Cholinergic suppression of visual responses in primate V1 is mediated by GABAergic inhibition

Anita A. Disney, Chiye Aoki, and Michael J. Hawken
Center for Neural Science, New York University, New York, New York

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There are two classes of ACh receptors: nicotinic (nAChR; ionotropic) and muscarinic (mAChR; G protein coupled). In rodents, activation of nAChRs increases the amplitude of thalamocortical postsynaptic potentials (Gil et al. 1997; Hasselmo and Bower 1992; Hsieh et al. 2000; Kimura et al. 1999). In macaque primary visual cortex (V1), activation of presynaptic nAChRs at thalamocortical synapses results in attention-like increases in responsiveness and contrast sensitivity (Disney et al. 2007). Response suppression, on the other hand, is rarely seen with nicotinic agonists (Disney et al. 2007), consistent with anatomical data showing that few inhibitory neurons in V1 express nAChRs (Disney et al. 2007). This is also consistent with data on the expression of nAChRs by interneurons in rodent neocortex (Christophe et al. 2002; Gulledge et al. 2007; Porter et al. 1999; Xiang et al. 1998).

When acting through mAChRs, ACh can have diverse effects depending on the receptor class and its downstream effector coupling (Brown et al. 1997). Determining which neurons in the circuit are receptive to ACh is a critical step in understanding cholinergic modulation. It is generally argued that suppressive effects of ACh in cortex result from mAChR-mediated reductions in glutamate release (Hasselmo and McGaughy 2004; Roberts et al. 2005; Yu and Dayan 2005). This argument is based on studies of the pharmacology of individual synaptic connections in the rodent cortex in vitro (Gil et al. 1997; Hasselmo and Bower 1992; Hsieh et al. 2000; McCormick and Prince 1986, 1985; Stone 1972a, 1972b; Wang and McCormick 1993), which show that many, perhaps most, excitatory neurons in the rodent cortex express AChRs and that ACh, acting via m2-type AChRs, inhibits glutamate release.

Quantitative neuroanatomical studies of AChR expression complement neuronal recording in probing the pharmacology of individual neurons and their synaptic connections. In our anatomical studies, we found that the majority of potentially cholinceptive neurons, those immunoreactive for β2 nAChR subunits or for m1- or m2AChRs, in macaque V1 are GABAergic (Disney and Aoki 2008; Disney et al. 2006, 2007). In macaque V1, fewer than 10% of excitatory neurons express m1AChRs, the receptor class expressed by the largest number of V1 neurons. This contrasts with data showing that between 25% (upper layer 2) and 95% (layer 5) of pyramidal neurons in rat cortex respond to ACh (Gulledge et al. 2007). Even more strikingly, the vast majority of parvalbumin-immunoreactive (PV-ir) neurons in macaque V1 express mAChRs (Disney and Aoki 2008), whereas ACh responses in fast-spiking (usually confirmed to be PV-ir) neurons are rarely seen in rat neocortex in vitro (Gulledge et al. 2007; Kawaguchi 1997; Xiang et al. 1998).

Thus there is an apparent incongruity between the common interpretation of physiological data on suppression by muscarinic agonists as reflecting presynaptic reductions in glutamate release and the anatomical data showing widespread expression of AChRs by GABAergic neurons in macaque V1 and perhaps in other species (McCormick and Prince 1986; Muller and Singer 1989). This incongruity led us to investigate whether local ACh release would lead to a net increase or decrease in spiking activity in macaque V1, and by what mechanism. We have shown previously that on the rare occasions when an effect of nicotine is seen outside layer 4c, visual responses are suppressed, an observation directly predictable from the anatomy (Disney et al. 2007). Muscarinic AChRs are

ACETYLCHOLINE (ACh) is released in cortex under the control of prefrontal areas involved in executive function (Sarter et al. 2005). Abnormal cholinergic function is implicated in dementias and their associated failures of vision and attention (Nobili and Sannita 1997) and in schizophrenia (Alexander et al. 2009; Hyde and Crook 2001). Neurophysiological and behavioral studies have begun to elucidate the modulatory effects of ACh in cortex and point to a role in attention (Arnold et al. 2002; Hasselmo and McGaughy 2004; Herrero et al. 2008; Himmelheber et al. 2000; McGaughy and Sarter 1998; Sarter et al. 2005).

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much more prevalent outside layer 4c (anatomical data) than are nAChRs and (being G protein coupled) are associated with more diverse effects on neuronal activity. The current experiment was therefore designed to look at both the direct effects of ACh in V1 (enhancement vs. suppression) and whether or not suppression, if seen, is mediated by changes in inhibitory synaptic transmission. There has been debate in the literature on this issue; McCormick and Prince (1986) reported that in the guinea pig neocortex, narrow-spiking (putatively somatotargeting inhibitory) neurons were excited by ACh and in turn inhibited nearby pyramidal cells. It has also been reported that ACh-mediated suppression in cat area 17 could be blocked by bicuculline (Muller and Singer 1989). However, bicuculline is known to act beyond the GABA<sub>A</sub> receptor (Heyer et al. 1981; Johnson and Seutin 1997; Olsen et al. 1976), and depolarization of putatively soma-targeting neocortical inhibitory neurons has not generally been observed, at least in the rat (Gulledge et al. 2007; Kawaguchi 1997; Xiang et al. 1998).

Here we report that a strong suppression by ACh in all cortical layers is mediated almost entirely by increased GABA<sub>A</sub> receptor activation. A recent study of ACh modulation in macaque visual cortex confirms our earlier results on presumptive nAChR facilitation but finds a higher proportion of facilitation by presumptive nAChR activation (Soma et al. 2012) than we report in this study; these differences are addressed in the results and discussion.

**EXPERIMENTAL PROCEDURES**

**Physiology and Pharmacology**

*Animals.* Eight adult (>2.5 kg body wt) male cynomolgus monkeys (*Macaca fascicularis*) were used in these experiments. Procedures were approved by the Institutional Animal Care and Use Committee of New York University (NYU), in accordance with National Institutes of Health (NIH) guidelines.

**Physiological recordings.** Details of surgical preparation, maintenance, and stimulus delivery and recording appear elsewhere (Xing et al. 2005). Animals were anesthetized with sufentanil citrate (6–18 μg·kg<sup>-1</sup>·h<sup>-1</sup>), and a craniotomy was made over V1. Animals were then paralyzed with vencuronium bromide (100 μg·kg<sup>-1</sup>·h<sup>-1</sup>), and anesthesia was maintained with sufentanil (6–24 μg·kg<sup>-1</sup>·h<sup>-1</sup>). Depth of anesthesia was assessed by continuously monitoring electroencephalograms from nearby electrodes (Carbostar, Kation Scientific), advanced through the tissue (minimum 3 min). Ninety seconds was the duration of stimulus steps, sequentially increasing the contrast from 2 to 96%, alternating with blank (mean gray) stimuli (i.e., a blank was presented after every 10 steps). The signal from the single fiber electrode was amplified differentially (Dagan, MN) with bandpass filtering (300 Hz to 10 kHz). The signal was then digitized using an analog-to-digital signal processing board (SGI). Spikes were discriminated and time-stamped by custom software running on a Silicon Graphics computer. Spike waveforms were selected for data collection with the use of a window discriminator, and spike times were stored for off-line analysis. We analyzed recordings from 63 recording sites; 53 were discriminated units, and 10 were thresholded “b ash.” Examination of interspike intervals indicated that some of the unit recordings might have been contaminated by spikes from another unit whose spike waveform could not be discriminated from that of the principal neuron being recorded. Thus we had in our population 28 single units (SU), 25 recordings of single-unit clusters (SUC; up to 2 or 3 neurons), and 10 multunit activity (MUA) recordings. The 10 MUA sites retained for analysis were all tightly tuned (i.e., there was a definable “optimal stimulus”). Both the SU and SUC recordings comprised waveforms that were many times larger than the hash and thus clearly discriminable from the latter in the window discriminator. In two cases, the GABA<sub>A</sub> antagonist gabazine caused the emergence of a new spike that could not be isolated from the original spike and sent to a separate channel. These recording sites were used in the suppression/enhancement analysis only, not for the analysis of gabazine effects. In most cases, changes in spike waveform due to pharmacological manipulation were small and did not cause the loss of isolation or the dropping of significant numbers of spikes. Our criterion for determining significant effects was stringent (P < 0.01) to ensure that a small number of dropped spikes could not cause a unit to be erroneously deemed “suppressed.” In the few cases where the waveform changed so substantially that isolation was lost during drug ejection, the recording was abandoned. Because SUs are particularly difficult to obtain and hold in layer 4c, limiting the analysis to only the 28 SUs reduces the number of layer 4c recordings to 3, only 1 of which was significantly enhanced. In addition, with analysis of SUs, layer 4b shows only enhancement (no suppression) and layer 6 shows only suppression (see Fig. 7 for SU analysis).

**Pharmacology.** Iontophoresis barrels (tip diameter 1–2 μm) were loaded with one of the following, all dissolved in deionized water: acetylcholine chloride (ACh; 1.0 M, pH 4.5–5.0) or carbamylcholine chloride (CCh; 0.25 M, pH 6.0–6.3), γ-aminobutyric acid (GABA; 0.2 M, pH 4.0), gabazine (SR-95531; 5 mM, pH 4.0), sodium chloride (0.25 M, pH 5.5–6.5), and either Alcian blue 8GX (8% in 0.5M sodium acetate, pH 5.8) or 10,000 mol wt biotinylated dextran amine (BDA; 10% in 0.01 M PBS, pH 7.2–7.4). In most experiments, nicotine hydrogen tartrate (0.25 M, pH 3.0) was also loaded. All chemicals were obtained from Sigma, with the exception of BDA, which was purchased from Invitrogen.

Drug ejection currents were kept below 160 nA, and in between ejection periods, −10-nA holding currents prevented barrel leak. A multichannel, nanoampere-range iontophoresis pump (Dagan) was used for current delivery.

**Visual stimulation, receptive field characterization, and data collection.** After a qualitative characterization of receptive field properties, quantitative measures of preference for orientation, spatial frequency, temporal frequency, and area were made using drifting gratings. Studies that when suppression beyond roughly 60% was allowed, a limit on suppression was imposed because we had observed in pilot studies that when suppression beyond roughly 60% was allowed, responses often took much longer (sometimes >30 min) to recover to baseline levels.
Where suppression with ACh or CCh ejection was evident (assessed by eye at the time of recording), after the return to a stable baseline (up to 30 min), gabazine was ejected, backed by increasing currents in the same baseline-run-recover sequence as described above, to determine the highest level of gabazine ejection possible without significantly increasing the spontaneous or driven firing rates (again determined by eye). Recovery periods for gabazine varied from site to site but were most often on the order of 10 min or so. Once a level of gabazine ejection was selected, a final sequence was run in which gabazine was ejected at this chosen level while ACh was co-ejected at the highest level used in the dose-response series.

At a subset of responsive recording sites, we collected data on the effects of nicotine and/or ACh/CCh on orientation and area tuning. In the orientation experiments, a full-contrast drifting grating, optimized for spatial and temporal frequency and area (classical receptive field stimulation only), was selected, and responses were recorded to 3 repeats of 18 randomly presented orientations (spanning 360° in steps of 20°), alternating with a blank screen (i.e., a blank was presented after every grating stimulus). The orientation response was then remeasured across these same values, accompanied by iontophoretic ejection of nicotine, ACh, or CCh. Constant-current ejection started concurrently with visual stimulation and lasted for 108 s (the duration required for measurement of 3 repeats). After each drug ejection period, three or more “recovery” orientation tuning profiles were recorded without drug application. We used the same sequence of increasing ejection currents as was used in the contrast study described above.

In the area-tuning experiments, a full-contrast drifting grating optimized for orientation and spatial and temporal frequency was used. Responses were recorded to 3 repeats of 10 randomly presented stimuli spanning radii of 0.1° to 5°, alternating with a blank screen. The center of a cell’s receptive field was carefully located by using a small circular patch (usually 0.2° radius or smaller) of drifting grating. The center of the stimulus was put at the center of the cell’s receptive field. The area-tuning responses were then remeasured across these same values accompanied by iontophoretic ejection of nicotine, ACh, or CCh. Constant-current ejection started concurrently with visual stimulation and lasted for 60 s (the duration required for measurement of 3 repeats). After each drug ejection period, three or more “recovery” tuning profiles were recorded without drug application. We used the same sequence of increasing ejection currents as was used in the contrast study described above. All recording locations were marked by ejecting either Alcian blue 8GX or BDA.

**Histological reconstruction.** At the end of physiological recording, animals were euthanized, exsanguinated with 0.01 M PBS, and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Blocks of V1 tissue were removed and sectioned in the sagittal plane at 50-μm thickness. Recording sites were identified using BDA or Alcian blue 8GX. For BDA experiments, the label was visualized by incubating the sections overnight in an avidin-horseradish peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories) at room temperature on a shaker. After primary incubation and thorough PBS rinses, the tissue was incubated in primary antibodies for 72 h at room temperature overnight in a 1% hydrogen peroxide wash (Sigma) in PBS. The tissue was then rinsed in PBS and incubated for 30 min in blocking solution: 1% IgG-free bovine serum albumin (BSA; Molecular Probes), 0.05% sodium azide, 0.04% Triton X-100 (Triton), and 0.1% PhotoFlo (Kodak) in PBS. Antibodies directed against the i3 loop of the m1 and m2 AChRs (Chemicon) were diluted 1:200 in 1% BSA and 0.05% sodium azide in PBS. Free-floating sections were incubated in primary antibodies for 72 h at room temperature on a shaker. Control experiments for these primary antibodies have been described previously and consisted of preadsorption with the antigen, omission of the primary antibody, or use of an inappropriate secondary antibody to verify complete absence of immunoreactivity, as well as Western blotting of macaque homogenates to verify immunoreactivity at a single band (Disney et al. 2006). After primary incubation and thorough PBS rinses, the tissue was incubated overnight at room temperature in a 0.8-nm gold-conjugated goat anti-rabbit IgG (Aurion, EMSciences) diluted 1:50 in PBS with 1% BSA and 0.05% sodium azide. The next day, the sections were rinsed, postfixed with 2% glutaraldehyde in PBS for 10 min, and rinsed in 0.2 M citrate buffer before silver enhancement of the gold particles [silver-intensified colloidal gold particles (SIG); Amersham IntenSE silver kit].

These sections were also used in an unrelated tract-tracing study; for further details of processing to visualize the tracer, see Disney et al. (2007). Tissue was processed and embedded using conventional electron microscopic methods. Data were collected under a JEOL 1200 XL transmission electron microscope. More details of the methods can be found in our prior publications (Disney et al. 2006, 2007).

**EM data collection.** Images were all taken from layers 2 and 3 at a magnification of ×40,000, in close proximity to the tissue/EPON interface (the region of high antibody penetration) but otherwise in strict sequence of encounter, to ensure random sampling. Images were captured on Kodak monochrome negatives and printed on Kodak paper. For quantitative analysis of immunoreactivity, neuronal profiles were counted if they contained one or more SIG particles. This permissive criterion was adopted because there were low overall levels of immunoreactivity accompanied by very low levels of non-specific labeling, as assessed by the complete absence of silver particles visible on the myelin sheaths of axons and in the immuno-
cytochemical control tissue. Altogether, 2,423 and 1,385 \( \mu \text{m}^2 \) were surveyed for the ultrastructural analyses of m1 and m2 mAChR localization, respectively.

RESULTS

The contrast-response function is a sensitive measure of visual gain used to examine visual responsiveness (Albrecht and Hamilton 1982; Sclar et al. 1990; Seguela et al. 1993). To determine the effect of cholinergic agonists on visual gain, we measured the extracellular spiking response to optimized drifting grating stimuli (see EXPERIMENTAL PROCEDURES) of increasing luminance contrast at 63 recording locations: 28 SU; 25 with 2 or 3 units, in multunit clusters, well above background but not individually discriminable (SUC); and 10 with MUA. Recordings were made across layers 2–6 of macaque V1 with and without application of either ACh (1 M, \( n = 38 \)) or CCh (0.25 M, \( n = 25 \)), a cholinergic agonist resistant to acetylcholinesterase (AChE). The 10 MUA recordings retained for analysis were tuned (i.e., there was an optimal stimulus). See EXPERIMENTAL PROCEDURES for a further discussion of the process of discriminating units and recording and analyzing MUA.

Population and Laminar Analyses

Initially, the effect of ACh or CCh on the contrast response was determined using a nonparametric (model free) analysis in which we summed the spike rate to each of 12 stimulus contrasts (spanning 2–96% contrast) and averaged that sum across three repeats (each repeat being 1 sequence of all 12 contrasts, with interleaved blanks). This measure is equivalent to the area under the contrast curve, the “response area,” and does not depend on fitting a model function to the data. This response area was determined at baseline (before drug application) and then again during and after (recovery) iontophoresis of cholinergic agonists. We defined an effect as significant if the response area at the maximum applied iontophoretic current (mean 85.5 nA, median 80 nA, range 20–160 nA) was more than three standard deviations (3 SD) from the mean response area for the immediately preceding baseline (no drug) condition. Suppression was the most prevalent effect. The magnitude of the change in response rate varied and included record-
in response suppression, representing 25% of all recordings and 36% of those outside layer 4c. Only units showing cholinergic effects on driven activity are included in the statistical analysis of response gain below.

ACh (and CCh) enhanced visual gain in layer 4c (mean response area 1.26, SD 0.43, $P = 0.04$, 1-tailed $t$-test). ACh significantly suppressed gain in layers 2–4a (mean response area = 0.57, SD = 0.32, $P < 0.001$, 1-tailed $t$-test) and 5 (mean response area 0.70, SD 0.31, $P = 0.006$, 1-tailed $t$-test). In layers 4b and 6 there was no net change in response area; a mixture of suppression and enhancement across different recordings canceled out in the population analyses (layer 4b: mean 1.07, range 0.43–1.86; layer 6: mean 0.919, range 0.61–1.37). In an earlier study we determined the mechanism behind the response enhancement seen with ejection of nicotine or ACh in layer 4c (Disney et al. 2007). The aim of the current experiment was to determine and characterize the effect of ACh outside layer 4c and to assess the contribution of GABA-ergic mechanisms to that effect. The enhancements seen in layers 4b and 6 are addressed in the DISCUSSION.

The suppression we observed was not an artifact of current ejection. We have shown previously that ejection of saline using currents up to 160 nA results in a small, but nonsignificant, suppression of spiking at ejection currents above 140 nA. The median ejection current required for a significant effect of ACh in the 18 suppressed recordings in this study was 80 nA. In addition, suppressive current effects can be ruled out for

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**Fig. 2.** Prevalence of cholinergic suppression and enhancement differs by cortical layer. Responses were deemed significantly enhanced or suppressed if the average area under the contrast-response curves (response area; see EXPERIMENTAL PROCEDURES) was at least 3 SD above or below baseline. A: a count of the number of recordings that fell into each class (enhanced, open bars; suppressed, black bars; or no effect, gray bars) in each cortical layer. Suppression was found across all layers of cortex, whereas enhancement was concentrated in the input layer (layer 4c). B: the average normalized response area for all cells within each layer. The data used in this average were from the condition where the maximum ejection current was applied; the actual value varies from case to case. Error bars represent SE. C: the suppression was dependent on ejection current and did not recover completely at the highest currents. The averaged normalized response area is shown as a function of ejection current for the 18 cases of suppression. We characterized the “dose-response” profile by ejecting ACh or CCh using an ascending series of ejection currents. Each current condition was always followed by recovery runs in which the contrast-response function was measured repeatedly without iontophoresis of any substance. The recovery data plotted are from the last recovery run collected after each iontophoretic current level. The timing between each plotted drug ejection run (filled squares, run type A) and its corresponding recovery run (open squares, run type R) varies from 10 to 60 min. The number of recordings contributing to each data point ($n$) decreases because we stopped testing higher currents when a “by-eye” assessment indicated that suppression of 60% or greater had been obtained. This was done because, as can be seen in C, at higher ejection currents it became increasingly difficult to achieve a return to baseline response levels. Error bars represent SE.
recordings in which the suppression was blocked using gabazine (see below); adding additional driving current to a second barrel would not abolish a current artifact. At the end of the experiment, if the recording was still stable, we performed current controls in which sodium chloride was ejected at the same current steps as were used in the assessment of the dose-response to ACh. These controls were performed for six suppressed neurons, including the only neuron for which the ACh-mediated suppression was not abolished by gabazine ejection. The mean normalized response area during ACh ejection for these six significantly suppressed neurons was 0.44 (median current 30 nA, range 20–80 nA), the mean response area for these same neurons during the highest level of saline ejection tested was 1.08 (median ejection current 80 nA, range 80–160 nA). For three (of 18) suppressed neurons, the possibility of current artifact cannot be directly ruled out on the basis of the data collected (i.e., there are no current controls because the recording was lost before completion of the gabazine experiment) but is unlikely because the observed suppression persisted after the driving currents were turned off (data not shown). If these three cells are excluded from the laminar analysis reported above, the conclusions of the study are not altered; the normalized response area in the superficial layers (2–4a) changes from 0.66 to 0.67, and the response area for layer 4b changes from 1.07 to 1.15.

Across the entire population, suppressive effects of ACh/CCh were “dose dependent,” i.e., there was a monotonic relationship between drug ejection current and normalized response area (Fig. 2C, filled squares). In addition, the response magnitude during the no-drug recovery trials, particularly for high ejection currents, was nonstationary (Fig. 2C, open squares). This baseline change is discussed below in Baseline Drift. As described in EXPERIMENTAL PROCEDURES, we delivered ACh or CCh at increasing ejection currents but stopped when a “by-eye” assessment indicated that we had achieved ~60% suppression (note the decreasing n in Fig. 2C). We did this because at levels of suppression >60%, recovery of baseline responses became increasingly difficult, and the goal of the study was to determine the prevalence of suppression rather than its magnitude. This experimenter-imposed “floor” on the data was reached most often in layers 2, 3, and 5. When considered alone, the recordings in which ACh (i.e., excluding recordings made with CCh) was used showed no non-monotonic relationship between response area and ejection current; this concentration effect is discussed below.

Concentration-Dependent Effects of ACh

A recent study, also conducted in V1 of the anesthetized macaque (Soma et al. 2012), reported that ACh usually enhances visual responses both within and outside layer 4c, a result that would appear to contrast with our own data showing enhancement within layer 4c and suppression in other layers. Before we turn to the issue of differences in the drug effects observed in the two studies, some differences in baseline response data should be noted. Figure 5 in Soma et al. provides population data on the $R_{\text{max}}$ and $C_{50}$ values of their fits to each cell’s contrast-response data. The data in their Fig. 5 indicate that 70% or more of their population had $R_{\text{max}}$ values of <25 spikes/s and about 9% (6 of 67) had $C_{50}$ values below 30% (it appears that only 1 or 2 cells had $C_{50}$ values below 10%). The unit data from our current and previous (Disney et al. 2007) studies differ from this population. Looking at the 66 unit recordings across our two studies (i.e., excluding MUA recordings), 20% (13 of 66) of our baseline fits resulted in $R_{\text{max}}$ values <25 spikes/s, and 59% (39 of 66) yielded $C_{50}$ values below 25%. If we compare these with the database of previous V1 recordings made in our laboratory using the same stimulus presentation and data collection conditions as the current experiment (M. Hawken and R. Shapley, unpublished data), we find that 23% of the function fits (125 of 540) yield $R_{\text{max}}$ values <25 spikes/s (compared with 70% in Soma et al. 2012). In 193 of 540 cases (36%), the fit yields an $R_{\text{max}}$ value between 25 and 50 spikes/s, and in 222 of 540 cases (41%), the value for $R_{\text{max}}$ is >50 spikes/s. In this same population, 62% (332 of 540, compared with 9% in Soma et al. 2012) of the fits yield $C_{50}$ values <30%, whereas $38%$ (208 of 540) yield $C_{50}$ values ≥30%. The proportion of neurons in our data with low response rates is comparable to that reported in other studies in V1 of awake macaques (Chen et al. 2009; B. G. Cumming, personal communication). It should therefore be kept in mind that although these low baseline response rates are unlikely to explain the different drug effects observed in the studies, the reported differences do occur in the context of a difference in baseline responses.

ACh has concentration-dependent effects that result from the differing affinities of the five muscarinic receptor subtypes (Kuczewski et al. 2005). We used 1 M ACh in our iontophoresis barrels and began ejection at 20 nA; Soma et al. (2012) used 0.5 M ACh, and the highest ejection current they used was 60 nA (median and mean values were not reported, but their figures indicate that currents below 15 nA were frequently used). Previous data suggest a good linear relationship between tissue concentration and barrel concentration/ejection current (Stone 1985), so Soma et al.’s highest current condition (60 nA) corresponds to a point between our lowest conditions (20 and 40 nA), and there is no equivalent in our data set for their current conditions below 20 nA. Nonetheless, when we examine enhancement vs. suppression at the lowest ejection currents we used, we also find that enhancement predominates (Table 1). We analyzed only the 38 recordings in which ACh was used as an agonist, because the facilitation requires AChE action to keep ACh levels low enough to prevent the emergence of suppression (Kuczewski et al. 2005). Using the 3 SD criterion, we saw enhancement in five recordings at low ejection current and in five recordings at high ejection currents (not the same 5 cells), but at low ACh concentration we saw suppression only twice. This increased to 13 recordings at high ACh concentration. These differences are significant by a chi-square analysis ($\chi^2 = 10.439, 2$ df, $P = 0.005$). It is important, however, to note that whereas Soma et al. reported enhanced cells in all layers, we

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Values are no. of recordings (of 38 total) that were enhanced, suppressed, or showed no effect at low vs. high ACh ejection current.
saw enhancement at low ACh concentrations only in layers 5 and 6. In one layer 6 neuron, we saw a switch from significant enhancement at 20 nA to significant suppression at 80 nA (normalized response areas at 20, 40, 80, and 120 nA, asterisks indicate >3 SD from baseline: 1.21*, 1.14*, 0.91, 0.89*). This was a unit with a baseline $R_{\text{max}}$ of 43 spikes/s and a baseline $C_{50}$ of 75%. The authors of the previous study did not, however, propose that the switch from enhancement to suppression must be mediated by effects on the same cells (Kuczewski et al. 2005).

**Response vs. Contrast Gain Effects**

The enhancement of layer 4c responses by ACh is best described as a multiplicative response gain (Disney et al. 2007). To determine the form of the suppression in other layers, we normalized for each recording the spike rate at each contrast by the spike rate obtained for the highest contrast at baseline (before drug ejection). We then averaged these normalized contrast responses (across significantly suppressed cells) and fit them with a Naka-Rushton function (see experimental procedures, Parametric analysis). These fits are shown by the solid black (baseline) and gray (drug) curves in Fig. 3. There are four fit parameters: $R_{\text{max}}$ (asymptote), $C_{50}$ (contrast at the half-maximum response), sFR (level of spontaneous activity), and $n$ (function exponent). In the superficial layers, the population effects of ACh are multifaceted (Fig. 3A): all four fit parameters ($R_{\text{max}}, C_{50}, \text{sFR}$, and exponent) change between the baseline and drug conditions. This result is consistent with both response and contrast gain effects. As to the mechanism, the translation of the curve along the $y$-axis is consistent with a subtractive mechanism. After we correct for this translation (see inset), we continue to see a difference in the function asymptote, suggesting a mixed subtractive/divisive effect. In contrast, when we look at the population data for neurons in the deep layers of V1 (Fig. 3B) and across the suppressed population as a whole (Fig. 3C), $C_{50}$ does not change with ACh/CCh, indicating a response gain effect. The response floor at the level of spontaneous activity (spontaneous firing rate is indicated in Fig. 3 by squares to the left of the function fits in all panels) makes subtractive and divisive mechanisms for this suppression indistinguishable (Ayaz and Chance 2009).

**Mechanism Behind the Observed Suppression**

We have shown previously that AChRs in macaque V1 are rarely expressed by excitatory neurons but are expressed by most GABAergic neurons (Disney and Aoki 2008; Disney et al. 2006, 2007). This anatomical pattern led us to hypothesize that the suppressive effects of ACh in macaque V1 result from increased GABA release. To test this hypothesis, we determined whether a GABA<sub>\alpha</sub> receptor antagonist would abolish cholinergic suppression of V1 neurons’ responses to optimal stimuli. This is, indeed, what we observed. An example single unit from layer 5 is shown in Fig. 4.

There were two series of experiments conducted on this neuron after characterization of optimal stimulus parameters. First, an initial contrast-response function to the optimal stimulus was recorded, with and without ACh iontophoresis at currents from 20 to 100 nA, interleaved with recovery runs (Fig. 4, A–E; recovery runs not shown). Having established a strong suppression at 100 nA, we ran a second series with 100 nA ACh combined with ejection of the GABA<sub>\alpha</sub> receptor antagonist gabazine, using currents ranging from 20 to 80 nA (Fig. 4, G–J).

Figure 4A shows the baseline contrast-response function in which a spontaneous activity of around 20 spikes/s (dashed line) was evident and the low and intermediate contrasts (2–32%) did not drive the neuron. At higher contrasts, the neuron reached a maximum driven response of around 70 spikes/s. As increasing ACh ejection currents were applied (Fig. 4, B–D; 20–80 nA), there was a progressive reduction in the driven and undriven responses. The contrast-response function with ACh at a 100-nA driving current (Fig. 4E) shows a reduction in the spontaneous firing rate to around 5 spikes/s and in the driven response to a maximum firing rate of 40 spikes/s.

We then established that for this cell, a relatively high ejection current (80 nA, which is more than twice the median current used to eject gabazine) of gabazine alone had a minimal effect on the contrast-response function (compare Fig. 4, A and F). The concurrent ejection of ACh at 100 nA with 20 nA for gabazine (Fig. 4G) still yielded a strong ACh effect. As the gabazine ejection current was increased, both the spontaneous and the contrast-evoked responses increased until at 80 nA, the...
Fig. 4. Gabazine blocks cholinergic suppression. A SU example from layer 5 (unit NA18 u004) is shown. In this case, ACh was used as the agonist. A: control contrast response. B–E: ACh ejected alone using increasing ejection currents from 20 to 100 nA. In between each of these ejection-current conditions are recovery runs, which are not shown. As ACh ejection is increased, there is greater suppression. F: the effect of ejecting gabazine alone (i.e., without co-ejection of ACh). The data in F should be compared with that in A in which no substance is being ejected (i.e., the baseline condition), demonstrating that gabazine ejected at 80 nA is not exciting the cell. G–J: the effect of pairing increasing levels of gabazine ejection (from 20 to 80 nA) with ejection of ACh at 100 nA (which as shown in E is strongly suppressive). Gabazine blocks suppression seen with ACh ejection in a dose-dependent fashion. Error bars represent SE. Gze, gabazine.

Co-ejection of gabazine with ACh (Fig. 4/J) yielded no discernible effect on the spontaneous or evoked response (compare Fig. 4, A and J). Note that these drug ejection conditions were all interleaved with recovery periods where no drug was ejected and responses returned to predrug control levels; at this site, recovery took ~3 min (data not shown).

Close examination of Fig. 4 suggests that ACh may have reduced this neuron’s response variability. However, no systematic change in Fano factor was evident across the population.

This entire sequence of recordings was completed for 13 of the 16 recordings in which suppression of visual responses was seen with ACh or CCh. Nine of these were unit recordings (3 SU, 6 SUC), and four were MUA. Four were in layers 2–4a, one in layer 4b, five in layer 5, and two in layer 6, and for one recording, layer information was not recovered. Figure 5A shows the mean normalized response area for each condition in this experiment. ACh suppression was strong (mean normalized response area for ACh condition 0.38, second bar in Fig. 5A, SD 0.2183, $P < 0.0001$, 2-tailed paired $t$-test), and this suppression was almost completely abolished with co-ejection of gabazine (median ejection current 30 nA, range 5–80 nA; mean area for ACh & gabazine condition 0.92, fourth bar in Fig. 5A, SD 0.306, no significant difference from baseline by 2-tailed paired $t$-test).

The levels of gabazine used were themselves not strongly excitatory (mean area gabazine condition 1.01, third bar in Fig. 5A, SD 0.312, no significant difference from baseline by 2-tailed paired $t$-test) at the population level. However, there were individual recordings in which excitation above baseline was observed with gabazine (range of normalized response areas 0.67–1.87). To confirm that the abolition of the ACh suppression could not be obtained by a simple linear combination of two pharmacological effects that were in fact independent, we subtracted, on a cell-by-cell basis and using normalized values, the excitation induced by gabazine ejected alone from the suppression induced by ACh ejected alone. The resulting mean normalized response area was 0.61, SD 0.3122 (ACh + gabazine condition, fifth bar in Fig. 5A). This value is significantly different from baseline ($P = 0.0008$, 2-tailed paired $t$-test), the mean for ACh ejected alone ($P = 0.0015$, 2-tailed paired test), and the mean for ACh and gabazine ejected together ($P = 0.030$, 2-tailed paired $t$-test). Thus the suppression cannot be accounted for solely by a linear combination of the independent pharmacological effects.

As before, we fitted the averaged normalized contrast responses with a Naka-Rushton function (Fig. 5B). There was strong suppression when ACh was ejected alone (Fig. 5B, red). When gabazine was co-ejected with ACh (Fig. 5B, blue), there was little difference from the baseline responses (Fig. 5B, black) or responses to gabazine alone (Fig. 5B, green). Further examination of the summary data reveals that the ACh & gabazine responses (Fig. 5B, blue) were slightly below those for baseline (black) and gabazine alone (green). Along with the normalized response area for ACh + gabazine of 0.92 (above

Fig. 5. Gabazine blocks the cholinergic suppression: population summary. A: the normalized response area for the suppression (averaged across all layers) at the baseline (B), ACh- or CCh-alone (ACh), and gabazine-alone (Gze) conditions, as well as for co-ejection of gabazine and ACh or CCh (AChGze) and for a linear sum of the effects of the 2 agonists ejected alone (ACh+Gze). The data plotted for the Gze- and ACh-alone conditions are for the ejection currents used in the co-ejection (AChGze) experiment. Gabazine alone produced no net increase in response at the current levels used in this experiment. The suppression by ACh alone was reversed when ACh and Gze were co-ejected. This block was seen in 12 of the 13 cases in which the full experiment was performed. Error bars represent SE. B: the normalized averaged contrast response for these 12 recordings under each condition, showing that the form of the contrast-response function is recovered with blockade of GABA_A receptors, not just the area under the curve.
and in Fig. 5A), this suggests there may have been a small ongoing suppression. This may be within expected recording and iontophoresis variability for such an experiment. On a site-by-site basis, suppression by ACh was blocked by gabazine co-ejection in 12 of 13 cases (the 12 recordings in Fig. 5B). For one unit near the layer 3b/4a border (assigned to layer 4a), the gabazine block failed entirely (data not shown).

The lack of a suppressive effect when ACh was co-ejected with gabazine was neither a current nor a pH effect. The average summed current across the barrels in the ACh/gabazine co-ejection condition was 111 nA (median 110 nA, range 50–180 nA). We have shown previously that ejection of a pH 3.0 solution of 0.9% saline using unbalanced currents up to 160 nA does not increase spike rate (Disney et al. 2007). If anything, the effect is suppressive. The ACh and gabazine solutions, pH 4.0–4.5, were less acidic than this control saline solution. In two recordings, the summed current for the co-ejection experiment was >160 nA (170 and 180 nA); in these cases, an opposing current (~170 and ~180 nA, respectively) was applied to a “balance barrel” that contained 0.9% saline at neutral pH. This balancing current was used in some, but not all, of the experiments with summed currents below 160 nA (in some cases, balancing was not possible due to a barrel block on the saline channel). No difference was seen between balanced and unbalanced recording runs when both were applied on the same units (data not shown).

Gabazine ejected alone did not change the mean normalized response area across the population (Fig. 5). The lack of a net gain effect for gabazine was the result of our having titrated the ejection current to achieve this effect. Our ejection current values (median 30 nA, mean 32 nA, range 5–80 nA) can be compared with those from a recent study in which the same Carbostar iontophoresis electrodes were used to record and eject in cat V1 (Katzner et al. 2011). In that study, gabazine effects were the focus of the experiments; a higher concentration was used (10 mM), and the drug was ejected using higher currents (50–150 nA). Although the levels of gabazine used for ACh co-ejection in the present study did not evoke an increase in contrast responses, when we used higher ejection currents in a complementary set of experiments on area tuning and in determining the optimal conditions for this co-ejection, we did evoke response gain-like effects in many units (Fig. 6). This gabazine effect on response gain has been previously reported (Katzner et al. 2011).

The above analyses combine SU, SUC, and MUA recordings. There were 28 clearly isolated single units in our data set and in Fig. 7 we reproduce the main analyses of these experiments for the single units alone. Cells for which track reconstruction was unsuccessful are included in this analysis under a category “u” (layer unknown). We also eased our significance testing criterion from 3 SD to 2 SD. This 95% confidence interval more closely matches that used in previous single-unit studies with which these data might be compared. In Fig. 7A, it can be seen that for the single units, there was only enhancement in the middle layers of cortex (layers 4b and 4c), only suppression in the deep layers (layers 5 and 6), and a mixture of enhancement and suppression in the superficial layers (layers 2–4a). This can be compared with Fig. 2A, in which the combined single- and multiunit data are assessed against a 3SD criterion and show a similar pattern of enhancement in the middle layers and mixed effects dominated by suppression elsewhere. This pattern of relative enhancement of layers 4b and 4c is clearer in the layer averaged data for the normalized response area (Figs. 2C and 7B). In the case of four single units, the entire experiment, including gabazine characterization, co-ejection, and recovery, was completed without losing unit isolation. These data are presented in Fig. 7C, in which it can be seen that, as for the population as a whole, gabazine was not itself exciting (average normalized response area 1.08), but was effective in blocking (average normalized area 1.08), the ACh-mediated suppression (average normalized area 0.31). Comparison of Figs. 5A and 7C shows that the results for single-unit recordings alone and the combined SU/SUC/MUA analysis are very similar.

Low Levels of Muscarinic Receptor Expression on Glutamatergic Axon Terminals

The primary competing explanation for the suppression that we observed would be that the mechanism reported in many cortical areas of the rat is also operating in V1 of the macaque. Suppression by ACh in the rat has been shown to be largely the
result of decreased glutamate release, following the activation of m2-type (G_{i} coupled) mAChRs expressed on the axons themselves (Gil et al. 1997; Hasselmo and Bower 1992; Hsieh et al. 2000). This reduced drive to excitatory neurons could lower the inhibitory tone without activation of mAChRs on inhibitory neurons themselves. To further examine the possibility that m2 AChRs might be reducing glutamate release, despite low levels of somatic immunoreactivity for m2 AChRs in excitatory neurons (Disney et al. 2006), we used immunoelectron microscopy to quantify expression of m2 (and m1)-type mAChRs at asymmetric (putatively excitatory) synapses across layers 2 and 3 of macaque V1, where the suppression observed in vivo was most prevalent (5 of 12, or 42% of recordings at a 0.01 criterion) and most profound (the experimenter-imposed floor of 60% suppression was applied most often in layers 2 and 3).

Micrographs of the tissue/EPON interface in layers 2 and 3 were taken at ×40,000 magnification and quantified off-line. All encountered synapses were included in the analysis. In the tissue processed to visualize the m1 AChR, there were 546 asymmetric synapses, 27 (4.9%) of which had membrane-associated presynaptic silver particles indicating the presence of an m1 AChR on the axon terminal. A further 15 (2.7%) asymmetric synapses had cytoplasmic (but not membranous) presynaptic m1 immunoreactivity. Thus a total of 42 (of 546, or 7.7%) asymmetric synapses expressed presynaptic m1 AChRs.

In the tissue processed to visualize the m2 AChR, there were 477 asymmetric synapses, 17 (3.6%) of which had membrane-associated silver on the presynaptic element. Eight (1.7%) asymmetric synapses were immunoreactive for m2 AChRs in the cytoplasm only. This gives 25 of 477 (5.2%) asymmetric synapses expressing presynaptic m2 AChRs. These numbers are similar to our previously reported somatic counts for these receptor types: 8% of excitatory somata for m2 and 10% for m1 (Disney et al. 2006).

**Baseline Drift**

For some neurons, responses during the “recovery” period did not return to baseline levels (Fig. 3, open squares). This nonstationarity was not observed in our previous study in which nicotine was used as a selective agonist for nAChRs (Disney et al. 2007). In a site-by-site analysis, we also found this “baseline drift” in recordings where ACh/CCh had had no significant effect on visual responses during drug ejection (the 36 "no effect" recordings). Figure 8A shows that no-drug response areas tended to drift upward (i.e., toward normalized response areas >1; Fig. 8A, triangles) in these otherwise no-effect or “unresponsive” recordings and downward (i.e., toward normalized response areas <1; Fig. 8A, circles) where ACh or CCh had an effect during drug ejection (regardless of whether that effect was a suppression or enhancement).

We again used a criterion of 3 SD of the initial (before any drug was ejected) response area to identify significant baseline

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**Fig. 7.** Data for 28 SUs. SUs were deemed significantly enhanced or suppressed if the average area under the contrast responses was at least 2 SD above or below the baseline average. Note that this criterion is not the same as that used for the population in Fig. 2A, where an effect was deemed significant if it changed the response by 3 SD. A: a count of the number of units that fell into each class (enhanced, open bars; suppressed, black bars; or no effect, gray bars) in each cortical layer. As was observed in the combined single- and multiunit data (Fig. 2), suppression was found in the supra- and infragranular layers of cortex, whereas enhancement was concentrated in the middle layers. B: the normalized average response area for all SUs within each layer. For all units, the data used in this average were from the condition where the maximum ejection current was applied; the actual value varies from recording to recording. C: the normalized response area for suppressed units in the baseline (B), gabazine-alone (Gze; response area 1.08, SD 0.18, no significant difference from baseline), and ACh- or CCh-alone conditions (ACh; response area 0.31, SD 0.21, P = 0.008, 2-tailed paired t-test) and for co-ejection of gabazine and ACh or CCh (ACh&Gze; response area 1.08, SD 0.43, no significant difference from baseline, significant difference from ACh alone P = 0.04, 2-tailed paired t-test). A linear sum of the conditions in which ACh and Gze were ejected alone cannot account for the abolished suppression (data not plotted; response area 0.42, SD 0.17, significant difference from co-ejection, P = 0.04, no significant difference from ACh alone). The data plotted for the Gze- and ACh-alone conditions are for the ejection currents used in the co-ejection (ACh&Gze) experiment. Gabazine alone produced no net increase in response at the current levels used in this experiment. The suppression by ACh alone is reversed when ACh and Gze are co-ejected. This block was seen for all 4 SUs for which the full experiment was performed. Error bars in B and C represent SE.
Cholinergic agonists act locally when they are applied by iontophoresis. However, in vivo ACh release probably occurs globally by volume transmission (Aoki and Kabak 1992; Descarries et al. 1997; Umbriaco et al. 1994). In vivo, ACh release would enhance the visual responses in layer 4c via presynaptic nAChRs on thalamic afferents (Disney et al. 2007). These enhanced responses in turn feed forward to neurons in other layers. Simultaneously, ACh will, in these other layers, increase inhibitory tone (present study). To address the co-action of these two apparently opposing effects, we sought to predict the resultant tuning from an initial facilitation of the responses at the input to the cortex, followed by a suppression that is contingent on the increased responsiveness of inhibitory interneurons (Fig. 9A).

As an example, we consider how the two stages of orientation selectivity in V1, tuned excitation and untuned suppression (Ringach et al. 2003; Xing et al. 2011), might change with ACh release. We first consider the influence of ACh on input layer neurons. We have shown previously that nicotine alters contrast responses in layer 4c and that this effect could best be described as a multiplicative increase in response gain (Fig. 9, B and C). Typically, the contrast-response function has been modeled by a saturating function with a fixed value of the maximum response (Albrecht and Hamilton 1982). However, in the case of ACh facilitation of layer 4c responses, there is a clear increase in the maximum response rate. Often, this increased responsiveness is accompanied by a change in slope (Disney et al. 2007). To confirm that this nAChR-dependent response enhancement can be accounted for by using a single multiplicative constant, we measured orientation tuning in layer 4c with and without nicotine ejection. We then took the ratio of the mean spiking response across the two directions of gratings at the optimal orientation in the baseline (no drug, Fig. 9B) and nicotine conditions (Fig. 9C, gray-shaded circles and solid lines). We applied this ratio to all the responses in the baseline condition, giving a simulated orientation tuning response (Fig. 9C, solid circles and dashed lines). The simulated (black) and actual (gray) nicotine responses in Fig. 9C are very similar, supporting the idea that the nicotinic effect can be thought of as a global multiplicative constant across all stimuli (not just the optimal stimulus used in the contrast gain experiments).

We then consider how the suppressive effects of ACh in layers 2 and 3 (which will receive this enhanced input from layer 4c) might influence orientation selectivity. We modeled this suppression as a subtractive constant and chose a value that returned responses at the nonpreferred (orthogonal) orientation to the baseline level of spontaneous activity (Fig. 9D). We note, however, that an underlying divisive shunting inhibitory mechanism can appear as subtractive on the spiking response of neurons (Holt and Koch 1997) while the inhibitory mechanism may be divisive at the membrane level. The final tuning function in layer 2/3 (Fig. 9D, dashed gray line, simulated) shows that cholinergic modulation in macaque V1 has the potential to enhance responsiveness at the peak of the tuning function without a concomitant loss of tuning selectivity. The effect of ACh on orientation tuning during simultaneous ACh application in the input and superficial layers has not been studied in any species so far; our model offers a clearly testable hypothesis for such an experiment.

DISCUSSION

We observed a prominent visual response suppression via increased GABA release in macaque V1. This is consistent with anatomical data showing that in macaque V1, GABAAergic neurons are more likely to express GABA receptors (muscarinic and nicotinic) than are excitatory neurons (Disney and Aoki 2008; Disney et al. 2006, 2007). Locally applied ACh is suppressive at 36% of V1 recordings outside layer 4c, and blocking GABA receptors virtually eliminates this suppression (Fig. 5). Nicotinic responses outside layer 4c are rarer in V1 (Disney et al. 2007): about 10% of neurons show suppression. Although

Fig. 8. The “baseline” response can drift following cholinergic agonist exposure. Interleaved between drug ejection trials, we made repeated measures of the contrast-response function in the absence of drug ejection. This was done to assess the extent to which we had recovered baseline responses following drug exposure. A and B both plot the normalized response area for the “recov- ery” runs that were measured during an ascending series of drug ejection currents. The data plotted show the response area without drug ejection, measured between 10 and 60 min after the last drug ejection run (i.e., the final recovery run before proceeding to the next ejection level). The values on the x-axis give the preceding drug ejection current. A: the average magnitude of baseline drift for the 27 cases in which ACh/CCh had an effect during drug ejection (open circles) and for the 36 cases where the agonists did not have a significant effect during drug ejection (open triangles). The no-drug response area was lower when ACh (or CCh) had a significant effect (previously referred to as “enhanced” and “suppressed”) and was dependent on prior ejection current. B: the same data in A, divided into groups based on significance testing on the drift effect itself. Eleven cases showed a significant elevation in response area relatively independent of ejection current (upward drift, open squares), whereas 8 cases showed decreases in response area (downward drift, open circles). In the remaining 42 recordings, there was no change (no drift, open triangles).

Descriptive Model

Cholinergic agonists act locally when they are applied by iontophoresis. However, in vivo ACh release probably occurs globally by volume transmission (Aoki and Kabak 1992; Descarries et al. 1997; Umbriaco et al. 1994). In vivo, ACh release would enhance the visual responses in layer 4c via presynaptic nAChRs on thalamic afferents (Disney et al. 2007). These enhanced responses in turn feed forward to neurons in other layers. Simultaneously, ACh will, in these other layers, increase inhibitory tone (present study). To address the co-action of these two apparently opposing effects, we sought to predict the resultant tuning from an initial facilitation of the responses at the input to the cortex, followed by a suppression that is
we did not pharmacologically dissect the suppression itself, on
the basis of the frequency of suppression to ACh/CCh com-
pared with that to nicotine (Disney et al. 2007), we propose that
the suppression by ACh is mediated largely by mAChRs.
Nonetheless, it will be important to determine the relative
contribution from the different receptor classes in future inves-
tigations. Within layer 4c, the predominant nicotinic effect is
an enhancement of thalamocortical transmission (Disney et al.
2007). This has been reported across species and sensory
systems, with the exception of olfaction (Hasselmo and Bower
2006). It is also unlikely that a substitution of the alternate
class of AChRs. We have reported previously that only 8%
of excitatory neurons in V1 express m2 AChRs (Disney et al.
2006). It is also unlikely that a substitution of the alternate
class of G_{i}-coupled muscarinic receptor has taken place in
macaque V1, because expression of the m4 receptor is also
low, in fact, lower than that of the m2 type (Tigges et al. 1997).
As shown by additional data we present here, the reduction
of glutamate release can be firmly rejected as the mechanism for
ACh-mediated suppression in macaque V1 on the basis of two
converging lines of evidence. We have shown that m2 AChRs
are expressed presynaptically at only 5% of glutamatergic
terminals in layers 2 and 3. On the basis of anatomical data
alone, it is unlikely that a suppression seen in 42% of record-
ings in layers 2–4a (Fig. 2A) could be mediated by a receptor
found on 5% of glutamatergic synapses or 8% of glutamatergic
somata. It is more plausible that the modulatory effects are
mediated by the 66% (~50% in layers 2 and 3) of inhibitory
neurons that express AChRs. These suggest anatomical
observations can now be combined with the present finding
that in 12 of 13 cases, suppression by ACh is abolished by
blocking inhibition (Figs. 4 and 5). This argues strongly that
the predominant mechanism behind cholinergic suppression in
macaque V1 is enhanced inhibition, not suppressed excitation.
Our finding that inhibition is a primary target for modulation
of visual processing by ACh is predicted not only by our own
anatomical data but also by a recent model of cholinergic
modulation in the context of a visual attention task by Deco
and Thiele (2011).

Species Differences in Cholinergic Mechanisms

We now have anatomical and physiological evidence that
inhibition is the primary target for cholinergic neuromodu-

Fig. 9. Integrating the 2 stages of cholinergic modulation. We present a conceptual model that considers the effects of the apparently opposing actions of ACh toward enhancing tuning of visual responses in macaque V1. The model provides a prediction of the tuning expected from an initial facilitation of the responses at the input to the cortex, followed by a suppression that is contingent on the increased responsiveness of inhibitory interneurons. A: outline of the conceptual model. In the first stage (left), there is a multiplicative increase in responsiveness that results from the activation of nicotinic ACh receptors located presynaptically on axons arriving in layer 4c from the lateral geniculate nucleus (LGN). This enhanced response is fed through to the next processing layer. In the second stage of the model (right), for example, in layer 2 or 3 of cortex, we introduce a suppressive component that corresponds to the observed results of applying ACh throughout the cortex. Layer 2/3 principal cells integrate the enhanced feedforward signal from layer 4c and the suppressive effects of ACh, which are mediated by inhibitory interneurons. CRF, classical receptive field. B: the measured orientation tuning for an isolated unit in layer 4c. C: these baseline responses are multiplied by a constant (black dashed line and circles; see RESULTS for how we obtained the multiplier), and the result is compared with the actual measured response of the neuron to ejection of nicotine (gray lines and circles). D: gray circles and dashed lines show the result of applying a subtractive constant to the simulated responses (i.e., to the data presented in black in C). The value of this constant was chosen such that it simply returned the response at the nonpreferred orientation to the level of spontaneous activity (dotted horizontal line) prior to the multiplication stage (for comparison, the baseline data from B are replotted in black). The resulting tuning shows a strikingly enhanced response (over baseline) at the optimal orientation but no change in bandwidth or optimal/orthogonal response ratio.
tion in macaque V1 outside layer 4c. There is also evidence that ACh increases inhibitory strength in cat (Erisir et al. 2001; Muller and Singer 1989) and guinea pig (McCormick and Prince 1986). Existing physiological data predict different results if the current experiments were repeated in rat V1. First, effects mediated by direct modulation of excitatory neurons would be more prevalent in rats, with 25–95% (depending on layer) of pyramidal neurons being ACh responsive (Gulledge et al. 2007). This direct modulation is rare in macaque V1 because so few excitatory neurons express AChRs. In addition, in rat V1, parvalbumin-immunoreactive (PV-ir) neurons either do not respond to (Gulledge et al. 2007) or are hyperpolarized by ACh (Xiang et al. 1998). In either case, the result would not be increased inhibitory tone. PV-ir neurons comprise about 50% of inhibitory neurons in rat V1 (Gonchar and Burkhalter 1997). In contrast, 74% of inhibitory neurons in macaque V1 express PV (Van Brederode et al. 1990), and the vast majority also express AChRs (Disney and Aoki 2008), as do most calbindin and calcerein neurons (Disney and Aoki 2008). In rats, cholecystokinin- and vasoactive intestinal polypeptide-immunoreactive neurons, most of which are GABAergic, do depolarize and spike in response to CCh (Kawaguchi 1997). These somewhat overlapping populations comprise perhaps 25% of inhibitory neurons in rat neocortex (Gonchar and Burkhalter 1997). Many of these cells are dendrite targeting and might be expected to influence input integration rather than control spike rate. Overall inhibitory tone has not been reported to increase with ACh release in the rodent cortex. Increased inhibitory tone in response to ACh has been observed in rat hippocampus (Cea-del Rio et al. 2010; Lawrence 2008), but the source and drivers of the cholinergic afferents there are different, as is the cortical structure, limiting its suitability as a model for primate neocortex.

Prevalence of Cholinergic Effects

Between studies, there is a range in the reported prevalence of ACh effects. This may be attributable in part to the criterion used to determine significance of effects and in part to the anesthetic agent used. We found no effect of ACh in 57% of our recordings. This is ~10–15% higher than in previous studies of marmoset V1 (Herrero et al. 2008; Roberts et al. 2005; Zinke et al. 2006) and much higher than has been reported for cats (Sato et al. 1987; Sillito and Kemp 1983). The higher rate of “responsiveness” in cats probably results from differences in significance testing. In those studies, a cell was deemed significantly modulated when its firing rate changed by a constant amount from baseline, without reference to baseline variability. The previous marmoset studies did account for variability in determining significance and set a 95% confidence interval. We used 99% confidence in the current study; if we ease our criterion to 95%, we obtain a “no-effect rate” of 40%, similar to these previous primate studies.

We found similar rates of suppression (28% of recordings) compared with earlier studies (Roberts et al. 2005; Sato et al. 1987; Sillito and Kemp 1983; Zinke et al. 2006) with the exception of the recent study in macaque V1 (Soma et al. 2012). Past studies in cats have reported suppression by ACh in between 16% and 31% of cells (Muller and Singer 1989; Murphy and Sillito 1991; Sato et al. 1987; Sillito and Kemp 1983). Studies in marmosets have reported suppression in 21% of cells (Roberts et al. 2005; Zinke et al. 2006). Our finding of 28% of sites suppressed is similar to these previous studies and unlike the recent report of 8% of sites suppressed (Soma et al. 2012). We do find a lower prevalence of enhancement (14%, compared with 34–39% in past studies in marmosets and 35–74% in cats). One explanation for this may be anesthesia, not the fact of anesthesia but the agents used. These previous studies all used anesthetic agents that antagonize AChRs: halothane in cats and saffan in marmosets (Mori et al. 2001; Puil and el-Beheiry 1990), and in the marmoset studies, agents (saffan and propofol) were also used that potentiate GABA, receptors (Lambert et al. 1995; Puil and el-Beheiry 1990; Trapani et al. 2000). Thus the modulatory system under study in the cat experiments and both the modulator and one of its primary effectors in the primate experiments were interacting with the mode of anesthesia. We used sufentanil, which is a μ-opioid agonist and has not been reported to interact directly with ACh or GABA receptors. This comment does not apply to the study by Soma et al. (2012) in which some recordings were made under pure fentanyl anesthesia. It also does not apply to a recent study of the awake macaque cortex by Herrero et al. (2008). The authors of the latter study did not directly report the number of neurons enhanced by ACh alone but reported that 55% of neurons altered their responses in the context of an attention task when ACh was applied. Given that some population of neurons in that study did not show attentional modulation in the first place, this proportion is a lower bound on the prevalence of excitation in response to ACh in the awake animal. Thus the prevalence of enhancement we report is lower than was reported in the awake animal in a study that also used much lower ACh concentration (0.1 M) and ejection currents (median 15 nA) than were used in the current study. The difference between the two studies could well be the result of the concentration dependence of ACh effects. This is probably also the primary explanation for the differences between the current study and that by Soma et al. (2012).

The idea that cholinergic signaling is concentration based is not new (Descarries et al. 1997). In particular, an interesting model was recently proposed (Hasselmo and McGaughy 2004) in which ACh concentration sets up circuit dynamics that facilitate memory consolidation and/or retrieval (at low ACh concentrations) or attention/memory encoding (at high ACh concentrations). Microdialysis studies have reported a wide range of basal ACh levels on cortex, from 0.5 nM to 3 μM (Mattinson et al. 2011), so the current study and the investigation by Soma et al. (2012) probably both fall within “naturalistic” levels of ACh exposure for the tissue, based on a transport number of 0.3–0.5 for 1 M ACh and high-resistance barrels resulting in a release of ~1.67 pmol/μC (Bradley and Candy 1970) for 1 M ACh and slightly less for 0.5 M ACh. The suggestive data we present on concentration effects clearly call for a comprehensive study of the relationship between ACh concentration and facilitation vs. suppression of spiking. The purpose of the current study, however, was to determine the mechanism behind suppression, when suppression is observed, not to determine the “true” prevalence or magnitude of suppression under conditions of natural release. Temporal limitations on microdialysis sampling mean that phasic ACh release has never been measured, so the problem of matching iontophoretic release to the natural range of concentrations ACh is underconstrained.
Baseline Shifts

In a number of our recordings, the normalized response area did not recover back to baseline levels in the absence of drug ejection (referred to above as “baseline drift”). ACh has a known role in cortical plasticity (Giocomo and Hasselmo 2007; Kilgard 2003; Weinberger 2007) involving baseline shifts (Brown and Adams 1980). Perhaps it is an early process related to this function that we observed in this study. We did not observe this phenomenon in our previous study in which we used nicotine as an ACh receptor agonist. Some of the neurons reported in this study as drifting with ACh ejection were also included in that previous study (Disney et al. 2007), so we can state that these individual neurons do not show altered baselines with exposure to nicotine, only with ACh. This may suggest an effect mediated specifically by mAChRs. However, drift occurred more often with ACh than with CCh; 75% of neurons showing downward drift and 82% showing upward drift were tested using the endogenous ligand. This observation may indicate an involvement of the low-affinity nAChR. CCh differs from ACh in its resistance to AChE, and as a result, the breakdown product, choline, will be present at lower levels in tissue exposed to CCh than in tissue exposed to ACh. The homomeric α7 nAChR is unique among AChRs in that it can bind choline (Mike et al. 2000) and is thus a possible contributor to any differences seen when local choline levels differ.

Lack of Strong Excitation by Gabazine

For each experiment, gabazine ejection was titrated to avoid large changes in spike rate. This was important because the possibility that our results could be explained by a linear combination of two independent pharmacological effects needed to be ruled out. The fact that this worked, however, is initially surprising; if the amount of gabazine ejected was so small that it did not increase visually evoked responses, what could be the mechanism behind that level being nonetheless sufficient to block the effects of cholinergic modulation? We think the most likely explanation is that the GABA_A receptors that are being blocked by gabazine are not usually contributing strongly to visual response gain. This implies that the GABA_A receptors activated are distinct and separable from those mediating inhibitory tone during less attentive visual stimulation. This could happen if GABAergic synapses with low probability of release are being activated, or if the GABA_A receptors themselves are extrasynaptic. Preferential gabazine antagonism at extrasynaptic receptors could result from physical exclusion from the synapse or because gabazine is a competitive antagonist; at low concentrations, it may not effectively compete for binding given what is probably saturating release of GABA (Mody et al. 1994).

If ACh is excitatory for GABAergic neurons, then some of the neurons in this study whose rates increased with ACh application may have been inhibitory. Although waveform analysis has been used to identify putatively inhibitory neurons (at least narrow-spike neurons) in some studies, waveform data in mammals other than rodents do not reliably distinguish inhibitory from excitatory neurons (Brumberg et al. 2000; Cardin et al. 2005; Gray and McCormick 1996; Nowak et al. 2003; Vigneswaran et al. 2011). In addition, there is anatomical evidence that in macaque V1, some excitatory neurons carry channels that can confer the ability to generate a narrow spike (Constantinople et al. 2009), and although we know that PV-ir neurons express mAChRs in macaque V1 (Disney and Aoki 2008), so do other (probably broad-spike) inhibitory neuron classes. It would be useful to find a method to reliably distinguish excitatory from inhibitory neurons in macaque V1 extracellular recordings; currently this is not possible.

Even if the cell under study can be identified, it is important to note that effects seen with iontophoresis in vivo cannot be attributed to direct action at receptors expressed by that identified and recorded cell, because the drug can diffuse away and influence a volume of tissue around the electrode tip. The distances involved are usually assumed to be on the order of 100–200 μm, but effective drug concentrations at distances up to 600 μm have been observed with prolonged iontophoretic ejection times (~10 min, much longer than were used in this study; Candy et al. 1974; Curtis et al. 1960; Stone 1985). Diffusion of compounds in brain tissue and the resulting local drug concentration are a function of the diffusion coefficient for that molecule and time, and the diffusion coefficient depends on molecular weight. ACh and CCh have similar molecular weights; however, in considering ACh, there will also be ACh breakdown by AChE, the distribution of which is not well-described for the central nervous system. The molecular weight of gabazine is more than two times that of ACh and CCh. Thus it is likely that for a given ejection current and duration, the drugs used in this study will have diffused over different distances from the electrode tip. The fact that higher ejection currents (80 nA and above, leading to high initial concentrations near the electrode tip) were needed to observe suppressive effects of ACh and CCh supports the conclusion that receptors on neuronal elements at some distance from the tip were involved. It is unlikely that the gabazine diffused over a matched volume, given the low ejection currents we used and the high molecular weight of the compound. This could have been the reason behind the small residual suppression observed in our recordings with combined iontophoresis of ACh and gabazine.

We cannot, however, with the techniques we used, distinguish between an ongoing suppression mediated by reduced glutamate release, as has been observed in other model systems, and an inability to get the two pharmacological agents bound to the necessary matched population of the receptors mediating the observed effects on inhibitory tone (AChRs on inhibitory neurons, probably at the soma, and GABA_A receptors at inhibitory synapses on excitatory neurons). The persistent suppression could also indicate an involvement of GABA_B receptors, which would not have been blocked by gabazine and could still contribute some response suppression. We ruled out a simple linear combination of independent effects as the explanation for the abolished suppression (the simple sum of the ACh- and gabazine-alone conditions was significantly different from the combined iontophoresis of ACh with gabazine). We cannot rule out more complex interactions between the two drugs and/or the populations of neurons and receptors they influenced; an examination of the cellular mechanisms behind the effects we observed, and the network interactions that result from these drugs being applied, would be best undertaken in an in vitro preparation.
Modulation of Inhibition vs. Excitation

Some suppression by ACh is reported in all species so far studied and is consistent with effects of elevating ACh levels in humans (Silver et al. 2008). We show that, as predicted by anatomical data, in macaque V1, this suppression is dominant and mediated by inhibition. How this modulation affects cortical function depends on the role ascribed to cortical inhibition; a full discussion of the role of inhibition in cortex is beyond the scope of this article. In the specific case of cortical receptive fields, we have applied a model for modulation to orientation tuning where we propose that the enhanced suppression maintains selectivity (Fig. 9) while allowing increased responsiveness. It has been proposed that cortical cholinergic modulation underlies attention (Arnold et al. 2002; Deco and Thiele 2011; Hasselmo and McGaughy 2004; Herrero et al. 2008; Himmelheber et al. 2000; McGaughy and Sarter 1998; Sarter et al. 2005). It also has been argued that changes in GABAergic inhibition could account for response gain changes seen in V1 during attention tasks (Katzner et al. 2011). The ability of ACh to modulate inhibition and enhance thalamic transmission (Disney et al. 2007) in V1 thus provides a plausible mechanism for response gain changes in visual attention without loss of selectivity for stimulus properties.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


