex such as those projecting back to the LGN (Yoshida et al., 2009). Also, the Vglut2 expression pattern in the brain has been correlated with cell types that have a high probability of release, a property that has been speculated to be important in driving projections (Fremeau et al., 2004; Sherman and Guillery, 2011). Nevertheless, not every driving connection expresses Vglut2, the central example being the layer V projection to the pulvinar (Wei et al., 2011). Additionally, in mouse some thalamocortical axons have been found to contain both Vglut1 and Vglut2 (Graziano et al., 2008). This finding does not discount Vglut2 as a mark of driving, but instead points to the possibility of more than one type of driving connection. This concept of multiple types of drivers is developing in the literature and will be discussed in greater detail below (Sherman and Guillery, 2011; Viaene et al., 2011; Wei et al., 2011). We find that most pulvinar projections to V2 express Vglut2 while V1 projections to V2 almost never express Vglut2. The frequency of expression was similar for the pulvinar and LGN projections to V2 and V1, respectively, but the intensity of expression was quite different, with the LGN projections showing stronger staining for Vglut2 in layer IV of V1. This novel finding fits with a recent *in situ* hybridization study in bush baby that showed mRNA expression of Vglut2 in both LGN and pulvinar cells with the LGN cells having substantially stronger expression (Balaram et al., 2011).

Multiple driver classes

This study grew out of the theoretical framework proposed by Sherman and Guillery (1998; 2002) that originally sought to define glutamatergic inputs as driving or modulatory using a parametric approach originally applied to the early stages of the visual system. In recent years the Sherman lab and other groups have begun to refine the original hypothesis. One important development has been data indicating that there are likely to be multiple types of cortical drivers (Sherman and Guillery, 2011; Viaene et al., 2011; Wei et al., 2011). Another development suggests that, at least in the mouse, there are both driver and modulator class reciprocal connections from the primary sensory cortices to adjoining cortices (Covic and Sherman, 2011; De Pasquale and Sherman, 2011). This second point finds some support from the work of Soares et al. (2004), who, working in the cebus monkey, showed a mixture of both excitation and inhibition of V2 responses after pulvinar blockade with GABA. It is easy to imagine a scenario where removal of one of two drivers to an area would result in a mix of excitation and inhibition. The latter results, however, are difficult to interpret given that, under normal circumstances, GABA effects last a very brief period (see Allison et al., 2000; Nealley and Maunsell, 1994), but much longer time periods were reported by Soares et al. (2004). Regardless, in the case of cortico-cortical connections the question remains whether the present work supports more than one class projecting from V1 and V2. If this were the case one would potentially expect to find a bimodal distribution in the sizes of boutons on V1 to V2 axons. The present data, however, are unimodal and so can not support this hypothesis, at least using this single original metric. Although our data do not support multiple populations within the V1 to V2 projections we cannot reject the possibility that V1 to V2 projections and pulvinar to V2 projections represent different types of driving connections particularly given the patchy distribution of projections from V1 and from pulvinar to V2 (see above).

Functional Considerations

Both the presence of Vglut2 and large bouton size have been linked to higher probability of transmitter release, a characteristic necessary for fidelity of information transfer (Covic and Sherman, 2011; Fremeau et al., 2004). By contrast, the presence of the other primary vesicular glutamate transporter (Vglut1) and small bouton size have been linked to low probability of release and various types of plasticity, especially potentiation (Balschun et al., 2010; Covic and Sherman, 2011; Fremeau et al., 2004). The latter appear to define, at least morphologically, the V1 to V2 axons. Caution is needed obviously in drawing functional conclusions from these metrics particularly given the compartmental specialization of V2 in primates and the possibility of multiple classes of drivers.. Nevertheless, our anatomical data on bouton sizes and axonal Vglut2 suggest that PL projections to V2 are more similar to geniculoc-V1 projections than are V1 projections to V2.

Traditionally, it has been thought that cortico-cortical feedforward projections, like the ones from V1 to V2, are responsible for driving a cortical area to respond to external sensory stimulation. Sherman and Guillery have hypothesized that secondary thalamic nuclei, such as the pulvinar, drive higher sensory cortex (e.g., V2) while cortico-cortical connections may modulate this driven activity (Sherman and Guillery, 1998; Sherman and Guillery, 2002). Our anatomical data on bouton sizes and axonal Vglut2 suggest that PL rather than V1 might be the driver of neural activity in V2. Furthermore, our recent study demonstrated that PL also powerfully controls and gates the information outflow from V1 to extrastriate areas (Purushothaman et al., 2012). Thus, it appears that PL is situated not only to directly activate V2 but also to control the V1 signals that influence V2. Taken together, these two studies emphasize a critical role for thalamocortical connections in determining the propagation of sensory information through the cortex.

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Figure 1.

Schematic summary of relevant connections between the eye (RET), lateral geniculate nucleus (LGN), lateral pulvinar (PUL) and primary (V1) and secondary (V2) visual cortical areas in bush baby. (A) Connections between areas are indicated by single or double-headed arrows. The connections compared in this study are indicated by green arrows. (B) Details of known primary connections between V1 and V2 and PUL and the individual layers of the LGN, the koniocellular (K), magnocellular (M) and parvocellular (P). Projections to cortex are indicated by horizontal line segments within the cortical layers which are indicated by roman numerals. Projections back to thalamus are indicated by connections from individual black cells in cortical layers V and VI. The open circle in V1 layers II/III indicates the

location of a cytochrome oxidase blob in V1. Data for this Figure were taken from Collins et al., 2003; Florence and Casagrande, 1987; Ichida and Casagrande, 1999; Lachica and Casagrande, 1992; Raczkowski and Diamond, 1981 and unpublished observations.

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Figure 2.

A coronal cytochrome oxidase (CO) section through the thalamus showing examples of injection sites in lateral geniculate nucleus (LGN) (green, biotinylated dextran [BDA]) and pulvinar (magenta, dextran conjugated to Alexafluor 568 [dex 568]) taken on the same section as a fluorescent image prior to CO staining. The LGN injection in this case involved all layers. The pulvinar injection was centered on the central vision representation in lateral pulvinar (PL). M, magnocellular; P, parvocellular; K, koniocellular; c, contralateral; i, ipsilateral; PI, inferior pulvinar; PL, lateral pulvinar; D, dorsal; L, lateral. Scale bar = 500 μ m.

Figure 3.

(A) Differential optical imaging of primary visual cortex (V1) viewed from the surface showing the activation resulting from the presentation of orthogonal drifting square wave gratings limited to a 5° circular window placed on *area centralis* (AC). This differential image allowed us to place the plant lectin *Phaseolus vulgaris* leucoagglutinin (PHAL) injections (red dots) near central vision. Scale bar = 2.75mm. (B) An example of an injection site (red) shown on a CO stained section. Since AC is represented laterally in V1 of bushbaby this section shows a slightly tangential view of the layers. CO blobs (arrow) can also be seen in layer III in the section. Scale bar = 250 μ m.

Figure 4.

Confocal photomicrographs of projections (green) to secondary visual cortex (V2) from pulvinar (**A**) and V1 (**B**) and from the LGN to V1 (**C**). Comparison of these projection patterns shows that all projections are centered in cortical layer IV but show different overall patterns of termination within layer IV and the other layers. Note that the LGN injection in this case was restricted to the ipsilateral parvocellular (P), koniocellular (K) and magnocellular (M) LGN layers given that ocular dominance columns can be seen clearly throughout the depth of layer IV. The K layer projections to layer III blobs and layer I of V1 were not present in this section. Images A and B are taken from a part of V2 on the dorsal

surface of the cortex while image C is taken from a part of V1 near the posterior pole located on the ventral surface of the brain. In image A and B dorsal is towards the top and lateral is towards the left. In image C dorsal is towards the bottom and lateral is towards the right. See text for details. Scalebars = 200 μ m

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Figure 5.

Pulvinar boutons were larger than V1 boutons in V2. **(A)** Boutons in V2 from the pulvinar (magenta, BDA) and V1 (green, PHAL) shown in a high power confocal photomicrograph (scale bar = 10 μm, flattened stack). **(B)** The cross-sectional areas of boutons on projections from pulvinar (magenta) and V1 (green) to V2 were quantified in three cases. A t-test revealed that the pulvinar boutons were significantly larger in every case ($p < 0.05$).

Figure 6.

LGN boutons in layer IV of V1 varied in size. Those from the M layers of LGN to IV α are shown in **(A)**. These boutons are significantly larger than any boutons within layer IV of V2 from either V1 or pulvinar. (scale bar = 10 μm , flattened stack). **(B)** LGN P projections to layer IV β of V1, however, did not differ in size from pulvinar to V2 boutons. (t-test $p > 0.05$).

Figure 7. Morphology revealed by dextran and PHAL tracing of the same projection is indistinguishable. **(A)** Projections from V1 labeled with dex 568 (magenta) and PHAL (green) in a high power confocal micrograph (scale bar = 10 μm , flattened stack) of V2. **(B)** The cross-sectional areas of projections from V1 to V2 traced with dex 568 are the same size as boutons on the same projections traced with PHAL (t-test $p > 0.05$).

Figure 8. Single plane confocal photomicrograph of LGN axons and parvalbumin (PV) in V1 (**A**). Higher magnification views from the same image (**B-D**) of a single axon indicated by the white arrowhead in all four panels. PV (green **A, B, D**) and LGN axons (Magenta **A, C, D**) co-localize (**A, D**). A striking structural congruency in the separate channels (compare **B** and **C**) was found for the majority of processes. See text for more details. Scale bars = 5 μ m.

Figure 9.

Single plane confocal photomicrograph of V1 axons and parvalbumin (PV) in V2 (A). Higher magnification views from the same image (B-D) of a single axon indicated by the white arrowhead in all four panels. PV (green A, B, D) and V1 axons (Magenta A, C, D) do not co-localize (A, D). See text for more details. Scale bars = 5 μ m.

Figure 10.

Single plane confocal photomicrograph of pulvina axons and parvalbumin (PV) in V2 (**A**). Higher magnification views from the same image (**B-D**) of a single axon indicated by the white arrowhead in all four panels. PV (green **A, B, D**) and pulvina axons (Magenta **A, C, D**) do not co-localize (**A, D**). See text for more details. Scale bars = 5 μm .

Figure 11.

LGN boutons in V1 (green, **A, C**) and vesicular glutamate transporter 2 (Vglut2) (magenta, **B, E, F**) co-localize (white, **C**). Similarly, pulvinar (green, **G, I**) boutons and Vglut2 co-localize in V2 (white, **I**) but V1 boutons in V2 (green, **D, F**) rarely co-localized with Vglut2 (**F**). Scale bar = 5 μ m.

Boutons in Layer IV

Figure 12.

Schematic summary of the main results. Axons with two boutons each from pulvinar and V1 in layer IV of V2 are shown on the left and an axon with two boutons from a P LGN cell in layer IV of V1 is shown on the right. The LGN boutons contain both PV and Vglut2, the pulvinar boutons contain only Vglut2 and the V1 boutons contain neither protein. See text for details.

Table 1

Primary Antibodies

Antigen	Immunogen	Manufacturer	Dilution factor
Parvalbumin	Purified frog muscle parvalbumin	Sigma-Aldrich, Inc (Saint Louis, MO), mouse monoclonal, lot #048k4752, cat # P3088	1:1000
<i>Phaseolus vulgaris</i> leucoagglutinin	Purified lectins	Vector labs (Burlingame, CA), Rabbit polyclonal, lot # T-1024, cat # AS-2300	1:2000
Vesicular glutamate transporter-2	Recombinant protein from rat VGluT2, full length	Chemicon now part of Millipore (Billerica, MA), mouse monoclonal, lot # Lv1823333, cat # MAB5504	1:5000

Table 2

Secondary Antibodies

Secondary antibody	type	Dilution	Source	Cat #
Alexa-fluor 488	Donkey anti-rabbit	1:400	Invitrogen	A10042
Alexa-fluor 568	Donkey anti-rabbit	1:400	Invitrogen	A21206
Alexa-fluor 647	Donkey anti-mouse	1:400	Invitrogen	A31571
DyLight 698	Donkey anti-mouse	1:500	Jackson	715495-150