Abstract

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 2-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.

Introduction

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-Alamconcos, 2003). Concurrently, neuronal populations become desynchronized and decorrelated (Goard and Dan, 2009; Pinto et al., 2013), which has also been attributed to attentional modulation (Mitchell et al., 2009; Cohen and Maunsell, 2009; Bauer et al., 2012; 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 (Hasselmo and Bower, 1992; Gil et al., 1997). 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 (Thiele et al., 2011) and coding capacity (Linster 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 2-photon calcium imaging to simultaneously record action potential activity of up to 900 neurons, in a field of view 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 ketamine (100 mg/kg)-xylazine (5 mg/kg). Brains were rapidly removed and sliced with a vibratome (VT1000S) in 0-4 degree high sucrose artificial cerebral spinal fluid (ACSF) (mM: CaCl₂ 0.5, MgSO₄ 3.5, KCl 3, NaHCO₃ 26, NaH₂PO₄ 1, Sucrose 205, Dextrose 25). 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-2AM (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-2AM, dissolved in a 13 μL of DMSO and 2 μL of Pluronic F-127. Fura-2AM 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, 10 Dextrose, aerated with 95% O₂ and 5% CO₂. Because the two ionic variants resulted in only 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 acetylcholine (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 the 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 minutes of ACh presence and washout was complete after 90 minutes. 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 was combined.

Experiments compared the same field of view (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 200μs pulses with a minimal amplitude of 10-30μA was delivered at 40Hz (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 SM, 2001) and these stimulation amplitudes minimized antidromic activation (Beierlein and Connors, 2002).

**Electrophysiology**

Whole cell current clamp recordings were made using 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 brightfield image along with morphology of surrounding neurons. Passive properties of neurons were assessed using 500ms hyperpolarizing and depolarizing DC current steps. All analyses were performed using 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 3 trails were excluded from analysis. UPstates (circuit events) were automatically detected as periods when membrane potential was elevated by at least 4mV for more than 500ms (MacLean et al., 2005). Neurons with no detected UPstates 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 UPstate depolarization in from current-clamp recordings.

Imaging

A 1.1mm circular diameter field of view (FOV) was scanned and each neuronal contour automatically selected. Then, a Lin-Kernighan heuristic (LKH) traveling salesman algorithm was used to generate a near optimal scan path that visits every contour (Fig. 1C; Sadovskey 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-2AM) within a single plane field of view, 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-2AM (Sippy and Yuste, 2013). Calcium signals were deconvolved into trains of action potentials using 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 4 neurons were active no more than 1.5 sec before the peak of population activity. A circuit event was defined as spiking within the population that lasted at least 500ms (Sadovsky and MacLean, 2013).

**Imaging Analysis**

All analyses were performed using custom written software (MATLAB). Unless stated otherwise, all reported values are mean ± standard deviation. The probability that a neuron was active was defined as the fraction of events, or UPstates, 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 datasets. We defined the joint probability that two neurons were co-active as the ratio of the number of circuit events that two neurons both fired at least one action potential, relative to the total number of events recorded in that dataset. To calculate pairwise intertrial correlations of first spike time, each neuron was represented as a vector of the peri-stimulus time of the first detected action potential during each circuit event. Then, for each neuronal pair, the vectors were confined to the events wherein 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 co-active 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, following 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 (Fig. 6). To calculate the average peri-stimulus time histogram (PSTH) across datasets (see Fig. 7 C, D), the spike times of all neurons during each circuit event were converted into seconds and then binned at 500ms intervals. The resulting discretized distributions were then normalized to the total number of active neurons in the dataset, so that each circuit event PSTH was represented as the fraction of neurons active over time. The fraction of neurons active in each 500ms 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 dataset from single frame lag correlation of spiking activity between all neurons (Sadovsky and MacLean, 2013). Only neurons that fired at least 4 spikes in the dataset, which itself is comprised of multiple circuit events, were included. A directed edge was drawn from the earlier spiking neuron to the latter 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 dataset. Modularity was calculated on binary (unweighted) directed graphs using 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.
Flow hierarchy was calculated on binary directed graphs using the function “flow_hierarchy” in the NetworkX Python module, which implements the method presented in Luo and Magee, 2011. The method quantifies 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 dataset. 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 dataset, 25 nulls were generated and modularity and flow hierarchy were calculated after each iteration of edge removal. By the tenth iteration, there was the same number of edges in the null graph as in the ACh graph from the dataset. 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.

**Bi-directional edges**

For each graph, we calculated the number of bi-directional edges that would be expected by chance connectivity based on the uniform probability of a connection. Thus, the probability of a bi-directional 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 uni-
directional 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 bi-directional edges observed was normalized to the number of bi-directional edges expected by chance. Thus, a ratio greater than one reflected more bi-directional 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 (Kurstal 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 2-photon calcium imaging, we recorded action potential activity of 600±165 neurons that spanned multiple columns and layers in a thalamocortical slice preparation (Agmon and Connors, 1991), at scan rates of 15±5Hz (Fig. 1; Sadovsky et al. 2011). Simultaneously, one or two neurons were recorded from 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 30-50uM 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 (>1mM), 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 (Luczak et al., 2007; Luczak et al., 2009; Ji and Wilson 2007; Hoffman and McNaughton 2002; Harvey et al., 2012) 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 UPstate (Fig. 1D,E; Shu et al., 2003; Cossart et al. 2003, MacLean et al. 2005). Consistent with previous reports (Cossart et al., 2003; Sadovsky and MacLean 2013), blockade of synaptic transmission via NMDA (50μM AP5) and AMPA (20μM CNQX), receptor antagonists completely halted spontaneous and thalamically stimulated circuit activity in both imaging and single cell recordings. Thus, the UPstate was the single cell substrate of multineuronal circuit activity.

ACh alters single cell activity during circuit events.

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

To quantify the effect of ACh on the reliability of neuronal recruitment into circuit activity, we calculated the fraction of UPstates during which a neuron fired at least one action potential. ACh decreased the probability that a neuron achieved threshold for action potential generation during spontaneous circuit activity (probability active, Control = 0.56± 0.31, ACh = 0.26 ± 0.32, Wilcoxon rank sum p = 0.0015; 2E). In contrast, when activity was evoked by thalamic stimulation, there was no significant change (Control = 0.47 ± -0.44, ACh = 0.41 ± 0.41,
Wilcoxon rank sum, p = 0.62; Fig. 2F). ACh also decreased the fraction of neurons with detected 
UPstates in electrophysiological recordings during spontaneous events (Spont., Control = 21/24 
patched neurons, ACh = 12/24; Evoked, Control = 10/14, ACh = 9/14). The decreased likelihood 
of a neuron being recruited into circuit activity was unique to the spontaneous condition, 
suggesting that ACh decreased the efficacy of intracortical synaptic connectivity, but the 
addition of thalamic drive overcame this impediment. The change in the likelihood of spiking 
was uncorrelated with the change in rheobase following application of ACh in single neurons 
(Spont., 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 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 2-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 (Control = 0.48 ± 0.61 
spikes, ACh = 0.29 ± 0.45, n = 4423 neurons, Wilcoxon signed rank test p = 0.0000), but not 
thalamically evoked (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 datasets). 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 datasets). This reflected a significant reduction in the number of neurons recruited into spontaneous circuit reactivations in the presence of ACh (Spont., Control = 43 ± 18% of neurons ever active per dataset, N = 80 circuit events, ACh = 30 ± 14, N = 77, Student’s t-test p = 9.6x10^{-7}, Fig. 3E; Evoked, Control = 46 ± 14, N = 59, ACh = 46 ± 14, N = 50, Student’s t-test p = 0.91; Fig. 3H). Thus, ACh resulted in significantly more unreliably active neurons (probability active < 0.25, Control=26±5 % of neurons ever active per dataset, ACh = 50±5%, Wilcoxon signed rank test p=0.0039, N=9 datasets), and fewer reliably active neurons (probability active 0.75-0.99, Control=16±4 percent of neurons, ACh=3±2%, Wilcoxon sign rank p=0.023), following application of ACh (Fig. 3C). We found that the population of neurons that were unreliable in ACh were less reliable in control. That is, compared to the rest of the neurons in the Control population (probability active = 0.53± 0.32, N = 1934), neurons that were active in less than 25% of trials in ACh were significantly less reliable in Control (prob. active =0.39±0.25, N = 1944; Student’s t-test p = 1.3x10^{-47}). 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 (Spont, r=0.33, p=0, Fig. 3F; Evoked, r=0.47, p=0, Fig. 3H). Thus, cholinergic 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 dataset, ACh = 6 ± 3%, Wilcoxon signed rank test p = 0.11, N = 9 datasets Fig. 3F,C).
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 ± 20ms; Fig. 4; see Methods). Edges were weighted according to the consistency of lagged activity, normalized to the number of events in that field of view. 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 (Ko et al. 2011; Ko et al., 2013, Honey et al., 2007; Bonifazi et al., 2009), these functional connections do not necessarily reflect direct synaptic connections (Gerstein, 1978). 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 =3388 ± 2548, ACh = 1351 ± 1329, 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 active neurons (weights>0.1; Control = 0.038 ± 0.19, NCtl = 162,228 edges, ACh = 0.018±0.13, NACH = 77,848, Wilcoxon Ranksum p = 6.6x10^{-97}). ACh pruned weak (weight<0.30) functional connections between neurons, while leaving strong connections intact (Fig. 4 A, 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 et al., 2013), functional circuits can be divided into discrete, non-shared 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, $Q = 0.14 \pm 0.03$; ACh $Q = 0.24 \pm 0.12$; Wilcoxon signed rank test $p = 0.0078$, $N = 9$ datasets), 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 manuscript
Sadovsky and MacLean, 2013), we have shown 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 dataset, we calculated the total number of edges with (ACh) and without (Control) acetylcholine, and then iteratively removed edges from the Control binary adjacency matrices until there was the same number of edges as in the ACh graphs (Fig. 4D; see Methods). Modularity was calculated at each iteration of edge removal. In all datasets, 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 non-random 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 backward 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 $h = 0.203 \pm 0.118$, Ach $h = 0.462 \pm 0.324$, 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 microcircuitry, 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. 4Eα), but does not necessarily have to (Fig. 4Eβ), reflect bi-directional 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 bi-directional edges (see 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 bi-directional edges to the number that would be expected by chance, given the total number of nodes and uni-directional edges in that graph. A ratio greater than one indicated the presence of more bi-directional connections in the data than would be expected by chance. We found that, in all graphs, there were many more bi-directional 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 bi-directional edges (e.g., Fig. 4Eα), but rather from changes in activity flow at a larger scale, such as illustrated in Fig. 4Eβ. 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 5 of 8 datasets, edge removal decreased flow hierarchy, making the graphs less feedforward, which is the opposite of the effect of ACh. In 3 of 8 datasets, edge removal increased flow hierarchy, but not
to the extent that ACh did. Thus, the enhanced flow hierarchy that we found following
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 (de la Rocha et al., 2007; Renart et al., 2010; Graupner and Reyes, 2013).

Using the thalamocortical slice preparation, we stimulated thalmo-cortical neurons (Fig. 41B)
and characterized the resulting cortical activity. We calculated the trial-to-trial correlation of first
spike times between reliably co-active pairs (see Methods) and found that ACh decreased the
average correlation in peri-stimulus 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 co-
active 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 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 to neuronal pairs that were reliably co-active following 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. 5B). This revealed that neuronal pairs that were positively correlated in the 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 zero in the presence of ACh (r = -0.10 ± 0.55, Wilcoxon signed rank test p = 0, N = 20,215 pairs). Despite this overall shift towards 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. 5A); 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 peri-stimulus 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 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 sec, N
= 19,496 neurons, ACh = 2.9 ± 1.3, N = 20,318, Student's t-test p = 0). To determine how the
shift in spike times of individual neurons manifested within the active circuit, we analyzed the
peri-stimulus time histograms (PSTH) of thalamically evoked events (Fig. 7). Specifically, we
binned the peri-stimulus spike times of all neurons active during each circuit event and
normalized to the total number of neurons in the field of view (see methods). We then calculated
the average fraction of neurons active in each 500ms bin and compared these values in Control
versus ACh. Compared to Control, significantly more neurons were active 4-6 seconds post
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
(Luczak et al., 2007; Kruskal et al., 2013; 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 Papura, 1997; see 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 seconds
post-stimulus (Wilcoxon rank sum p<0.05 in each 500ms time bin 2-5.5sec post-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 second following 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 Maas, 2009). We found that the presence of acetylcholine
(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, this
indicates 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 UPstate 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 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 signal-to-noise ratio (SNR) of sensory input
in primary sensory cortex (Oldford and Castro-Alamonsos, 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 thalamo-cortical 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 (50-100ms), 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 ACh receptors (nAChs)
directly conduct ions and desensitize quickly (Barrantes, 1978), while muscarinic ACh receptors (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 \textit{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 2-photon calcium imaging is well suited.

The large spatial scale and periods of quiescence provided by slice preparation allows 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 peri-stimulus first spike times; these correlations capture shared trial-trial variability in the temporal recruitment of neurons. Imaging somatic calcium transients at 15±5Hz, 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 (Mitchell et al., 2009; Cohen and Maunsell, 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 seconds post-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 wherein 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 acetylcholine, 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 (Goard and Dan, 2009) and attention (Mitchell et al., 2009; Cohen and Maunsell, 2009).
References


Luczak A, Maclean JN. Default activity patterns at the neocortical microcircuit level. Front Integr Neurosci. 6:30. 2012


**Figure Legends:**

**Figure 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 box indicates cortical field of view for multiphoton imaging.
shown in C; Orange circle corresponds to thalamic FOV shown in B. B, whole cell recording of
a thalamic neuron (above) responding to four 200ms extracellular stimuli (below) pulses of 10-
30μA, delivered at 40Hz. Blue inset C, Automated detection of neuronal contours and
Heuristically Optimal scan path. Insert is magnified view of region in blue circle D, Top,
Multineuronal raster of a spontaneous circuit event in ACSF containing 50μM of acetylcholine
(ACh). Spike trains were deconvolved from calcium fluorescence signals. D (bottom)
Simultaneous whole cell recordings of two neurons engaged in the local circuit event. E, Same
neurons and FOV shown in D, but without ACh.

**Figure 2.** ACh alters circuit-dependent activity of single neurons in a source dependent
manner. A, Whole cell voltage clamp recording of a spontaneous circuit event in regular
ACSF; inset, magnified view. B, the same neuron in A engaged in spontaneous activity in the
presence of 50μM bath applied ACh. C, mean UP state amplitude is reduced by ACh in both
spontaneous (*p = 0.0081) and thalamically evoked events (*p = 0.0025). D, UPstate amplitude
was not correlated with the change in resting membrane potential of neurons (Spont., r=0.053,
p=0.80; Evoked, r=0.34, p=0.28). E, ACh reduced the average probability that a neuron fired an
action potential during a spontaneous UPstate (*p=0.0015), but not when activity was
thalamically evoked (F; *p=0.62). G, The ACh-induced change in the probability of spiking
during an UPstate was not correlated with the ACh-induced change in rheobase (Spont., r=-
0.10, p=0.63; Evoked, r=-0.13, =0.66).
Figure 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 field of view in Control (top) and ACh (bottom); inset (right), magnified view of boxed segment to the left. Color bar indicates the fraction of events at least one action potential was detected in the neuron. B, Same layout as in A, but from an experiment where activity was evoked by thalamic stimulation. C, D, Binned probability that a neuron was active, expressed as the percent of neurons that were ever active in the field of view per experiment. Errorbars are mean ±SEM across spontaneous (C) and evoked (D) datasets, 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,G, Distribution of the percent of neurons that were active in a circuit event across combined datasets, during spontaneous (E) and evoked (G) events. The number of neurons active in each event was normalized to the total number of neurons ever active in the field of view of the dataset. F, H, Neurons were binned based on the probability they were active in Control (x-axis) versus ACh (y-axis). Heatmap indicates the percent of neurons that belonged to each probability bin across combined datasets, so that the sum of all bins in the plot is 100 percent. Colorbar is on a log scale. Neurons on the diagonal (black line) did not change in reliability by more than 0.25 with ACh.

Figure 4. Functional connectivity of spontaneous circuit activations in the presence and absence of ACh. A, Single dataset 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 the 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 dataset illustration of modularity $Q$ value in the same field of view before (left) and after (right) application of ACh. Individual communities are indicated by color; total degree is indicated by node size, relative to each independent control/ACh case. Graphs are spatially organized to illustrate distinct modules and do not reflect the distances between neurons in anatomical space. D, Single dataset 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 Methods). As $\sigma$ 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. When $\sigma = 1$, the number of edges in the null graphs equaled the number of edges in the ACh graph. E, Cartoon example of flow hierarchy calculation. The bottom illustrates a network with a hierarchy degree of 1, indicating complete feedforward flow. The top illustrates what happens when cycles (red edges) are introduced to the graph. A backwards cycle can consist of a bi-directional edge (\(\alpha\)), but does not have to (\(\beta\)). F, Single dataset example of binary directed graph matrices with (ACh, left) and without (Control, left) acetylcholine. Bi-directional edges were colored red for illustration.
**Figure 5. ACh reduces statistical dependencies between neuronal pairs.** A, Neuronal pairs were binned based on the trial-to-trial correlation of peri-stimulus first spike times in Control (y-axis) versus ACh (y-axis). Heat map indicates the percent of neurons that belonged to each probability bin across combined datasets. Data was confined to the intersection of pairs reliably co-active in both ACh and Control. Color bar is on a log scale. Neurons on the diagonal (black line) did not change in Pearson correlation coefficients by more than 0.25 with ACh. B, Distribution of Pearson correlation coefficients from data in A; *p=0, Wilcoxon signed rank test.

**Figure 6. ACh restructures temporal recruitment of a subset of neurons.** A, Peri-stimulus raster of a single neuron that did not change in first spike time with the presence of ACh, *p=0.90. Raster is Gaussian-convolved in 100 ms bins for illustration. B, Another neuron from the same FOV that was recruited significantly later presence of ACh, *p=0.0040. C, Histogram from combined datasets showing the average change in first spike time of each neuron that changed significantly at the 0.05 confidence level, with ACh application.

**Figure 7. Stereotyped activity is extended in time in the presence of ACh.** A, (Top) Population peristimulus time histogram (PSTH) of a thalamically evoked circuit activation in Control (light blue) and ACh (purple) from an example dataset. Dashed box indicates segment shown in B. A, (Bottom) Spike train rasters used to generate the PSTH above. B, Spatial-temporal projection of boxed segment in A (Control, Top; ACh, Bottom). Each panel encompasses four imaging frames. Heat map indicates the peri-stimulus frame that the neuron fired. If a neuron fired more than once in a single panel, the neuron was colored according to
only the first spike. C, Average PSTH across all evoked datasets. Each circuit event was normalized to the total number of neurons ever active in that dataset; (*) denotes significantly more neurons active in ACh at 0.05 confidence level. D, Average PSTH of only significantly stereotyped neurons (see Methods); (+) indicates significantly less neurons active in ACh at 0.05 confidence level.
A Control Adjacency Matrix

B ACh Adjacency Matrix

C Control Modularity Graph
Modularity = 0.1593

D ACh Modularity Graph
Modularity = 0.4119

E Flow hierarchy = 5/10 = 0.5
Flow hierarchy = 8/8 = 1.0

F Control

G ACh
Fires Sooner with ACh

ACh - Neuron #543

Fires Later with ACh

ACh - Neuron #171

Control - Neuron #543

Control - Neuron #171

Number of Neurons with Significant Change

Change in Average First Spike Time (seconds)