The Mouse Pulvinar Nucleus Links the Lateral Extrastriate Cortex, Striatum, and Amygdala

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The pulvinar nucleus is a large thalamic structure involved in the integration of visual and motor signals. The pulvinar forms extensive connections with striate and extrastriate cortical areas, but the impact of these connections on cortical circuits has not previously been directly tested. Using a variety of anatomical, optogenetic, and *in vitro* physiological techniques in male and female mice, we show that pulvinocortical terminals are densely distributed in the extrastriate cortex where they form synaptic connections with spines and small-diameter dendrites. Optogenetic activation of these synapses *in vitro* evoked large excitatory postsynaptic responses in the majority of pyramidal cells, spiny stellate cells, and interneurons within the extrastriate cortex. However, specificity in pulvinar targeting was revealed when recordings were targeted to projection neuron subtypes. The neurons most responsive to pulvinar input were those that project to the striatum and amygdala (76% responsive) or V1 (55%), whereas neurons that project to the superior colliculus were rarely responsive (6%). Because the pulvinar also projects directly to the striatum and amygdala, these results establish the pulvinar nucleus as a hub linking the visual cortex with subcortical regions involved in the initiation and control of movement. We suggest that these circuits may be particularly important for coordinating body movements and visual perception.

Key words: corticocortical; corticostriatal; corticotectal; electron microscopy; interneuron; pulvinocortical

Significance Statement

We found that the pulvinar nucleus can strongly influence extrastriate cortical circuits and exerts a particularly strong impact on the activity of extrastriate neurons that project to the striatum and amygdala. Our results suggest that the conventional hierarchical view of visual cortical processing may not apply to the mouse visual cortex. Instead, our results establish the pulvinar nucleus as a hub linking the visual cortex with subcortical regions involved in the initiation and control of movement, and predict that the execution of visually guided movements relies on this network.

Introduction

Vision is a very active process in that we constantly scan our surroundings using eye and body movements. As a consequence, there must be a tight coordination between the visual and motor circuits of the brain. A variety of studies suggest that the dorsal thalamus is involved in this visuomotor coupling. For example, in the primate, inactivation of the pulvinar nucleus disrupts the planning of visually guided eye and hand movements (Wilke et al., 2010), and inactivation of the mediodorsal nucleus can both

disrupt sequential saccadic eye movements and shift visual receptive fields in the frontal cortex (Sommer and Wurtz, 2002, 2006). Most recently, projections to the striate cortex from the mouse lateral posterior nucleus (considered a homolog of the tectorecipient zones of the primate pulvinar nucleus) (Zhou et al., 2017) have been shown to signal discrepancies between optic flow and running speed (Roth et al., 2016). Therefore, pulvinar activity conveys visual information that relates to movement, and this activity appears to be crucial for the subsequent planning and execution of appropriate visually guided actions. However, the synaptic mechanisms underlying these complex functions are currently unknown.

In particular, the impact of pulvinar projections on cortical circuits has not been directly tested. Indeed, conventional hierarchical views of cortical organization imply a relatively minor impact of pulvinar projections on activity in extrastriate regions (Van Essen, 2005). Instead, corticocortical transfer of visual information from V1 is thought to drive receptive field properties in extrastriate regions of the visual cortex because lesions of the striate cortex greatly diminish visually driven activity in these

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areas (Girard and Bullier, 1989; Girard et al., 1991, 1992; Kaas and Krubitzer, 1992; Collins et al., 2003, 2005). Nevertheless, visually evoked activity can still be recorded in extrastriate areas following lesions or cooling of the striate cortex (Rodman et al., 1989; Girard et al., 1992; Azzopardi et al., 2003). Moreover, inactivation of the pulvinar nucleus can suppress visual activity within V1 (Purushothaman et al., 2012). Thus, pulvinar projections could effectively regulate cortical processing.

In the current study, we sought to directly test the impact of pulvinar projections on cortical circuits by examining the synaptic properties of terminals that originate from this thalamic region. Using a combination of anatomical and optogenetic techniques, we found that, in mice, the pulvinar nucleus can greatly impact the activity of the extrastriate cortex, and recordings targeted to specific projection neuron subtypes suggest that the pulvinar may act as a hub to dynamically coordinate body movements with the perception of visual signals.

Materials and Methods

Animals. All breeding and experimental procedures were approved by the University of Louisville Institutional Animal Care and Use Committees. Experiments were performed using mice, of either sex, of a C57BL/6 line (Jackson ImmunoResearch Laboratories, stock #000664), or a line in which neurons that contain the 65KD isoform of glutamic acid decarboxylase (GAD65) express GFP (López-Bendito et al., 2004). A calretinin-cre driver line (Calb2-IRES-Cre, Jackson ImmunoResearch Laboratories, stock #010774, B6(Cg)-Calb2^{tm1(cre)Zjh/J}) was used to generate a map of pulvinocortical projections.

Biotinylated dextran amine (BDA) injections. To label thalamocortical axon projections via anterograde transport, C57BL/6 mice ranging in age between postnatal day 22 (P22) and P35 were deeply anesthetized with a mixture of ketamine (100–150 mg/kg) and xylazine (10–15 mg/kg). The analgesic meloxicam (1–2 mg/kg) was also injected before surgery. The animals were then placed in a stereotaxic apparatus (Angle Two Stereotaxic, Leica). An incision was made along the scalp, and a small hole was drilled in the skull. A glass pipette (20–40 μ m tip diameter) containing a 5% solution of BDA (Invitrogen) in saline was lowered into the dorsal lateral geniculate nucleus (dLGN) (from bregma: 2.14 posterior, 2.0 lateral, 2.89 ventral) or pulvinar (from bregma: 2.11 posterior, 1.73 lateral, 2.73 ventral), and BDA was iontophoretically ejected using 3 μ A continuous positive current for 20 min. After removal of the pipette, the scalp skin was sealed with tissue adhesive (*n*-butyl cyanoacrylate), lidocaine was applied to the wound, and the animals were placed on a heating pad until mobile. After surgery, animals were carefully monitored for proper wound healing, and oral meloxicam (1–2 mg/kg) was administered for 48 h.

Cholera toxin subunit B (CTB) injections. To label cortical projection cells via retrograde transport, P22–P35 C57BL/6 mice were prepared as described above. Either a glass pipette (20–40 μ m tip diameter), or a Nanofil syringe with an attached 34 gauge needle, containing a 0.2% solution of cholera toxin subunit B conjugated to AlexaFluor-488, -546, or -633 (CTB-488, CTB-546, CTB-633) in PBS (0.01 M phosphate buffer with 0.9% NaCl) was lowered into cortical area V1 (from bregma: 4.02 posterior, 2.5 lateral, 1.81 ventral), striatum and/or amygdala (from bregma: 1.55 posterior, 3.58 lateral, 4.5 ventral), or superior colliculus (from bregma: 4.09 posterior, 0.68 lateral, 1.7 ventral). CTB was iontophoretically ejected using 3 μ A continuous positive current for 15 or 30 min, or 100–210 nl was ejected at a rate of 25 nl/min using an ultramicropump. After removal of the pipette or needle, the wound was closed and the animals were treated during recovery as described above.

Herpes simplex virus (HSV) injections. The HSV virus hEF1a-EYFP-IRES-cre (obtained from the Massachusetts Institute of Technology viral vector core) was injected into the striatum and/or amygdala to label projection cells in the lateral extrastriate cortex (LES) via retrograde uptake. This virus induced the infected cells to express a yellow fluorescent protein (YFP). P22–P60 C57BL/6 mice were deeply anesthetized and prepared as described above, and a Nanofil syringe and ultramicropump

were used to deliver volumes of 100–250 nl at a rate of 10 nl/min. The wound was then closed, and the animals monitored during recovery as described above.

Adeno-associated virus (AAV) injections. To label and activate the projections of the pulvinar, an AAV serotype 2/1 carrying a vector for the Channelrhodopsin variant Chimera EF with I170 mutation (ChIEF) fused to the red fluorescent protein, tdTomato (for production details, see Jurgens et al., 2012) was injected unilaterally or bilaterally into the pulvinar. To label projections from the pulvinar to subsequently construct a map of cortical areas in the coronal plane, a cre-dependent virus, Flex-*rev-oChIEF*-tdTomato (Plasmid #30541, Addgene), packaged using AAV serotype 9, was injected unilaterally into the pulvinar of calretinin-cre mice. For virus delivery, P22–P60 C57BL/6 or GAD65-GFP mice were deeply anesthetized with a mixture of ketamine and xylazine as described above. An incision was made along the scalp, and a small hole created in the skull above the left and/or right pulvinar. Virus was delivered via a 34-gauge needle attached to a Nanofil syringe inserted in an ultramicropump. A volume of 75 nl was injected into each pulvinar at a rate of 20 nl/min.

AAV and CTB or HSV injections. In some cases, in the same animals that received unilateral AAV injections in the pulvinar, an additional injection of CTB-488 or HSV-YFP was placed in the ipsilateral cortex, striatum/amygdala, or SC as described above. The wound was then closed, and the animals monitored during recovery as described above.

Slice preparation and optogenetic stimulation. Eight to 12 d following virus injections, mice were deeply anesthetized with avertin (0.5 mg/kg). Mice used for slice preparation ranged in age from P29 to P37 (average age P31). Mice were either directly decapitated or transcardially perfused with cold (4°C), oxygenated (95% O₂/5% CO₂) slicing solution containing the following (in mM): 2.5 KCl, 26 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 10 MgCl₂, 2 CaCl₂, 234 sucrose, and 11 glucose, before rapid decapitation (in mice older than P35). The brain was removed from the head, chilled in the cold slicing solution described above for 2 min, and was quickly transferred into a Petri dish with room temperature slicing solution to block the brain for subsequent sectioning. Coronal slices (300 μ m) were cut in room temperature slicing solution using a vibratome (Leica VT1000 S). Then slices were transferred into an incubation solution of oxygenated (95% O₂/5% CO₂) ACSF containing the following (in mM): 126 NaCl, 26 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 2 MgCl₂, 2 CaCl₂, and 10 glucose at 32°C for 30 min, and later maintained at room temperature.

Individual slices were transferred into a recording chamber, which was maintained at 32°C by an inline heater and continuously perfused with room temperature oxygenated ACSF (2.5 ml/min, 95% O₂/5% CO₂). Slices were stabilized by a slice anchor or harp (Warner Instruments, 64–0252). Neurons were visualized on an upright microscope (Olympus, BX51WI) equipped with both differential interference contrast optics and filter sets for visualizing CTB-488 and YFP (Chroma 49002) or tdTomato (Chroma 49005) using a 4 $\times$ or 60 $\times$ water-immersion objective (Olympus) and a CCD camera. Recording electrodes were pulled from borosilicate glass capillaries (World Precision Instruments) by using a Model P-97 puller (Sutter Instruments). The electrode tip resistance was 4–6 M Ω when filled with an intracellular solution containing the following (in mM): 117 K-gluconate, 13.0 KCl, 1 MgCl₂, 0.07 CaCl₂, 0.1 EGTA, 10 HEPES, 2 Na₂-ATP, and 0.4 Na₂-GTP, with pH adjusted to 7.3 with KOH and osmolarity 290–295 mOsm. Biocytin (0.5%) was added to this intracellular solution to allow morphological reconstruction of the recorded neurons.

Whole-cell recordings were obtained from the LES regions of the cortex. For single-injection experiments (in which only the pulvinar was injected with virus), cells in layers IV and V were targeted for recording within the pulvinar termination zones. For GAD65-GFP experiments, interneurons labeled with GFP with overlapping pulvinar terminals were targeted for recording. For double-injection experiments (in which the pulvinar was injected with virus and the SC, striatum, or cortex were injected with CTB-488 or HSV-YFP to label LES projection cells via retrograde transport), labeled and unlabeled cells were targeted for recording with the pulvinar termination zones. Video images of the patched cell locations, and the CTB/YFP/GFP within patched cells, were recorded using the CCD camera.

Recordings were obtained with an AxoClamp 2B amplifier (Molecular Devices), and a Digidata 1440A was used to acquire electrophysiological signals. The stimulation trigger was controlled by Clampex 10.3 software (Molecular Devices). The signals were sampled at 20 kHz, and data were analyzed offline by pClamp 10.0 (Molecular Devices). Series resistance was compensated by a bridge protocol and only recordings with stable series resistance and overshooting action potentials were included in the analysis. For current-clamp recordings, voltage signals were obtained from cells with resting potential of -60 mV to -75 mV. For voltage-clamp recordings, membrane currents were obtained at -65 mV to -75 mV.

For photoactivation of pulvinocortical terminals, light from a blue light-emitting diode (Prizmatix UHP 460) was reflected into a $60\times$ water-immersion objective. This produced a spot of blue light onto the submerged slice with a diameter of ~ 0.3 mm. Pulse duration and frequency were under computer control. For repetitive stimulation, pulse duration was either 1 or 10 ms. Synaptic responses were recorded using light intensities of 10 – 112 mW/mm² (the intensity was measured using a light meter placed under the dry objective), and light pulse frequencies of 1, 2, 5, 10, and 20 Hz.

To test whether the responses were monosynaptic, TTX (1 μ M; Alomone Labs, catalog # T-550) was added to the bath to block action potentials, and 4-aminopyridine (4-AP, 1 mM; Sigma, catalog #275875-5G) was added to augment depolarization of the terminals. In other experiments, APV (10 μ M; Sigma, catalog #A-5282) and/or CNQX (8 μ M; Tocris Bioscience, catalog #0190)/DNQX (80 μ M; Sigma, catalog #D0540-50MG) were added to the bath to block NMDA and AMPA receptors. GABA receptors (GABA_A) were blocked via bath application of the antagonist 2-(3-carboxypropyl)-3-amino-6-(4-methoxyphenyl)-pyridazinium bromide (SR95531, 20 μ M; Tocris Bioscience, catalog #1262).

Processing of cells filled during physiological recording. Following recording, slices were placed in a fixative solution of 4% PFA in 0.1 M phosphate buffer (PB), pH 7.4, for at least 24 h. The sections were then rinsed in PB and incubated overnight in a $1:1000$ dilution of streptavidin-conjugated to AlexaFluor-633 (Invitrogen) in PB containing 1% Triton X-100. The following day, the slices were washed in PB, preincubated in 10% normal goat serum in PB, and then incubated overnight in a $1:500$ dilution of a rabbit anti-DSred antibody (Clontech, catalog #632496) in PB with 1% normal goat serum. The following day, the sections were rinsed in PB and incubated for 1 h in a $1:100$ dilution of a goat-anti-rabbit antibody conjugated to AlexaFluor-546 (Invitrogen). The sections were then rinsed in PB and mounted on slides to be imaged with a confocal microscope.

Histology for tissue used for anatomical analyses. For animals that were not used for physiological experiments, 2 d to 2 weeks following injection of tracers and/or viruses, mice were deeply anesthetized with Avertin (0.5 mg/g) and transcardially perfused with a fixative solution of 4% PFA, or 2% PFA, and 2% glutaraldehyde in PB. Additional C57BL/6 or GAD65-GFP mice that were not injected were also perfused for immunocytochemistry. In each case, the brain was removed from the skull and 70 – 100 μ m-thick coronal sections were cut using a vibratome (Leica Microsystems). Sections that contained fluorescent labels were mounted on slides and imaged using a confocal microscope (Olympus, FV1200BX61), or additionally stained using antibodies as described below.

Selected sections were incubated overnight in antibodies against parvalbumin (made in mouse, Sigma, catalog P3088, $1:2000$) or calretinin (made in mouse, Millipore, catalog MAB1568, $1:1000$). The following day, the sections were incubated in a $1:100$ dilution of a goat-anti-mouse antibody that was directly conjugated to fluorescent compounds (AlexaFluor-488, -546, or -633; Invitrogen). The sections were then mounted on slides and imaged using a confocal microscope (Olympus).

To label tissue for viewing in a transmitted light microscope or transmission electron microscope, sections that contained CTB-labeled cells were incubated overnight in a rabbit anti-CTB antibody (Sigma, catalog #C3062; $1:10,000$). Sections that contained TdTomato were incubated overnight in a rabbit anti-DSred antibody ($1:500$). Sections that contained YFP or GFP were incubated overnight in a rabbit anti-GFP antibody (Millipore, catalog #AB3080, $1:1000$). Sections incubated in the

antibodies or sections that contained BDA were incubated in a $1:100$ dilution of a biotinylated goat-anti-rabbit antibody (1 h), followed by avidin and biotinylated HRP (ABC solution, Vector Laboratories, 1 h) and reacted with nickel-enhanced DAB. The sections were then mounted on slides and imaged using transmitted light, or processed for electron microscopy as described below.

Electron microscopy. Sections that contained terminals labeled by the anterograde transport of BDA, or cells and terminals labeled with the GFP antibody, were postfixed in 2% osmium tetroxide, dehydrated in an ethyl alcohol series, and flat-embedded in Durcupan resin between two sheets of Aclar plastic (Ladd Research). Durcupan-embedded sections were first examined with a light microscope to select areas for electron microscopic analysis. Selected areas were mounted on blocks, ultrathin sections (70 – 80 nm, silver-gray interference color) were cut using a diamond knife, and sections were collected on Formvar-coated nickel slot grids. Selected sections were stained for the presence of GABA. A postembedding immunocytochemical protocol described previously (Chomsung et al., 2008, 2010; Day-Brown et al., 2010) was used. Briefly, we used a rabbit polyclonal antibody against GABA that was tagged with a goat-anti-rabbit antibody conjugated to 15 nm gold particles (BBI Solutions). The sections were air dried and stained with a 10% solution of uranyl acetate in methanol for 30 min before examination with an electron microscope.

Experimental design and statistical analyses. Three general types of experiments were developed and reported in this manuscript: (1) electron microscopic analysis of labeled profiles, (2) *in vitro* electrophysiological analysis of responses to photoactivation of pulvinocortical terminals, and (3) morphological analysis of cortical neurons filled with biocytin during recording.

1. For electron microscopic analysis of tracer-labeled thalamocortical terminals and their postsynaptic targets ($n = 7$ animals), ultrathin tissue sections were examined using an electron microscope, and every labeled terminal involved in a synapse was imaged ($n = 501$ terminals). The presynaptic and postsynaptic profiles were characterized on the basis of size (measured using ImageJ, RRID:nif-000-30467, Maxim DL 5 software) and the presence or absence of synaptic vesicles. To analyze tissue from a GAD65-GFP mouse that was stained for GABA using postembedding immunocytochemical techniques, GFP-labeled profiles ($n = 136$) were imaged and the overlying gold particle density was quantified. One-way ANOVA with Tukey's Multiple Comparisons Post Test were used for statistical analyses of ultrastructural data and plotted as column box-and-whisker graphs using Prism 6.0.

2. For *in vitro* electrophysiological analysis of responses to photoactivation of pulvinocortical terminals in labeled or unlabeled neurons, 108 animals of either sex were used and a total of 501 neurons were patched in slices of the cortex (4 or 5 cortical slices were used per animal). The majority of recorded neurons ($n = 425$) were tested to determine their response to activation of surrounding pulvinocortical terminals. Baseline values were measured just before the onset of photostimulation, and the amplitude of synaptic responses was measured from this baseline value. Further analysis was limited to neurons with response amplitudes >7 times the SD of the baseline ($n = 278$). Neurons below this conservative threshold were considered nonresponsive and excluded from further electrophysiological analysis. Electrophysiological measurements were tested for normality using the D'Agostino-Pearson Omnibus Test, and appropriate statistical analyses were chosen based on the Gaussian or non-Gaussian distribution of the data. For each variable, differences between the groups were assessed using nonparametric one-way ANOVA followed by *Post hoc* Tukey's Multiple Comparison Test. For comparisons of response probability from contingency graphs, Fisher's Exact Test was used. All data were presented as mean $\pm$ SD, except the vertical scatter plots, which were plotted as mean $\pm$ SEM. Significance was set at $\alpha = 0.05$ for all statistical tests. Prism 6.0 was used to generate vertical scatter plots, bar graphs, column mean $\pm$ SD connected graphs, or grouped interleaved bar graphs.

3. Confocal images of labeled cells were categorized based on the following criteria: location of soma and dendrites relative to labeled thalamocortical terminals, location of the neuron within a map of the cortex (see Fig. 5), the presence or absence of an apical dendrite, or the

Figure 1. Laminar distribution and ultrastructure of thalamocortical terminals originating from the dLGN and pulvinar. *A*, In V1, thalamocortical terminals originating from the dLGN primarily innervate layers I and IV, and corticogeniculate cells are concentrated in layer VI. *B*, In V1, sparser thalamocortical terminals originating from the pulvinar innervate layers I and Va. *C*, In LM/LI, dense terminals originating from the pulvinar innervate layers I and IV. Corticothalamic cells that project to the pulvinar are seated in layer VI. *D–F*, Examples of (Figure legend continues.)

Figure 2. Optogenetic activation of thalamocortical terminals that originate from the pulvinar. **A**, Schematic illustrates the experimental protocol. Virus injections in the pulvinar induced expression of ChIEF and TdTomato in pulvinar-cortex terminals (red). Ten days later, slices of the cortex were prepared for *in vitro* whole-cell patch recordings in the area of labeled terminals. Biocytin (green) was included in the pipettes to fill cells while recording their responses to blue light pulses. **B**, Injection site in the pulvinar. **C**, Induced TdTomato expression in pulvinar-cortex terminals in V1 and LES. **D**, **E**, Higher magnification of pulvinar terminals in V1 and LES. **F**, **G**, Pulvinar-LES responses were light intensity-dependent (100% light intensity equals 112 mW/mm²) and could be elicited in the presence of 1 μ M TTX when paired with 1 mM 4-AP, and were blocked by the application of 80 μ M DNQX and 10 μ M APV, indicating that glutamate release from pulvinar terminals activates AMPA and NMDA receptors. **H**, Pyramidal cells (green) that responded to light activation of surrounding pulvinar-cortex terminals (red). Scale bars: **B**, **C**, 250 μ m; **D**, **E**, 100 μ m; **H**, 50 μ m. PM, Posteromedial cortex; PT, pretectum; vLGN, ventral lateral geniculate nucleus; PUL, pulvinar nucleus.

presence or absence of markers in the soma (retrograde markers CTB or YFP, or GFP labeling in GAD65-GFP mice). A total of 356 cells were recovered, and the labeling of 316 of these cells was sufficiently complete to categorize their morphology.

Results

Extrinsic projections of the mouse pulvinar: comparison with the dLGN

Laminar distribution of pulvinar and dLGN projections

Iontophoretic injections of BDA in the mouse dLGN or pulvinar label distinct bands of terminals in V1. Terminals labeled by injections in the dLGN are concentrated in layers IV and I, with sparser terminations located in layers II/III and VI (Fig. 1A). Terminals labeled by injections in the pulvinar are concentrated in layers I and Va (Fig. 1B), as previously described (Herkenham, 1980; Roth et al., 2016). In addition to V1, the pulvinar projects densely to the lateral extrastriate cortex: LES, corresponding to the posterior (P), postrhinal (POR), lateromedial (LM), and laterointermediate (LI) cortex, defined by Wang and Burkhalter (2007). Within the LM, LI regions, projections from the pulvinar

are concentrated in layers IV and I (Fig. 1C). Indeed, the laminar distribution of pulvinocortical terminals in the LM and LI is very similar to that of geniculocortical terminals in V1 (Fig. 1A). Pulvinar projections are most densely distributed within the P and POR. In these cortical regions, projections from the pulvinar are concentrated in layers I and IV but also extend into layers V and VI (Roth et al., 2016).

Ultrastructure of geniculocortical and pulvinocortical terminals

Using electron microscopy, we examined a total of 647 labeled geniculocortical or pulvinocortical terminals involved in synapses across cortical areas and lamina: dLGN-V1 terminals in layer I, $n = 84$; dLGN-V1 terminals in layer IV, $n = 94$; pulvinar-V1 terminals (layers not differentiated), $n = 83$; pulvinar-LM/LI terminals (layers not differentiated), $n = 118$; pulvinar-P/POR terminals (layers not differentiated), $n = 122$; pulvinar-P/POR terminals in layer I, $n = 70$; and pulvinar-P/POR terminals in layer IV, $n = 76$ terminals. Labeled synaptic terminals in all areas contained densely packed synaptic vesicles and made synaptic contacts on small dendrites and spines with thick postsynaptic densities (Fig. 1D–F).

Measurements of the size of presynaptic terminals (Fig. 1G) established that, as a group, the smallest presynaptic profiles were pulvinocortical terminals in V1 ($0.25 \pm 0.20 \mu\text{m}^2$). These terminals were significantly smaller than geniculocortical terminals in layer IV of V1 ($0.36 \pm 0.19 \mu\text{m}^2$, one-way ANOVA with Tukey's post-test, $p = 0.004$), but not significantly different from dLGN-V1 terminals in V1 layer I ($0.28 \pm 0.15 \mu\text{m}^2$, $p = 0.969$). Surprisingly, the largest presynaptic terminals were found to be pulvinocortical terminals in layer I of the P/POR region ($0.43 \pm 0.25 \mu\text{m}^2$). These terminals were significantly larger than pulvinocortical terminals in layer IV of the P/POR ($0.30 \pm 0.20 \mu\text{m}^2$, $p =$

(Figure legend continued.) thalamocortical terminal ultrastructure. **D**, dLGN terminal in V1 layer I. **E**, pulvinar terminal in LM/LI. **F**, pulvinar terminal in P/POR. **D–F**, Dark gray represents labeled terminals. Light blue represents postsynaptic dendrites. Arrows indicate synapses. **G**, **H**, Comparisons of the size of presynaptic thalamocortical terminals and their postsynaptic dendrites. Horizontal bar within each box represents the mean terminal/dendrite size. Box boundaries represent the lower and upper quartiles (25% and 75%, respectively). Vertical lines (whiskers) indicate the full range of terminal/dendrite sizes. * $p < 0.05$ (one-way ANOVA, *post hoc* Tukey's Multiple Comparison Test). ** $p < 0.01$ (one-way ANOVA, *post hoc* Tukey's Multiple Comparison Test). *** $p < 0.001$ (one-way ANOVA, *post hoc* Tukey's Multiple Comparison Test). **** $p < 0.0001$ (one-way ANOVA, *post hoc* Tukey's Multiple Comparison Test). Scale bars: **A–C**, 50 μ m; **D–F**, 600 nm.

Figure 3. Pulvinar activation of pyramidal cells. **A, B**, Responses of a pyramidal cell in layer 4 in area POR to photoactivation of pulvinar terminals. EPSPs (**A**) and EPSCs (**B**) were induced by a train of 10 light pulses (10 ms duration; blue bars) at 1, 2, 5, 10, and 20 Hz. **C–E**, Examples of biocytin-filled pyramidal cells that were recovered after recording. **F, G**, Plots of the ratio of the amplitude of the second EPSP divided by the first EPSP (paired pulse ratio; **F**), or the average of the amplitudes of the second to 10th EPSPs divided by the amplitude of the first EPSP (train/first pulse ratio; **G**) for 48 pyramidal cells. **H**, Of 174 recorded pyramidal cells, 118 (68%) responded to photoactivation of pulvinar terminals. Scale bars: **C–E**, 50 μm .

0.0024), as well as pulvinocortical terminals in the LM/LI ($0.33 \pm 0.20 \mu\text{m}^2$, $p = 0.0092$) and V1 ($0.25 \pm 0.20 \mu\text{m}^2$, $p = 0.0001$). Notably, the sizes of geniculocortical terminals in V1 layer IV were not found to be significantly different from the sizes of pulvinocortical projections to any regions of the LES. These results provide further evidence for the unique organization of pulvinar projections to V1 versus the LES and, in addition, suggest that the impact of the pulvinar on LES activity could potentially be as robust as the impact of geniculocortical projections on V1 activity.

The majority of profiles postsynaptic to geniculocortical and pulvinocortical terminals were small dendrites and spines. As a group, the largest postsynaptic profiles were located in the P/POR, whereas the smallest postsynaptic profiles were located in V1. Indeed, we found no significant difference in the size of postsynaptic profiles in V1 (dLGN to layer I, $0.19 \pm 0.23 \mu\text{m}^2$; dLGN to layer IV, $0.14 \pm 0.07 \mu\text{m}^2$; pulvinar to V1, $0.14 \pm 0.06 \mu\text{m}^2$), whereas in P/POR layer 1, the postsynaptic profiles ($0.28 \pm 0.02 \mu\text{m}^2$) were found to be significantly larger than postsynaptic

profiles in V1 (dLGN to layer I, $p = 0.0007$; dLGN to layer IV, $p < 0.0001$; pulvinar to V1, $p < 0.0001$) and LM/LI ($0.17 \pm 0.10 \mu\text{m}^2$, $p < 0.0001$). Additionally, for a sample of P/POR synapses in which the layers were not differentiated (included terminals in both layer I and IV), the postsynaptic profiles ($0.21 \pm 0.14 \mu\text{m}^2$) were also found to be significantly larger than postsynaptic profiles in V1 (dLGN-V1 layer IV, $p = 0.0112$; pulvinar-V1, $p = 0.0253$). These results could potentially reflect differences in the proportions of cell types in V1 versus the P/POR. For example, as discussed below, the P/POR contains the greatest density of cells that project to the striatum and amygdala.

Optogenetic activation of thalamocortical terminals that originate from the pulvinar

To activate thalamocortical terminals that originate from the pulvinar, viral vector injections were placed in the caudal and lateral parts of the pulvinar (Fig. 2A,B) to induce the expression of TdTomato and Chief in pulvinar cells and their axon projec-

Figure 4. Pulvinar activation of spiny stellate cells. **A, B**, Responses of spiny stellate cells to photoactivation of pulvinar terminals. EPSPs (**A**, in layer V of the POR region) or EPSCs (**B**, in layer IV of the P region) were induced by a train of 10 light pulses (1–10 ms duration; blue bars) at 1, 2, 5, 10, and 20 Hz. **C–E**, Examples of biocytin-filled spiny stellate cells that were recovered after recording. **F, G**, Plots of the ratio of the amplitude of the second EPSP divided by the first EPSP (paired pulse ratio; **F**), or the average of the amplitudes of the second to 10th EPSPs divided by the amplitude of the first EPSP (train/first pulse ratio; **G**) for 18 spiny stellate cells. **H**, Of 46 recorded/recovered spiny stellate cells, 30 (65%) responded to photoactivation of pulvinar terminals. Scale bars: **C–E**, 20 μ m.

tions; this resulted in a dense band of terminals in the LES and sparser terminations in V1 (Fig. 2C–E), similar to the patterns observed following BDA injections in the pulvinar (Fig. 1B, C). In coronal slices of the cortex containing V1 and LES, whole-cell recordings were obtained from neurons in the regions innervated by the pulvinar ($n = 501$). The majority of these recorded neurons ($n = 425$) were tested to determine their response to activation of surrounding pulvinocortical terminals. The remaining recorded neurons were simply filled with biocytin to augment our morphological analysis of cell types in the extrastriate cortex.

Pulses of blue light (1 or 10 ms in duration) through the microscope objective were used to activate the light-sensitive channels expressed by the pulvinar terminals (Fig. 2A). These induced robust responses, with short (<6.5 ms), fixed latencies in the majority of neurons (278 of 425 or 65% of recorded neurons). The responses of neurons to activation of surrounding pulvinar terminals increased in amplitude as the intensity of the blue light pulses was increased (Fig. 2F), and action potentials could be elicited in postsynaptic neurons with light intensities as low as (14 mW/mm²; Fig. 2F).

To ensure that activation of pulvinar terminals elicited monosynaptic responses, the sodium channel blocker TTX was added

to the bath to block action potentials, paired with the potassium channel blocker 4-AP to enhance the depolarization of the terminals ($n = 38$). In the presence of TTX and 4-AP, large-amplitude responses could be elicited (Fig. 2G), which were abolished by subsequent application of the AMPA and NMDA receptor antagonists DNQX and APV ($n = 12$; Fig. 2G).

Morphology and location of recorded cortical neurons

Pipettes included biocytin so that the location and morphology of recorded cells could be established after recording (Fig. 2H; 356 cells were recovered, and the labeling of 316 of these cells was sufficiently complete to categorize their morphology). We found that the majority of these (220 of 316 or 70%) were pyramidal cells, characterized by the presence of apical dendrites that extended from the soma toward the cortex surface (Fig. 3C–E). Pyramidal cells were further subdivided based on the branching patterns of their apical dendrites. Most pyramidal cells had a single apical dendrite (157 of 220 or 71%). Other pyramidal cells had two or three apical dendrite branches, in which each branch made up greater than half the total length of the apical arbor (two branches, 58 of 220 or 26%; three branches, 5 of 220 or 2%). The remaining recovered cells were either spiny stellate cells (51 of

Figure 5. Distribution of cells activated by the pulvinar. To create a flattened cortical map from coronal sections, one in two series of sections through the caudal regions of cortex (**A**) was processed to reveal the projections labeled by injecting a cre-dependent virus in the pulvinar nucleus of a calretinin-cre mouse (**B**). **C**, The approximate location of each coronal section in **A** is indicated by dotted lines in the flattened cortex map. Defined by Wang and Burkhalter (2007). **D–G**, The distribution of recovered cells converted from coronal sections to the flattened cortex map. Red dots indicate cells that responded to pulvinar innervation. Green dots indicate unresponsive cells. Black dots indicate cells filled with biocytin but not tested for pulvinar input. A, Anterior; AL, anterior lateral; AUD, auditory cortex; D, dorsal; L, lateral; MGN, medial geniculate nucleus; PUL, pulvinar nucleus; TeA, temporal association area; V, ventral. Scale bars, 1 mm; P, posterior; M, medial.

316 or 16%), characterized by radially oriented spiny dendrites (Fig. 4C–E), interneurons (24 of 316 or 8%; see Fig. 8C–E), characterized by thin nonspiny dendrites, or cells that did not clearly fit into any of these categories (21 of 316 or 7%).

The locations of morphologically categorized cells were plotted on coronal sections, and these locations were transferred to a flattened representation of cortical areas (Fig. 5). To correlate cortical areas within coronal sections with cortical areas depicted on a flattened map, pulvinocortical projections were labeled (Fig. 5A) by injecting a cre-dependent virus in the pulvinar nucleus of calretinin-cre mice (Fig. 5B), and section spacing was overlaid on

the flattened map (Fig. 5C; defined by Wang and Burkhalter, 2007). Variations in the distribution of pulvinocortical terminals were used to define cortical areas (Fig. 5A), and cell locations were plotted on the flattened map using cortical area and section spacing as coordinates (Fig. 5D–G).

Recording sites were concentrated in layers IV and V of the LM/LI and P/POR, where terminals originating from the pulvinar are most densely distributed. V1 recordings were limited to cases in which no spread of virus into the adjacent dLGN could be detected, and TdTomato-labeled terminals were confined to layers I and Va within V1. Of the morphologically categorized cells,

Figure 6. Pulvinocortical responses: comparison of cortical area, lamina, and cell types. Histograms compare the probability (percentage of recorded cells that responded to photoactivation of pulvinocortical terminals; **A, D, G**) and strength (maximum EPSP elicited at 1 Hz photoactivation of pulvinocortical terminals; **B, E, H**) and frequency dependence (**C, F, I**) of pulvinocortical connections across cortical areas (**A–C**), cortical layers (**D–F**), and neuron morphology (**G–I**). Connection probability was greater in the P/POR region compared with V1 ($p = 0.0125$) and lower in layer V compared with either layer II–III ($p = 0.0034$) or layer IV ($p < 0.0001$). **A, D, *** $p < 0.05$ (Fisher test). ****** $p < 0.01$ (Fisher test). ******** $p < 0.0001$ (Fisher test). No significant differences in the strength or frequency dependence of pulvinocortical responses were detected (one-way ANOVA, *post hoc* Tukey's Multiple Comparison Test).

similar proportions of pyramidal cells (Fig. 5D) and spiny stellate cells (Fig. 5E) were found across cortical areas (of 152 filled cells in P/POR 28 or 18% were spiny stellate and 100 or 66% were pyramidal; of 67 filled cells in LM/LI, 10 or 15% were spiny stellate and 51 or 76% were pyramidal; of 44 filled cells in V1, 8 or 18% were spiny stellate and 28 or 63% were pyramidal).

Pulvinocortical responses across cortical areas, lamina, and cell types

Cells within the P/POR region were found to be the most responsive to optogenetic activation of pulvinar terminals (104 of 142 or 73.24%). In the LM/LI region, 35 of 55 recorded cells (63.64%) were responsive to activation of pulvinar terminals; and in V1, 21 of 41 (51.22%) were responsive (Fig. 6A). However, there was no significant difference in the maximum EPSP amplitudes elicited

by activation of pulvinar terminals in V1, LM/LI, or P/POR (Fig. 6B). Cells in layer V were found to be least responsive to photactivation of pulvinocortical terminals (40 of 89 recorded cells or 44.94%). As illustrated in Figure 6D, in the other targeted layers, the majority of recorded cells were responsive to activation of pulvinar terminals (layer II/III, 17 of 21 or 80.95%; layer IV, 109 of 141 or 77.30%). However, the maximum EPSP amplitudes elicited by activation of pulvinar terminals in layers II/III, IV, and V were not found to be statistically different (Fig. 6E). Finally, when pulvinar responses were analyzed based on postsynaptic cell type, response rates were similar (Fig. 6G; 120 of 176 or 68.18% of pyramidal cells responded, 30 of 47 or 63.83% of spiny stellate cells responded, and 28 of 37 or 75.68% of interneurons responded), and no difference in maximum EPSP amplitudes was detected (Fig. 6H).

Figure 7. Characterization of GAD65-GFP cells in V1. V1 sections that contain GFP-labeled interneurons (green; **A**, **C**, **D**, **F**) were stained with antibodies against calretinin (purple; **B**, **C**), parvalbumin (purple; **E**, **F**), or GFP (dark reaction product; **G**–**I**) and GABA (gold particles; **G**–**I**). GFP often colocalized with calretinin (**C**, white cells indicated by arrows) but rarely with parvalbumin (**F**). GFP-labeled somata (**G**) and larger dendrites (**H**) contained detectable levels of GABA (high density of gold particles), but GABA was often undetectable in smaller GFP-labeled profiles (**I**). Scale bars: **C** (applies to **A**–**F**), 20 μ m; **I** (applies to **G**, **H**), 600 nm.

Activation of pulvinar terminals with train-of-light pulses (1, 2, 5, 10, 20 Hz) revealed an overall frequency-dependent depression of synaptic responses (see Figs. 3*A,B*, 4*A,B*, 8*A,B*). This is illustrated for individual cells by plotting the ratio of the amplitude of the second EPSP divided by the first EPSP (paired pulse ratio; Figs. 3*F*, 4*F*, 8*F*) or the average of the amplitudes of the second to 10th EPSPs divided by the amplitude of the first EPSP (train/first pulse ratio; see Figs. 3*G*, 4*G*, 8*G*). Comparison of paired pulse ratios of pulvinocortical responses recorded in different cortical areas (Fig. 6*C*), in different cortical lamina (Fig. 6*F*), or in different cortical cell types (Fig. 6*I*) revealed no significant differences.

Optogenetic activation of pulvinar terminals: effects on cortical interneurons

To determine whether cortical interneurons receive input from the pulvinar, we used a transgenic mouse line in which GFP is expressed in a subset of GABAergic interneurons (GAD65-GFP). In this line, GFP is expressed in interneurons that contain the calcium-binding protein calretinin (Fig. 7*A–C*) and/or other interneuron subtypes (López-Bendito et al., 2004), but GFP is not

expressed in interneurons that contain parvalbumin (Fig. 7*D–F*). Electron microscopic evaluation of GAD65-GFP-labeled profiles, in tissue additionally stained with a GABA antibody tagged with gold particles, revealed that GFP-labeled somata (Fig. 7*G*) and the majority of larger dendrites (Fig. 7*H*) contained GABA (gold particle density overlying GFP-labeled profiles $>0.35 \mu\text{m}^2$ was 52.60 ± 30.33 gold particles/ μm^2). However, GFP-labeled profiles within the size range of dendrites postsynaptic to pulvinar terminals (Fig. 7*I*) were often devoid of any overlying gold particles (no gold particles were found overlying 70 of 136, or 52%, of GFP-labeled profiles $<0.35 \mu\text{m}^2$). This indicates that GABA is not consistently detectable in smaller dendrites using standard postembedding immunohistochemical techniques. For this reason, we were unable to determine whether pulvinar-cortex terminals contact GABAergic interneurons using anatomical techniques alone.

Virus injections were placed in the pulvinar of GAD65-GFP mice; and in subsequent slice experiments, whole-cell recordings were obtained from GFP-labeled neurons (Fig. 8*A,B*; $n = 34$, locations plotted in Fig. 5*C*), and surrounding pulvinar terminals were activated with blue light. The input resistance of responsive

Figure 8. Pulvinar activation of GAD65 interneurons. **A, B**, In GAD65-GFP transgenic mice, responses of an interneuron in layer V of V1 to photoactivation of pulvinar terminals. EPSPs (**A**) and EPSCs (**B**) were induced by a train of 10 light pulses (10 ms duration; blue bars) at 1, 2, 5, 10, and 20 Hz. **C–E**, Examples of GFP-labeled (green) biocytin-filled interneurons (red) that were recovered after recording. **F, G**, Plots of the ratio of the amplitude of the second EPSP divided by the first EPSP (paired pulse ratio; **F**), or the average of the second to 10th EPSPs divided by the amplitude of the first EPSP (train/first pulse ratio, **G**) for 25 interneurons. **H**, Of 34 recorded interneurons, 25 cells (74%) responded to photoactivation of pulvinar terminals. Scale bars: **C–E**, 50 μ m.

GFP-labeled interneurons (340.9 ± 237.9) was significantly greater than that of responsive pyramidal cells (154.1 ± 75.31) and responsive spiny stellate cells (148.5 ± 66.94 ; Fig. 9A), and the morphology of successfully recovered biocytin-filled GFP-labeled cells ($n = 24$) was consistent with previous descriptions of interneurons (i.e., smooth dendrites; Fig. 8C–E). We found that the majority (25 of 34 or 74%; Fig. 8H) of GFP-labeled cells responded to optogenetic activation of pulvinar terminals (1 or 10 ms duration, $10\text{--}112$ mW/mm² blue light pulses through the microscope objective) with large-amplitude EPSPs or EPSCs ($1.064\text{--}26.22$ mV; $35.72\text{--}699.7$ pA), with short (<4.6 ms), fixed latencies. Example responses are illustrated in Figure 8A, B. When pulvinar terminals were stimulated with train-of-light pulses, the interneuron responses generally exhibited frequency-dependent depression (Fig. 8A, B). However, paired pulse ratio plots (Fig. 8F) and train/first pulse ratio plots (Fig. 8G) demonstrate the variability observed between neurons. Further dem-

onstrating the involvement of interneurons in pulvinar-cortex circuits, application of a GABA_A receptor antagonist (SR95531, 20 μ M, $n = 6$) during photostimulation of pulvinocortical terminals increased the EPSPs up to fourfold (Fig. 9B, $n = 2$), or induced spikes (Fig. 9C, $n = 4$).

Optogenetic activation of pulvinar terminals: effects on cortical output neurons

To determine whether terminals that originate from the pulvinar can directly influence the activity of cortical projection neurons, we paired pulvinar virus injections with CTB-488 or HSV injections in the ipsilateral SC, V1, or striatum/amygdala. The CTB or HSV injections labeled corticotectal, corticocortical, or corticostriatal/amygdala cells, which we then targeted for recording using epifluorescent visualization of cortex slices maintained *in vitro*. Recordings were limited to cortical regions that contained overlapping distributions of CTB- or HSV-labeled cells and TdTomato-

Figure 9. Interneuron properties. **A**, The membrane resistance of interneurons is significantly larger than that of both spiny stellate and pyramidal cells. **** $p < 0.0001$ (one-way ANOVA, *post hoc* Tukey's Multiple Comparison Test). Data are mean $\pm$ SD. Interneurons: 340.9 ± 237.9 , $n = 36$; spiny stellate cells: 148.5 ± 66.94 , $n = 29$; pyramidal cells: 154.1 ± 75.31 , $n = 122$. **B**, The amplitude of EPSPs evoked in interneurons via photoactivation of pulvinocortical terminals was increased by the bath application of the GABA_A blocker SR95531 ($n = 6$) and induced firing in 4 of the 6 tested neurons (example shown in **C**).

labeled thalamocortical terminals originating from the pulvinar (Fig. 10A–C), and in which responses could be evoked in non-CTB-labeled cells within the same experiment. CTB- or virus-induced labeling of recorded cells was confirmed by video recordings of the patched cells and/or subsequent imaging of CTB-488 or YFP within recovered biocytin-filled cells using a confocal microscope (Fig. 10Ai–Aiii, Bi–Biv, Ci–Civ).

The distribution of corticotectal cells was limited to layer V in V1 and the LES. Corticocortical cells projecting to V1 were distributed in layers V and VI of the LES (Fig. 10Bi). Corticostriatal/amygdala cells were confined to layer V within V1, but their distribution expanded in the more ventral and caudal regions of the LES; in the LM and LI, most corticostriatal/amygdala cells were confined to layer V, but within P and POR, corticostriatal/amygdala cells were distributed throughout most layers (Fig. 10Ci, Ciii). The increase in the density of corticostriatal/amygdala cells in ventral/caudal cortical areas mimicked the increase in the density of pulvinar projections in ventral/caudal cortical areas.

Whole-cell recordings were obtained from a total of 120 cells labeled by retrograde transport (31 corticotectal, 34 corticocortical, and 68 corticostriatal/amygdala cells; Fig. 10F). Of these three cell groups, corticostriatal/amygdala cells were by far the most responsive group; 52 of 68 or 76% of corticostriatal/amygdala cells responded to activation of surrounding pulvinar terminals with short latency (≤ 5.7 ms). Using these same criteria,

18 of 34 corticocortical cells (53%), and 2 of 31 corticotectal cells (6%) were categorized as responsive. Comparison of the maximum EPSP or EPSC amplitudes elicited in these cell groups via activation of pulvinar terminals also revealed significant differences, with corticocortical cells exhibiting the most robust responses (Fig. 10D, E).

Activation of pulvinar input to projection cells with train-of-light pulses revealed a frequency-dependent depression of synaptic responses (Fig. 10G, H). Furthermore, in the presence of TTX and 4-AP, large-amplitude responses could be elicited (Fig. 10I; corticostriatal/amygdala, $n = 10$; corticocortical, $n = 5$), which were decreased by the application of the NMDA antagonist APV, and abolished by subsequent application of the AMPA receptor antagonist CNQX (Fig. 10I; corticostriatal/amygdala, $n = 4$; corticocortical, $n = 1$). The locations of all recovered corticostriatal/amygdala cells were plotted (Fig. 5G). Most responsive cells were concentrated in the P/POR region. The majority of corticostriatal/amygdala cells were pyramidal cells, but four were categorized as spiny stellate cells.

Discussion

We found that the mouse pulvinar projects densely to interconnected regions of the LES, striatum, and amygdala. The laminar distribution and ultrastructure of pulvinocortical terminals in the LES were found to be nearly identical to that of geniculocortical terminals in V1, and optogenetic activation of pulvinocortical

Figure 10. Pulvinar activation of extrinsic projection cells. **A–C**, Schematics illustrate the experimental protocol. Virus injections in the pulvinar were paired with CTB-488 injections into superior colliculus, V1, or striatum to label corticotectal, corticocortical, or corticostriatal cells for targeted recordings. **A_i**, Example of filled corticotectal cells. **A_{ii}**, **A_{iii}**, Higher magnification of the CTB within the somata of the two biocytin-filled cells in **A_i**. **B_i**, Example of filled corticocortical cells. **B_{ii}–B_{iv}**, Higher magnification of the three cells in **B_i**. **C_i**, **C_{iii}**, Example of filled corticostriatal cells. **C_{ii}**, **C_{iv}**, Higher magnification of cells in **C_i** and **C_{iii}**, respectively. **D**, **E**, Maximum EPSP/EPSC amplitude for responsive corticotectal, corticocortical, and corticostriatal (Figure legend continues.)

Figure 11. Summary of pulvinocortical circuits. Schematic of the interconnected circuits that involve the pulvinar nucleus; the pulvinar may serve as a hub to coordinate body movements with the perception of visual signals. Solid arrows indicate excitatory connections. Dashed arrows indicate inhibitory connections. PUL, Pulvinar; SGI, stratum griseum intermediale; SGS, stratum griseum superficiale; ST, striatum.

terminals strongly depolarized pyramidal cells, spiny stellate cells, and interneurons in the LES. Furthermore, recordings targeted to specific projection neuron subtypes within layer V revealed that the pulvinar strongly affects the activity of corticostriatal, corticoamygdala, and corticocortical cells in the LES via direct and indirect synaptic contacts. This information, coupled with the input and output organization of the pulvinar nucleus, suggests that the pulvinar is a pivotal component of circuits used for the visual guidance of movement (Fig. 11).

Distribution and morphology of pulvinocortical terminals

The distribution of pulvinocortical terminals that we observed in the mouse is similar to that described in other species, including primates. That is, pulvinocortical projections to V1 are relatively sparse, whereas projections outside V1 are dense and primarily target the middle layers, similar to geniculocortical projections (Glendenning et al., 1975; Ogren and Hendrickson, 1977; Curcio and Harting, 1978; Rezak and Benevento, 1979; Marion et al., 2013). However, individual pulvinocortical axons innervate multiple lamina, multiple cortical areas, as well as the striatum and amygdala (Rockland et al., 1999; Nakamura et al., 2015), whereas geniculocortical axons are more restricted topographically, and individual axons innervate distinct lamina (Humphrey et al., 1985; Raczkowski and Fitzpatrick, 1990; Ding and Casagrande, 1997; Casagrande et al., 2007; Cruz-Martín et al., 2014; Bickford et al., 2015). Previous studies have also found differences in the ultrastructure of pulvinocortical and geniculocortical terminals. In the tree shrew, pulvinocortical terminals in the temporal cortex are smaller than layer IV geniculocortical terminals in V1 (Chomsung et al., 2010; Familtsev et al., 2016), and other features, such as dendritic protrusions (Erisir and Dreusicke, 2005)

are found within geniculocortical terminals, but not pulvinocortical terminals. In the Galago, pulvinocortical terminals in area V2 are smaller than layer IV magnocellular geniculocortical terminals, but not significantly different from parvocellular geniculocortical terminals (Marion et al., 2013). Our extensive ultrastructural comparison of geniculocortical and pulvinocortical terminals in mouse V1 and LES revealed a number of distinctions in the size of presynaptic and postsynaptic profiles across cortical areas and lamina. Notably, pulvinocortical terminals were largest in the P/POR region, where pulvinar projections were most densely distributed, and the response probability was greatest. However, differences in the size of pulvinocortical terminals did not appear to influence synaptic strength or dynamics.

Frequency dependence of pulvinocortical responses

In the majority of recorded neurons, we found that the amplitude of pulvinocortical responses decreased with stimulation frequency, similar to that previously noted for geniculocortical responses (Kloc and Maffei, 2014). Although our optogenetic techniques could have introduced an artificial response depression (Jackman et al., 2014), the same virus and photoactivation techniques revealed a robust frequency-dependent facilitation of corticogeniculate responses (Jurgens et al., 2012). This suggests that our optogenetic techniques reflect the dynamics of different synapses, and predict that the firing frequency of pulvinar neurons will affect the efficacy of their cortical synaptic connections (Swadlow et al., 2005).

Pulvinocortical terminals contact interneurons

In the mouse and tree shrew, pulvinocortical terminals contact spines and other small dendritic profiles in which GABA is not detected using immunocytochemical techniques (Chomsung et al., 2010). However, our optogenetic experiments in mice identified interneurons as targets of pulvinocortical terminals, which prompted us to critically evaluate the ability to detect GABA in small dendritic profiles of cortical interneurons. By comparing GAD65-GFP-labeled elements with GABA-stained elements, we conclude that, in contrast to thalamic interneurons which release GABA from dendritic terminals (Govindaiah and Cox, 2004; Bickford et al., 2010), cortical interneurons do not accumulate detectable levels of GABA within small-diameter dendrites, presumably because they release GABA only from axon terminals (Nahmani and Turrigiano, 2014).

Distribution of spiny stellate cells

Spiny stellate cells are a major target of thalamocortical terminals in the mouse somatosensory cortex (White and Rock, 1979; Benshalom and White, 1986). Spiny stellate cells are also densely packed in V1 of nonrodent species (Muly and Fitzpatrick, 1992; Fitzpatrick, 1996; Callaway and Borrell, 2011; da Costa and Martin, 2011). However, our study provides the first evidence that spiny stellate cells are also located in the mouse V1 and LES. In the ferret striate cortex, spiny stellate cells develop from pyramidal neurons by a visual experience-dependent process of apical dendrite pruning (Callaway and Borrell, 2011). It is possible that our detection of spiny stellate cells was facilitated by the fact that we used mice at older ages than previous studies of mouse V1 (e.g., Kloc and Maffei, 2014). However, spiny stellate cells in the mouse somatosensory cortex exhibit their mature morphology by P5 (Callaway and Borrell, 2011). Therefore, it is most likely that detection of spiny cells was simply the result of our large sample size (316 neurons with complete filling); in all visual cortical areas, spiny stellate cells made up only 15%–18% of sampled neurons.

(Figure legend continued.) cells. **F**, Of 31 tested corticotectal cells, 2 cells (6%) were responsive to pulvinar innervation. Of 33 tested corticocortical cells, 18 cells (55%) were responsive to pulvinar innervation. Of 68 corticostriatal cells, 52 cells (76%) were responsive to pulvinar innervation. **G, H**, Comparison of the paired pulse ratio and train/first pulse ratio for 17 corticocortical cells, 44 corticostriatal cells, and 25 interneurons. **I**, Responses of a corticostriatal cell to photoactivation of pulvinar terminals in the presence of TTX and 4-AP. This response was blocked by the sequential application of APV and CNQX. Scale bars: **Ai–Ci, Cii**, 50 μ m; **Aii, Aiii, Bii–Biv, Cii, Cii**, 10 μ m.

Projection neurons of the mouse visual cortex

Recently, three genetically distinct types of layer 5 projection neurons were identified in V1 (Kim et al., 2015): (1) neurons that project to the striatum and other cortical areas, (2) neurons that project to the superior colliculus, thalamus, brainstem, and striatum, and (3) neurons that project locally within the cortex, but not to striatum. We find that pulvinocortical projections primarily target cortical cells in LES that project to the striatum/amygdala (76%) or to V1 (55%). Thus, it is possible that pulvinocortical projections target a single class of cells that have branching projections to the striatum and V1. However, we focused most or our recordings on extrastriate areas of the cortex. Because the genetic labeling patterns that classify cell types in V1 likely differ in extrastriate areas of cortex, it remains to be determined whether pulvinocortical terminals preferentially target a single genetically defined cell type. Our study of the morphology and projection patterns of neurons targeted by the pulvinar nucleus lays the groundwork for further thalamocortical circuit analysis that includes genetically defined cell types.

The pulvinar nucleus and anesthesia

We found that pulvinocortical projections can strongly impact the activity of neurons in the extrastriate cortex, supporting the view that visually evoked activity in extrastriate areas may be primarily relayed via the pulvinar nucleus. This view is at odds with the conventional hierarchical organization of the primate cortex, in which the transfer of visual information to extrastriate cortical areas is considered to be primarily relayed from V1 via corticocortical connections (Van Essen, 2005). One conclusion to be drawn from this study is that, compared with primates, the extrastriate cortex of the mouse is more dependent on subcortical input. Alternatively, anesthesia could at least partially account for these disparate views. Recordings in anesthetized animals support a hierarchical view in that silencing V1 activity results in a profound depression of responses in extrastriate areas (Girard et al., 1992; Kaas and Krubitzer, 1992; Azzopardi et al., 2003). However, in anesthetized mice, spontaneous activity in the pulvinar nucleus is significantly lower than that recorded in the dLGN (Roth et al., 2016), and the proportion of pulvinar neurons that respond to simple visual stimuli is approximately half that of dLGN neurons (Allen et al., 2016). Even in awake but inactive primates, the spontaneous activity of pulvinar neurons is less than half that of dLGN neurons (Ramcharan et al., 2005). Moreover, recordings in awake running mice have revealed that visual activity in V1 is affected by movement of the animal even though dLGN activity is unaffected (Niell and Stryker, 2010). These various pieces of evidence suggest that the full influence of pulvinocortical projections can only be assessed in awake, active animals.

Is the pulvinar nucleus a hub for visually triggered action selection?

The mouse pulvinar nucleus receives dense input from wide-field vertical cells in the superior colliculus (Zhou et al., 2017). The large dendritic fields of wide-field vertical cells have been described as motion detectors (Major et al., 2000); they respond preferentially to small objects moving across the visual field in any direction (Gale and Murphy, 2014, 2016). Our results indicate that, in addition to the direct projections of the pulvinar nucleus to the striatum and amygdala (Day-Brown et al., 2010), pulvinar projections to the cortex preferentially target corticostriatal and corticoamygdala cells. These input and output connections of the pulvinar nucleus suggest that it can be viewed as a hub involved in the initiation or alteration of the appropriate

actions in response to the detection of visual movement. Recent optogenetic studies support this idea. Activation of an SC-pulvinar-amygdala pathway has been shown to elicit freezing responses, whereas inactivation of this pathway inhibits innate freezing response elicited by overhead looming stimuli (Wei et al., 2015). Given the large repertoire of behaviors that can now be quantified using mice (Yilmaz and Meister, 2013; De Franceschi et al., 2016; Hoy et al., 2016), future optogenetic and/or chemogenetic manipulations may help to unravel the specific contributions of the pulvinocortical circuits to the initiation of apt behavioral responses.

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