Intracortical convergence of layer 6 neurons

Charles C. Lee[a], Ying-Wan Lam[b] and S. Murray Sherman[b]

Axonal branches from a subset of neurons in cerebral cortical layer 6 innervate both cortical layer 4 and the thalamus. As such, these neurons are poised to modulate thalamocortical transmission at multiple forebrain sites. Here, we examined the functional organization of the layer 6 intracortical projections in auditory, somatosensory, and visual cortical areas using an optogenetic approach to specifically target these neurons. We characterized the anatomical and physiological organization of these projections using laser-scanning photostimulation to functionally map the elicited postsynaptic responses in layer 4. We found that these responses originated from regions over 1 mm in width, eliciting short-term facilitating responses. These results indicate that intracortical modulation of layer 4 occurs through widespread layer 6 projections in each sensory cortical area. ***NeuroReport*** **23:736–740 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

NeuroReport 2012, **23**:736–740

Keywords: auditory, cortex, Cre–Lox recombination, layer 4, layer 6, optogenetics, somatosensory, thalamus, visual

[a]Department of Comparative Biomedical Sciences, Louisiana State University, School of Veterinary Medicine, Baton Rouge, Louisiana and [b]Department of Neurobiology, University of Chicago, Chicago, Illinois, USA

Correspondence to Charles C. Lee, PhD, Department of Comparative Biomedical Sciences, Louisiana State University, School of Veterinary Medicine, Skip Bertman Drive, Baton Rouge, LA 70803, USA
Tel: +1 225 578 9032; fax: 1 225 578 9895; e-mail: cclee@lsu.edu

Received 26 May 2012 **accepted** 7 June 2012

Introduction

In the cerebral cortex, a subset of cerebral cortical layer 6 neurons sends branched projections to the thalamus and cortical layer 4 [1–4]. Anatomically, the layer 6 to layer 4 projections are particularly robust, contributing ~30% of the synapses onto a layer 4 neuron [5]. Physiologically, these projections in both the cortex and the thalamus elicit responses showing paired-pulse facilitation and, at sufficiently high level, activate metabotropic glutamate receptors [1,6–9]. Because of their potential roles in modulating cortical and thalamic activity [6,8,10], we aimed to specifically study the functional organization of these layer 6 projections in the sensory cortical areas of the mouse using an optogenetic approach based on Cre–Lox recombination [11].

An adeno-associated virus, inserted with a 'floxed' construct containing the fused sequence of channelrhodopsin and YFP (ChR–YFP), was transfected in various cortical areas (auditory, somatosensory, visual) of B6.Cg-Tg(Ntsr1-cre)GN220Gsat/Mmcd mice, which express Cre-recombinase only in these layer 6 intracortical and corticothalamic projection neurons. We characterized the anatomical patterns of the layer 6 ChR–YFP-expressing fibers in each cortical area injected. We then assayed the functional topography and the short-term synaptic properties of the layer 6 projections using laser-scanning photostimulation to specifically activate ChR–YFP-expressing fibers. In general, we found that these layer 6 fibers arborize markedly in layer 4 and thalamus. Moreover, photostimulation of layer 6 fibers expressing ChR–YFP led to a broad pattern of convergent excitation in layer 4, supporting a potential role for the widespread subgranular modulation of layer 4 thalamorecipient neurons.

Materials and methods

Surgery and injections

Adult mice [B6.Cg-Tg(Ntsr1-cre)GN220Gsat/Mmcd; MMRRC-UC Davis, Davis, California, USA] expressing Cre-recombinase in intracortical and corticothalamic projection neurons of layer 6 were used in these experiments. All procedures were approved by the Institutional Animal Care and Use Committee. To anesthetize the animal, an intraperitoneal injection of ketamine (100–120 mg/kg) and xylazine (5–10 mg/kg) was administered and deemed sufficient when no responses were elicited to strong toe pinches. The animal was then placed in a stereotactic device (Stoelting, Wood Dale, Illinois, USA) and a craniotomy was performed above the injection sites. A pAAV-Ef1a-double floxed-hChR2(H134R)-EYFP-WPRE-pA vector (200 µl) (UNC Vector Core, Chapel Hill, North Carolina, USA) was pressure injected using a nanoliter injector (Drummond Scientific, Broomall, Pennsylvania, USA) into either the primary auditory (A1: $n = 4$), somatosensory (S1: $n = 4$), or visual (V1: $n = 4$) cortices. The craniotomy site was covered and the injection site was sutured with nonabsorbable monofilament nylon. An injection of buprenorphine (0.05–0.1 mg/kg) was administered for postoperative analgesia. Following recovery and a 14–21-day survival to allow the adequate expression and transport of ChR–YFP, the animals were sacrificed and the brain was removed and processed using the techniques for slice preparation described below.

Slice preparation and recording

After deep anesthetization of the animals with isofluorane, followed by decapitation, the brains were submerged in cool, oxygenated, artificial cerebral spinal fluid [(ACSF): 125 mM

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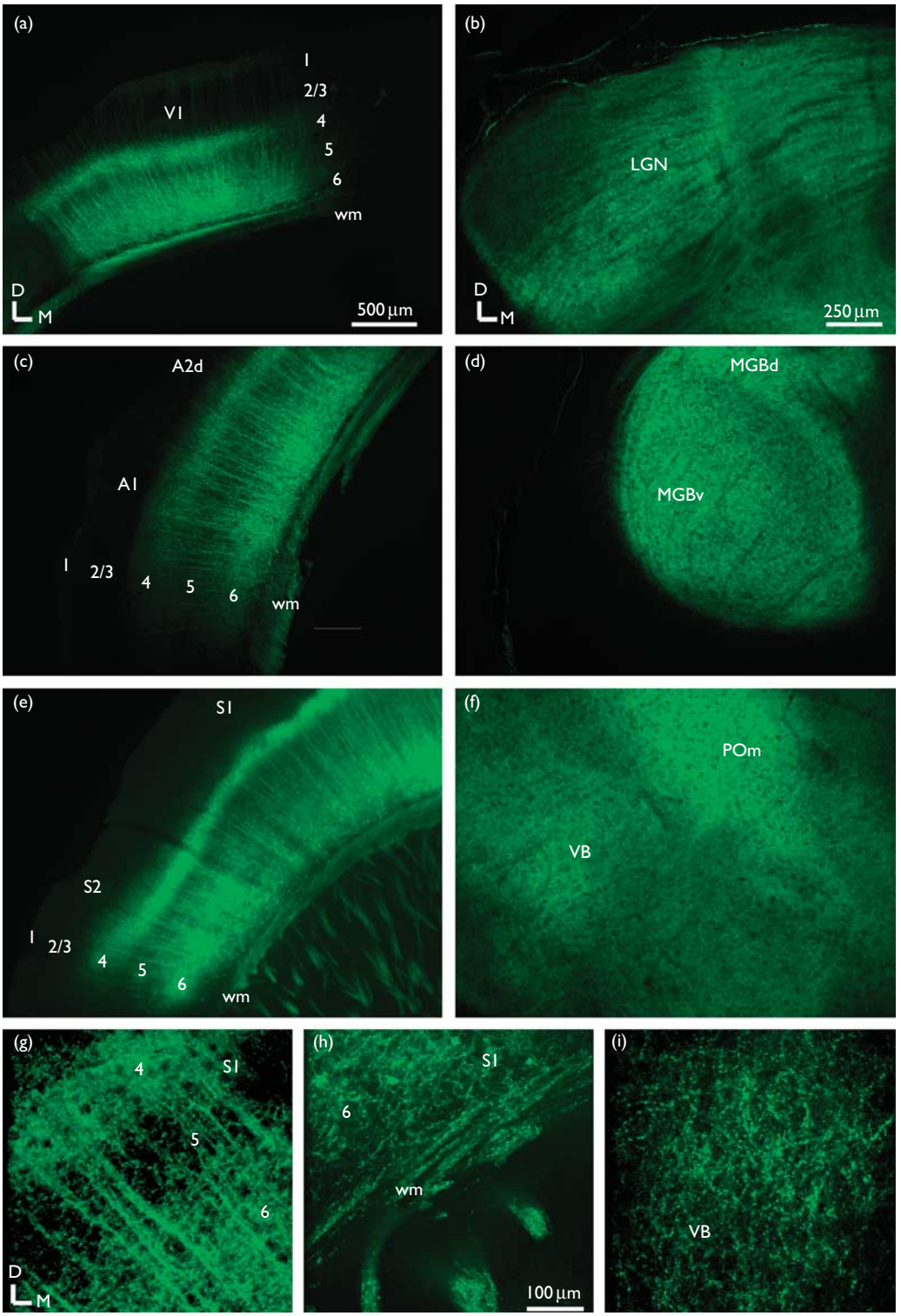
Fig. 1



ChR–YFP expression in layer 6 corticothalamic neurons in the visual (a, b), auditory (c, d), and somatosensory (e–i) cortices and thalamic nuclei. Neuronal cell bodies in layer 6 project to layer 4 (a, c, e, g) and the thalamus (b, d, f, i), arborize broadly, and terminate in small boutons. 1-6, layers 1-6; A1, primary auditory cortex; A2d, dorsoposterior auditory area; ChR–YFP, channelrhodopsin conjugated to YFP; D, dorsal; LGN, lateral geniculate nucleus; M, medial; MGBv, ventral division of the medial geniculate body; POm, posterior medial nucleus; S1, primary somatosensory cortex; S2, secondary somatosensory area; V1, primary visual cortex; VB, ventrobasal; wm, white matter.

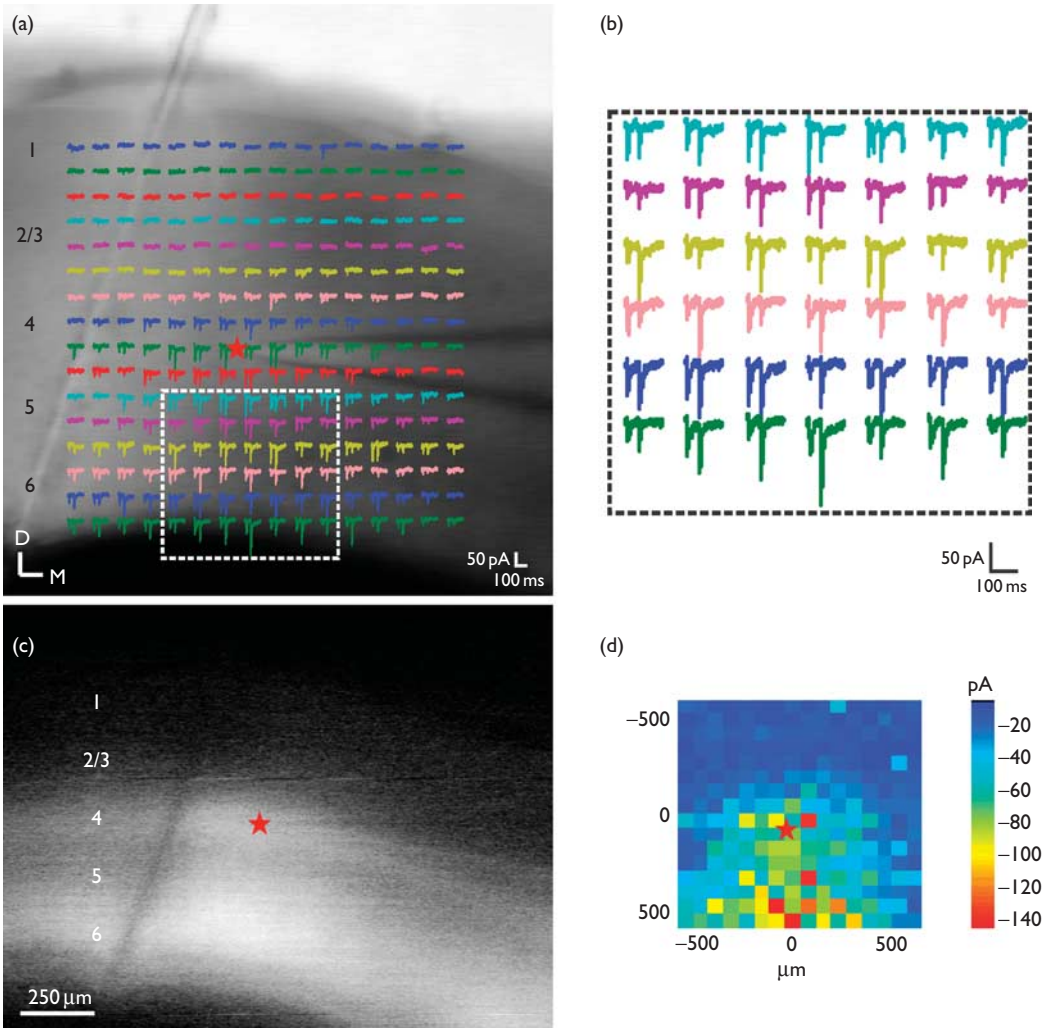
NaCl, 25 mM HCO_3, 3 mM KCl, 1.25 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 25 mM glucose]. Whole brains were then blocked coronally and affixed to a vibratome stage to collect 500-μm-thick sections, which were placed in a holding chamber containing physiological ACSF to recover for 1 h at 32°C, and then returned to room temperature for the rest of the experiment.

A recording chamber perfused with ACSF was used for whole-cell recordings of the slice preparation under DIC optics using recording pipettes with tip resistances of 4–8 MΩ filled with an intracellular solution (135 mM K-gluconate, 7 mM NaCl, 10 mM HEPES, 1–2 mM Na_2ATP, 0.3 mM GTP, and 2 mM $MgCl_2$ at a pH of 7.3 obtained with KOH, and osmolality of 290 mOsm obtained with distilled water). This solution results in ~10 mV junction potentials that are uncorrected for in voltage measurements. Current or voltage clamp recordings were performed using the Axoclamp 2A amplifier and pCLAMP software (Molecular Devices, Sunnyvale, California, USA). The data acquired were digitized using a Digidata 1200 board and then stored in a computer for later analysis.

Laser-scanning photostimulation

Laser-scanning photostimulation was used to activate ChR–YFP-labeled fibers using an average beam intensity of 5 mW to yield a 1 ms, 100-pulse light stimulus by Q-switching the pulsed UV laser (355 nm wavelength, frequency-tripled Nd: YVO4, 100 kHz pulse repetition

Fig. 2



Repetitive photostimulation elicits robust, facilitating EPSCs in layer 4 neurons across broad regions of layers 4–6. (a) Overlay of stimulation sites and current responses on an acute slice photomicrograph. (b) Expanded traces from the boxed region in (a). (c) ChR–YFP expression in the acute slice preparation. (d) Mean amplitude of elicited first EPSCs. 1-6, layers 1-6; ChR–YFP, channelrhodopsin conjugated to YFP; D, dorsal; EPSCs, excitatory postsynaptic currents; M, medial.

rate; DPSS Lasers Inc., Santa Clara, California, USA). Responses were analyzed using custom software written in Matlab (MathWorks Inc., Natick, Massachusetts, USA), and the traces were superimposed on a photomicrograph corresponding to the stimulation sites, as described previously [3,8,12].

Histology

Following physiological recordings, the acute slices were fixed overnight in 4% paraformaldehyde/30% sucrose and then resectioned at 40 μm using a freezing microtome. The sections were mounted on gelatinized slides and confocal images were acquired with a 3I Marianas Yokogawa-type spinning disk confocal microscope (Intelligent Imaging Innovations, Denver, Colorado, USA).

Results

In the Cre-transgenic mice [B6.Cg-Tg(Ntsr1-cre) GN220Gsat/Mmcd] that we transfected with the 'floxed' ChR-YFP construct, robust expression of ChR–YFP was observed in layer 6 neuronal cell bodies and in dense fibers that terminated in layer 4 and thalamic nuclei corresponding to the cortical areas injected (Fig. 1). Thus, labeling in the visual (V1: Fig. 1a), auditory (A1: Fig. 1c), and somatosensory (S1: Fig. 1e) areas corresponded with labeling in modality-appropriate thalamic nuclei, that is lateral geniculate nucleus (LGN: Fig. 1b), medial geniculate body (MGB: Fig. 1d), and the ventrobasal and posterior medial nuclei (VB and POm: Fig. 1f). Apical dendrites and axonal fibers originating in layer 6 cell bodies coursed through layer 5, ramifying sparsely, and terminated robustly in layer 4 (Fig. 1g). More rarely, labeled apical fibers traversed and arborized in upper layer 1, being slightly more prevalent in the primary visual cortex. Corticothalamic axons traversed from the layer 6 neuronal cell bodies through the white matter (Fig. 1h), before arborizing profusely in the thalamus, terminating in small boutons (Fig. 1i).

To examine the physiological properties of these ChR–YFP-labeled projections, whole-cell slice recordings from neurons in layer 4 and the thalamus were obtained in response to paired-pulse photostimulation (50 ms ISI) of these ChR–YFP-labeled fibers. The excitatory postsynaptic currents elicited in both layer 4 and the thalamus generally occurred in response to repetitive photostimulation (Fig. 2), similar to previous studies that used electrical stimulation methods [1,6,9,13,14]. In addition, layer 4 neurons were excited by photostimulation across a broad area (Fig. 2), suggesting a high degree of convergence of layer 6 inputs. Laser-scanning photostimulation only induced facilitating responses from regions where labeled fibers were primarily present (i.e. layers 4–6; Fig. 2a and c). The most robust responses, on the basis of the amplitude of the first excitatory postsynaptic current, were found within a narrow column (~200 μm) beneath the recorded neuron; however, prominent responses could be induced across the entire span of the stimulation map (~1.3 mm), putatively encompassing several cortical columns.

Discussion

Our results expand upon previous studies that suggest that layer 6 plays a modulatory role in sensory information processing by regulating the efficacy of thalamocortical transmission [1,6,9,10,13–16]. Our findings here indicate that layer 6 can potentially modulate layer 4 neurons across an extensive cortical domain greater than 1 mm in width. The breadth of these layer 6 inputs to layer 4 determined using our optogenetic approach contrasts with those obtained using laser-scanning photostimulation through uncaging of glutamate, which instead showed highly focal regions of layer 6 projections [8,17–21]. We posit that the maps obtained through uncaging of glutamate could potentially underestimate the breadth of these relatively weak inputs from layer 6, because of the inadequate recruitment of neurons sufficient to induce detectable responses in layer 4. Conversely, our optogenetic approach may overestimate the breadth of these connections, as the stimulation of axon collaterals could antidromically elicit responses from neurons far from the stimulation site. As such, the breadth of the functional layer 6 intracortical projections is likely broader than indicated previously, but presumably less than the upper boundary established here.

Conclusion

Our results indicate substantial convergence of facilitating synaptic inputs from layer 6 to layer 4 using an optogenetic approach to specifically target this defined population of cortical neurons. Although this in-vitro characterization of synaptic properties is invaluable for dissecting the functional topography of neuronal circuitry, the application of this cell-type specific optogenetic approach *in vivo* enables the specific examination of the role of these layer 6 neurons in receptive fields and behavior [10]. The methods used here should thus continue to determine unexpected functional relationships among otherwise obfuscated or intractable neuronal circuits.

Acknowledgements

The authors thank Dr Vytas Byndokas for his valuable assistance with the confocal microscope and image acquisition. This work was supported by NIH/NIDCD grant R03 DC 011361, SVM CORP grant LAV3202, and NSF-LA EPSCoR grant PFUND276 (CCL) and NIH/NIDCD grant R01 DC 008794 (SMS).

Conflicts of interest

There are no conflicts of interest.

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