Differential effects of cholinergic and noradrenergic neuromodulation on spontaneous cortical network dynamics

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Abstract

Cholinergic and noradrenergic neuromodulation play a key role in determining overall behavioral state by shaping the underlying cortical network dynamics. The effects of these systems on synaptic and intrinsic cellular targets are quite diverse and a comprehensive understanding of how these neuromodulators regulate (spontaneous) cortical network activity has remained elusive. Here, we used multielectrode electrophysiology in vitro to investigate the effect of these neuromodulators on spontaneous network dynamics in acute slices of mouse visual cortex. We found that application of Carbachol (CCh) and Norepinephrine (NE) both enhanced the spontaneous network dynamics by increasing (1) the activity levels, (2) the temporal complexity of the network activity, and (3) the spatial complexity by decorrelating the network activity over a wide range of neuromodulator concentrations (1 μM, 10 μM, 50 μM, and 100 μM). Interestingly, we found that cholinergic neuromodulation was limited to the presence of CCh in the bath whereas the effects of NE, in particular for higher concentrations, induced plasticity that caused outlasting effects most prominently in the deep cortical layers. Together, these results provide a comprehensive network-level understanding of the similarities and differences of cholinergic and noradrenergic modulation of spontaneous network dynamics.

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1. Introduction

Cortical networks exhibit a broad range of different activity states that range from slow, rhythmic discharges during slow-wave sleep to fully desynchronized activity patterns during periods of behavioral arousal and focused attention (Steriade and Amzica, 1998; Steriade et al., 2001). The neuromodulators acetylcholine (ACh) and norepinephrine (NE) play a key role in controlling this activation which leads to desynchronization of cortical network dynamics (Armitage et al., 1969; Celesa and Jasper; 1966; Constantinople and Bruno, 2011; Jouvet, 1969; Kalmbach et al., 2012). However, phasic release of these neuromodulators also occurs in the awake state in response to different behavioral triggers. ACh appears to be tightly linked to attentional processing and learning whereas NE is released under circumstances of substantial changes in the environment that need overall behavioral adjustment (Baxter and Chiba, 1999; Dalley et al., 2001; Vankov et al., 1995; Yu and Dayan, 2005). Both neuromodulators target a wide range of cellular targets that affect intrinsic and synaptic excitability in a complex way (Armstrong-James and Fox, 1983; Giocomo and Hasselmo, 2005; Hasselmo et al., 1997; Sato et al., 1989; Sillito and Kemp, 1983). Furthermore, both ACh and NE have the potential to introduce long-term circuit modification due to their ability to alter the rules by which synaptic weights are altered during experimentally-induced long-term potentiation (LTP) and long-term depression (LTD) (Huerta and Lisman, 1995; Kirkwood et al., 1999; Seol et al., 2007; Thomas et al., 1996). Despite this growing understanding of context-dependent release of ACh and NE and the

Abbreviations: ACh, Acetylcholine; aCSF, artificial Cerebral Spinal Fluid; ApEn, Approximate Entropy; CCh, Carbachol; FR, Firing Rate; LTD, Long Term Depression; LTP, Long Term Potentiation; NE, Norepinephrine; PCA, Principal Component Analysis; PSD, Power Spectral Density; V1, Primary Visual Cortex.

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corresponding cellular targets, very little is known about the direct
effect of these neuromodulators on cortical network dynamics. We
here examined what the net effects of neuromodulation on cortical
networks are in absence of experimental and potentially unphy-
siological stimulation paradigms. To this purpose, we studied how
spontaneous, desynchronized cortical network dynamics are
modulated by using multichannel electrophysiology in acute
cortical slices combined with quantitative strategies from infor-
mation theory (Pincus and Goldberger, 1994) and network science
(Tononi and Sporns, 2003) to elucidate the effects on the spatio-
temporal network dynamics.

2. Materials and methods

2.1. Ethical statement

All animal procedures were approved by the Institute of Animal Use and Care of
the University of North Carolina — Chapel Hill and were in compliance with the
National Institutes of Health guide for the care and use of Laboratory animals (NIH
Publications No. 8023, revised 1978). All efforts were made to minimize animal
suffering, to reduce the number of animals used, and to utilize alternative ap-
proaches where available.

2.2. Solutions

All chemicals were purchased from Sigma (St. Louis, MO). Sucrose solution:
83.0 mM NaCl, 2.5 mM KCl, 0.5 mM CaCl2, 3.3 mM MgSO4, 1.0 mM NaH2PO4,
26.2 mM NaHCO3, 22.0 mM NaHCO3, 22.0 mM Dextrose Anhydrose, 72.0 mM Sucrose. Artificial cere-
bral spinal fluid (aCSF): 119.0 mM NaCl, 2.5 mM KCl, 1.0 mM NaH2PO4, 26.2 mM NaHCO3,
22.0 mM glucose, 1.0 mM MgSO4, and 1.0 mM CaCl2. Incubation solution:
aCSF modified to contain 2.0 mM MgSO4 and 2.0 mM CaCl2. Control aCSF with
added KCl to a final concentration of 3.5 mM KCl. Nicotine aCSF: control aCSF with
10 µM nicotine added. Pirenzepine aCSF: control aCSF with 50 µM pirenzepine
added. L-Phenylephrine aCSF: control aCSF with 50 µM L-Phenylephrine. Clonidine
aCSF: control aCSF with 50 µM clonidine. Isoproterenol aCSF: control aCSF with
40 µM isoproterenol added. Prazosin aCSF: control aCSF with 20 µM prazosin added.
Yohimbine aCSF: control aCSF with 40 µM yohimbine. Propranolol aCSF: control
aCSF with 40 µM propranolol added.

2.3. Slice preparation

Adolescent (p15-p36) C57BL/6J mice were deeply anesthetized with Euthasol and
decapitated. Brains were removed and quickly placed in ice-cold sucrose so-
lution bubbled with carbogen (95% O2, 5% CO2) and 200 µm coronal slices were then
cut from the primary visual cortex (V1) using a VT1000S (Leica Microsystems,
Wetzlar, Germany). Slices recovered in incubation solution bubbled with carbogen
for at least 45 min at 34 °C before placement on the array.

2.4. Drug application and experiment design

Slices were placed on a MEA 2100 (Multichannel Systems, Reutlingen, Germany)
with perforated arrays of 59 electrodes of 30 µm diameter and 200 µm spacing. The array was perfused from both sides with control aCSF bubbled with carbogen at 36 °C (Fig. 1A). After a 1800s control epoch the perfusion was switched
to control aCSF with 1 µM, 10 µM, 50 µM, or 100 µM of carbachol (CCh) or NE which
was then followed by a 1800s washout epoch with control aCSF. Providing
oxygenated aCSF to both sides of the slice together with a high perfusion flow rate
(>4 mL/min) in a relatively small chamber around the array enabled the occurrence
of spontaneous activity without any pharmacological manipulations in control
conditions. Control aCSF differed from the aCSF commonly used in slice experiments
by containing only 1 mM Mg2+ and 1 mM Ca2+. Reduced Mg2+ and Ca2+ concen-
trations more closely resemble the values of CSF in vivo (Sanchez-Vives and
McCormick, 2000). We found that switching to the higher, standard

Fig. 1. Use of a multielectrode array (MEA) allows simultaneous readings of neuronal activity on 59 channels. A. 200 µm × 200 µm MEA. B. Cortical slice on the MEA with electrode locations superimposed (red); electrodes [2, 1] (top, leftmost) and [8, 7] (bottom, rightmost) indicated. C. Nissl stained slice of mouse primary visual cortex with cortical layers and pia indicated. D. A 10 s trace of all channels simultaneously recorded (top left: electrode [2, 1]; bottom right electrode [8, 7]). E. Expanded traces from a single channel of panel (D)
with many action potentials visible (Top: 1 s; Bottom: 250 ms). F. Spontaneous activity (spikes per second) by cortical depth exhibited laminar profile with deeper layers more active
than superficial layers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
to determine the receptor subtypes responsible for the effects studied here, we used specific receptor agonists and antagonists. The cholinergic signaling was dissected by nicotine as a selective agonist for nicotinic cholinergic receptors and M1-specific muscarinic agonistspirenzepine. We isolated the three noradrenergic receptor subtypes α2, α2, and β by agonist p-phenylephrine, clonidine, isoproterenol, respectively, and agonist prazosin, yohimbine, and propranolol, respectively. The agonists were applied to the aCSF instead of CCh or NE; antagonists were added to the aCSF for the entire experiment in addition to 50 μM of CCh or NE that were applied after 1800 s. To ensure the measured response was indeed mediated by the applied compound and not an artifact of the perfusion system or the experimental setup, we conducted control experiments where the perfusion was switched between two heakers of control aCSF (“placebo”).

2.5. Histology and electrode locations

Typically, about half of the electrodes (channels) were positioned over V1. Electrode locations were determined by superimposing the image of the slice on the array to that of the array alone (Fig. 1B). Cortical layers were then mapped to the electrodes by measuring the relative position between pia and white matter and comparing to Nissl-stained reference sections of age-matched mice (Fig. 1C).

2.6. Multunit and single unit data acquisition

Raw traces were recorded from 59 channels simultaneously, sampled at 25 kHz (Fig. 1D–E, example traces for the entire array, many channels show robust action potential firing, enlarged individual traces show prototypic action potential waveforms, spikes) with MC_Rack (Multichannel Systems). The raw data was high-pass filtered with a 300 kHz 4th order Butterworth filter and the noise level determined by calculating the standard deviation of the recorded signal on each channel. Spikes were identified by crossing – 4 times the noise level of that channel (with 1 ms dead time after each threshold crossing). As described in Section 2.5, the location of each channel was determined by percentage depth from pia (Fig. 1F, average firing rate by depth shows higher activity in deep layers as typically seen in vivo recordings of cortex). To identify single units, spike-sorting was performed for each channel using a random subset of 3000 spikes which were overclustered using the kmeans algorithm. Clusters were manually combined after examining wave shape and the distance between individual clusters determined by linkage analysis (Fec et al., 1996) as shown in Fig. 2A. After spike-sorting, reference waveforms were determined by averaging the waveforms for each unit. All spikes from the corresponding electrode were then assigned to one of the reference units by determining the minimal distance between the actual spike and the reference waveform (template matching). The resulting units had to contain a minimum of 100 spikes and the distribution of the negative peak values of the spikes had to resemble a normal distribution (Hill et al., 2011). Fig. 2B displays a representative subset of units from 8 channels (clusters from the same channel are displayed in the same color, different slice from Fig. 2A). On average, 52 clusters were isolated from each slice or 2.97 units per channel.

2.7. Data analysis

The data were analyzed using custom-written scripts in Matlab (Mathworks, Natick, MA). Only electrodes in V1 were spike-sorted and therefore data from other brain regions were not included in the single unit data. Raster plots were created by sorting units by their distance from pia and plotting the last 20 s of each epoch (“control”, “drug application”, “washout”). Multunit channels were identified as stable and included if 3 of 5 evenly spaced overlapping lines fitted to the firing-rate time-course had a slope of less than 0.006 spikes per second in the control epoch. Channels that had no spikes for a period of at least 600 s were excluded. The time-courses for each electrode were built by counting spikes in 20 s bins for the duration of the experiment. The time-courses of each channel were then z-scored which removed biases from our calculations of medians due to different intrinsic firing rate levels across channels. Furthermore, the z-score also normalized the standard deviation. Then, the median of all channels in V1 was computed for each 3 s bin. The last electrode (channels 47–59) was treated separately and peak amplitude (control, drug applied and washout) was used for all subsequent analysis and discrete spike trains were constructed for each channel by binning spike times (1 ms bins). To determine if the application of neuromodulators caused a significant increase or decrease of the firing rate (FR), respectively, we created a shuffled control in which the FRs of both the control and neuromodulated epochs were split into 5 s windows and recombined. The modulation index was then calculated as follows:

$$\text{Modulation Index} = \frac{(\text{FR}(\text{NM}) - \text{FR}(\text{Ctrl})) / (\text{FR}(\text{NM}) + \text{FR}(\text{Ctrl}))}{\text{FR}(\text{Ctrl})},$$

where FR(NM) denotes the FR during neuromodulator application and FR(Ctrl) denotes the FR during the preceding control epoch. The modulation index of the measured firing rate was compared to the modulation-index distribution of the 500 shuffles: our significance criterion required that the true modulation index was significantly different from the median of the shuffled value ($p > 0.05$ after a Bonferroni correction for the number of channels being compared). Channels not in V1 were then excluded using the method described in Section 2.4.

We calculated the average FR of each single unit for each epoch window. The effect of the neuromodulator was determined by dividing the neuromodulator epoch FR by the control epoch FR. Similar to the neuromodulator effect, the plasticity effect of each neuron was calculated by dividing the washout epoch FR by the control epoch FR. Single units where this ratio was either zero or undefined due to the complete absence of spikes in either of the epochs were excluded. These ratios are reported on a log10 scale (i.e. no effect corresponds to a value of zero). We determined skewness of effect distributions using the skewness function of Matlab. We calculated the standard error of skewness using SE = s($\text{ssq}(n)$), any distribution with a skewness greater than 2$\text{SE}$ was considered significantly skewed. The correlation between effect of FR and effect of each neuromodulator was determined using the correlation function of Matlab on all cells that had a non-zero FR for all epochs. To examine the change caused by neuromodulators as a function of cortical depth we then grouped units in layers 1–IV and layers V–VI.

Approximate entropy (ApEn) is a measure of signal irregularity based on how frequently individual temporal patterns occur in the data (Pincus and Goldberger, 1994). ApEn was calculated using an additional script for Matlab written by Kijoon Lee based on (Chon et al., 2009). ApEn requires three input parameters: $m$, $r$, and $N$: the length of the pattern, a tolerance threshold, and the length of the data to be analyzed. Briefly, the algorithm that determines ApEn scans the data and looks for approximate matches for every pattern detected. Tolerance threshold $r$ determines how close this match must be. For each pattern, all matches are then evaluated by considering their first subsequent data point. If this data point is different from the first subsequent data point of the original pattern, ApEn is accordingly increased. In order to normalize the number of times with zero match, we still fulfilling the minimum data-length requirement (Pincus and Goldberger, 1994) of the ApEn algorithm (typically $n \geq 1000$), spike trains were binned with 300 ms resolution. The following parameter values were used: $m = 2$, $r = 0.1 \times \text{std}(\text{FR})$, and $N = 1000$. Pairwise correlation was calculated by applying the corrcnt function in Matlab to spike trains binned with 100 ms resolution. This correlation coefficient was computed for each pair of multunit channels. Compensated power spectral densities (PSDs) of single units were calculated using Thomson’s multitaper method and compared to a shuffle control as in Rivlin-Etzion et al. (2006). Briefly, the frequency spectrum of each single unit was calculated from the discrete spike train using with the Matlab function pcorr.m. The raw frequency spectrum is divided by the average spectrum of 20 SS shuffled versions of the spike train. Principal component analysis (PCA) was performed on z-scored, 10 ms binned spike trains and then broken into 5 s non-overlapping windows using the princomp function in Matlab. PCA transformed the binned FR of all units in a slice to decorrelated component dimensions. We then sorted the component dimensions by percent variance accounted and determined the number of dimensions required to account for 50% of the total variance. Due to the discretization of this measure, we used bootstrapping on the windowed results to estimate the mean percent of total dimensions for each epoch. We then examined change in the percentage of total dimensions to account for 50% of the variance between neuromodulator applied and control epochs to assess the effect of the neuromodulators.

2.8. Statistics

All data are expressed as median values ± SEM unless otherwise noted. Medians of data shown on log10 scale were taken as the log10 of the median, while SEM for data on log10 scale was determined by:

$$\text{SEM} = \text{std}((\text{log10(data)}) / \sqrt{n}).$$

In general, FR data are typically not normally distributed (Shafti et al., 2007); we confirmed that effect size did not follow a normal distribution and therefore statistical significance was determined using Kruskal–Wallace tests in conjunction with Tukey’s multiple comparison tests at $p < 0.05$, unless otherwise noted. These test compute confidence intervals at $p = 0.05$, if the confidence intervals of two groups do not overlap then $p < 0.05$. However, exact $p$ values for each comparison were not computed.

3. Results

3.1. Neuromodulators CCh and NE increase spontaneous activity levels

To assess the effects of cholinergic and noradrenergic neuromodulators on network dynamics in sensory cortex, we performed multi-electrode array recordings from slices of visual cortex maintained in an environment that mimicked the ion concentrations of the cerebral spinal fluid. We found that the slices exhibited sparse spontaneous action potential firing. We first asked whether CCh and NE modulated this spontaneous activity. To this end, we...
Fig. 2. Single units. A. Left: Dendrogram of similarity between combined clusters. Clusters are grouped by linkage analysis. Right: 16 clusters from 3000 spike waveforms. B. Representative units from 8 channels of a representative example slice. Units from the same channel are in the same color; channel numbers and number of spikes (n) for each unit are indicated. C. Raster plots of all single units isolated in 50 μM CCh (top) and 50 μM NE (bottom) for the final 20 s of control, neuromodulator, and washout epochs (left to right), indicating increased FR for 50 μM CCh, 50 μM NE, and NE washout epochs.
recorded a stable control baseline of 1800s and added either CCh or NE to the bath for an additional 1800s before switching back to control for another 1800s to probe for long-term network effects that outlast the presence of the neuromodulators. Raster plots of individual units (n = 726 units pooled from 13 slices for CCh, and n = 512 units from 10 slices for NE) for initial control, neuromodulator application, and subsequent washout showed that application of 50 μM CCh increased the spontaneous activity which returned to baseline after washout (Fig. 2B, Top, left to right). In the case of 50 μM NE, activity also increased upon application but remained elevated after washout (Fig. 2B, bottom, left to right). Given these distinct effects of CCh and NE on spontaneous network dynamics, we next examined the concentration-dependence of the time-course of action of CCh and NE.

3.2. Temporal response profile

We determined the effect of the neuromodulators CCh and NE on network dynamics (z-scored time-course of average firing rate) as a function of time and concentration (1, 10, 50 and 100 μM for both CCh and NE). For CCh experiments (Fig. 3A 1, 10, 50 and 100 μM CCh from top to bottom. n = 10, 13 13 and 15 slices), the FR increased and remained elevated for the duration of CCh present in the bath. For higher concentrations (50 and 100 μM CCh), the initial transient of the FR response to CCh became steeper and exhibited an overshoot in comparison to the steady-state response. In summary, application of CCh caused a pronounced, reversible increase in spontaneous activity.

Similarly, NE also caused an FR increase for all concentrations (Fig. 3B, 1 and 10, 50 and 100 μM NE from top to bottom. n = 13, 11, 10 and 14 slices) with a pronounced overshoot only for 50 μM NE. When the perfusion was returned to control aCSF, we found different responses as a function of concentration: 1 μM NE allowed a quick return to baseline, 10 and 100 μM NE concentrations caused a slower return to baseline, whereas 50 μM NE caused an outlasting, stable elevation of the FR for the remaining 1800 s of the experiment. Thus, spontaneous activity markedly increased with application of NE; higher concentrations caused FR increases that outlasted the application of NE. This sustained elevation of spontaneous activity motivated the further consideration of not only the control and neuromodulator data but also the washout ("plasticity") epoch.

3.3. Concentration-dependent modulation of spatial spread of spontaneous activity

Based on the time-course plots (Fig. 3), we next analyzed the last 300 s before the end of each epoch to quantify the steady-state response to the neuromodulators. All subsequent analysis was limited to these analysis windows. We first asked if individual channels (locations in the slice) exhibited a significant FR increase or decrease with the application of neuromodulators as measure of the spatial spread of the effect. An increase in channels with altered FR indicated that a larger fraction/area of the network exhibited a response to the applied neuromodulator. The percentages of channels (locations in the slice) exhibited a significant FR increase or decrease with the application of neuromodulators as measure of the spatial spread of the effect. An increase in channels with altered FR indicated that a larger fraction/area of the network exhibited a response to the applied neuromodulator. The percentages of channels on each slice were then calculated for each of the three neuromodulator effect types (Fig. 4, increase, no effect, decrease; CCh left, NE right). We first applied this measure to multiunit channels of control ("placebo") slices where no neuromodulators were applied to exclude false positives (all values are mean

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*Fig. 3. Time-courses of spontaneous activity in response to neuromodulator application. A. Time-course of median z-score of FR for all 1, 10, 50 and 100 μM CCh slices (top to bottom). Dashed lines indicate times of aCSF switches. Analysis windows were taken from last 300 s before aCSF switch (between dotted and dashed lines). CCh response begins with a large increase in FR around 700 s. After washout (about 2500 s here) the FR begins to return to baseline. By the washout analysis window all FRs have returned to baseline. B. Time-course of median z-score of FR for all 1, 10, 50 and 100 μM NE slices (top to bottom). Around 700 s there is an increase in FR which remains until 2500 s where 1 μM NE slices return to baseline FR. 10, 50 μM NE slices do not return to baseline for the duration of the experiment (at least 1800 s washout).*
50.4 ± 1.75 for 1 μM, 42.1 ± 1.86 for 10 μM, 24.2 ± 1.07 for 50 μM, and 49.4 ± 1.94 for 100 μM, all values are mean ± SEM). The percentage of channels with an FR increase was significantly larger than those of control slices for 10, 50 and 100 μM CCh. All concentrations of CCh had significantly fewer channels with no effect than control slices (ANOVA and post-hoc Tukey's test \( p < 0.05 \)). Of the four concentrations of CCh used, only 1 μM had significantly more channels with an FR decrease than control (15.9 ± 1.41 for 1 μM, 10.7 ± 0.50 for 10 μM, 0.72 ± 0.50 for 50 μM, and 10.2 ± 0.67 for 100 μM; all values are mean ± SEM).

NE had a more complex effect on the spatial spread of activity; as for CCh, we found that for all concentrations NE had fewer channels with no effect than the control slices (51.5 ± 1.30 for 1 μM, 49.6 ± 1.56 for 10 μM, 52.8 ± 1.74 for 50 μM, and 44.8 ± 1.90 for 100 μM; \( n = 10, 13, 13 \) and 15 slices). However, in contrast to the CCh experiments, slices did not exhibit significantly more channels with FR increases than the control slices (32.6 ± 1.44 for 1 μM, 36.4 ± 1.72 for 10 μM, 31.3 ± 1.49 for 50 μM, and 32.7 ± 2.07 for 100 μM). Only 100 μM NE had significantly more channels with an FR decrease (15.9 ± 1.29 for 1 μM, 13.9 ± 0.90 for 10 μM, 15.9 ± 1.19 for 50 μM, and 22.4 ± 1.73 for 100 μM). Therefore, NE modulated the network dynamics compared to control yet the effects on the FR were bidirectional.

### 3.4. Magnitude of neuromodulator and plasticity effects

The above analysis examined the effect of the neuromodulators on overall multunit FR which consists of the action potential firing of all neurons close to the recording electrode site. We next sorted the action potential waveforms into single units (presumed individual neurons) to test for concentration-dependent effects on the FR of individual neurons. We defined neuromodulator effect as the FR in presence of neuromodulator divided by the control FR for each single unit. The range of neuromodulator effect sizes covered six orders of magnitude since many units were very quiet during control. **Fig. 5A** shows a histogram of the logarithmic effect size for increasing concentration from left to right. FR of cells in slices exposed to CCh exhibited large increases for all concentrations (0.327 ± 0.0407 for 1 μM, 0.612 ± 0.0389 for 10 μM, 1.39 ± 0.0433 for 50 μM, and 0.730 ± 0.0450 for 100 μM; all values \( \log_{10} \); \( n = 395, 581, 635 \) and 665 units across 9, 13, 13 and 14 slices; all significantly different from each other with the exception of the comparison of 10 μM–100 μM CCh). This result is in close agreement with the above-shown time-course and multunit FR data. Since the multunit data for NE failed to show a significant increase in the number of channels with increased FR, we predicted that the effect of NE at the level of single units would be bidirectional such that some units had an FR increase whereas others had an FR decrease. Indeed, FR in units of NE-exposed slices displayed smaller median logarithmic effect sizes (value ± SES: 0.168 ± 0.0328 1 μM, 0.398 ± 0.0342 for 10 μM, 0.416 ± 0.0438 for 50 μM, and 0.280 ± 0.0393 100 μM; \( n = 685, 650, 472 \) and 470 units across 13, 11, 10 and 15 slices). Some concentration dependence existed for this measure: 1 μM NE was significantly different from 10 μM NE as well as 100 μM NE. To determine the uniformity of the neuromodulator effects, we next examined the skewness of the effect distributions in **Fig. 5A**. For this measure, if the effect of the neuromodulator increases the spontaneous activity by some factor (with Gaussian noise) the effect size distribution is normal with a skewness of zero. We found that the effect size distributions of 10, 50 and 100 μM concentrations of CCh had a significant negative skewness (0.148 ± 0.123 for 1 μM, −0.228 ± 0.102 for 10 μM, −0.459 ± 0.097 for 50 μM, and −0.319 ± 0.096 for 100 μM). Skewness of NE effect size distributions varied by concentration with significant positive skewness for the effect size of 1 μM NE and negative skewness for 50 μM NE. (0.431 ± 0.094 for 1 μM, 0.181 ± 0.096 for 10 μM, −0.623 ± 0.113 for 50 μM, and −0.137 ± 0.113 for 100 μM). Therefore, a simple model of neuromodulator effect size on cellular excitability cannot explain the experimentally determined effects.
Due to the observed outlasting effect of NE application on multunit firing rate, we next determined the single unit behavior for the last 300 s of the “wash out” epoch after drug application. We defined the “plasticity effect” by calculating the change of the washout epoch FR relative to (divided by) the control epoch FR. We first examined the correlation between the neuromodulator effect size and the plasticity size for CCh (red, left) and NE (blue, right) (Figs. 5A, B). For CCh, correlation coefficients between neuromodulator effect and plasticity were low (0.066–0.261 for 1 μM, 0.310–0.463 for 10 μM, 0.306–0.445 for 50 μM, and 0.222–0.369 for 100 μM (95% confidence interval); n = 384, 474, 561 and 581 single units across 9, 13, 13 and 14 slices). Conversely, NE had higher correlation for concentrations greater than 1 μM (0.165–0.308 for 1 μM, 0.549–0.653 for 10 μM, 0.603–0.708 for 50 μM, and 0.485–0.616 for 100 μM (95% confidence interval); n = 659, 567, 456 and 432 units across 13, 11, 10 and 15 slices). The higher correlation for 10, 50 and 100 μM indicated that the plasticity effect seen in the time courses (Fig. 3) was mediated by neurons continuing to maintain their increased FR rather than different neurons responding to washout of NE. We next examined the plasticity size in terms of effect size for all units. This result further confirms that CCh had little plasticity effect for any concentration. Conversely, NE had higher correlation for concentrations greater than 1 μM (0.165–0.308 for 1 μM, 0.549–0.653 for 10 μM, 0.603–0.708 for 50 μM, and 0.485–0.616 for 100 μM (95% confidence interval); n = 659, 567, 456 and 432 units across 13, 11, 10 and 15 slices).
effect for all single unit channels (Fig. 5D, increasing concentration from left to right). This plot visualizes whether the increased FR during the washout epoch was mediated by units maintaining their neuromodulator-epoch FR or by different units becoming active after the washout. In the case of maintained FR, we would expect many points on the unity line of the plot. However, if different cells responded to washout, we would expect the data to contain two groups; the first group would contain cells that increased their FR with NE but exhibited an FR decrease after wash-out (above unity line in Fig. 5D) and the second group would contain cells that failed to directly respond to NE application with an increase in FR yet exhibited a delayed response after NE wash-out (below unity line). For the NE concentrations that appeared to have an outlasting effect as determined by the time-course data (Fig. 3), we noted an increased number of cells on the unity line (blue dots, 3 rightmost panels), further supporting that the units which directly responded to NE were the ones to exhibit an outlasting FR increase.

After we determined that the FR of slices subjected to higher concentrations of NE remained elevated for the remaining 1800s of the experiments after washout of the drug, we wanted to determine how long this plasticity can remain in effect. To do so we performed 9000 s experiments using 1800 s of control, then 1800 s with established by the control epoch (1800 s), followed by 5400 s of washout. We then examined 300 s windows at the end of control epoch (1800 s), end of the NE epoch (3600 s), and 5 washout epochs (5400 s, 6000 s, 7000 s, 8000 s, and 9000 s). We compared the distribution of FRs of each of the washout epochs to that of the same channels in control. We found that the plasticity effect was detectable for least 40 min after switching to control aCSF (Figure S2).

3.5. Effects of neuromodulators by cortical depth

Having described the effects of both neuromodulators on average FR, we further divided the data to examine if these results depended on cortical depth. We used histological methods to determine the cortical layer of each electrode and by extension each cell recorded from that electrode. We separated cells into superficial (layers I–IV) and deep layers (V and VI) to examine the differential effect of CCh and NE between superficial and deep layers of cortex. In Fig. 6A, we display the median neuromodulator effect size of both superficial and deep layers for CCh (top) and NE (bottom) for all concentrations (increasing left to right). Only 1 and 100 μM concentrations showed a significant difference between superficial and deep layers (Table 1, two leftmost columns). However, for all concentrations of CCh the data trended toward deeper layers exhibiting a larger response. When examining NE slices, we found that for both 1 and 50 μM NE concentrations the median neuromodulator effect size of deep layers was significantly larger than that of superficial layers. As with CCh slices, all concentrations of NE trended toward a larger effect in deeper layers, however the bidirectional modulation of FR by NE obscured the results. Deeper layers of cortex exhibited a greater response to 1 and 50 μM NE than those in superficial layers.

We were interested in examining both the neuromodulator and washout epochs of our experiment to determine if the response observed during the neuromodulator epoch was reversible by washout. We examined the median plasticity size of both CCh (top) and NE (bottom) for all concentrations (increasing from left to right) in Fig. 6B. We found that when evaluating washout, single units for all CCh concentrations return to, or undershoot baseline established by the control epoch (Table 1, two rightmost columns). The small difference between control and washout FR confirmed the return to baseline observed in Figs. 2B and 3A. Additionally, the layer dependence of the neuromodulator effect observed in the neuromodulator epoch was not present during the washout epoch for all concentrations. After washout of CCh, there was no significant difference between superficial and deep layers for any of the concentrations studied. Having established that the same cells stay active during the plasticity epoch after washout of NE (Fig. 5B), we expected to see one or both layer-groups remain at an elevated FR for higher concentrations of NE. Indeed, after washout of 10 or 50 μM NE, the FR of neurons in deep layers remained significantly elevated in comparison to those in superficial layers. This indicates that the elevated spontaneous activity visible in the plasticity epoch of the time-courses for these concentrations was mediated by cells in deeper layers of cortex.

3.6. Network dynamics

So far, we have presented analysis of the changes in FR induced by a wide-range of CCh and NE concentrations. However, signaling in cortex is assumed to rely on more sophisticated temporal and spatial features of network activity. We therefore employed four additional approaches to provide more detailed insights into cholinergic and noradrenergic modulation of spontaneous cortical network dynamics with regards to temporal and spatial activity structure. In particular, it has been shown that information processing in cortex is dependent on not only the number of spikes but also the timing of those spikes (Gerstner et al., 1997). To investigate the amount of information contained in the spontaneous activity we examined the ApEn of each channel. ApEn is a nonlinear measure of variance that when applied to a spike train measures the amount of information that can be contained in that spike train. When we plotted the change in ApEn versus the neuromodulator
effect size (the change in FR) (Fig. 7A, CCh top, NE bottom, increasing concentration from left to right), we found that ApEn is not correlated with neuromodulator effect size and therefore indeed a measure for temporal structure and not simply of activity frequencies (up to 1554, 1368, 972 and 368). 1 and 10 μM CCh both significantly decreased correlations, indicating that neural activity on different channels became more independent with application of NE. This decorrelation led us to conclude that the temporal and spatial patterning of the spontaneous activity increased the complexity of the network dynamics within the slices.

We next examined the effect of CCh and NE on the power spectrum since these neuromodulators typically reduce low frequency oscillations in vivo yet the effect of CCh, only in presence of blocked synaptic inhibition, has been reported to enhance rhythmic neocortical activity in vitro (Lukatch and Maclver, 1997). We examined the compensated power spectral densities (PSDs) of the discrete spike trains of single units obtained in each epoch. Fig. 8A displays the median compensated PSD of single units in neuromodulator minus the control compensated PSD. Both neuromodulators in all concentrations decrease the power in low frequencies (up to ~8 Hz) as indicated by a negative difference between neuromodulator and control.

Since we were able to simultaneously record spikes from over 50 units per slice on average, we were able to use measures of the dynamics of the slice as a whole which can uncover fundamental changes in the network dynamics (Schiff et al., 2007). To examine the complexity of the network as a whole, we performed PCA on the spike trains of all single units that together form time-dependent activity vectors (5 s non-overlapping windows). As applied here PCA uses singular value decomposition to transform spike trains of measured single units into theoretical activity generators, each of which explain some amount of the variance of the original activity vectors. We first examined percent variance explained by each component dimension and determined the percent of total dimensions required to explain at least 50% of the total variance (Fig. 8B top left; high variance dimensions in black, low variance dimensions in gray). The mean of all windows was determined for both control and neuromodulator epochs and the difference calculated (Fig. 8B bottom left; green percent of total dimensions for each window in control, red in 50 μM CCh). From the increased temporal and spatial complexity of multiunit channels as measured by ApEn and correlation coefficients, we expected addition of neuromodulators to increase the spatiotemporal complexity of the whole-slice network dynamics. Indeed, for all concentrations of both neuromodulators the data trended toward increased complexity. When we applied CCh the percentage of total dimensions needed to explain 50% of the variance was significantly increased for 50 and 100 μM concentration (5.16 ± 1.17 for 1 μM CCh, 6.72 ± 1.42 for 10 μM CCh, 15.80 ± 1.59 for 50 μM CCh, 7.30 ± 1.49 for 100 μM CCh; n = 9, 13, 13 and 13 slices). Similarly 1 and 10 μM concentrations of NE caused a significant increase in dimensionality (5.37 ± 1.23 for 1 μM NE, 4.00 ± 0.86 for

### Table 1

<table>
<thead>
<tr>
<th>Effect I–IV</th>
<th>Effect V–IV</th>
<th>Plasticity I–IV</th>
<th>Plasticity V–VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 μM CCh</td>
<td>0.065 ± 0.070; n = 120</td>
<td>0.433 ± 0.048; n = 278</td>
<td>−0.178 ± 0.051; n = 125</td>
</tr>
<tr>
<td>10 μM CCh</td>
<td>0.544 ± 0.081; n = 135</td>
<td>0.640 ± 0.044; n = 446</td>
<td>−0.160 ± 0.063; n = 126</td>
</tr>
<tr>
<td>50 μM CCh</td>
<td>1.290 ± 0.080; n = 194</td>
<td>1.449 ± 0.051; n = 438</td>
<td>0.150 ± 0.063; n = 179</td>
</tr>
<tr>
<td>100 μM CCh</td>
<td>0.398 ± 0.071; n = 194</td>
<td>0.879 ± 0.056; n = 462</td>
<td>0.000 ± 0.053; n = 184</td>
</tr>
<tr>
<td>1 μM NE</td>
<td>0.088 ± 0.046; n = 334</td>
<td>0.280 ± 0.046; n = 359</td>
<td>0.101 ± 0.036; n = 339</td>
</tr>
<tr>
<td>10 μM NE</td>
<td>0.276 ± 0.059; n = 173</td>
<td>0.449 ± 0.041; n = 477</td>
<td>0.000 ± 0.053; n = 169</td>
</tr>
<tr>
<td>50 μM NE</td>
<td>0.135 ± 0.077; n = 130</td>
<td>0.545 ± 0.051; n = 342</td>
<td>−0.015 ± 0.053; n = 137</td>
</tr>
<tr>
<td>100 μM NE</td>
<td>0.199 ± 0.085; n = 109</td>
<td>0.322 ± 0.044; n = 361</td>
<td>−0.109 ± 0.070; n = 111</td>
</tr>
</tbody>
</table>
10 μM NE; 6.16 ± 2.11 for 50 μM NE, and 3.59 ± 1.47 for 100 μM NE; n = 13, 11, 10 and 14 slices). The increase in the number of dimensions required to explain 50% of the variance indicates that the complexity of the network as a whole is increased when CCh or NE was applied.

3.7. Pharmacological agents identify receptor specific effects

Thus far we have characterized the effects of CCh and NE on overall cortical network dynamics in visual cortex. We next used selective agonist and antagonists to determine the receptor types involved in these responses. Based on the literature (Kirkwood et al., 1999; Metherate et al., 1990), we expected that muscarinic M1 receptors mediated the increase in FR by the cholinergic agonist CCh. Indeed, we found that the muscarinic M1 receptor antagonist pirenzepine completely blocked the CCh (50 μM) mediated enhancement of spontaneous activity (5 μM; logarithmic effect size: −0.085 ± 0.043, n = 5 slices; Fig. 9A, left). In addition, we also did not find a response to nicotine (10 μM; −0.052 ± 0.019 n = 12, Fig. 8A, right). Together, in agreement with the literature, these experiments suggest that the CCh response was predominantly mediated by M1 muscarinic receptors.

We dissected the response to NE by using both selective agonists and antagonists (in presence of 50 μM NE) for α1, α2, and β receptors. Inspection of the z-scored time-courses for the agonists supported a potential role of both α1 and β signaling in mediating the outlasting increase in FR (Fig. 9B, left, top to bottom). We found that application of the α1 agonist L-Phenylephrine slowly increased FR which remained elevated after washout (10 μM; 0.245 ± 0.048, n = 7 slices). Clonidine, an α2 agonist, caused a small reduction in
the spontaneous FR ($-0.044 \pm 0.022, n = 6$ slices). Using a β agonist, isoproterenol (40 μM; $0.104 \pm 0.048, n = 5$ slices), we observed an initial transient response to the agent similar to that of 50 μM E (Figure 3B, 3rd from top) and again an outlasting response. We then used selective antagonists (Fig. 8B, right, top to bottom) together with 50 μM NE to further verify these results. Indeed, blocking $\alpha_1$ and $\alpha_2$ receptors did not suppress the enhancing effect of NE based on the time-course but the application of the $\alpha_2$ antagonist caused an overall run down of the activity which lead to decrease in FR in our measure (20 μM, 40 μM; $0.040 \pm 0.030$, $-0.044 + 0.025 n = 6$ and 8 slices, respectively). However, the β antagonist propranolol completely blocked the enhancement which suggests that the NE response shown in this study was mostly mediated by this receptor type (40 μM; $-0.0335 + 0.0261, n = 7$ slices). The activated yet delayed response to the $\alpha_1$ agonist, however, hints at extra complexity that may warrant further future studies with techniques complementary to the ones used here.

4. Discussion

4.1. Summary of findings

In this study, we compared the concentration-dependent network effects of cholinergic and noradrenergic neuromodulators that are associated both with determining overall cortical state and with synaptic plasticity. We found that application of CCh greatly increased spontaneous activity of most active
cells predominately via modulation of muscarinic M1 receptors. In contrast, the effect of NE was more diverse with cells increasing or decreasing their firing rate. Overall, NE mediated only a modest instantaneous increase in network activity when compared to CCh. However, NE induced plastic reorganization of the network (particularly in infragranular layers) that was maintained after wash-out for at least 40 min. We found this plasticity to be mediated by β noradrenergic receptors with a potential (smaller) role for α1 receptors. Interestingly, we found significant effects of even just 1 μM for both CCh and NE, concentrations which are likely close to physiological concentrations in vivo. Changes in activity level were accompanied by changes in the complexity of the spatiotemporal structure of the network activity. Both neuromodulators increased temporal complexity of individual channels as measured by ApEn. As would be expected for neuromodulators responsible desynchronizing networks (Armitage et al., 1969; Celesia and Jasper, 1966; Constantinople and Bruno, 2011; Jouvet, 1969; Kalmbach et al., 2012), lower concentrations of CCh and all concentrations of NE increased the spatial complexity by causing a decrease in the pairwise correlation between activity in different network locations. Similarly, measurements of dimensionality of the whole-slice network dynamics by PCA also indicated an increase in complexity. Together, these results indicate that there are fewer similarities between network locations and more complexity in each individual location and in the overall spatio-temporal structure. Therefore, we concluded that both cholinergic and adrenergic neuromodulation increases overall network complexity yet CCh had a more pronounced, instantaneous effect whereas NE had a more moderate but outlasting effect.

4.2. Studying network dynamics in vitro with multielectrode arrays

To our knowledge, this is the first study that combined the use of a multielectrode array with sophisticated network-level analysis strategies to investigate the role of neuromodulation in acute cortical slices. The study of spontaneous network dynamics in acute cortical slices has emerged as a promising approach to understanding the synaptic mechanisms of rhythmic activity in cortex (Frohlich and McCormick, 2010; Sanchez-Vives and McCormick, 2000; Traub et al., 1994). Here, we used similar adaptions to the aCSF to make the conditions more in vivo-like and found robust yet sparse spontaneous activity in control conditions. Importantly, the spontaneous activity in our slices exhibited a layer-dependent FR profile very similar to what is typically found in vivo (Ruthazer et al., 1999; Schroeder and Foxe, 2002). Oxygenation of slices had been reported to be of particular importance for the generation and maintenance of spontaneous activity in slice (Hajos et al., 2009; Hajos and Mody, 2009) and the perfusion system in our MEA experiments likely provided enhanced oxygenation in comparison to the standard submerge slice chamber. The new generation of MEAs (Boppart et al., 1992; Huang et al., 2012; Stett et al., 2003) has provided important insight into the genesis of hypersynchronized, epileptiform activity patterns in acute hippocampal and cortical slices (Beggs and Plenz, 2003; Gonzalez-Sulser et al., 2011; Motamed et al., 2012, 2006; Yang et al., 2012). Previously, MEA studies mostly focused on cultured neuronal networks due to the better signal quality to be obtained from neurons directly grown on the MEA (Becchetti et al., 2012; Beggs and Plenz, 2004; Egert et al., 1998; Gullo et al., 2009; Nikonenko et al., 2003; Okawara et al.,...
4.3. Neurounodulation in acute cortical slices

The literature on the effects of the neuromodulators ACh and NE on cellular and synaptic excitability is inhomogeneous. Most recently (Favero et al., 2012) have noted that ACh caused a decrease in excitability of excitatory cells of layers II/III and IV of somatosensory cortex in vivo. Conversely, Eggermann and Feldmeyer (2009) uncovered an excitatory effect of acetylcholine in layer II/III and V while confirming a decrease in excitability of layer IV spiny neurons. Earlier, pioneering studies have already pointed to how both increases and decreases of excitability can occur in response to ACh in vitro (McCormick and Prince, 1986). Adding to this complexity, studies in vivo have yielded a wide range in percentage of neurons excited by cholinergic modulation (Bassant et al., 1990; Lamour et al., 1982, 1988; McKenna et al., 1988; Metherate et al., 1990, 1988; Muller and Singer, 1989; Sillito and Kemp, 1983). We have shown here that the net effect of CCh acting on cholinergic receptors is an increase of overall spontaneous activity. For NE, previous work (Favero et al., 2012) suggests a decrease in excitability of excitatory cells accompanied by an increase in excitability of fast-spiking interneurons. This result, which is in agreement with (Kawaguchi and Shindou, 1998) may explain the diversity of changes in FR when we applied NE to the slices in this study. When NE is applied, the FR of interneurons would increase while postsynaptic excitatory cells would decrease their FR, and indeed we noted that 100 μM NE caused a significant number of channels with an FR decrease (Fig. 4, bottom right). However, a recent human study, administration of an NE reuptake inhibitor (thereby increasing extracellular NE) was shown to increase excitability in visual cortex (Hoffken et al., 2012). Therefore, no consensus has yet been reached on the net effect of NE on cortical network activity; our results support a net enhancement of the activity levels for the wide range of concentrations evaluated in our study. Some of the differences to these studies might be explained by differences in the methods used; in our study due to the arrangement of electrodes we performed a sampling of spontaneously active cells and by means of extracellular recordings we did not perturb the intracellular milieu (and therefore signaling) as is the case for intracellular recordings. Extracellular recordings of action potentials also have potential biases toward (1) neurons with high spontaneous firing rates such as fast-spiking inhibitory interneurons (McCormick et al., 1985) and (2) pyramidal cells that exhibit larger extracellular spike waveforms due to their cell morphology (Holt and Koch, 1999).

4.4. Response dynamics

Despite the similarities in effect between the cholinergic and noradrenergic systems, there are important functional contrasts (Aston-Jones and Cohen, 2005; Baxter and Chiba, 1999; Bouret and Sara, 2005; Vankov et al., 1995; Yu and Dayan, 2005). In particular, the cholinergic system has been linked to tasks where there is an expected uncertainty, for example the state of a traffic light on a daily commute. On the other hand, NE is released in response to an unexpected uncertainty or object novelty, for example a new traffic light on the commute. Therefore, while both systems are tuned to enable privileged processing of incoming input by for example increasing the signal-to-noise ratio in primary sensory cortices, our results indicate that the modulation of spontaneous activity is different for the two systems, in particular with regards to the duration of the effect. An overall (and pronounced) increase in overall excitability as found for CCh may subserve the processing of weaker stimuli that otherwise would have remained subthreshold events. Scenarios that lead to an increase in NE release in the behaving animal are likely to be of fundamental importance to the animal where a change to the circuitry beyond the presence of the increased NE release is beneficial and may correspond to the plasticity effect found in this study for NE. Importantly, we did not employ any artificial (electric) stimulation of the network to induce changes to the network or measure changes to evoked responses in the network with application of CCh or NE, rather we studied how the endogenously generated, spontaneous cortical activity was modulated.

4.5. Plasticity

Both cholinergic and noradrenergic systems have been implicated in learning and memory because both systems affect mechanisms of synaptic plasticity that cause long-term changes in synapse strength such as long-term potentiation (LTP) and long-term depression (LTD) for example by spike-time dependent plasticity (STDP) (Bear and Singer, 1986; Bi and Poo, 1998; Froemke and Dan, 2002; Fu et al., 2002; Kirkwood et al., 1996; Markram et al., 1997; Sjostrom and Nelson, 2002). Typically, synaptic plasticity is studied in vitro with different protocols of presynaptic and postsynaptic stimulation. Given that the detailed temporal structure of the pre-synaptic and postsynaptic spiking pattern determines synaptic plasticity (Froemke and Dan, 2002; Sjostrom and Nelson, 2002), our results on NE-induced plasticity are particularly intriguing since we did not apply any exogenous electrical stimulation. Rather, the spontaneous activity in our slice preparation was sufficient to support changes in its activity structure in response to NE application. Supportive of such an underlying mechanism are the findings that when neuromodulators are added, the temporal windows between pre- and postsynaptic action potentials for plasticity were increased in hippocampus (Lin et al., 2003) and visual cortex (Seol et al., 2007). Alternatively, the plasticity effects in our study could also be explained by intrinsic plasticity. In this scenario, the increased firing rate caused by NE triggered a reorganization of intrinsic excitability (Desai et al., 1999).

While we do not have evidence for the specific role of synaptic or intrinsic plasticity in our experiments, possibly the most parsimonious explanation is indeed that CCh did not trigger synaptic plasticity but NE triggered LTP. Previous studies (Choi et al., 2005; Kirkwood et al., 1999; Seol et al., 2007) found LTD caused by cholinergic M1 receptors. At the level of spontaneous cortical network activity, we did not find any response indicative of LTD. Therefore, either the spontaneous activity was not appropriately patterned to induce LTD or the synapses that were indeed subject to LTD played a minor role in the generation of the spontaneous activity. In contrast, it has been shown that NE causes LTD (Thomas et al., 1996; Yang et al., 2002), which is independent of the order of pre- and post-synaptic spikes (Lin et al., 2003). In the case of higher concentrations (in particular 10 and 50 μM), we observed that the application of NE had an outlasting, enhancing effect on the spontaneous activity. This outlasting effect is likely caused by such LTD where the temporal patterning of the spontaneous activity is not specific enough to induce a measurable change in activity levels but when the STDP rule is loosened such that any temporal order induces LTP, the same structure is sufficient to induce long-lasting changes to synaptic strength.
4.6. Conclusions and outlook

Acetylcholine and norepinephrine are two well-studied neuromodulators due to their global importance in regulating synaptic and neuronal excitability as a function of behavioral state. Importantly, aberrations in these neuromodulatory systems have been implicated in a range of neuro-psychiatric disorders such as autism (Bodner et al., 2012; Deutsch et al., 2010; Perry et al., 2001) and attention-deficit disorder (Michelson et al., 2003; Milberger et al., 1997; Wijesuriya et al., 1999). The vast number of different targets for these neuromodulators even just within cortex and the often seemingly contradicting findings on their effect on excitability has prevented an overall understanding of the effect on cortical network dynamics from emerging. Here we made use of recent technological innovation for the study of network dynamics with single-unit resolution in vitro (Egert et al., 2002). We found that the cholinergic and the noradrenergic system both increased overall network complexity and activity levels with the important difference that the effects by norepinephrine outlasted the presence of the neuromodulators, indicative of a plastic reorganization of the underlying cortical circuit.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http:// dx.doi.org/10.1016/j.neuropharm.2013.04.045.

References


