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Multichannel activity propagation across an engineered axon network

H. Isaac Chen^{1,2}, John A. Wolf^{1,2}, and Douglas H. Smith¹

¹Department of Neurosurgery, University of Pennsylvania, Philadelphia, PA 19104

²Philadelphia Veterans Affairs Medical Center, Philadelphia, PA 19104

Abstract

Objective—Although substantial progress has been made in mapping the connections of the brain, less is known about how this organization translates into brain function. In particular, the massive interconnectivity of the brain has made it difficult to specifically examine data transmission between two nodes of the connectome, a central component of the “neural code.” Here, we investigated the propagation of multiple streams of asynchronous neuronal activity across an isolated *in vitro* “connectome unit.”

Approach—We used the novel technique of axon stretch growth to create a model of a long-range cortico-cortical network, a modular system consisting of paired nodes of cortical neurons connected by axon tracts. Using optical stimulation and multi-electrode array recording techniques, we explored how input patterns are represented by cortical networks, how these representations shift as they are transmitted between cortical nodes and perturbed by external conditions, and how well the downstream node distinguishes different patterns.

Main results—Stimulus representations included direct, synaptic, and multiplexed responses that grew in complexity as the distance between the stimulation source and recorded neuron increased. These representations collapsed into patterns with lower information content at higher stimulation frequencies. With internodal activity propagation, a hierarchy of network pathways, including latent circuits, was revealed using glutamatergic blockade. As stimulus channels were added, divergent, non-linear effects were observed in local versus distant network layers. Pairwise difference analysis of neuronal responses suggested that neuronal ensembles generally outperformed individual cells in discriminating input patterns.

Significance—Our data illuminate the complexity of spiking activity propagation in cortical networks *in vitro*, which is characterized by the transformation of an input into myriad outputs over several network layers. These results provide insight into how the brain potentially processes information and generates the neural code and could guide the development of clinical therapies based on multichannel brain stimulation.

Keywords

Activity propagation; connectome; neural code; neural interfaces; neural networks; optogenetics; tissue engineering

Since it was proposed over a decade ago (Sporns et al., 2005), the brain connectome has transformed our conceptualization of how neural activity propagates through the brain (Bock et al., 2011; Van Essen et al., 2012; Oh et al., 2014; Zingg et al., 2014). However, these intricate maps do not explain the processing, coding, and transfer of data between discrete nodes in the connectome (Kumar et al., 2010; Bargmann and Marder, 2013; Kopell et al., 2014). Examining these questions in the brain is particularly challenging because of the rapid spread of activity over multiple overlapping networks (Stroh et al., 2013), which obscures the relationship between paired nodes. Similar problems are associated with *ex vivo* brain slices, although on a smaller scale.

In vitro models are commonly used to simplify the study of neural network behavior. Typically consisting of uniform networks of dissociated neurons, these models have helped to elucidate intrinsic neuronal firing properties (Gal et al., 2010), mechanisms of network plasticity (Bakkum et al., 2008; Fong et al., 2015), and facets of short-term memory (Dranias et al., 2013). However, the random geometry of these culture systems does not resemble the highly organized architecture of paired nodes in the brain, thereby limiting examination of internodal communication (Shein-Idelson et al., 2011). Modular structure is an intrinsic feature of the brain that permits complex information processing, including parallel and hierarchical computation (Meunier et al., 2010). It is thus of great importance to reproduce this modularity in *in vitro* networks.

Building upon prior work on modular networks (Peyrin et al., 2011; Kanagasabapathi et al., 2012; Levy et al., 2012; Pan et al., 2015), we have created the concept of an *in vitro* “connectome unit” to investigate the transmission of neural activity across long-range cortico-cortical networks in isolation. These long-range circuits integrate information from different regions of the brain, which facilitates the emergence of higher-order cortical functions (Mao et al., 2011; Zhang et al., 2014). The connectome unit is comprised of two nodes of cortical neurons connected by a long axon tract consisting of multiple fascicles. The design of this structured culture is based on a novel tissue-engineering technique for generating long axon tracts using mechanical tension (Smith et al., 2001; Pfister et al., 2004; Pfister et al., 2006). This method exploits a natural “second phase” of axon development that is induced purely by tensile forces on axons as they are stretched to match the growth of an organism’s body (Smith, 2009).

Understanding the propagation of spiking activity through the brain is central to unraveling the neural code (Kumar et al., 2010) and the effects of external stimulation. Prior studies have examined the spread of spontaneous network bursts (Baruchi et al., 2008; Shein Idelson et al., 2010; Kanagasabapathi et al., 2011), but there is a relative dearth of data on the transmission of asynchronous firing activity (Reyes, 2003). Here, using multichannel optical stimulation and multisite electrical recordings (Figure 1A), we investigated the representation of stimulation-based inputs in cortical networks, factors modifying these

representations during transmission to a downstream node, and the degree to which the downstream node can discriminate among different input patterns. Neuronal responses to stimulation of different regions of the connectome unit were examined to identify changes in response representations as a function of stimulus distance. Subsequently, parameters reflecting the quality and quantity of information content (i.e., stimulation frequency, glutamatergic tone, and number of input channels) were evaluated for their influence on these representations. Lastly, the ability of the downstream node to differentiate among input patterns was probed using pairwise difference analysis of individual cell response rates and summed neuronal responses.

Materials and Methods

Multi-electrode array & elongation bioreactor chamber preparation

A planar multi-electrode array (MEA) probe with 32 contacts (312 μm^2 surface area) distributed as tetrodes at the end of 8 individual shanks (NeuroNexus; Ann Arbor, MI) was rinsed 3 times with tissue culture-grade water (Lonza; Basel, Switzerland). The probe was then attached to the internal wall of a previously described axon elongation bioreactor chamber (Pfister et al., 2006) using a silicone adhesive (NuSil; Carpinteria, CA). The towing membrane of the bioreactor chamber was positioned adjacent to the tips of the probe shanks (Figure S1). Sterilization of the probe and chamber was achieved using overnight irradiation with ultraviolet light. The interface between the probe and the towing membrane was coated first with polyethyleneimine (0.005% w/v; Sigma-Aldrich; St. Louis, MO) overnight at room temperature and then a 100 μL bubble of laminin (5 $\mu\text{g}/\text{cm}^2$; BD Biosciences; San Jose, CA) for 2–3 hours at 37°C.

Primary neuron isolation & maintenance

Pregnant Sprague-Dawley rats were euthanized with CO_2 , and embryonic day (E18) fetuses were harvested. Cortical tissue was isolated under a dissecting microscope, rinsed twice in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hank's Balanced Salt Solution (HBSS; Life Technologies; Grand Island, NY), and incubated with trypsin (0.25% + 1 mM EDTA; Life Technologies) for 10 minutes at 37°C. DNase I (Roche; Indianapolis, IN) was added for a final concentration of 0.3 mg/mL, and the cortices were vortexed briefly to break up the pieces of tissue. The cell suspension was then centrifuged at 1,000 rpm for 3–4 minutes. After the supernatant was aspirated, the cells were resuspended in Neurobasal base media (Neurobasal media (Life Technologies), 2% B27 (Life Technologies), and 0.25% Glutamax (Life Technologies), supplemented with 5% fetal bovine serum (FBS; Atlanta Biologicals; Flowery Branch, GA) and 1 $\times$ penicillin-streptomycin (Life Technologies). The inclusion of serum in the cell culture media results in a mixed neuronal-glial culture that supports long-term neuronal activity (Wagenaar et al., 2006). The laminin bubble on the probe was carefully aspirated, and 250,000 cells in 125 μL of media were plated in the same region as the bubble, covering a circular area with a diameter of roughly 8 mm (surface area = 50 mm^2). Following 3 hours of incubation at 37°C to allow the cells to adhere, an additional 1 mL of above media was added. On day 1 *in vitro* (DIV), a full media change was performed using 1.5 mL Neurobasal base media (NB) with 2% FBS. A half media change with NB+2% FBS was performed every other day thereafter.

Viral transduction of neuronal cultures

On DIV 5, the neurons were transduced with AAV2/1.hSynapsin.ChR2(H134R).eYFP.WPRE.hGH (Penn Vector Core; Philadelphia, PA) in a volume of 1 mL (titer: 6.71×10^9 genome copies/mL, multiplicity of infection: $2.24 - 2.68 \times 10^4$) over a period of 16–24 hours at 37°C. This vector expresses channelrhodopsin-2, a blue light-sensitive opsin, fused to a fluorescent tag (enhanced yellow fluorescent protein) under the control of the human synapsin I promoter. This promoter is known to drive expression in both excitatory and inhibitory neurons (Nathanson et al., 2009). On DIV 6, the virus solution was completely removed, and the culture was rinsed with 0.75 mL of NB+2% FBS. Then, 2.25 mL NB+2%FBS was added to the culture.

Axon elongation

The elongation bioreactor chamber was connected to a stepper motor (Servo Systems; Montville, NJ) on DIV 6, and the axons were elongated at a rate of 0.5 mm/day for 24 hours, 0.8 mm/day for 24 hours, and then 1.7 mm/day until the desired length was reached. Typically, elongation was stopped on DIV 12, resulting in 8 mm long axon tracts. A 1/3 media change with NB+2%FBS was performed every 6 days after the initiation of elongation until the culture was used for immunocytochemistry or electrophysiology.

Immunocytochemical analysis of connectome units

For immunocytochemistry, elongated axon cultures were prepared in the absence of the MEA probe and ChR2 viral transduction. To stabilize them for subsequent steps, the axons were coated with 80 μ L of 1% Seaprep agarose (Lonza). The agarose gel was solidified by cooling the bioreactor chamber at –20°C for 3 minutes and then 4°C for 30 minutes. Then, the cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences; Hatfield, PA) in phosphate buffered saline (PBS; Roche) for 1 hour at room temperature and rinsed 4 times with PBS. Blocking was performed with a PBS solution containing 4% normal goat serum (NGS; Vector Labs; Burlingame, CA) and 0.3% Triton X-100 (Sigma-Aldrich) for 1 hour at room temperature. The cells were incubated with primary antibodies diluted in 4% NGS/PBS overnight at 4°C. Following 5 rinses with PBS the next day, the cells were incubated with secondary antibodies diluted in 4% NGS/PBS for 2 hours at room temperature. After another 5 rinses with PBS, coverslips were mounted using Prolong Antifade Gold (Life Technologies). Epifluorescence microscopy was performed 24 hours after coverslip mounting using an Eclipse TE300 inverted microscope system (Nikon, Inc.; Melville, NY) and NIS-Elements software (Nikon).

Primary antibodies used in this study included β -III tubulin (1:500; T8578, Sigma-Aldrich), MAP2 (1:1000; ab5392, Abcam; Cambridge, MA), and GAD65+GAD67 (1:250; ab49832, Abcam). Secondary antibodies were all diluted at 1:500 and included 488 goat anti-rabbit, 594 goat anti-mouse, 647 goat anti-chicken, and 647 goat anti-rabbit (Life Technologies).

MEA recordings of connectome unit activity

For electrophysiological recording studies, the bioreactor chamber was removed from the stepper motor and placed on a water-heated hard pad (Hallowell EMC; Pittsfield, MA) to maintain a media temperature of 37°C. The MEA probe was then connected to a Digital

Lynx SX system (Neuralynx; Bozeman, MT) running Cheetah 5 software (Neuralynx) for data acquisition. No changes in media were performed. Recordings were done on DIV 21–23.

Blockade of glutamate receptors was achieved using additive boluses of MK-801 (Sigma-Aldrich) or NBQX (Sigma-Aldrich). Stock solutions were diluted 1:1000 in the culture media to obtain a final concentration of 5 μ M for both blockers. In a similar manner, blockade of sodium voltage-gated ion channels and of sodium-dependent action potential initiation was performed using boluses of tetrodotoxin (TTX; Sigma-Aldrich) for a final concentration of 0.5 μ M. Additional boluses of blockers were added at 5-minute intervals as necessary.

Optical stimulation of connectome units

Light stimulation was performed using a custom-made four-channel fiber optic array (Figure S2) coupled to 470-nm light-emitting diodes (LED; Thorlabs, Newton, NJ). Individual fibers had a 200 μ m diameter core with a numerical aperture of 0.39. We estimated that each fiber illuminated a circular area with a diameter of approximately 500 μ m and that each area of illumination was separated from neighboring areas by about 400 μ m (Figures S2 and S3). By visual inspection, we confirmed that there was no overlap between the regions illuminated by adjacent fibers. Assuming that each node of the connectome unit covered 25 mm² of surface area (see above), each fiber illuminated approximately 0.8% of a particular node. The system was controlled by a Matlab script (Mathworks; Natick, MA) run through a data acquisition board (National Instruments; Austin, TX) and a multichannel LED driver (DC4104, Thorlabs). During stimulation studies, the fibers were positioned approximately 0.5 mm above the cells using a micromanipulator. Stimulation was performed at 2, 5, 10, and 20 Hz for various experiments using various combinations of the four-channel optical array. For a given stimulus period using a defined combination of LED channels, a minimum of 120 stimulation pulses was presented.

Electrophysiological data analysis

Tetrode-based cluster cutting allowed us to resolve single units from the multi-unit signals recorded from an electrode contact. Individual clusters on each tetrode were resolved using SpikeSort3D software (Neuralynx) running the KlustaKwik algorithm option (Peaks 0 0, 1 1, and 2 2; Valleys 4 0, 5 1; maximum of 10 clusters). Each cluster was then manually evaluated to determine if it was a unique neuronal waveform, a duplicate waveform, or noise. Duplicate waveform clusters were merged, and noise clusters were discarded. The remaining neuronal clusters were optimized via manual thresholding. Each neuron was named after the tetrode from which it was recorded, corresponding to the MEA shank on which the tetrode was located, and the cluster number assigned by the KlustaKwik algorithm (i.e., tetrode.cluster). Subsequent analyses were done with NeuroExplorer (Nex Technologies; Madison, AL) and Matlab scripts. Post-stimulus time histograms (PSTHs), rate histograms, raster plots, and average waveforms were created using Matlab. Box-and-whisker plots were created using Prism 5 (GraphPad Software; La Jolla, CA). To enable accurate comparisons across experimental conditions, the PSTHs in Figure 4 were normalized to account for differences in the number of stimulation pulses. The number of

spikes per bin was multiplied by the quotient of 1,500 pulses divided by the number of pulses used to generate the PSTH data under consideration.

The response latency for a given neuron represented the time difference between the start of the light stimulation pulse and the peak of the response identified on a post-stimulus time histogram. We estimated the axonal conduction velocity for an individual neuron by dividing the length of the elongated cortical axons by the conduction time (remote node response latency – recording node response latency). The remote node and recording node response latencies represent the latency of a neuron's response to optical stimulation of the remote node (towing membrane side) and the recording node (MEA side), respectively. Axon lengths were determined by measuring how far the towing membrane had moved.

Response rates were calculated by dividing the spike count by the number of stimulation pulses within a specified stimulus period. For the experiments depicted in Figures 2C & S8, the time window was 100 ms except for 20 Hz stimulation data, for which the time window was 50 ms. The selection of this time window was made to permit meaningful comparisons among 2, 5, and 10 Hz data. A 50 ms time window was not applied to all stimulation frequencies because significant response peaks were often observed 50–100 ms post-stimulus.

Multichannel stimulation and data analysis—Remote node neurons were illuminated with seven combinations of the four-channel LED array at 2 Hz (each individual channel alone, channels 1 & 2 together, channels 3 & 4 together, and all four channels together). Separate analyses were done for two time windows. We initially planned to separate direct and indirect responses. However, because of variability in conduction velocities across the axon tract of the connectome unit, we could not reliably differentiate between direct and indirect/short latency responses with a fixed post-stimulus time threshold. We thus decided to combine direct and indirect/short latency responses in the first time window, which reflected “local” circuit responses to stimulation. An interval of 20–80 ms post-stimulus was chosen for the first time window based upon our results of remote node stimulation (Figures 3C, S6). The second time window (>80 ms post-stimulus) represented indirect/long latency responses, i.e., broader network activity elicited by stimulation. While previous studies (Bakkum et al., 2008; Kermany et al., 2010) informed these methodological decisions, we could not directly use their established post-stimulus time intervals because of variable time delays introduced by the axon tract of the connectome unit.

In each time window, neuronal responses were analyzed at the individual cell and summed population levels. For an individual cell, the response rate derived from the entire stimulus period was used as a surrogate for that cell's stimulation response. Response rates for a given cell were ranked, 1 for the greatest response and 7 for the least response, in order to compare multichannel responses across different cells and experiments. At the population level, sum scores were calculated by adding together the response rates of all individual cells for a given LED channel combination. Sum scores were also ranked using the same system described above for individual cell response rates. Pairwise difference analysis was performed by calculating the differences in individual cell response rates or sum scores between every possible pair of LED channel combinations (21 total pairs).

Results

Structure of the electronics-integrated connectome unit

To generate an isolated two-node cortical network, E18 rat cortical neurons were used to produce axon tracts up to 1 cm in length over 1 week at a maximum elongation rate of 1.7 mm/day. These connectome unit cultures consisted of two neuronal clusters with their associated dendritic processes (MAP2+/ β -III tubulin+) connected by dense axon fascicles (β -III tubulin+ only) aligned in a parallel orientation (Figure 1B). A minority of the neurons in the connectome unit was positive for GAD65/67, consistent with an interneuron identity (Figure S4).

To record the activity of multiple neurons within the connectome unit simultaneously, we integrated one neuronal population, designated the “recording node,” with a 32-channel multi-electrode array (MEA) prior to axon elongation (Figure S1). Gradual mechanical separation of these neurons from the second neuronal population, called the “remote node,” resulted in stretch growth of the spanning axons, which coalesced into dense fascicles as elongation progressed (Figures 1C & S5). Serendipitously, we found that the elongated axons preferentially coalesced into larger fascicles at the points of the MEA shanks (Figure 1D).

Recording node stimulation

As a first step, we evaluated how local stimulation was represented by neurons within the recording node of the connectome unit (i.e., neurons on or around the MEA shank tips; * in Figure 1C). In the absence of external stimulation, these neurons exhibited the typical bursting activity that has been documented previously for dissociated cortical neuron cultures (data not shown) (Wagenaar et al., 2006). Stimulation of the connectome unit was achieved by transducing the culture with channelrhodopsin-2 (ChR2) before axon elongation and with a four-channel light-emitting diode (LED) fiber optic array. This approach permitted us to interrogate points in close proximity to each other with minimal cross-stimulus contamination. With simultaneous stimulation by all four channels of the LED array, we observed several response patterns, including some that were similar to prior data from electrical stimulation studies (Bakkum et al., 2008; Kermany et al., 2010). In addition to direct action potential responses (very short latency and minimal response variability), we identified two categories of indirect responses that were separated on the basis of their response latencies (short versus long; Figure 2A). Both of these indirect responses had a larger degree of response variability that was consistent with synapse-mediated activity. Lastly, some neurons exhibited multiple response peaks to stimulation, including combinations of direct and indirect responses (Figure 2A). Most multiple responses contained two distinct peaks of activity, although a few neurons exhibited three peaks.

Stimulation frequency is a commonly manipulated variable in brain stimulation interventions, and its variable effects on neuronal activity can help probe the processing capacity of a network (Gal et al., 2010). Thus, we tabulated the numbers of neurons falling into each response category (i.e., direct, indirect/short latency, indirect/long latency, and multiple) and examined how this distribution changed with stimulation frequency. A total of

176 unique neurons were recorded (Table S1). As the stimulation frequency increased, short-latency indirect responses increased while all other response categories decreased (Figure 2B, Table S2). Of the 119 neurons that responded to more than one frequency of stimulation, the majority of the cells (96 neurons, 80.7%) stayed within the same response category with changes in stimulation frequency. Twenty-three cells (19.3%) switched to a different response category with changes in stimulation frequency (Figure 2C). Most of these changes involved the simplification of a multiple response into a single response (19/23); 13 neurons lost their second response peak while 6 neurons lost their first response peak. In a few cells (4/23), narrow response peaks at lower stimulation frequencies became broader with higher stimulation frequencies.

We further characterized some basic properties of neuronal responses to recording node stimulation. Response latencies were generally less than 20 ms, although some latencies stretched upwards of 80 ms (Figures 3C, S6). Response rates, defined as the number of spikes per stimulation pulse, fell with increasing stimulation frequency, irrespective of the response category (Figure 2D). In some cases, response rates exceeded 1, indicative of a larger number of spikes than the number of stimulation pulses (2 Hz: 8 of 54 neurons [14.8%], 5 Hz: 10 of 164 neurons [6.1%], 10 Hz: 2 of 102 neurons [2.0%], 20 Hz: 1 of 47 neurons [2.1%]). Of note, no background activity correction was performed for response rates, both here and in subsequent experiments, because of the low rate of baseline activity, especially between stimulation epochs (Figure S7).

Axonal and remote node stimulation

Subsequently, we illuminated the elongated axons and remote node neurons (** and ^ in Figure 1C, respectively) using all four optical channels simultaneously to ascertain how recording node neurons responded to increasingly distant stimulation. In addition to direct and indirect responses, multiple responses were present, often comprised of more peaks than seen in recording node stimulation (Figure 3A). Moreover, a new category emerged, consisting of near-continuous activity throughout the stimulus period (Figure 3A, second panel from bottom). This activity was not seen in the absence of optical stimulation (Figure S7) and was sometimes associated with a superimposed activity peak (Figure 3A, bottom panel).

We again tabulated the numbers of neurons falling into each response category to examine changes in this distribution as a function of stimulation frequency (Tables S1 & S2). We recorded 94 and 120 neurons from axonal and remote node stimulation, respectively. Although direct responses could still be found, they were less common for axon stimulation and rare for remote node stimulation in comparison to recording node stimulation (Figure 3B). With increasing stimulation frequency, indirect and multiple responses decreased while cells exhibiting continuous firing activity increased in occurrence, findings that were more pronounced with remote node stimulation (Figure 3D). In 61.7 % (58/94, axon) and 50.0% (60/120, remote) of recorded neurons, responses were present at multiple stimulation frequencies. For axon stimulation, 19 neurons demonstrated changes in response categories with 6 peak dissolutions as described in Figure 2C and 13 transitions to continuous activity. For remote node stimulation 33 neurons demonstrated changes in response categories with 5

peak dissolutions and 27 transitions to continuous activity. Moreover, for both axon and remote node stimulation, the ratio of cells exhibiting continuous activity plus a superimposed peak versus continuous activity alone generally fell with increasing stimulation frequency (Table S2).

Increasing response latency variability was seen for axon and remote node stimulation (Figures 3C, S6). We estimated the axonal conduction velocity to be 0.26 ± 0.10 m/s (mean $\pm$ standard deviation, $n=12$ neurons over 3 separate experiments). Tetrodotoxin effectively ablated these responses, demonstrating the sodium channel dependence of the transmitted activity (Figure S8). As with recording node stimulation, response rates generally decreased with increasing stimulation frequency (Figure S9)..

Emergence of hidden circuits with glutamatergic inhibition

In order to better understand the circuits underlying activity propagation across the connectome unit, we successively applied NMDA and AMPA receptor antagonists while optically stimulating the remote node with all four LED channels and recording from the recording node. Several stereotyped patterns of neural responses emerged from these experiments with variations in the onset and latency of the response and the number of neurons involved (Figure 4A). Most pathways existed before the addition of receptor blockers, but others appeared only after the addition of NMDA receptor antagonists. AMPA receptor antagonists alone did not appear to elicit these changes. We observed three distinct categories in terms of the number of neurons engaged in the circuit. A narrow response peak that was not eliminated by glutamate receptor blockade was indicative of retrograde axonal stimulation. Interestingly, this pattern only appeared in a delayed fashion. The other two pathways were consistent with anterograde synaptic transmission. In pathways with few synaptic relays (“oligo-synaptic”), receptor blockers caused a response peak to become broader and acquire progressively longer response latencies until it disappeared altogether. In pathways with multiple synaptic relays (“poly-synaptic”), we observed an initially broad response peak with a relatively long response latency, which became increasingly disorganized in the presence of receptor blockers until it completely faded away. This change was similar to the transition to continuous firing activity at higher stimulation frequencies seen with axon and remote node stimulation in the absence of glutamate receptor antagonists. These findings suggested the presence of hidden networks within the nodes of the connectome unit that are held latent by inhibitory circuits.

Multichannel stimulation across the connectome unit

We next studied how multiple streams of independent data were transmitted between and interpreted by the cortical nodes. After stimulating the remote node with seven different channel combinations of the LED array, we analyzed individual and summed neuronal responses within the recording node. Two post-stimulus time windows were used for this analysis, one representing local responses (direct and indirect/short latency) and the other reflecting broader network activity elicited by stimulation (indirect/long latency). In the first time window, 2/89 recorded cells (4.1 in Experiment 1 [Figure 4A] and 7.3 in Experiment 4 [Figure S10]) appeared to respond robustly in the presence of one specific LED channel, acting as a signature for that channel. For instance, cell 4.1 in Experiment 1 responded well

to any channel combination including channel 4 (4, 34, 1234) but poorly to all other combinations (1, 2, 3, 12). The other cells in the first time window and all cells in the second time window had more complex, mixed responses to multichannel stimulation. Both within and between experiments, we observed substantial variability in the neuronal response rates to stimulation by a given channel combination, such that standard deviations were often greater than means (Table S3). Thus, in order to compare multichannel responses across neurons and experiments, we analyzed response rate ranks rather than response rates themselves. In the first time window, mean ranks decreased as stimulation channels were added, indicating an increase in response activity (Figure 4B). This additive effect was non-linear. For example, for cell 2.1 in Figure 4A, the response rate to channel one and two separately was 1.54 and 1.85, respectively, while the response rate to channels one and two combined was 1.92. Interestingly, we found an inverse relationship in the second time window, suggesting that response activity generally decreased with the addition of more stimulation channels (Figure 4C).

To examine the extent to which different stimulus patterns could be resolved by the connectome unit, we used the concept of pairwise differences in response rates to different LED channel combinations. In this analysis, stimulation inputs were differentiated based upon differences in response rates rather than the absolute value of the response rates. The greater the difference, the more easily the input patterns could be discriminated. The vast majority of response rate differences in both time windows were low, falling below 0.05 (Figures 4D–E). We hypothesized that combining the responses of multiple neurons could improve upon this level of discrimination. For each channel combination, response rates were summated across all neurons recorded in an experiment to obtain a sum score (Figure 4A, Table S4), and pairwise differences of these sum scores were calculated in a similar manner to response rates. We found a distinct change in the shape of the distribution and a clear shift towards larger pairwise differences with sum score analysis compared to response rate analysis. (Figures 4D–E).

Discussion

An *in vitro* “connectome unit,” consisting of paired nodes of cortical neurons spanned by stretch-grown axon tracts, was developed to model an isolated long-range cortico-cortical network. This modular culture permitted investigation of the representation and modification of patterned inputs as they were transmitted between the nodes of a neuronal network. In contrast to prior binary classifications of neuronal stimulation responses in *in vitro* cultures, a more complex set of post-stimulus activity was identified, including several types of synaptic and multiplexed responses. Prolonged glutamate receptor blockade revealed a hierarchy of network pathways within the connectome unit, some of which were initially latent. Furthermore, multiple input streams elicited divergent response patterns in different network layers, and ensembles of neurons were more effective at discriminating among input patterns than individual cells. These data provide a glimpse of the remarkable complexity that characterizes the process of transforming inputs into outputs across two cortical nodes.

Modeling brain modularity in *in vitro* networks

Generated using lithographic or microfluidic techniques (Wheeler and Brewer, 2010), patterned neuronal cultures in the form of bi-nodal configurations or grids have contributed to a better understanding of neuronal network communication. These modular networks have been useful in experimentally identifying gating mechanisms that control the spread of neural activity among network nodes (Yvon et al., 2005; Feinerman et al., 2008; Levy et al., 2012; Pan et al., 2015), a phenomenon that typically has been studied through computational modeling (Shinozaki et al., 2007; Vogels and Abbott, 2009). In addition, modular networks have been used to study the initiation and maintenance of asymmetries in communication between network nodes, such as master-slave relationships (Baruchi et al., 2008; Shein Idelson et al., 2010; Kanagasabapathi et al., 2012).

In the current study, we used the technique of axon stretch growth to generate a unique structured culture that is substantially different from previous *in vitro* modular systems. Our internodal axon tracts achieved lengths of up to 1 cm, at least one order of magnitude greater than previous modular cultures. Notably, with shorter distances between nodes, neuritogenesis led to the formation of new inter-modular connections, thereby converting the culture to a uniform network (Pan et al., 2015). Furthermore, in contrast to the segregation of axon fibers formed via patterned channels in previous modular cultures (Peyrin et al., 2011; le Feber et al., 2015; Pan et al., 2015), the axon tract of the connectome unit was composed of a near-continuous array of naturally fasciculated axons (Figure S5). The mean conduction velocity across the engineered axons of 0.26 m/s likely underestimates the actual value, since the calculation method did not remove the temporal effect of synaptic activity within the remote node. Nonetheless, it is in agreement with reported speeds for unmyelinated intracortical axons (Lohmann and Rorig, 1994). Thus, the connectome unit provides a platform for studying internodal activity propagation in the context of axon lengths and architecture that more closely reflect long-range cortico-cortical circuits in the brain.

Activity propagation across the connectome unit

The present data demonstrated a more complex set of stimulation responses than the binary schema (i.e., direct versus synapse-mediated) proposed by previous studies using dissociated neuronal cultures (Bakkum et al., 2008). Within the category of indirect responses, we observed varied latencies and histogram peak shapes, suggestive of different classes of synaptic responses. We also observed multiple responses combined in a single cell and continuous spiking activity with axonal/remote node stimulation. These more complex responses could be the result of the activation of multiple pathways to the recorded neuron or network reverberations (Durstewitz et al., 2000; Lau and Bi, 2005; Kermany et al., 2010; Volman and Gerkin, 2011). The possibility of binodal “ping-pong” activity could be explored in subsequent studies using MEAs integrated into both nodes of the connectome unit or optical imaging techniques. These results indicate that asynchronous inputs are converted into a variety of neuronal response types, especially after they are transmitted to a downstream node.

At higher stimulation frequencies, the connectome unit exhibited a finite capacity to process information. Of the neurons that switched response categories in recording node stimulation,

the majority involved the devolution of multiple responses to a single response, perhaps the more efficient pathway of neural activation. Of the neurons that changed response categories in axonal/remote node stimulation, most underwent a transition from a distinct response peak to continuous firing activity. We interpreted this latter change as the loss of the ability to transmit a neural rate code with the preservation of the capacity to reflect state changes (i.e., on versus off stimulation). Previously, stimulation frequency thresholds for cortical neurons have been described above which response failures become more common, resulting in erratic response patterns (Gal et al., 2010). Our data support the notion that there are inherent boundaries to neural activity propagation between the nodes of a cortical network.

Multichannel data transmission

Divergent neuronal responses across two post-stimulus time windows were observed as additional stimulation channels were added. In the first time window, which represented local circuit activity, the addition of stimulation channels led to non-linear additive responses for both individual cells and summed neuronal populations. Sublinear summation or “normalization” is thought to underlie the integration of multiple sensory inputs into complex representations and perception (Carandini and Heeger, 2012), although the summation of weak stimuli may operate through linear or supralinear mechanisms (Heuer and Britten, 2002; Lavzin et al., 2012). In contrast, the same addition of stimulation channels often resulted in response suppression in the second time window, which reflected recruitment of broader network activity. This observation is reminiscent of surround suppression, although the mechanisms that explain this phenomenon involve more complex architecture than is present in the connectome unit (Webb et al., 2005). Because stimulus responses may shift over time through the process of accommodation or other mechanisms, investigating response variations within a stimulus period could lead to further insights on the effects of multichannel stimulation.

Data from multichannel stimulation in the connectome unit also suggested that neuronal ensembles were generally superior to individual cells for discriminating among input patterns. While certain cells exhibited high-fidelity responses to specific channels, the majority responded in a mixed manner to stimulation and could not readily distinguish different stimulation patterns. Using pairwise difference analysis, we further showed that sum score differences for neuronal populations were larger and had a less skewed distribution than response rate differences for individual cells. The sum score difference analysis could be interpreted as a hypothetical neuron downstream from the recording node that integrates neuronal output from that node, a variation of the integrate-and-fire model (Burkitt, 2006). Although it is well established that neurons are exquisitely sensitive to incoming synaptic input (Magee, 2000), the thresholds relevant for differentiating various input patterns are not clear. The efficiency of propagating different multichannel inputs between network nodes could be examined by iteratively feeding decoded outputs back into the connectome unit as new inputs. This strategy was previously used to identify the evolution of synchrony in the propagation of a firing rate code (Reyes, 2003) but has not been applied to determining the minimal differences in neuronal responses that are needed to propagate independent input patterns.

Network layers within the connectome unit

Multiple processing layers are important components of several computational algorithms, including perceptrons and other feed-forward networks (Hornik et al., 1989), and may have been present within the connectome unit in at least two ways. First, the time windows delineated for multichannel stimulation experiments contained different neuronal responses and could represent distinct network layers. Second, some pathways were immediately activated upon stimulation while others were exposed only in the presence of glutamatergic blockade, a dichotomy reminiscent of the essential versus compensatory networks described in human brain disorders (Chen et al., 2014). One possible explanation for this latter observation is that the receptor blockers mediated a preferential decrease in excitatory input to inhibitory interneurons, allowing downstream neurons to fire (Figure 4B). Cortical disinhibition via preferential NMDA antagonism of interneurons has been observed in the prefrontal cortex (Homayoun and Moghaddam, 2007) and posterior cingulate/retrosplenial cortices (Li et al., 2002). Synaptic plasticity is an alternative explanation for this observation, although long-term potentiation is typically impaired in the presence of NMDA receptor blockade (Morris et al., 1986) and shifts in neuronal activation typically require longer periods of stimulation *in vitro* (Lignani et al., 2013). While it is presumed that multiple processing layers exist *in vivo*, demonstrating their presence and performing experimental manipulations on them is difficult. As an alternative, the connectome unit may possess the layered substrate necessary to test precepts of computational neuroscience in a biological context.

Implications for clinical neuromodulation

The complexity of multichannel stimulation responses across multiple network layers has implications for next-generation neuromodulation therapies. Current interventions stimulate large volumes of brain tissue in a uniform manner, but future technologies may enable multiple channels of stimulation with finer spatial resolution (Pisanello et al., 2014). Such advances could improve the degree of control over neural circuits and permit gain modulation during stimulation of specific pathways. However, they also could lead to unintended consequences due to the intricate, nonlinear effects of multichannel stimulation. Adjustments of stimulation parameters would need to take this complexity into account to optimize functional outcomes. As an example, stimulation frequency could be varied to either maximize information transfer or possibly create information lesions at higher rates of stimulation (Grill et al., 2004). Parsing the effects of multichannel stimulation on the brain will require further use of models such as the connectome unit to develop a finer understanding of the principles governing internodal activity propagation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Structure of connectome units

(A) This schematic depicts a connectome unit integrated with an LED optical array on left and an MEA on right. The raster plots above demonstrate hypothetical responses to stimulation from different combinations of independent light channels. In each row of plots, the rasters depict the light responses of a set of spike waveforms that correspond to a single neuron. The blue line represents the light stimulus. Each row of the raster plots represents a light stimulus trial, and the dots illustrate detected spikes at the specified latency. In the first column of plots, only light channel 1 is on, which results in a narrow response with a short latency from neuron 1 but no response from neuron 2. In the second column, only light channel 2 is on. There is no response from neuron 1, but neuron 2 displays a broad response with a longer latency, more consistent with general network activation. When both light channels 1 and 2 are on, both neurons respond in an additive manner. (B) This fluorescence micrograph depicts two distinct neuronal clusters that are MAP2- (red) and β -III tubulin- (green) positive separated by a region composed primarily of elongated axons that are only β -III tubulin-positive. The axonal rippling seen on the left is an artifact of agarose stabilization. (C) This phase contrast image shows a connectome unit with an MEA integrated on the right of the elongated axon construct. Four electrode contacts arranged in a tetrode configuration are situated at the tip of each probe shank. Neurons grow around and on top of the shanks. The blue symbols indicate the location of recording node (*), axon (**), and remote node (^) optical stimulation. (D) Elongated axons transduced with ChR2-yellow fluorescent protein (green) coalesce at the tips of the MEA shanks, leading to thicker axon fascicles. Scale bars: 500 μ m (B, C), 100 μ m (D).

Figure 2. Recording node stimulation responses

(A) With illumination of the recording node, several types of neuronal responses are evident, consistent with direct neuronal stimulation (top), indirect stimulation through synaptic relays with short latencies (second from top), indirect stimulation with long latencies (second from bottom), and multiple responses (bottom). Each post-stimulus time histogram (PSTH) represents data from a different neuron. The blue line at time 0 ms represents the stimulation pulse (pulse width: 5 ms). The blue traces in the upper right corner of each histogram represent the mean neuronal waveforms, and the dotted red lines are one standard deviation above and below the means. (B) This graph depicts the number of neurons falling into each response category divided by the total number of neurons recorded at a given stimulation rate (pooled data across all recorded neurons, see Table S2 for raw data). (C) In 119 of the 176 recorded neurons, responses were present at multiple stimulation frequencies. Twenty-three neurons demonstrated changes in response category. In neurons with multiple responses, 13 neurons lost their second peak (left) and 6 lost their first peak (middle) at higher stimulation frequencies. Four neurons exhibited dissolution of a narrow peak into a broader peak at higher stimulation frequencies. Each column displays a representative individual neuron with the top row showing responses at a lower stimulation frequency and the bottom row showing responses at a higher stimulation frequency. As in (A), the blue bar depicts the stimulation pulse, and the right-sided blue traces represent neuronal waveforms. (D) This graph depicts response rates (mean + standard deviation) for neurons in each response category for different stimulation frequencies. For (B) and (D), 1: direct response, 2: indirect response with short latency, 3: indirect response with long latency, 4: multiple responses.

Figure 3. Axon and remote node stimulation responses

(A) Optical stimulation of the axon tracts or remote node neurons resulted in response patterns consistent with direct responses (top), indirect responses (second from top), multiple responses (middle), and continuous network activity (bottom two). In the case of continuous network activity, some cells exhibited a superimposed peak of activity (bottom). Each PSTH represents a different neuron. All panels depict remote node stimulation responses but are representative of axon stimulation responses. (B) These graphs depict the frequency of neurons in each response category at different stimulation frequencies (pooled data across all recorded neurons, see Table S2 for raw data). 1: direct response, 2+3: indirect response, 4: multiple responses, 5+6: continuous activity. (C) Plotting the distribution of response latencies reveals a large degree of response heterogeneity at each position of illumination. An expected increase in the response latency occurs as the LED array is moved from the recording node (top) to the remote node (bottom). Data are expressed as the percentage of cells within 2 ms bins. The stimulation frequency of all three plots is 5 Hz. Recording node: 128 neurons from 9 experiments, axon: 42 neurons from 5 experiments, remote node: 37 neurons from 8 experiments. (D) The PSTH's show the recording node stimulation responses of 1 cell at 3 different stimulation frequencies, depicting a transition from multiple responses to continuous activity with a superimposed peak to only continuous activity. In (A), (B), and (D), the light blue bars at time 0 ms represent stimulation pulses (pulse width: 5 ms). In (A) and (D), the blue traces in the upper right corner of each histogram represent the mean neuronal waveforms, and the dotted red lines are one standard deviation above and below the means.

Figure 4. Layered networks revealed by glutamate receptor antagonists

While optically stimulating neurons in the remote node, MK801 and NBQX were added sequentially to the media bath (final bath concentration of 5 μ M per bolus) to block glutamate receptors in an incremental fashion. (A) Four distinct response patterns emerged, two of which were immediately present (“initial”) and two of which appeared only after the addition of MK801 (“delayed”). Direct axonal stimulation (“axonal”) was only observed in a delayed fashion. “Oligo” and “poly” refer to the postulated few or many neurons present in the observed neural circuit, respectively. Each column represents data from an individual neuron with a unique response pattern. The top row depicts rate histograms for each neuron while the bottom five rows show PSTHs corresponding to the condition labeled on the left. For the rate histograms, the colored arrows on the x-axis indicate the time at which glutamate receptor blockers were added (color-coded legend on the left). In the PSTHs, the blue bars represent the timing of the optical stimulation (pulse width = 5 ms). Data in each PSTH has been normalized to 1,500 stimulation pulses (300 s of data acquisition time at 5 Hz stimulation) to enable comparisons among experimental conditions. Mean neuronal waveforms with standard deviations for each neuron are shown at the bottom. (B) These diagrams illustrate simplified archetypes of the neuronal connections for one potential explanation of the response patterns. For the “delayed” patterns, glutamate receptor blockade could preferentially affect interneuron firing, resulting in net disinhibition. The “N” under each pattern heading denotes the aggregate number of cells identified for that pattern over 3 experiments. For the sake of simplicity in these models, all intermediate neurons are depicted in the remote node.

Figure 5. Multichannel stimulation

Remote node neurons were stimulated at 2 Hz with seven combinations of the LED array channels. (A) The results of one experiment, which correspond to “Experiment 1” below and in Tables S3 and S4, are depicted in this heat map of response rates (legend on right). Each box in the heat map represents the response rate of an individual neuron to stimulation from a specific combination of LED channels. Cell designations are the tetrode number followed by the cluster number on that tetrode. The LED array fibers providing stimulation are listed on the left (“Channel On”). The data are divided into two time windows, 20–80 ms and 80–200 ms after the stimulus. The sum of the individual neuronal response rates across all cells in the experiment is listed on the right, as well as the ranks of the sum scores. (B, C) In order to compare responses across different experiments, mean ranks for the response rates of individual cells were plotted for each channel combination across Experiments 1–6 (89 neurons). While ranks decreased (i.e., response rates increased) when additional LED channels were turned on in the 20–80 ms time window, ranks increased with additional LED channels in the 80–200 ms time window. (D, E) To assess how different stimulation patterns could be differentiated after propagation across the connectome unit, the difference between individual cell response rates (single) and sum scores (sum) for each pair of LED channel combinations was calculated. The frequency of pairwise differences in five bins was determined within each Experiment, and histograms for each time window were created using the mean frequencies across Experiments 1–6. This pairwise comparison suggested

that the combined response of multiple neurons (sum scores) was superior to the response rates of individual neurons for the purpose of distinguishing different combinations of LED channels.