Cholinergic suppression of visual responses in primate V1 is mediated by GABAergic inhibition Anita A. Disney, Chiye Aoki and Michael J. Hawken

J Neurophysiol 108:1907-1923, 2012. First published 11 July 2012; doi: 10.1152/jn.00188.2012

You might find this additional info useful...

- This article cites 86 articles, 35 of which you can access for free at: http://jn.physiology.org/content/108/7/1907.full#ref-list-1
- Updated information and services including high resolution figures, can be found at: http://jn.physiology.org/content/108/7/1907.full
- Additional material and information about *Journal of Neurophysiology* can be found at: http://www.the-aps.org/publications/jn

This information is current as of October 2, 2012.

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

Submitted 2 March 2012; accepted in final form 10 July 2012

Disney AA, Aoki C, Hawken MJ. Cholinergic suppression of visual responses in primate V1 is mediated by GABAergic inhibition. J Neurophysiol 108: 1907–1923, 2012. First published July 11, 2012; doi:10.1152/jn.00188.2012.—Acetylcholine (ACh) has been implicated in selective attention. To understand the local circuit action of ACh, we iontophoresed cholinergic agonists into the primate primary visual cortex (V1) while presenting optimal visual stimuli. Consistent with our previous anatomical studies showing that GABAergic neurons in V1 express ACh receptors to a greater extent than do excitatory neurons, we observed suppressed visual responses in 36% of recorded neurons outside V1's primary thalamorecipient layer (4c). This suppression is blocked by the GABA_A receptor antagonist gabazine. Within layer 4c, ACh release produces a response gain enhancement (Disney AA, Aoki C, Hawken MJ. Neuron 56: 701-713, 2007); elsewhere, ACh suppresses response gain by strengthening inhibition. Our finding contrasts with the observation that the dominant mechanism of suppression in the neocortex of rats is reduced glutamate release. We propose that in primates, distinct cholinergic receptor subtypes are recruited on specific cell types and in specific lamina to yield opposing modulatory effects that together increase neurons' responsiveness to optimal stimuli without changing tuning width.

visual cortex; neuromodulation; muscarinic receptor

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).

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 Mc-Gaughy 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 cholinoceptive neurons, those immunoreactive for β 2 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

Address for reprint requests and other correspondence: A. A. Disney, Systems Neurobiology Laboratory, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037 (e-mail: anita@salk.edu).

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 somatargeting 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_A 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_A 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 mAChR 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 $\mu g k g^{-1} h^{-1}$, and a craniotomy was made over V1. Animals were then paralyzed with vencuronium bromide (100 μ g·kg⁻¹·h⁻¹), and anesthesia was maintained with sufertanil (6–24 μ g·kg⁻¹ h⁻¹). Depth of anesthesia was assessed by continuously monitoring electroencephalogram (EEG), end-tidal CO2, and heart rate. Extracellular recordings were made using a six-barrel combined iontophoresis/physiology electrode (Carbostar, Kation Scientific), advanced through the tissue using a motorized microdrive (Narishige, Japan). The signal from the single carbon 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 "hash." 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 multiunit 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 GABAA 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 $1-2 \mu m$) 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 grating stimuli (Xing et al. 2005). MUA sites were abandoned if the tuning was not tight enough to allow determination of a best stimulus. A drifting grating, optimized for orientation, spatial and temporal frequency, and area (classical receptive field stimulation only), was then selected and the contrast response measured in 12 logarithmic steps, sequentially increasing the contrast from 2 to 96%, alternating with blank (mean gray) stimuli (i.e., a blank was presented after every grating stimulus). The contrast response across these same values was then measured accompanied by iontophoretic ejection of ACh or CCh. Constant-current ejection started concurrently with visual stimulation. Ejection times never exceeded 90 s without a period of recovery (minimum 3 min). Ninety seconds was the duration of stimulus presentation required for measurement of the contrast-response function over three repeats and was the same for every recording. A baseline contrast response was recorded before each ejection period, and three or more "recovery" contrast-response profiles were recorded after drug application. We refer to this as a "baseline-run-recover" sequence. This series of recordings was repeated with increasing ejection currents, beginning at 20 nA up to a limit of 160 nA (always with interleaved recovery). The sequence of dose conditions was: 20, 40, 80, 120, and 160 nA. Occasionally, a 10-nA condition was included. If spike rates appeared to be suppressed by the drug ejection, the limit for current ejection was 160 nA, or >60% suppression of the response at 96% contrast. We set no upper limit for excitation. The 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- μ m 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. The next day, after PBS rinses, the sections were reacted for 15–20 min using the ABC-diaminobenzidine (DAB) technique (Hsu et al. 1981). Laminar reconstruction was done by camera lucida aided by cytochrome oxidase histochemistry (Wong-Riley et al. 1998) with a cresyl violet counterstain for Nissl bodies.

Parametric analysis. For each condition (baseline, run, recovery), responses (R) were averaged across three repeats of the increasing sequence of stimulus contrasts (C). A hyperbolic ratio function (Naka and Rushton 1966),

$$\mathbf{R} = \mathbf{R}_{\max} \frac{C^n}{C^n + C_{50}^n} + \mathrm{sFF}$$

was fit to these data, and the parameters R_{max} (maximum response), C_{50} (contrast value at half-maximum response), and *n* (exponent) were obtained, along with the parameter sFR, capturing the offset of

this equation attributable to spontaneous firing (see Disney et al. 2007 for further details).

Electron Microscopy

Animals. Two adult (4.2 and 4.9 kg body wt) male rhesus monkeys (*Macaca mulatta*) were used following chronic electrophysiology experiments (Platt and Glimcher 1997). Procedures for our own experiments and those of our donor laboratory were approved by the Institutional Care and Use Committee for NYU, in accordance with NIH guidelines.

Histological preparation. Animals were deeply anesthetized by intravenous pentobarbital sodium and transcardially exsanguinated with heparinized lactated Ringer, followed by 4 liters of chilled fresh 4% paraformaldehyde-0.25% glutaraldehyde (both from Electron Microscopy Services) in 0.1 M PB. The visual cortex was blocked at the lunate sulcus, postfixed overnight in 4% paraformaldehyde at 4°C, and then vibratome-sectioned at 40- μ m thickness. After the sections were reacted in 1% sodium borohydride (Sigma) in 0.1 M PB, a cytochrome oxidase reference set was prepared (Wong-Riley et al. 1998) and the remaining sections were stored at 4°C in 0.01 M PBS with 0.05% sodium azide (Sigma).

Immunoelectron microscopy. The antibodies directed against the m1 and m2 AChR used in this study have been characterized thoroughly for their specificity (Levey et al. 1991). A freeze-thaw technique was used to improve antibody penetration (Wouterlood and Jorritsma-Byham 1993), and endogenous peroxidase activity was blocked by a 30-min room temperature incubation in 1% hydrogen peroxide (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 \times 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 nonspecific labeling, as assessed by the complete absence of silver particles visible on the myelin sheaths of axons and in the immunocytochemical control tissue. Altogether, 2,423 and 1,385 μ m² 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 multiunit 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 recordings showing almost complete suppression (Fig. 1, A-D). Facilitation was observed, most often in the thalamic recipient layer 4c (Fig. 1, E and F). Note that the apparently pure contrast gain effect (a shift in gain with no change in function asymptote) shown in Fig. 1F is unique within our data set and is presented for completeness. Figure 1E is more representative of the enhancement seen in our population. Further individual examples of gain enhancements with nicotine and ACh can be found in Disney et al. (2007).

Across the population, using this 3 SD (P = 0.01) criterion, there was a significant suppression compared with baseline in 18 recordings (28%; 5 tested with CCh, 13 with ACh). In nine cases we observed enhancement (14%; 4 CCh, 5 ACh). There was no significant drug effect in the remaining 36 recordings (57%). We have shown previously that contrast-response area in V1 is stationary under repeated measurement without drug application and that, with the use of these electrodes, ejection currents up to 160 nA neither stimulate nor suppress extracellularly recorded spike rates (Disney et al. 2007). The prolonged recovery times we observed (i.e., drug effects persist well after



Downloaded from http://jn.physiology.org/ at Salk Institute on October 2, 2012

Fig. 1. Cholinergic suppression and enhancement. Naka-Rushton functions (see EXPERIMENTAL PROCEDURES) were fit to contrast-response data obtained under baseline conditions (no ejection current, black circles/lines) and during ejection of acetylcholine (ACh) or carbamylcholine (CCh) (gray circles/lines). In each case, the agonist ejection current shown is the highest delivered (see EXPERIMENTAL PROCEDURES for details on how maximum ejection currents were determined). *A–D*: 4 examples showing suppression, which was the most prevalent effect of ejecting cholinergic agonists in macaque V1, occurring in 28% of recordings. *A* shows a single-unit cluster (SUC) recorded from layer 5 (unit NA15 u013); *B–D* show single-units (SUs) recorded in layers 4b, 3, and 5, respectively). *E* and *F*: 2 examples showing enhancement, which was seen in 14% of recordings. *E* shows an SU recorded in layer 4b (unit NA13 u029), and *F* shows a SUC recorded in layer 4c (unit NA21 u010). Error bars represent SE.

ejection has stopped, sometimes up to 60 min, see below) also indicate that the effects we report are not a result of the iontophoretic currents themselves; further current control experiments are presented below. Except as noted in the sections on baseline drift and drug concentration, in the analysis that follows, data from the two agonists, CCh and ACh, are combined because their effects were indistinguishable.

Successful track reconstructions allowed layer assignment for 57 recordings. A laminar analysis is presented in Fig. 2, *A* and *B*. Suppression was evident in all layers of cortex (Fig. 2*A*, solid black bars) but in only 1 of 12 recordings in layer 4c (the primary thalamic recipient layer). Enhancement, on the other hand, was prevalent in layer 4c (Fig. 2*A*, open bars) and was not seen in layers 2 through 4a or 5. After identifying significantly affected neurons, we calculated for each a "normalized response area," which is the area under the contrast curve for the maximum ejection current condition, normalized by the area under the curve for the immediately preceding no-drug baseline. Averaged over all cells within a layer, there was a net suppression in layers 2, 3, and 5 and enhancement in layer 4c (Fig. 2*B*).

In addition to differences in stimulus-evoked activity, there was often a decrease in the spontaneous activity with ACh/CCh ejection. Our goal was to study cholinergic effects on visual responses, not spontaneous activity, so we separated the spontaneous and stimulus-evoked components by subtracting the summed spike rate across the no-stimulus periods interleaved between stimulus presentations from the area under the contrast responses. We found that changes in spontaneous activity accounted for the entire ACh/CCh-induced suppression in four (of 18) examples of suppression. One of these was the only



example of suppression in layer 4c. One was a recording for which laminar position could not be firmly established (at a depth of 525 μ m from the estimated pial surface, it is probably layer 2). The other two were in layers 2 and 5. This blank subtraction was done for all recordings and revealed a further two cases (in layers 4b and 5) in which an increase in spontaneous activity had masked a significant suppression of visually driven responses. Thus, whereas effects on undriven activity explained the suppression entirely for 4 units, after these were accounted for there remained 16 units with significant visual 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 GABAergic 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

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 "doseresponse" 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 $\sim 60\%$ 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 show a 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_{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_{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 $R_{\rm max}$ values <25 spikes/s, and 59% (39 of 66) yielded C₅₀ 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_{max} values <25 spikes/s (compared with 70% in Soma et al. 2012). In 193 of 540 cases (36%), the fit yields an R_{max} value between 25 and 50 spikes/s, and in 222 of 540 cases (41%), the value for R_{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 C₅₀ values \geq 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

 Table 1.
 Significant enhancement and suppression (by 3 SD criterion) at low vs. high ACh concentrations

	Median Current, nA	Enhanced	Suppressed	No Effect
Low ACh	20	5	2	31
High ACh	80	5	13	20

Values are no. of recordings (of 38 total) that were enhanced, suppressed, or showed no effect at low vs. high ACh ejection current.

Downloaded

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_{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 EXPERI-MENTAL 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_{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_{max}, C₅₀, 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), C₅₀ 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_A 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_A 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. 4*E*) 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. 3. Mixed gain effects across layers. For each neuron that was significantly suppressed by ACh/CCh, the raw spike data were normalized by the maximum firing rate obtained under baseline conditions. The normalized responses across neurons were averaged and fit. *A*: fits for the superficial layers 2–4a. *B*: fits for layers 5 and 6. *C*: averaged responses from all layers (including layers 4b and 4c). In *A*–*C*, baseline data are shown in black and ACh/CCh data are shown in gray. The level of spontaneous activity for each condition is given by the filled squares to the *left* of the contrast-response fits ("sFR" on the *x*-axis; error bars represent SE). Also given are the response gain (R_{max}) and contrast gain (C_{50}) parameters for each fit. C_{50} changes are only observed in layers 2 and 3, whereas R_{max} reductions are evident in all layers. The translation of the curve along the *y*-axis in *A* is consistent with a subtractive mechanism, with an R_{max} change still evident after this translation has been removed (*inset* in *A*).



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 \sim 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. 5*B*). There was strong suppression when ACh was ejected alone (Fig. 5*B*, red). When gabazine was co-ejected with ACh (Fig. 5*B*, blue), there was little difference from the baseline responses (Fig. 5*B*, black) or responses to gabazine alone (Fig. 5*B*, green). Further examination of the summary data reveals that the ACh & gabazine responses (Fig. 5*B*, 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 (ACh&Gze) 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 (ACh&Gze) 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. 5*A*), 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. 5*B*). 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 coejection 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



Fig. 6. Gabazine produces a response gain increase at ejection currents higher than were needed to block ACh-mediated suppression. A: the non-normalized (i.e., total spike count) response areas are shown for 6 pharmacological conditions. Recording is from a SU in layer 6 (unit NA21 u026). The mean baseline (B) response area was 438 spikes. The response area increased with increasing levels of gabazine ejection at 10 nA (421 spikes), 15 nA (497 spikes), and 20 nA (723 spikes). ACh (120 nA) suppressed this unit's responses (317 spikes), and this suppression was blocked by co-ejection of gabazine at 10 nA (421 spikes, "co-eject"). Error bars represent SD. B: a second example SU, for which layer information was not recovered (unit NA17 u027), in which CCh strongly suppressed contrast responses (120 nA; inverted triangles, with solid line giving Naka-Rushton fit to the data). This suppression was blocked by gabazine (CCh&Gze, circles and solid fit line). The effective dose of gabazine did not itself strongly excite the neuron (Gze 50 nA; asterisks and dotted fit line). C: at the same site as recorded in B, higher ejection currents were used to deliver gabazine in an area-tuning experiment. The 80-nA (squares and dotted line) and 120-nA gabazine ejection currents (stars and dashed line) resulted in a response gain increase, as expected from previous data (Katzner et al. 2011). Lines in C are for visualization only; they are not a fit to the data. Error bars in B represent SE.

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 \times 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 membraneassociated 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 membraneassociated 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

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 coejection, 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.



Prior ejection current (nA)

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 "recovery" 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 (unresponsive, 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. The no-drug response area was higher following the highest ejection current when the cholinergic agonists had no significant effect. 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).

drift. Figure 8*B* plots the magnitude of this effect for each group as a function of the preceding drug ejection current. By this measure, there was a significant upward drift in 11 cases (17%: 2 enhanced, 2 suppressed, and 7 no effect) and a significant downward drift in 8 cases (13%: 2 enhanced, 3 suppressed, and 3 no effect). The magnitude of these effects was not monotonically related to preceding ejection current. The baseline nonstationarity was not simply a failure of recovery. Of 19 instances in which we observed significant drift, 10 were recordings in which there was no significant effect during drug ejection from which to recover. Also, in four of the remaining nine cases, the drift was in the direction opposite to the effect on visual responses.

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 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 grating motion 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, GABAergic neurons are more likely to express AChRs (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_A 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

ACH SUPPRESSION OF VISUAL GAIN RECRUITS INHIBITION



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; *D*: gray circles and dashed lines show the result is compared with the actual measured 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.

we did not pharmacologically dissect the suppression itself, on the basis of the frequency of suppression to ACh/CCh compared 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 investigations. 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 1992), and was again observed in 50% of layer 4c recordings in the present study. There were two examples of enhancement in layer 4b and one in layer 6. This could be due to local spread of ACh to nAChRs on lateral geniculate nucleus (LGN) afferents in layers $4c\alpha$ and 6 and to subsequent feedforward excitation. It also could be due to direct excitation of inhibitory neurons from which we were recording.

The principal alternate hypothesis for the current study is that muscarinic suppression is mediated by inhibition of glutamate release. Preexisting data have already indicated this interpretation is unlikely for macaque V1. The suppression of cortical glutamate release reported in other species is mediated by G_i-coupled receptors, which for ACh are the m2 and m4 classes 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 recordings 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% (\sim 50% in layers 2 and 3) of inhibitory neurons that express AChRs. These suggestive 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 neuromodula-

1919

Downloaded from http://jn.physiology.org/

at Salk Institute on October 2,

, 2012

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 calretinin neurons (Disney and Aoki 2008). In rats, cholecystokinin- and vasoactive intestinal polypeptideimmunoreactive 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 $\sim 10-15\%$ 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_A 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 μ M (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/ μ C (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 α 7 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-spiking 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-spiking) 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 μ m, but effective drug concentrations at distances up to 600 μ m 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.

Downloaded

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.

ACKNOWLEDGMENTS

We thank C. Henry, S. Joshi, P. Williams, and D. Xing for assistance with data collection. We also thank M. Schiff, K. Nielsen, and E. Anderson for helpful comments on previous versions of this manuscript.

GRANTS

This work was supported by National Institutes of Health Grants EY8300 and EY15549 (to M. J. Hawken), 1 R01 EY13145 and NS41091 (to C. Aoki), and Core Grant P30-EY13079.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

A.A.D., C.A., and M.J.H. conception and design of research; A.A.D., C.A., and M.J.H. performed experiments; A.A.D. analyzed data; A.A.D. interpreted results of experiments; A.A.D. prepared figures; A.A.D. drafted manuscript; A.A.D., C.A., and M.J.H. edited and revised manuscript; A.A.D., C.A., and M.J.H. approved final version of manuscript.

REFERENCES

- Albrecht DG, Hamilton DB. Striate cortex of monkey and cat: contrast response function. J Neurophysiol 48: 217–237, 1982.
- Alexander KS, Brooks JM, Sarter M, Bruno JP. Disruption of mesolimbic regulation of prefrontal cholinergic transmission in an animal model of schizophrenia and normalization by chronic clozapine treatment. *Neuropsychopharmacology* 34: 2710–2720, 2009.
- Aoki C, Kabak S. Cholinergic terminals in the cat visual cortex: ultrastructural basis for interaction with glutamate-immunoreactive neurons and other cells. *Vis Neurosci* 8: 177–191, 1992.
- Arnold HM, Burk JA, Hodgson EM, Sarter M, Bruno JP. Differential cortical acetylcholine release in rats performing a sustained attention task versus behavioral control tasks that do not explicitly tax attention. *Neuroscience* 114: 451–460, 2002.
- Ayaz A, Chance FS. Gain modulation of neuronal responses by subtractive and divisive mechanisms of inhibition. J Neurophysiol 101: 958–968, 2009.

- **Bradley PB, Candy JM.** Iontophoretic release of acetylcholine, noradrenaline, 5-hydroxytryptamine and D-lysergic acid diethylamide from micropipettes. *Br J Pharmacol* 40: 194–201, 1970.
- Brown DA, Abogadie FC, Allen TG, Buckley NJ, Caulfield MP, Delmas P, Haley JE, Lamas JA, Selyanko AA. Muscarinic mechanisms in nerve cells. *Life Sci* 60: 1137–1144, 1997.
- **Brown DA, Adams PR.** Muscarinic suppression of a novel voltage-sensitive K⁺ current in a vertebrate neurone. *Nature* 283: 673–676, 1980.
- **Brumberg JC, Nowak LG, McCormick DA.** Ionic mechanisms underlying repetitive high-frequency burst firing in supragranular cortical neurons. *J Neurosci* 20: 4829–4843, 2000.
- Candy JM, Boakes RJ, Key BJ, Worton E. Correlation of the release of amines and antagonists with their effects. *Neuropharmacology* 13: 423–430, 1974.
- **Cardin JA, Palmer LA, Contreras D.** Stimulus-dependent gamma (30–50 Hz) oscillations in simple and complex fast rhythmic bursting cells in primary visual cortex. *J Neurosci* 25: 5339–5350, 2005.
- Cea-del Rio CA, Lawrence JJ, Tricoire L, Erdelyi F, Szabo G, McBain CJ. M3 muscarinic acetylcholine receptor expression confers differential cholinergic modulation to neurochemically distinct hippocampal basket cell subtypes. J Neurosci 30: 6011–6024, 2010.
- Chen Y, Anand S, Martinez-Conde S, Macknik SL, Bereshpolova Y, Swadlow HA, Alonso JM. The linearity and selectivity of neuronal responses in awake visual cortex. J Vis 9: 12 11–17, 2009.
- Christophe E, Roebuck A, Staiger JF, Lavery DJ, Charpak S, Audinat E. Two types of nicotinic receptors mediate an excitation of neocortical layer I interneurons. *J Neurophysiol* 88: 1318–1327, 2002.
- **Constantinople CM, Disney AA, Maffie J, Rudy B, Hawken MJ.** Quantitative analysis of neurons with Kv3 potassium channel subunits, Kv3.1b and Kv3.2, in macaque primary visual cortex. *J Comp Neurol* 516: 291–311, 2009.
- **Curtis DR, Perrin DD, Watkins JC.** The excitation of spinal neurones by the ionophoretic application of agents which chelate calcium. *J Neurochem* 6: 1–20, 1960.
- **Deco G, Thiele A.** Cholinergic control of cortical network interactions enables feedback-mediated attentional modulation. *Eur J Neurosci* 34: 146–157, 2011.
- **Descarries L, Gisiger V, Steriade M.** Diffuse transmission by acetylcholine in the CNS. *Prog Neurobiol* 53: 603–625, 1997.
- Disney AA, Aoki C. Muscarinic acetylcholine receptors in macaque V1 are most frequently expressed by parvalbumin-immunoreactive neurons. J Comp Neurol 507: 1748–1762, 2008.
- **Disney AA, Aoki C, Hawken MJ.** Gain modulation by nicotine in macaque V1. *Neuron* 56: 701–713, 2007.
- **Disney AA, Domakonda K, Aoki C.** Differential expression of muscarinic acetylcholine receptors across excitatory and inhibitory cells in visual cortical areas V1 and V2 of the macaque monkey. *J Comp Neurol* 499: 49–63, 2006.
- **Erisir A, Levey AI, Aoki C.** Muscarinic receptor m2R distribution in the cat visual cortex: laminar distribution, relationship to GABAergic neurons, and the efect of cingulate lesions. *J Comp Neurol* 441: 168–185, 2001.
- Gil Z, Connors BW, Amitai Y. Differential regulation of neocortical synapses by neuromodulators and activity. *Neuron* 19: 679–686, 1997.
- Giocomo LM, Hasselmo ME. Neuromodulation by glutamate and acetylcholine can change circuit dynamics by regulating the relative influence of afferent input and excitatory feedback. *Mol Neurobiol* 36: 184–200, 2007.
- **Gonchar Y, Burkhalter A.** Three distinct families of GABAergic neurons in rat visual cortex. *Cereb Cortex* 7: 347–358, 1997.
- **Gray CM, McCormick DA.** Chattering cells: superficial pyramidal neurons contributing to the generation of synchronous oscillations in the visual cortex. *Science* 274: 109–113, 1996.
- Gulledge AT, Park SB, Kawaguchi Y, Stuart GJ. Heterogeneity of phasic cholinergic signalling in neocortical neurons. J Neurophysiol 97: 2215– 2229, 2007.
- Hasselmo ME, Bower JM. Cholinergic suppression specific to intrinsic not afferent fiber synapses in rat piriform (olfactory) cortex. J Neurophysiol 67: 1222–1229, 1992.
- Hasselmo ME, McGaughy J. High acetylcholine levels set circuit dynamics for attention and encoding and low acetylcholine levels set dynamics for consolidation. *Prog Brain Res* 145: 207–231, 2004.
- Herrero JL, Roberts MJ, Delicato LS, Gieselmann MA, Dayan P, Thiele A. Acetylcholine contributes through muscarinic receptors to attentional modulation in V1. *Nature* 454: 1110–1114, 2008.

- Heyer EJ, Nowak LM, Macdonald RL. Bicuculline: a convulsant with synaptic and nonsynaptic actions. *Neurology* 31: 1381–1390, 1981.
- Himmelheber AM, Sarter M, Bruno JP. Increases in cortical acetylcholine release during sustained attention performance in rats. *Brain Res Cogn Brain Res* 9: 313–325, 2000.
- Holt GR, Koch C. Shunting inhibition does not have a divisive effect on firing rates. *Neural Comput* 9: 1001–1013, 1997.
- Hsieh CY, Cruikshank SJ, Metherate R. Differential modulation of auditory thalamocortical and intracortical synaptic transmission by cholinergic agonist. *Brain Res* 880: 51–64, 2000.
- Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem* 29: 577–580, 1981.
- Hyde TM, Crook JM. Cholinergic systems and schizophrenia: primary pathology or epiphenomena? J Chem Neuroanat 22: 53–63, 2001.
- **Johnson SW, Seutin V.** Bicuculline methiodide potentiates NMDA-dependent burst firing in rat dopamine neurons by blocking apamin-sensitive Ca²⁺-activated K⁺ currents. *Neurosci Lett* 231: 13–16, 1997.
- Katzner S, Busse L, Carandini M. GABA_A inhibition controls response gain in visual cortex. *J Neurosci* 31: 5931–5941, 2011.
- Kawaguchi Y. Selective cholinergic modulation of cortical GABAergic cell subtypes. J Neurophysiol 78: 1743–1747, 1997.
- **Kilgard M.** Cholinergic modulation of skill learning and plasticity. *Neuron* 38: 678–680, 2003.
- Kimura F, Fukuda M, Tsumoto T. Acetylcholine suppresses the spread of excitation in the visual cortex revealed by optical recording: possible differential effect depending on the source of input. *Eur J Neurosci* 11: 3597–3609, 1999.
- Kuczewski N, Aztiria E, Gautam D, Wess J, Domenici L. Acetylcholine modulates cortical synaptic transmission via different muscarinic receptors, as studied with receptor knockout mice. J Physiol 566: 907–919, 2005.
- Lambert JJ, Belelli D, Hill-Venning C, Peters JA. Neurosteroids and GABA_A receptor function. *Trends Pharmacol Sci* 16: 295–303, 1995.
- Lawrence JJ. Cholinergic control of GABA release: emerging parallels between neocortex and hippocampus. *Trends Neurosci* 31: 317–327, 2008.
- Levey AI, Kitt CA, Simonds WF, Price DL, Brann MR. Identification and localization of muscarinic acetylcholine receptor proteins in brain with subtype-specific antibodies. *J Neurosci* 11: 3218–3226, 1991.
- Mattinson CE, Burmeister JJ, Quintero JE, Pomerleau F, Huettl P, Gerhardt GA. Tonic and phasic release of glutamate and acetylcholine neurotransmission in sub-regions of the rat prefrontal cortex using enzyme-based microelectrode arrays. *J Neurosci Methods* 202: 199–208, 2011.
- McCormick DA, Prince DA. Mechanisms of action of acetylcholine in the guinea-pig cerebral cortex in vitro. J Physiol 375: 169–194, 1986.
- McCormick DA, Prince DA. Two types of muscarinic response to acetylcholine in mammalian cortical neurons. *Proc Natl Acad Sci USA* 82: 6344–6348, 1985.
- McGaughy J, Sarter M. Sustained attention performance in rats with intracortical infusions of 192 IgG-saporin-induced cortical cholinergic deafferentation: effects of physostigmine and FG 7142. *Behav Neurosci* 112: 1519–1525, 1998.
- Mike A, Castro NG, Albuquerque EX. Choline and acetylcholine have similar kinetic properties of activation and desensitization on the alpha7 nicotinic receptors in rat hippocampal neurons. *Brain Res* 882: 155–168, 2000.
- Mody I, De Koninck Y, Otis TS, Soltesz I. Bridging the cleft at GABA synapses in the brain. *Trends Neurosci* 17: 517–525, 1994.
- Mori T, Zhao X, Zuo Y, Aistrup GL, Nishikawa K, Marszalec W, Yeh JZ, Narahashi T. Modulation of neuronal nicotinic acetylcholine receptors by halothane in rat cortical neurons. *Mol Pharmacol* 59: 732–743, 2001.
- **Muller CM, Singer W.** Acetylcholine-induced inhibition in the cat visual cortex is mediated by a GABAergic mechanism. *Brain Res* 487: 335–342, 1989.
- Murphy PC, Sillito AM. Cholinergic enhancement of direction selectivity in the visual cortex of the cat. *Neuroscience* 40: 13–20, 1991.
- Naka KI, Rushton WA. S-potentials from luminosity units in the retina of fish (Cyprinidae). J Physiol 185: 587–599, 1966.
- Nobili L, Sannita WG. Cholinergic modulation, visual function and Alzheimer's dementia. *Vision Res* 37: 3559–3571, 1997.
- Nowak LG, Azouz R, Sanchez-Vives MV, Gray CM, McCormick DA. Electrophysiological classes of cat primary visual cortical neurons in vivo as revealed by quantitative analyses. *J Neurophysiol* 89: 1541–1566, 2003.

- Olsen RW, Ban M, Miller T. Studies on the neuropharmacological activity of bicuculline and related compounds. *Brain Res* 102: 283–299, 1976.
- Platt ML, Glimcher PW. Responses of intraparietal neurons to saccadic targets and visual distractors. J Neurophysiol 78: 1574–1589, 1997.
- **Porter JT, Cauli B, Tsuzuki K, Lambolez B, Rossier J, Audinat E.** Selective excitation of subtypes of neocortical interneurons by nicotinic receptors. *J Neurosci* 19: 5228–5235, 1999.
- Puil E, el-Beheiry H. Anaesthetic suppression of transmitter actions in neocortex. Br J Pharmacol 101: 61–66, 1990.
- Ringach DL, Hawken MJ, Shapley R. Dynamics of orientation tuning in macaque V1: the role of global and tuned suppression. *J Neurophysiol* 90: 342–352, 2003.
- Roberts MJ, Zinke W, Guo K, Robertson R, McDonald JS, Thiele A. Acetylcholine dynamically controls spatial integration in marmoset primary visual cortex. J Neurophysiol 93: 2062–2072, 2005.
- Sarter M, Hasselmo ME, Bruno JP, Givens B. Unraveling the attentional functions of cortical cholinergic inputs: interactions between signal-driven and cognitive modulation of signal detection. *Brain Res Rev* 48: 98–111, 2005.
- Sato H, Hata Y, Masui H, Tsumoto T. A functional role of cholinergic innervation to neurons in the cat visual cortex. *J Neurophysiol* 58: .765–780, 1987.
- Sclar G, Maunsell JH, Lennie P. Coding of image contrast in central visual pathways of the macaque monkey. *Vision Res* 30: 1–10, 1990.
- Seguela P, Wadiche J, Dineley-Miller K, Dani JA, Patrick JW. Molecular cloning, functional properties, and distribution of rat brain alpha 7: a nicotinic cation channel highly permeable to calcium. *J Neurosci* 13: 596–604, 1993.
- Sillito AM, Kemp JA. Cholinergic modulation of the functional organization of the cat visual cortex. *Brain Res* 289: 143–155, 1983.
- Silver MA, Shenhav A, D'Esposito M. Cholinergic enhancement reduces spatial spread of visual responses in human early visual cortex. *Neuron* 60: 904–914, 2008.
- Soma S, Shimegi S, Osaki H, Sato H. Cholinergic modulation of response gain in the primary visual cortex of the macaque. *J Neurophysiol* 107: 283–291, 2012.
- Stone TW. Cholinergic mechanisms in the rat cerebral cortex. *J Physiol* 222: 155P–156P, 1972a.
- Stone TW. Cholinergic mechanisms in the rat somatosensory cerebral cortex. *J Physiol* 225: 485–499, 1972b.
- Stone TW. Microiontophoresis and Pressure Ejection. New York: Wiley, 1985.
- Tigges M, Tigges J, Rees H, Rye D, Levey AI. Distribution of muscarinic cholinergic receptor proteins m1 to m4 in area 17 of normal and monocularly deprived rhesus monkeys. *J Comp Neurol* 388: 130–145, 1997.
- **Trapani G, Altomare C, Liso G, Sanna E, Biggio G.** Propofol in anesthesia. Mechanism of action, structure-activity relationships, and drug delivery. *Curr Med Chem* 7: 249–271, 2000.
- Umbriaco D, Watkins KC, Descarries L, Cozzari C, Hartman BK. Ultrastructural and morphometric features of the acetylcholine innervation in adult rat parietal cortex: an electron microscopic study in serial sections. J Comp Neurol 348: 351–373, 1994.
- Van Brederode JF, Mulligan KA, Hendrickson AE. Calcium-binding proteins as markers for subpopulations of GABAergic neurons in monkey striate cortex. J Comp Neurol 298: 1–22, 1990.
- Vigneswaran G, Kraskov A, Lemon RN. Large identified pyramidal cells in macaque motor and premotor cortex exhibit "thin spikes": implications for cell type classification. J Neurosci 31: 14235–14242, 2011.
- Wang Z, McCormick DA. Control of firing mode of corticotectal and corticopontine layer V burst-generating neurons by norepinephrine, acetylcholine, and 1*S*,3*R*-ACPD. J Neurosci 13: 2199–2216, 1993.
- Weinberger NM. Auditory associative memory and representational plasticity in the primary auditory cortex. *Hear Res* 229: 54–68, 2007.
- Wong-Riley M, Anderson B, Liebl W, Huang Z. Neurochemical organization of the macaque striate cortex: correlation of cytochrome oxidase with Na⁺K⁺ATPase, NADPH-diaphorase, nitric oxide synthase, and N-methyl-D-aspartate receptor subunit 1. Neuroscience 83: 1025–1045, 1998.
- Wouterlood FG, Jorritsma-Byham B. The anterograde neuroanatomical tracer biotinylated dextran-amine: comparison with the tracer *Phaseolus* vulgaris-leucoagglutinin in preparations for electron microscopy. J Neurosci Methods 48: 75–87, 1993.
- Xiang Z, Huguenard JR, Prince DA. Cholinergic switching within neocortical inhibitory networks. *Science* 281: 985–988, 1998.

- Xing D, Ringach DL, Hawken MJ, Shapley RM. Untuned suppression makes a major contribution to the enhancement of orientation selectivity in macaque v1. *J Neurosci* 31: 15972–15982, 2011.
- Xing D, Shapley RM, Hawken MJ, Ringach DL. Effect of stimulus size on the dynamics of orientation selectivity in Macaque V1. J Neurophysiol 94: 799–812, 2005.
- Yu AJ, Dayan P. Uncertainty, neuromodulation, attention. *Neuron* 46: 681–692, 2005.
- Zinke W, Roberts MJ, Guo K, McDonald JS, Robertson R, Thiele A. Cholinergic modulation of response properties and orientation tuning of neurons in primary visual cortex of anaesthetized Marmoset monkeys. *Eur J Neurosci* 24: 314–328, 2006.

