Acetylcholine functionally reorganizes neocortical microcircuits

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Acetylcholine (ACh) functionally reorganizes neocortical microcircuits. J Neurophysiol 112: 1205–1216, 2014. First published May 28, 2014; doi:10.1152/jn.00071.2014.—Sensory information is processed and transmitted through the synaptic structure of local cortical circuits, but it is unclear how modulation of this architecture influences the cortical representation of sensory stimuli. Acetylcholine (ACh) promotes attention and arousal and is thought to increase the signal-to-noise ratio of sensory input in primary sensory cortices. Using high-speed two-photon calcium imaging in a thalamocortical somatosensory slice preparation, we recorded action potential activity of up to 900 neurons simultaneously and compared local cortical circuit activations with and without bath presence of ACh. We found that ACh reduced weak pairwise relationships and excluded neurons that were already unreliable during circuit activity. Using action potential activity from the imaged population, we generated functional wiring diagrams based on the statistical dependencies of activity between neurons. ACh pruned weak functional connections from spontaneous circuit activations and yielded a more modular and hierarchical circuit structure, which biased activity to flow in a more feedforward fashion. Neurons that were active in response to thalamic input had reduced pairwise dependencies overall, but strong correlations were conserved. This coincided with a prolonged period during which neurons showed temporally precise responses to thalamic input. Our results demonstrate that ACh reorganizes functional circuit structure in a manner that may enhance the integration and discriminability of thalamic afferent input within local neocortical circuitry.

acetylcholine; functional connectivity; graph theory; thalamus; cortex; two-photon imaging

INFORMATION PROCESSING in the neocortex is dynamic and state dependent. Neuromodulators influence cortical activity patterns (Harris and Thiele 2011), suggesting that the functional organization of cortical circuits is also modified (Quilichini and Bernard 2012). Functional connectivity within a circuit can reflect the reliable propagation of activity through the underlying synaptic structure (Ko et al. 2011, 2013), and state-dependent modulation of this structure is a potential mechanism through which information processing can be dynamically regulated (Quilichini and Bernard 2012). Acetylcholine (ACh) is the major neurochemical substrate underlying attention (Herrero et al. 2008; Paolone et al. 2013), behaviorally defined as enhanced discriminability of select sensory stimuli (Cohen and Maunsell 2009; Pinto et al. 2013). In vivo neuronal recordings show that ACh is sufficient to immediately improve sensory discriminability within neuronal populations and behaviorally (Pinto et al. 2013), consistent with the hypothesis that ACh enhances the signal-to-noise ratio (SNR) of sensory representation in primary sensory cortex (Oldford and Castro-Alamancos 2003). Concurrently, neuronal populations become desynchronized and decorrelated (Goard and Dan 2009; Pinto et al. 2013), which has also been attributed to attentional modulation (Bauer et al. 2012; Cohen and Maunsell 2009; Mitchell et al. 2009; Pinto et al. 2013). It is unclear how changes in functional cortical circuitry produce these altered population patterns that underlie behavioral modulation.

Studies at the single-cell level have demonstrated that ACh can differentially modulate neuronal properties (Alitto and Dan 2012) and synaptic transmission (Hasselmo and Bower 1992), making it difficult to predict ACh’s impact on microcircuit dynamics. Source-dependent effects of ACh have been identified at particular synapses, namely, intrinsic intracortical inputs are suppressed and thalamic afferent inputs are enhanced (Gil et al. 1997; Hasselmo and Bower 1992). These data have contributed to the hypothesis that ACh increases the SNR of thalamic inputs, but it is unclear how these monosynaptic and cellular effects influence the activity of highly recurrent and densely interconnected cortical circuits. The difficulty in bridging these multiple observations has inspired the use of computational models to predict how cholinergic modulation manifests at the network level to enhance sensory representation (Deco and Thiele 2011) and coding capacity (Linstead et al. 2003) in primary sensory cortices. Densely sampling cortical microcircuit activity provides the data necessary to bridge these multiple levels of investigation.

Here we experimentally address the hypothesis that ACh reorganizes functional circuit structure to enhance the cortical representation of thalamic afferent input. We used high-speed two-photon calcium imaging to simultaneously record action potential activity of up to 900 neurons, in a field of view (FOV) spanning multiple columns and layers (Sadovsky et al. 2011). Combining this technique with patch-clamp electrophysiology allowed us to compare ACh-mediated changes in single-cell properties with modifications in circuit-dependent activity. To identify changes within the functional architecture of local circuits, we recorded spontaneous cortical activity with and without ACh. We quantitatively evaluated the functional organization of cortical microcircuitry by applying graph metrics to functional wiring diagrams that were generated from correlations in spiking activity in cortical circuits. Using thalamocortical slice preparations, we evaluated how these ACh-modulated circuits differentially represented input from the thalamus. This approach enabled us to identify changes in functional circuit organization that may underlie the increased SNR of thalamic input in the cortex induced by ACh.

MATERIALS AND METHODS
Slice preparation. Experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of
Chicago. C57BL/6 mice of both sexes, postnatal ages P14–P17, were anesthetized with intraperitoneal injection of ketamin(100 mg/kg)-xylazine (5 mg/kg). Brains were rapidly removed and sliced with a vibratome (VT1000S) in 0–4° high-sucrose artificial cerebrospinal fluid (ACSF, mM: 0.5 CaCl₂, 3.5 MgSO₄, 3 KCl, 26 NaHCO₃, 1 NaH₂PO₄, 205 sucrose, 25 dextrose). Thalamocortical slices (450 μm thick) were cut at an angle to maintain effective connectivity of axonal projections from the thalamus to somatosensory “barrel” cortex (Agmon and Connors 1991; Fig. 1). After dissection, slices were incubated at 34°C for 40 min and then transferred to an oxygenated loading chamber for bulk loading of the calcium indicator fura-2 AM (Invitrogen) (MacLean et al. 2005). Slices were incubated in 2 ml of ACSF at 30°C for 20–30 min in the presence of 50 μg of fura-2 AM dissolved in 13 μl of DMSO and 2 μl of Pluronic F-127. Fura-2 AM is selectively loaded into both excitatory and inhibitory neurons indiscriminately (Sippy and Yuste 2013). Experiments were performed in ACSF containing either (mM) 2 CaCl₂, 2 MgSO₄, and 3 KCl or 1.2 CaCl₂, 1.0 MgSO₄, and 3.5 KCl (Shu et al. 2003), along with 123 NaCl, 1.25 NaH₂PO₄, 26 NaHCO₃, and 10 dextrose, aerated with 95% O₂-5% CO₂. Because the two ionic variants resulted in differences in the interval between circuit events (Sippy and Yuste 2013) but not the dynamics within the events themselves, all data were combined.

**Experimental procedure.** All experiments involved bath application of 30–50 μM ACh (Sigma). Fresh ACh was prepared daily from a frozen 100 mM stock and dissolved into the same ACSF that was being used that day for Control, kept at 25°C. Solutions were changed by manual replacement into an aerated perfusion bottle. To establish the time course for application and washout, whole cell recordings were monitored for change and recovery of resting membrane potential; spontaneous activity was monitored for change and recovery of the number of neurons active. We determined that application took full effect after 15 min of ACh presence and washout was complete after 90 min. For a subset of experiments (6/17, imaging), chronology was reversed so that recordings were first made in the presence of ACh and then in Control (washout). Because the two orders did not yield different results in the analyses that we present here, data were combined.

Experiments compared the same FOV in Control (ACSF) versus ACh (ACSF + ACh); the same scan path was used so that neuronal label was maintained. Activity was either evoked by thalamic stimulation or allowed to arise spontaneously; experiments were exclusive to a single activity source (evoked or spontaneous) to maximize sample size for analysis. To evoke orthodromic thalamocortical input, an extracellular electrode was placed in the ventroposterior medial nucleus (VPm) of the thalamus and a train of four 200-ms extracellular stimuli with a minimal amplitude of 10–30 μA was delivered at 40 Hz (Fig. 1B). As previously reported (MacLean et al. 2005), whole cell patch-clamp experiments demonstrated that this frequency was sufficient to evoke a burst of action potentials in thalamic relay neurons (Fig. 1B; Sherman 2001) and these stimulation amplitudes minimized antidromic activation (Beierlein and Connors 2002).

**Electrophysiology.** Whole cell current-clamp recordings were made with Multiclamp 700B amplifiers and custom software implemented in LabVIEW. Patched neurons were in either cortical layer 4 or 5; lamina was assigned based on distance from pia in the brightfield image along with morphology of surrounding neurons. Passive properties of neurons were assessed with 500-ms hyperpolarizing and depolarizing DC current steps. All analyses were performed with custom programming in MATLAB. The P values reported with Pearson correlation coefficients reflect the probability that the observed correlation could occur from random chance when the true correlation value is zero (MATLAB). Rheobase was defined as the average current required to drive a neuron to fire at least one action potential. Neurons that did not fire in at least three trials were excluded from analysis. UP states (circuit events) were automatically detected as periods when membrane potential was elevated by at least 4 mV for >500 ms (MacLean et al. 2005).

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- Fig. 1. Local populations of neurons are activated in spontaneous and thalamically evoked circuit reactivations. A: brightfield image of a thalamocortical slice of murine somatosensory “barrel” cortex with extracellular electrode in the ventroposterior medial nucleus (VPm) of the thalamus. Magenta circle indicates cortical field of view (FOV) for multiphoton imaging shown in C. B: whole cell recording of a thalamic neuron (top) responding to four 200-μs extracellular stimuli (bottom) pulses of 10–30 μA, delivered at 40 Hz. Blue inset in C: automated detection of neuronal contours and heuristically optimal path scan. Inset is magnified view of region in blue circle. D, top: multineuronal raster of a spontaneous circuit event in ACSF containing 50 μM acetylcholine (ACh). Spike trains were deconvolved from calcium fluorescence signals. Bottom: simultaneous whole cell recordings of 2 neurons engaged in the local circuit event. E: same neurons and FOV shown in D but without ACh.

Neurons with no detected UP states in either condition were removed from analysis. The firing rate of a neuron was defined as the number of action potentials divided by the duration of UP state depolarization in from current-clamp recordings.

**Imaging.** A 1.1-mm-circular diameter FOV was scanned and each neuronal contour automatically selected. Then a Lin-Kernighan heu-
ristic (LKH) traveling salesman algorithm was used to generate a near-optimal scan path that visits every contour (Fig. 1C; Sadovsky et al. 2011), yielding a scan rate inversely proportional to the number of contours selected. The experiments in this manuscript covered the range of 302–906 neurons loaded with calcium indicator (fura-2 AM) within a single plane FOV, at respective scan rates of 23.1–9.2 Hz. No discrimination was made between excitatory and inhibitory neurons, as both types are permeated by the calcium indicator dye fura-2 AM (Sippy and Yuste 2013). Calcium signals were deconvolved into trains of action potentials with an algorithm modified from Vogelstein et al. (2010). For temporal analyses, spike times were defined relative to the stimulus TTL in evoked experiments. For spontaneous events, onset was defined as the point in time when at least four neurons were active no more than 1.5 s before the peak of population activity. A circuit event was defined as spiking within the population that lasted at least 500 ms (Sadovsky and MacLean 2013).

Imaging analysis. All analyses were performed with custom-written software (MATLAB). Unless stated otherwise, all reported values are means ± SD. The probability that a neuron was active was defined as the fraction of events, or UP states, that at least one action potential was detected in that neuron. A contour, corresponding to a neuron, was included in analysis if at least one action potential was detected in a minimum of 20% of events in either ACh or Control, which facilitated comparisons between data sets. We defined the joint probability that two neurons were coactive as the ratio of the number of circuit events in which two neurons both fired at least one action potential, relative to the total number of events recorded in that data set. To calculate pairwise intertrial correlations of first spike time, each neuron was represented as a vector of the peristimulus time of the first detected action potential during each circuit event. Then, for each neuronal pair, the vectors were confined to the events in which both neurons were active. The vectors were then de-meaned, and the Pearson correlation coefficient was calculated. Both neurons in the pair had to be coactive during at least three events for inclusion.

A Student’s t-test was then used to identify the neurons with a significant change in first spike time at the $P < 0.05$ confidence level after application of ACh. Only neurons that were active in at least three circuit events in both ACh and Control conditions were included for analysis of changes in first spike time (see Fig. 6). To calculate the average peristimulus time histogram (PSTH) across data sets (see Fig. 7, C and D), the spike times of all neurons during each circuit event were converted into seconds and then binned at 500-ms intervals. The resulting discretized distributions were then normalized to the total number of active neurons in the data set, so that each circuit event PSTH was represented as the fraction of neurons active over time. The fraction of neurons active in each 500-ms bin was then averaged across all circuit reactivations. Statistical comparisons between Control and ACh conditions were made by performing a Wilcoxon rank sum test in each bin, with a sample size equal to the number of circuit reactivations across combined data.

Graph theoretic metrics. Functional graphs, represented by adjacency matrices, were generated for each experimental data set from single-frame lag correlation of spiking activity between all neurons (Sadovsky and MacLean 2013). Only neurons that fired at least four spikes in the data set, which itself is comprised of multiple circuit events, were included. Directed edges were drawn from the earlier-spiking neuron to the later-spiking neuron, and edge weight was defined by the relative number of occurrences of that time-lagged relationship. Thus each graph captured temporal relationships between neurons over multiple circuit activations in a data set. Modularity was calculated on binary (unweighted) directed graphs with the function “modularity_dir” implemented in MATLAB based on the method presented in Leicht and Newman (2008). This algorithm quantifies modularity in directed graphs by comparing the fraction of edges within communities and the expected fraction of those edges given a random graph with the same degree sequence (Leicht and Newman 2008). Flow hierarchy was calculated on binary directed graphs with the function “flow_hier” in the NetworkX Python module, which implements the method presented in Luo and Magee (2011). The method counts the fraction of nodes that are not included in any backwards cycles in a directed graph, capturing the extent to which the flow propagates in a unified direction (Luo and Magee 2011).

To test whether the removal of edges by ACh accounted for the observed values of modularity or flow hierarchy, we generated null graphs based on the experimental data. To generate these null graphs, edges were removed from Control graphs in 10 iterative steps until the number of remaining edges in the null graph was equal to the number of edges found in the experimental ACh graph for the corresponding data set. This resulted in null graphs that had the same number of edges as in the ACh condition but were not topologically identical to the ACh graphs. For each data set, 25 nulls were generated and modularity and flow hierarchy were calculated after each iteration of edge removal. By the 10th iteration, there was the same number of edges in the null graph as in the ACh graph from the data set. The purpose of removing edges in iterative steps was to assess whether modularity or flow hierarchy changed as a continuous function of the total number of edges in a graph.

Bidirectional edges. For each graph, we calculated the number of bidirectional edges that would be expected by chance connectivity based on the uniform probability of a connection. Thus the probability of bidirectional connection between two nodes, by chance, is equal to the probability of a unidirectional connection between any two nodes squared. We calculated the probability of a unidirectional edge for each graph by dividing the total number of observed edges by the total number of possible edges between active nodes. Only nodes with at least one incoming or outgoing edge were included, so that the number of expected connections was not biased by a change in total active nodes. For each graph, the number of bidirectional edges observed was normalized to the number of bidirectional edges expected by chance. Thus a ratio >1 reflected more bidirectional edges in the data than expected by chance connectivity.

Temporal stereotypy. A spike train distance metric was used to quantify spike train similarity over multiple events (Kruskal et al. 2013; Victor and Purpura 1996). Briefly, spike trains were aligned, and a cost was assigned to either jitter a spike in time or to add or remove a spike. To establish statistically significant stereotypy, the spike train of a neuron was compared to a reshuffling of the spike train. Reshuffling was performed according to the probability distribution of the population to test against the null hypothesis that the same spike train would result if that neuron was following the activity of the population; significance was established as $P < 0.05$. This analysis was run on ACh and Control trials separately and confined to neurons active at least four circuit events in that condition.

RESULTS

Using high-speed two-photon calcium imaging, we recorded action potential activity of 600 ± 165 neurons that spanned multiple columns and layers in a thalamocortical slice preparation (Agnon and Connors 1991) at scan rates of 15 ± 5 Hz (Fig. 1; Sadovsky et al. 2011). Simultaneously, one or two neurons were recorded using whole cell patch-clamp electrophysiology. Local circuit activation was either evoked by a short train of minimal electrical stimulation applied to thalamocortical neurons (Fig. 1B) or allowed to arise spontaneously, both in the presence (Fig. 1D) and absence (Fig. 1E) of 30–50 μM ACh. This allowed direct comparison of circuit dynamics in the same population of neurons over multiple circuit reactivations, with and without ACh (Fig. 1). This comparison was not possible at very high concentrations of ACh (>1 mM), which silenced spontaneous, but not thalamically evoked, cortical activity. Circuit reactivations are discrete periods of action potential generation within local subsets of neurons (Fig. 1).
Work in acute slice (Kruskal et al. 2013; Sadovsky and MacLean 2013) and in vivo (Harvey et al. 2012; Hoffman and McNaughton 2002; Ji and Wilson 2007; Luczak et al. 2007, 2009) has demonstrated that cortical neurons demonstrate stereotyped temporal structure in spike timing over multiple circuit reactivations. Imaging conducted simultaneously with whole cell patch-clamp recordings demonstrated that multineuronal firing was coincident with sustained depolarization in single cells, termed an UP state (Fig. 1, D and E; Cossart et al. 2003; MacLean et al. 2005; Shu et al. 2003). Consistent with previous reports (Cossart et al. 2003; Sadovsky and MacLean 2013), blockade of synaptic transmission via NMDA (50 μM AP5) and AMPA (20 μM CNQX) receptor antagonists completely halted spontaneous and thalamically stimulated circuit activity in both imaging and single-cell recordings. Thus the UP state was the single-cell substrate of multineuronal circuit activity.

*AC*H alters single-cell activity during circuit events. We characterized the effect of *AC*H on both the intrinsic properties and circuit-dependent activity of individual neurons, using whole cell patch-clamp recordings. *AC*H has been reported to alter the conductances of single neurons, which can vary depending on the *AC*H receptor (AChR) subtype and cellular location (Giocomo and Hasselmo 2007), as well as neuronal cell type (Alitto and Dan 2012; Eggermann and Feldmeyer 2009). Consistent with these reports, we found that neurons could hyperpolarize or depolarize in the presence of *AC*H (Fig. 2D). We also found that neurons could either increase or decrease in firing rate in the presence of *AC*H during both spontaneous and thalamically evoked circuit activations (spontaneous: 11/26 patched neurons increased in firing rate, 15/26 decreased; evoked: 2/14 increased, 11/14 decreased, 1/14 no change). Regardless of the heterogeneity of changes in resting membrane potential and firing rate, there was a consistent and significant reduction in the amplitudes of sustained membrane potential depolarization during both spontaneous (Control = 7.28 ± 1.61 mV, *AC*H = 5.94 ± 1.59 mV, Wilcoxon rank sum *P* = 0.0081, *N* = 24; Fig. 2C) and thalamically evoked (Control = 7.93 ± 1.63 mV, *AC*H = 5.47 ± 1.19 mV, Wilcoxon rank sum *P* = 0.0025, *N* = 11; Fig. 2C) UP states. We found that there was no correlation between changes in resting membrane potential and UP state amplitude (spontaneous: *r* = 0.053, *P* = 0.80; evoked: *r* = 0.34, *P* = 0.28; Fig. 2D; see MATERIALS AND METHODS), indicating that the reduction of UP state amplitude was not the result of the hyperpolarization. Rather, this implied that the global reduction in amplitudes of sustained depolarization produced by *AC*H application could be due to changes in synaptic drive during circuit activity.

To quantify the effect of *AC*H on the reliability of neuronal recruitment into circuit activity, we calculated the fraction of UP states during which a neuron fired at least one action potential. *AC*H significantly decreased the probability that a neuron achieved threshold for action potential generation during spontaneous circuit activity (probability active: Control = 0.56 ± 0.31, *AC*H = 0.26 ± 0.32, Wilcoxon rank sum *P* = 0.0015; Fig. 2E). In contrast, when activity was evoked by thalamic stimulation, there was no significant change (Control = 0.47 ± 0.44, *AC*H = 0.41 ± 0.41, Wilcoxon rank sum, *P* = 0.62; Fig. 2F). *AC*H also decreased the fraction of neurons with detected UP states in electrophysiological recordings during spontaneous events (spontaneous: Control = 21/24 patched neurons, *AC*H = 12/24; evoked: Control = 10/14, *AC*H = 9/14). The decreased likelihood of a neuron being recruited into circuit activity was unique to the spontaneous condition, suggesting that *AC*H decreased the efficacy of intracortical synaptic connectivity but the addition of thalamic drive overcame this impediment.

![Graphs and figures](https://example.com/fig2.png)
change in the likelihood of spiking was uncorrelated with the change in rheobase following application of ACh in single neurons (spontaneous: \( r = -0.10, P = 0.63 \); evoked: \( r = -0.13, P = 0.66 \); Fig. 2G). This demonstrates that the reduced reliability of recruitment into spontaneous circuit reactivation could not be fully accounted for by an increase in the current required to drive a neuron to threshold. These results indicate that cholinergic modulation of intrinsic conductances alone did not fully account for modifications in circuit-dependent activity.

Spontaneous circuit activity is more sparse in the presence of ACh. Next, we used two-photon imaging to determine how ACh-induced changes in single-cell behavior manifested at the local circuit level. We found that the average number of detected action potentials per neuron decreased in the presence of ACh in spontaneous (Fig. 3, A and C) (Control = 0.48 ± 0.61 spikes, ACh = 0.29 ± 0.45, \( N = 4,423 \) neurons, Wilcoxon signed-rank test \( P = 0.0000 \)) but not thalamically evoked (Fig. 3, B and D) (Control = 0.55 ± 0.81, ACh = 0.58 ± 1.00, Wilcoxon signed-rank test \( P = 0.4831 \)) circuit events. Consistent with patch-clamp physiology data, calcium imaging confirmed that there was no significant difference in the average probability that a neuron fired at least once during thalamically evoked circuit activity in the presence of ACh (Control = 0.51 ± 0.14, ACh = 0.45 ± 0.085, Wilcoxon rank sum \( P = 0.44, N = 8 \) data sets). Also consistent with the results of our single-cell physiology, ACh significantly reduced the probability that an imaged neuron was active during spontaneous circuit events (Control = 0.47 ± 0.13, ACh = 0.30 ± 0.098, Wilcoxon rank sum \( P = 0.014, N = 9 \) data sets). This reflected a significant reduction in the number of neurons recruited into spontaneous circuit reactivations in the presence of ACh (spontaneous: Control = 43 ± 18% of neurons ever active per data set (\( N = 80 \) circuit events), ACh = 30 ± 14 (\( N = 77 \)), Student’s \( t \)-test \( P = 9.6 \times 10^{-7} \) (Fig. 3, E and F); evoked: Control = 46 ± 14 (\( N = 59 \)), ACh = 46 ± 14 (\( N = 50 \)), Student’s \( t \)-test \( P = 0.91 \) (Fig. 3, G and H)). Thus ACh resulted in significantly more unreliable active neurons (probability active < 0.25: Control = 26 ± 5% of neurons ever active per data set, ACh = 50 ± 5%, Wilcoxon signed-rank test \( P = 0.0039, N = 9 \) data sets), and fewer reliably active neurons (probability active 0.75–0.99: Control = 16 ± 4% of neurons, ACh = 3 ± 2%, Wilcoxon signed-rank \( P = 0.023 \)), during spontaneous cir-

![Fig. 3](image-url)

Fig. 3. Spontaneous circuit activity is more sparse in the presence of ACh. A: neuronal contours from imaging of multiple spontaneous circuit activations in the same FOV in Control (top) and ACh (bottom). Inset, right: magnified view of boxed segment on left. Color bar indicates the fraction of events in which at least 1 action potential was detected in the neuron. B: same layout as in A but from an experiment in which activity was evoked by thalamic stimulation. C and D: binned probability that a neuron was active, expressed as % of neurons that were ever active in the FOV per experiment. Error bars are means ± SE across spontaneous (C) and evoked (D) data sets, comparing Control and ACh in that bin [Spont., unreliable neurons (probability active < 0.25, \( *P = 0.0034 \)], reliable neurons (probability active 0.75–0.99, \( *P = 0.0042 \)]. E and G: distribution of % of neurons that were active in a circuit event across combined data sets, during spontaneous (E; \( *P = 9.6 \times 10^{-7} \), Student’s \( t \)-test) and evoked (G) events. The number of neurons active in each event was normalized to the total number of neurons ever active in the FOV of the data set. F and H: Neurons were binned on the basis of the probability that they were active in Control (x-axis) vs. ACh (y-axis). Heat map indicates % of neurons that belonged to each probability bin across combined data sets, so that the sum of all bins in the plot is 100%. Color bar is on a log scale. Neurons on the diagonal (black line) did not change in reliability by >0.25 with ACh.

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circuit events (Fig. 3C). We found that the population of neurons that were unreliable in ACh were less reliable in Control. That is, compared with the rest of the neurons in the Control population (probability active = 0.53 ± 0.32, N = 1,934), neurons that were active in <25% of trials in ACh were significantly less reliable in Control (probability active = 0.39 ± 0.25, N = 1,944; Student’s t-test P = 1.3 × 10^{-4}). These data indicate that the neurons that dropped out of spontaneous activity in the presence of ACh were already unreliable in its absence (Fig. 3F). Consistent with this, there was a significant correlation between the probability that a neuron spiked in the presence of ACh and the probability it spiked in Control (spontaneous: r = 0.33, P = 0, Fig. 3F; evoked: r = 0.47, P = 0, Fig. 3H). Thus ACh modulation resulted in the exclusion of unreliable neurons from spontaneous circuit activity. Note that there was conservation of neurons that were active in every event in the presence of ACh (probability active = 1, Control = 9 ± 5% of neurons ever active per data set, ACh = 6 ± 3%, Wilcoxon signed-rank test P = 0.11, N = 9 data sets; Fig. 3, C and F).

**Weak functional connections are pruned by ACh.** To more thoroughly evaluate the effect of ACh on spontaneous cortical activity, we used graph theory as a mathematical framework to compare the topologies of functional wiring diagrams generated from correlated spiking between pairs of neurons (Bullmore and Sporns 2009; Sadovsky and MacLean 2013). Active neurons were represented as nodes in each graph, and directional connections, or edges, between nodes were formed according to lagged correlation of a single imaging frame (frame duration 70 ± 20 ms; Fig. 4; see MATERIALS AND METHODS). Edges were weighted according to the consistency of lagged activity, normalized to the number of events in that FOV. The resulting edge weights represented the reliability of a functional connection, reflecting the statistical dependency of activation between neurons (Pajevic and Plenz 2009; Sadovsky and MacLean 2013). While related to synaptic connectivity between neurons (Bonifazi et al. 2009; Honey et al. 2007; Ko et al. 2011, 2013), these functional connections do not necessarily reflect direct synaptic connections (Gerstein et al. 1978).

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**Fig. 4.** Functional connectivity of spontaneous circuit activations in the presence and absence of ACh. **A:** single data set example of an inferred graph connectivity adjacency matrix in Control (left) and ACh (right) conditions. Directed connections are represented by row (source), column (target) values. The strength of these weighted directed connections is indicated by color in a logarithmic scaling from 0 (no edges) to 1 (always reliable edge). **Insets to right** of each graph are magnification of the area on the graph squared in white. **B:** change observed in the distribution of edge weights for all possible edges between ACh and Control conditions are displayed with 0.05 weight-sized bins on the x-axis and the numerical difference in the number of edges observed in ACh and Control conditions on the y-axis. The dotted red line demarks no change in the number of edges in a bin, and values below it show a lower count in ACh conditions. **C:** single data set illustration of modularity Q value in the same FOV before (left) and after (right) application of ACh. Individual communities are indicated by color; total degree is indicated by node size, relative to each Control or ACh graph independently. Graphs are spatially organized to illustrate distinct modules and do not reflect the distances between neurons in anatomical space. **D:** single data set example of modularity as a function of the number of edges in a graph. Colored lines plot the modularity of null graphs generated from random iterative removal of edges from the Control graph (see MATERIALS AND METHODS). As α increases along the x-axis, the total number of edges in a null graph is reduced. Blue asterisk indicates the modularity observed in the data in Control, and red cross marks modularity in ACh. **E:** cartoon example of flow hierarchy calculation. Flow hierarchy = 8/8 = 1.0; **F:** cartoon example of flow hierarchy calculation. Flow hierarchy = 5/10 = 0.5. **G:** cartoon example of flow hierarchy calculation. Flow hierarchy = 8/8 = 1.0.
Consistent with a decrease in active neurons, ACh reduced the number of nodes (Control = 527 ± 176, ACh = 400 ± 269, Wilcoxon signed-rank test $P = 0.020, N = 9$) and edges (Control = 3,388 ± 2,548, ACh = 1,351 ± 1,329, Wilcoxon signed-rank test $P = 0.0078, N = 9$) in the functional graphs of spontaneous activity. This resulted in a significant reduction in the overall mean functional weights of the inferred graphs, even when zero weights were excluded to account for the difference in the number of active neurons [weights $> 0.1$: Control $= 0.038 ± 0.19$ ($N_{\text{Cu}} = 162,228$ edges), ACh $= 0.018 ± 0.13$ ($N_{\text{ACh}} = 77,848$), Wilcoxon rank sum test $P = 6.6 \times 10^{-97}$]. ACh pruned weak (weight $< 0.30$) functional connections between neurons, while leaving strong connections intact (Fig. 4, A and B). In this way, ACh increased the mean reliability of networks by removing edges that were weak, reflecting large variability in their patterned firing.

ACh renders functional topology more modular and feedforward. We next asked whether the removal of weak functional connections between pairs of neurons impacted the global organization of cortical circuits. By representing each functional connectivity map as a graph of nodes and weighted edges, we were able to quantify and compare the complex dynamics of local circuit activity (Bullmore and Sporns 2009; Sadovsky and MacLean 2013). We first applied a test of directional modularity to the functional connectivity maps (Leicht and Newman 2008) generated from spontaneous circuit activations in the presence and absence of ACh. As previously demonstrated (Sadovsky and MacLean 2013), functional circuits can be divided into discrete, nonshared groups of cells on the basis of maximal within-group and minimal between-group connectivity. Maximum directional modularity, $Q$, is a statistic that quantifies the confinement of these communities. Values near 0 indicate that edges between neurons are random, and values approaching 1 indicate maximum within-community structure. We found that ACh significantly increased the functional network $Q$ value (Control $Q = 0.14 ± 0.03$; ACh $Q = 0.24 ± 0.12$; Wilcoxon signed-rank test $P = 0.0078, N = 9$ data sets), which transformed the weak community structure found in Control conditions into more modular networks. Thus activity propagated through more structured communities of neurons when ACh was present.

Next, we wanted to address whether the removal of weak functional connections by ACh could account for the increase in network modularity that was observed. In a previous study (Sadovsky and MacLean 2013), we showed that reshuffling spike times or randomizing functional topologies reduced values of graph metrics, reflecting a loss of structure in general. This is in contrast to the effect of ACh, which increased graph metric values. To determine whether the removal of edges alone could increase modularity, we generated null graphs by removing edges from the Control graphs. For each data set, we calculated the total number of edges with (ACh) and without (Control) ACh and then iteratively removed edges from the Control graphs until there was the same number of edges as in the ACh graphs (Fig. 4D; see MATERIALS AND METHODS). Modularity was calculated at each iteration of edge removal. In all data sets, modularity increased as the number of edges in the null graphs decreased (Fig. 4D). Edge removal always produced graphs that were more modular than the Control graph (Fig. 4D). This demonstrates that the targeted removal of the edges by ACh from the nonrandom functional graphs increased the modularity of the observed topology.

To further quantify the consequence of the reorganization of functional circuit structure, we used a graph metric of flow hierarchy (Luo and Magee 2011). This measure indicates how activity will propagate through a circuit according to circuit topology. Specifically, it quantifies the relative number of backwards cycles present in a graph, which indicates recurrent activity flow (Fig. 4E). ACh modified the functional graphs, rendering them significantly more hierarchical, and thus feedforward (Control $h = 0.203 ± 0.118$, ACh $h = 0.462 ± 0.324$, Wilcoxon signed-rank test $P = 0.027$), indicating that the reorganization observed in the presence of ACh reduced the number of cycles present in the resulting directed graphs. Together, these analyses demonstrate that ACh modifies the functional organization of neocortical microcircuity, reducing overall network recurrence and promoting the feedforward flow of activity through modular cortical circuits.

Flow hierarchy quantifies the total number of backwards cycles in a directed graph, which captures the emergent flow of activity within the graph as a whole. This can (Fig. 4E, $\alpha$), but does not necessarily have to (Fig. 4E, $\beta$), reflect bidirectional connections between neuronal pairs. To address whether ACh increased flow hierarchy by reducing the number of reciprocal functional connections between pairs, we used a probabilistic ratio to capture changes in the relative portion of bidirectional edges (see MATERIALS AND METHODS). It was necessary to use a relative measure because ACh decreased the total number of edges and nodes. For each graph, we compared the number of bidirectional edges to the number that would be expected by chance, given the total number of nodes and unidirectional edges in that graph. A ratio $> 1$ indicated the presence of more bidirectional connections in the data than would be expected by chance. We found that, in all graphs, there were many more bidirectional edges than expected by chance (ratio = 3.53 ± 1.38, $N = 18$ graphs) and that ACh did not significantly change this ratio (Control = 3.35 ± 0.89 ($N = 9$ graphs), ACh = 3.71 ± 1.78 ($N = 9$); Wilcoxon signed-rank $P = 0.57$). This analysis indicated that the increase in flow hierarchy observed in the presence of ACh was not accounted for by a significant decrease in bidirectional edges (e.g., Fig. 4E, $\alpha$) but rather by changes in activity flow at a larger scale, such as illustrated in Fig. 4E, $\beta$. As was done with modularity, we generated null models for flow hierarchy by removing edges from Control graphs. In contrast to modularity, edge removal had variable effects on the values of flow hierarchy. In five of eight data sets edge removal decreased flow hierarchy, making the graphs less feedforward, which is opposite of the effect of ACh. In three of eight data sets edge removal increased flow hierarchy, but not to the extent that ACh did. Thus the enhanced flow hierarchy that we found after application of ACh could not be accounted for by a reduction in reciprocal connections between pairs or the removal of edges.

ACh reduces pairwise dependencies. After establishing that ACh modified the functional organization of cortical microcircuits, we set out to evaluate how these changes impacted the cortical representation of thalamic afferent input. In vivo studies have identified reductions in pairwise activity correlations with attention (Cohen and Maunsell 2009; Mitchell et al. 2009) and ACh release into the cortex (Goard and Dan 2009; Pinto et al. 2013). Multiple mechanisms can affect the firing rate
correlation between neuronal pairs. Firing rate decorrelation does not necessarily occur through a reduction of shared input but can also result from greater negative correlations due to fast inhibitory feedback or reduced firing rates (Graupner and Reyes 2013; Renart et al. 2010; de la Rocha et al. 2007). Using the thalamocortical slice preparation, we stimulated thalamocortical neurons (Fig. 1B) and characterized the resulting cortical activity. We calculated the trial-to-trial correlation of first spike times between reliably coactive pairs (see MATERIALS AND METHODS) and found that ACh decreased the average correlation in peristimulus first spike times [Control = 0.24 ± 0.57 (N = 154,238 pairs), ACh = 0.12 ± 0.57, Wilcoxon rank sum P = 0 (N = 126,769 pairs)]. When calculating intertrial dependencies, we found that ACh reduced the number of neuronal pairs that were reliably coactive during the same circuit event. Although ACh did not significantly change the number of neurons active in response to thalamic input (Fig. 3G), it reduced the joint probability that two neurons both fired action potentials during the same circuit event (Control = 0.45 ± 0.26, ACh = 0.37 ± 0.26, N = 203,796 pairs, Student’s t-test P = 0; see MATERIALS AND METHODS). These data indicate that, on average, ACh reduced statistical dependencies between neuronal pairs.

To see how ACh changed shared variance between individual pairs of neurons, we confined our analysis to the population of neuronal pairs that were reliably coactive after thalamic stimulation in both ACh and Control and then compared the Pearson correlation coefficient of the pairs with and without ACh (N = 64,869 pairs; Fig. 5). This revealed that neuronal pairs that were positively correlated in Control (r = 0.54 ± 0.30) were significantly less correlated in the presence of ACh (r = 0.13 ± 0.54; Wilcoxon signed-rank test P = 0, N = 44,628 pairs). Pairs that were negatively correlated in Control (r = −0.38 ± 0.28) had correlation values closer to 0 in the presence of ACh (r = −0.10 ± 0.55, Wilcoxon signed-rank test P = 0, N = 20,215 pairs). Despite this overall shift toward zero correlations with the addition of ACh, a subset of large positive (r > 0.8, Control = 17.8% of pairs, ACh = 13.5%) and negative (r < −0.8, Control = 3.5% of pairs, ACh = 8.7%) correlations were maintained (Fig. 5); 24.3% of the neurons with large positive correlation coefficients (r > 0.8) with ACh also had large values in Control. By decreasing moderate correlations, ACh caused the population to become more bimodal, with a subset of neurons displaying strong dependencies, in contrast to the remainder of the more independent local population.

ACh modifies temporal recruitment of neurons. We next evaluated the effect of ACh on spike timing in single neurons. To do so we compared the peristimulus first spike time of neurons before and after ACh application (Fig. 6). Even though a subset of neurons always fired within a frame after thalamic stimulation, activity was sustained cortically for several seconds within the imaged area that spanned multiple cortical columns and layers. To characterize changes in first spike time, we confined our analysis to the intersection of neurons reliably active in response to thalamic stimulation in both ACh and Control. We found a significant change (P < 0.05, see MATERIALS AND METHODS). In Control, 17.8% of neurons fires sooner with ACh application, a subset with an average change in first spike time of 0.03 s (Fig. 6A). In ACh, 13.5% of neurons fires later with ACh application, a subset with an average change in first spike time of 0.05 s (Fig. 6B). The ACh-induced change in average first spike time is significant (Fig. 6C).
AND METHODS) in the first spike times in a subset of neurons that were reliably active in both conditions (96/680 = 14% of neurons; Fig. 6). The majority of these neurons had a delay in first spike time in the presence of ACh (71/96 = 74% of the subset with significant change; Fig. 6C). We also found that the mean spike time within the entire active population was significantly later in the presence of ACh [Control = 2.4 ± 1.3 s (N = 19,496 neurons), ACh = 2.9 ± 1.3 s (N = 20,318 neurons), Student’s t-test P = 0.05; Fig. 7C]. To determine how the shift in spike times of individual neurons manifested within the active circuit, we analyzed the PSTHs of thalamically evoked events (Fig. 7). Specifically, we binned the peristimulus spike times of all neurons active during each circuit event and normalized to the total number of neurons in the FOV (see MATERIALS AND METHODS). We then calculated the average fraction of neurons active in each 500-ms bin and compared these values in Control versus ACh. Compared with Control, significantly more neurons were active 4–6 s after stimulus with ACh (Wilcoxon rank sum P < 0.05, N_{Ctl} = 59 circuit events, N_{ACh} = 50; Fig. 7C). Thus on average ACh resulted in the delayed recruitment of spiking neurons relative to thalamic stimulation in the imaged neuronal population.

Cortical neurons have been shown to demonstrate conserved temporal structure in spike timing (Kruskal et al. 2013; Luczak et al. 2007; Sadovsky and MacLean 2013) during both spontaneous and thalamically evoked circuit activations (MacLean et al. 2005; Luczak et al. 2009). We next evaluated whether the modifications in functional organization induced by ACh influenced temporal stereotypy in response to thalamic input. To do so, we used a spike distance metric to identify neurons significantly stereotyped across multiple circuit activations (Kruskal et al. 2013; Sadovsky and MacLean 2013; Victor and Purpura 1996; see MATERIALS AND METHODS). This metric established statistical significance by comparing experimentally measured spike times to spike times resampled from an inhomogeneous Poisson distribution that was defined by the population firing rate. Similar to Control, almost one-third of reliably active neurons were significantly stereotyped with ACh (Control = 27 ± 7%, ACh = 29 ± 18%, Wilcoxon rank sum P = 0.8182, N = 8 experiments). However, with ACh stereotyped activity was more evenly distributed over time, so that a larger fraction of stereotyped neurons were active with ACh in the epoch 2–5.5 s after stimulus (Wilcoxon rank sum P < 0.05 in each 500-ms time bin 2–5.5 s after stimulus, N_{Ctl} = 59 circuit events, N_{ACh} = 50; Fig. 7D). This occurred despite the fact that, in ACh, fewer neurons were active 0.5–1 s after thalamic stimulation (Wilcoxon rank sum P < 0.05; Fig. 7D). Thus the presence
of ACh prolonged the time window during which temporally stereotyped activity propagated through local circuits.

**DISCUSSION**

Sensory processing is influenced by the interaction between incoming stimuli and the internal state of the cortex (Buonomano and Maass 2009). We found that the presence of ACh modified the functional organization of cortical microcircuits, and the response to thalamic stimulation, in a manner that could enhance the discriminability of thalamic afferent input within local neocortical circuitry. ACh made spontaneous intracortical activity more sparse, excluding weak functional connections and unreliable neurons and rendering functional circuits more modular and predisposed to feedforward relay of information. Strong correlations were maintained within a subset of neurons, but a single neuron’s activity was overall more independent from the majority of the population. Stereotyped activity evoked by thalamic drive was sustained within these modified circuits for a prolonged period of time. Together, these findings indicate that the pruning of weak correlations by ACh could permit a more stable sustained representation of sensory information, potentially increasing the time window for the integration of inputs across cortical columns. We also found that changes in intrinsic properties following the application of ACh, such as resting membrane potential and rheobase, were not correlated with changes in circuit-dependent activity in patched neurons, such as UP state depolarization amplitude and the probability of spiking. Rather, ACh reorganized functional circuitry that was observed in the Control condition, suggesting that the dominant holistic action of ACh is to modulate existing local microcircuitry.

The results of our study bridge observations made at various spatial levels of investigation. Single-neuron studies in slice have demonstrated that cholinergic receptor agonism selectively reduces excitatory postsynaptic potentials (EPSPs) evoked by intracortical (Gil et al. 1997) or intrinsic fiber (Hasselmo and Bower 1992) stimulation but does not abate EPSPs evoked by thalamic (Gil et al. 1997) or afferent fiber (Hasselmo and Bower 1992) stimulation. This source-dependent modulation offers one mechanism through which ACh could increase the SNR of sensory input in primary sensory cortex (Oldford and Castro-Alamons 2003). In vivo studies in visual area V4 have shown that reduction in ongoing, low-frequency (Mitchell et al. 2009) or noise (Cohen and Maunsell 2009) correlations is the dominant contributor to the enhanced SNR of sensory input seen with attentional modulation. By combining whole cell recordings with simultaneous calcium imaging in a thalamocortical slice, we were able to isolate the source of activity and show that tonic presence of ACh dampens spontaneous activity that arises naturally from within the cortex, while having no effect on the number of neurons recruited by thalamic input. This observation helps to bridge prior in vitro single-cell studies of ACh to in vivo studies of attention and demonstrates that the tonic presence of ACh is capable of selectively sparsifying stimulus-independent intercortical activity.

By recording from large populations of neurons, we were also able to address cholinergic modulation of large-scale dynamics of intercortical activity in cortical circuitry. We found that weak functional connections, which reflect positive fixed-lagged correlations of a single imaging frame (50–100 ms), were eliminated and strong connections remained intact. Using graph theoretic metrics, we found that the removal of weak functional connections, or edges, by ACh yielded a more modular circuit structure. This isolation of activity to smaller communities of neurons could potentially increase SNR by reducing interference from global activity, generating a more stable cortical representation. When ACh was present, activity also propagated in a more feedforward direction, which could increase SNR by reducing feedback noise. This phenomenon was observed at the global circuit level and could not be accounted for by loss of edges or a reduction in reciprocal functional connections between neuronal pairs. This illustrates that there were changes in emergent circuit dynamics induced by ACh that could not be captured by analysis of pairwise relationships alone.

Depending on which cholinergic receptor(s) a neuron expresses, the endogenous release of ACh can alter conductance to sodium, potassium, and/or calcium ions at a range of different timescales of activation and desensitization. For example, nicotinic AChRs (nAChRs) directly conduct ions and desensitize quickly (Barrantes 1978), while muscarinic AChRs (mAChRs) desensitize slowly and indirectly alter channel conductances via G protein-coupled cascades (Gigout et al. 2012). Bath application of ACh does not reflect the rapid timescale of basal forebrain activation or active control of attention in vivo. As a result, we are unable to evaluate the differential contributions of specific receptor subtypes or the spatial-temporal profile of cholinergic receptor activation. Rather, our experiments were designed to address the holistic effect of tonic ACh on local circuit dynamics to better understand the relationship between neuromodulation, functional connectivity, and information processing. The time course of calcium imaging also places an upper bound on the temporal resolution over which neuronal activity can be studied. Thus our analyses are customized to quantify the relative timing and reliability of action potentials within large and densely sampled cortical populations, for which two-photon calcium imaging is well suited.

The large spatial scale and periods of quiescence provided by slice preparation allow us to image thalamically evoked activity as it propagates from the primary recipient column across multiple cortical columns and layers. We found that ACh reduced intertrial correlations in peristimulus first spike times; these correlations capture shared trial-to-trial variability in the temporal recruitment of neurons. Imaging somatic calcium transients at 15 ± 5 Hz, the correlation coefficients we observe cannot be directly compared in value to firing rate correlations from electrophysiological studies, although decorrelation in general has been reported with cholinergic receptor agonism (Goard and Dan 2009) and attention (Cohen and Maunsell 2009; Mitchell et al. 2009). Similar to the loss of weak functional connections, we see that weak positive intertrial correlations are removed by ACh, which could increase the information coding capacity of local circuits by reducing redundancies and providing more orthogonal dimensions to encode information. We additionally found that the relative enhancement of a thalamic input by ACh can be sustained cortically and does not necessarily require continuous thalamic drive. On the contrary, we observed fewer neurons active immediately after thalamic stimulation in the presence of ACh relative to the control and more neurons active 2–5 s after...
stimulus. This demonstrates that a consequence of reorganization within cortical circuitry is the more even distribution of temporally precise activity evoked by thalamic drive. As a substrate for working memory, computational models of recurrent networks have shown that information can be maintained in persistent neocortical activity (Hopfield 1982; Lim and Goldman 2013). By prolonging temporally precise activity, as we demonstrate here, ACh could increase the time window in which information can be integrated from multiple thalamic sources.

The neurochemical environment within the cortex regulates how inputs are translated and transmitted through cortical circuitry. In this way, the synaptic structure serves as a dynamic substrate for information processing, where both the neuromodulatory environment and glutamatergic drive shape cortical activity (Buonomano and Maass 2009; Harris and Thiele 2011). We show that systematic changes in functional circuitry occur in response to the neuromodulator ACh, which offers insight into the relationship between circuit wiring and information processing in the brain. We hypothesize that ACh's ability to parse information into insular representations, with less interference from global activity, could contribute to the increased discriminability of sensory stimuli reported with ACh (Board and Dan 2009) and attention (Cohen and Maunsell 2009; Mitchell et al. 2009).

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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