Two distinct profiles of fMRI and neurophysiological activity elicited by acetylcholine in visual cortex

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Acetylcholine (ACh) is a key neuromodulator in the brain and is involved in many aspects of cognition, including sensory processing (14–16), attention (14), and learning and memory (17). Nearly all cortical regions receive cholinergic projections that originate from the basal forebrain (BF; ref. 18), and histological evidence has shown differential distributions of cholinergic receptors (AChRs; refs. 19, 20) and acetylcholinesterase (AChE) in cortex (21). However, despite this anatomical evidence, it has generally been assumed that the cholinergic system exerts uniform effects across all cortical structures (22).

Studies using electrical microstimulation of BFB neurons (16, 22) and systemic injection of cholinergic agonists (23) have shown increases in sensory-evoked neural activity in visual cortex as well as in cerebral blood flow (CBF; refs. 22–25). This effect of ACh has also been observed in other sensory modalities, such as the somatosensory (26) and the auditory cortices (27). However, it is unclear to what extent these changes are the result of local cholinergic effects or caused by a chain of events instantiated by global changes in ACh levels (28).

In this study, we combined intracortical injections of ACh with pharmaco-MRI (BOLD and CBF) and electrophysiology [single-electrode physiology and microelectrocorticography (μECoG) electrode arrays] to investigate the impact of cholinergic neuromodulation on neural responses and neurovascular coupling in macaque V1. Applying ACh in anesthetized animals allowed us to investigate the mechanisms by which ACh affects neural and fMRI responses without the potentially confusing effects of...
behavioral processes like attention (15) or learning (29). We found two distinct cholinergically induced effects in the fMRI and neurophysiological responses in V1. Near the injection site, the amplitude of the BOLD, CBF, and neural responses to the visual stimulus was decreased and the baseline increased. Further away from the injection site, visually induced modulation of the BOLD, CBF, and neural responses increased, with no changes in the baseline, and preserved neurovascular coupling.

**Results**

Our goal was to investigate the effects of cholinergic neuromodulation on neurovascular coupling. For this, we combined functional imaging in V1 with neurophysiology and pharmacology in anesthetized nonhuman primates (Macaca mulatta). We acquired BOLD, functional CBF (fCBF), and neurophysiological data, the latter using single electrodes as well as μECoG arrays, while animals viewed a rotating checkerboard (Fig. 1 A and B). We mimicked cholinergic neuromodulation by locally applying ACh in V1. To ensure that ACh was reliably delivered, we monitored the changes in the concentration of ACh in the extracellular space using microsampling in conjunction with HPLC coupled to mass spectrometry (SI Appendix, Fig. S1). Following the injection of ACh, we found that levels of ACh immediately increased from baseline levels and returned to baseline levels 10 min after the end of the injection (SI Appendix, Fig. S1B).

**ACh Alters Sensory Evoked Neural Activity in Primary Visual Cortex.** We first investigated the effects of ACh on neural activity, because studies have shown that at the neuronal population level, ACh reduces excitability (30–33). We were interested to know if our pharmacological approach yielded similar results to those reported. We used neurophysiology data that was simultaneously collected with fMRI data and calculated the absolute power spectral density (PSD) from the LFPs and multiunit activity (MUA) using previously described methods (11, 34). Changes in the PSD are good indicators of changes in neural activity, as these reflect changes in the coordinated activity of excitatory and inhibitory postsynaptic potentials (1, 3, 5, 36). Our analysis revealed that injection of ACh decreased the power of the neuronal activity across a wide frequency range (1–250 Hz; SI Appendix, Fig. S2). However, given that it has been shown that the gamma (γ; 50–90 Hz), high-gamma (hγ; 91–150 Hz), and MUA (900–3,000 Hz) bands carry most information about the sensory stimulus and are most strongly correlated with the fMRI signal (4, 5, 37), we focused our analysis on these bands.

Fig. 2 illustrates the effects of ACh injection on the LFP and MUA: Fig. 2 A–C show the average time courses over 15 experimental sessions for the extracted frequency bands. The visual stimulus caused an increase in power/amplitude of >10% compared with baseline in all frequency bands. A substantial effect of the ACh injection is evident in the γ, hγ, and MUA bands (Fig. 2 A–D) as a decrease in visual modulation in the γ (MODγ,γ < 2%; FAR < 0.25%; P = 0.028), in the hγ (MODhγ,γ = 62 ± 2%; P = 0.031), and in the MUA (MODhMUA,γ = 64 ± 1%; P = 0.025) bands. The effects of ACh on the γ, hγ, and MUA amplitude returned to baseline ~22 min after the ACh infusion was stopped.

Moreover, it has been shown that ACh can affect the amplitude of the response as well as the variability of the neural activity, which impacts the information encoding in the visual cortex (8, 9, 15, 16, 38). Therefore, we calculated the signal-to-noise ratio (SNR). This showed an increase in SNR in the hγ (SNR hγ = 11.5 ± 2.4 dB; P = 0.031, paired t test) and MUA (SNR hMUA,γ = 11 ± 2.2 dB; P = 0.027, paired t test) bands starting shortly after the beginning of the injection. The SNR in the γ band was not significantly increased (SNR γ = 10.4 ± 2.8 dB; P = 0.59, paired t test). None of these effects were observed during control injection of artificial cerebrospinal fluid (ACSF; see SI Appendix, Figs. S2 and S3).

**Evoked BOLD Responses Under Cholinergic Influence.** The neural data in Fig. 2 were recorded simultaneously with BOLD responses. Fig. 3 A shows an example of a typical functional activation map showing the selected V1 regions before and during injection of ACh. We selected two regions of interest (ROIs) (Fig. 1C) according to their location relative to the injector: ROI<sub>NEAR</sub> (voxels located within a ~2-mm diameter surrounding the electrode) and ROI<sub>FAIR</sub> (voxels located farther away from the injector, 3–5 mm). These criteria led us to identify two distinct effects of ACh in the BOLD signal. The average visually induced BOLD change in V1 was 5.8 ± 0.25% (15 experimental sessions) in both ROIs. The activated voxels in both selected regions are color-coded according to their percentage changes (Fig. 3A) and location in the near or far ROI. Fig. 3 B and C show the time courses of the response of the BOLD modulation over the course of the ACh injection in both ROIs. The time course for the voxels in the ROI<sub>NEAR</sub> (BOLD<sub>NEAR</sub>; red) exhibited an increase in the baseline following the start of the injection, which reached its maximum ~2 min after the start of the injection. In contrast, the time course of the ROI<sub>FAIR</sub> (Fig. 3C, BOLD<sub>FAIR</sub>) did not show this shift in the baseline and remained stable throughout the experiment. ACh also elicited different
Evoked CBF Responses Under Cholinergic Influence. The BOLD signal reflects the deoxyhemoglobin concentration [dHB] and thus is affected by changes in the cerebral blood volume (CBV), CBF, and cerebral metabolic rate of oxygen consumption (CMRO2). In earlier work, we showed that these fMRI signals were differentially affected by dopamine (10) and found that BOLD-fMRI alone cannot be used to make inferences about changes in neural activity and metabolism under neuromodulation. Following this line of argument, we performed CBF measurements using the same pharmacological approach. Using arterial spin labeling (ASL) in combination with BOLD-fMRI and electrophysiology may offer better insights into the changes in energy metabolism following changes in neural activity. Fig. 4A shows activation patterns of the CBF in V1 before and during injection of ACh. As for the BOLD responses, we generated two ROIs relative to the location of the injector and computed the visually induced modulation and baseline changes. The mean CBF change in the ROIs was 36 ± 2.1% (seven experimental sessions), in line with earlier CBF changes in anesthetized monkeys V1 (39–41). The average time course of the CBF\textsubscript{NEAR} across experiments (Fig. 4B) showed a positive shift in the baseline and a decrease in visually induced modulation similar to the changes in the BOLD activity. When comparing the CBF\textsubscript{NEAR} “predrug” period with the “drug” period, we observed a mean decrease in the visually induced modulation (CBF\textsubscript{drug, NEAR} = 65 ± 5%; \( P = 0.043, \) paired \( t \) test, \( n = 7 \)). The largest CBF decrease was observed toward the end of the infusion (62% at \( \sim \) 8 min after the start of the infusion; Fig. 4D). The increase in the baseline started \( \sim 1.5 \) min after the onset of the injection (CBF\textsubscript{drug, NEAR} = 118 ± 4%; \( P = 0.038, \) paired \( t \) test). In contrast, the CBF\textsubscript{FAR} showed a mean increase in the visually induced modulation (CBF\textsubscript{drug, FAR} = 123 ± 5%; \( P = 0.042, \) paired \( t \) test) and no changes in the baseline (Fig. 4D and E), similar to those reported for BOLD. To summarize, the CBF experiments also revealed two distinct changes in the CBF responses and mirrored the BOLD and neural responses. An increase of the baseline and a decrease in visually evoked modulation was observed in the CBF\textsubscript{NEAR}, whereas we observed an increase in the visually induced modulation and no changes in the baseline for CBF\textsubscript{FAR}.

ACh Does Not Change the Correspondence Between Neural Activity and fMRI Responses. An important question was to establish whether the two distinct cholinergically induced profiles of fMRI activity, in the different spatial regions, corresponded with the local neural activity. To record neural responses over an area corresponding to the extent of the fMRI responses, we acutely implanted \( \mu \)ECoG arrays on the surface of the brain (Fig. 5A, \( \mu \)ECoG 32 channels). We selected the electrode contacts according to the following criteria: (i) electrodes that were located within a 1-mm ratio close to the injector were assigned as ECoG\textsubscript{NEAR} (Fig. 5A, shown in red); (ii) electrodes that were within a ratio of 1.5–2.5 mm were assigned as ECoG\textsubscript{FAR} (Fig. 5A, shown in teal); and (iii) electrodes that were located on top of a blood vessel were not considered in the analysis. We first determined if there were differences between the LFP power in the two regions by computing the strength of the oscillatory activity across electrodes in ECoG\textsubscript{NEAR} and ECoG\textsubscript{FAR}. Fig. 5B denotes a typical average LFP spectrum in V1 over electrode sites located in ECoG\textsubscript{NEAR} (six experimental sessions) and electrode sites in ECoG\textsubscript{FAR} shown in red and teal, respectively. Similar to what we observed during single-electrode recordings, the broadband LFP power in the near region decreased following the injection of ACh (SI Appendix, Fig. S2, shown in red). Moreover, the power in the broadband LFP in the region far from the injector increased (SI Appendix, Fig. S2, shown in blue).

Fig. 5 C–F illustrates the effects of ACh on the γ LFP bands: Fig. 5 C and D shows the average time courses across experiments for the γ and hy. We focused on the γ and hy, given that these bands originate from different sources. For instance, γ is
closely linked to changes in excitation/inhibition balance, while the hy has a closer relation to the MUA (42, 43). The average visually induced modulation in the γ range was increased >13% relative to the baseline. The effects of ACh on activity recorded by electrodes in ECoG<sub>Near</sub> were similar to the effects described in Fig. 2—that is, we observed that during the injection period, the visual modulation in the γ band decreased (MOD<sub>γ</sub><sub>Near</sub> 63 ± 3%; P = 0.031; Fig. 5 C and E, dark red), as well as in the hy band (MOD<sub>γ</sub><sub>Hy</sub><sub>Near</sub> 72 ± 4%; P = 0.038; Fig. 5 C and E, red). In contrast, the electrodes located in ECoG<sub>Far</sub> showed an increase in the amplitude of the γ band during the injection period (MOD<sub>γ</sub><sub>Far</sub> 123 ± 2%; P = 0.033; Fig. 5 D and E, blue) and hy band (MOD<sub>γ</sub><sub>Hy</sub><sub>Far</sub> 124 ± 2%; P = 0.038; Fig. 5 D and E, teal). The SNR in the γ range at the ECoG<sub>Near</sub> electrodes was unaffected by the ACh injection (SNR<sub>γ</sub><sub>Near</sub> = 0.95 ± 0.15 dB; P = 0.38; Fig. 5F, dark red). However, the SNR in the hy range increased (SNR<sub>Hy</sub><sub>Near</sub> = 1.14 ± 0.18 dB; P = 0.033; Fig. 5F, light red). The SNR of the γ band recorded by electrodes in the ECoG<sub>Far</sub> region was unaffected by the injection (SNR<sub>γ</sub><sub>Far</sub> = 0.98 ± 0.25 dB; P = 0.33 paired t test; Fig. 5F, dark blue), while the SNR in the hy showed a decrease (SNR<sub>Hy</sub><sub>Far</sub> = 0.83 ± 0.35 dB; P = 0.031 paired t test; Fig. 5F, green). Overall, these results reveal (i) a reduced neural activity at the population level (γ) in the region near the injector while neural activity was increased far from the injector; and (ii) a direct cholinergic influence in shaping the fidelity (SNR) of neural γ activity in the region near the injector, while fidelity appears to decrease away from the injector.

ACh Pharmacodynamics. The profiles of fMRI and neurophysiological activity described above can be caused by the effects of ACh or could be due to the influence of choline (Ch). Ch is the immediate metabolite of ACh and has been shown to activate nAChRs and to increase neural activity (44, 45). By extracting extracellular brain fluid (EBF) using microdialysis (see detailed information in SI Appendix; ref. 46), we simultaneously monitored how our injections influenced the concentration of Ch and ACh in the EBF in V1. We found that, following the ACh injection, the concentrations of ACh and Ch increased (SI Appendix, Fig. S1B, shown in blue and red, respectively). However, ACh and Ch exhibited different pharmacodynamics (for details, see SI Appendix, Fig. S1B).

Discussion

We used pharmaco-MRI (BOLD and CBF), neurophysiology (single-electrode recording and μECoG arrays), and intracortical injections to understand the effects of ACh on neural and fMRI activity in V1. The main finding is that the injection of ACh elicits two distinct fMRI and neural responses in V1 depending on the distance to the injector: (i) near the injection site, the stimulus-induced fMRI responses (BOLD and CBF) decreased with a concomitant increase in baseline activity, while the power of the neural activity decreased and its SNR increased; and (ii) further away from the injector we observed an increase in the visually induced modulation of the hemodynamic signals with a concomitant increase in LFP power but a decrease in the SNR of the neural activity. The decreased cell-population activity concomitant with the BOLD and CBF responses in the region near the injector might be an indication of decreased energy demands, while further away from the injector, the increased neural activity and fMRI responses might suggest increased energy demands. The increases in modulation of the hemodynamic and neural responses in the region far from the injector suggest an increased metabolic demand due to the increased neural activity, which might be due to the activation of nAChRs caused by Ch. Overall, our findings indicate that the effects of neuromodulators can vary considerably, depending on properties of local circuits and neuromodulators, such as the distance from...
release of a neuromodulator, concentration differences, and the kinetics of the neuromodulator.

**Cholinergically Induced Changes in Neural Activity.** ACh decreased the power in the \( \gamma \), \( \beta \), and MUA bands near the injection site. This decrease in power is in good agreement with electrophysiological studies in monkeys, which demonstrated strong inhibition of V1 activity after ACh injection (21, 32, 33). This has been ascribed to the activation of muscarinic receptors (mACHRs, particularly type 1), which are the most abundant AChRs in V1 (47) and are mostly expressed on parvalbumin-immunoreactive (PV-ir) interneurons (19, 31, 32), which in monkeys constitute 78% of the interneurons in cortex (48).

Although our results are in good agreement with findings reported in the sensory cortices of monkeys (32, 33), cats (49), and guinea pigs (50), our results contrast with evidence showing increased sensory-evoked activity in the sensory cortices of rats (22, 51, 52). A possible explanation for this discrepancy may be interspecies differences (33, 53). For instance, 25–95% of pyramidal neurons in the rodent sensory cortex are responsive to ACh, leading to an increase in neural activity (53). This neuromodulatory effect is rare in monkeys because few excitatory neurons express mACHR (19, 31). Moreover, in rat visual cortex, <50% of inhibitory neurons are PV-ir (54), and they either do not respond to (53) or are hyperpolarized by ACh (55).

The effects of ACh are multiple and complex and are ascribed to various factors, including the concentration of ACh (17, 56), the receptor types mediating cholinergic effects (15, 31, 32), and density and location of cholinergic inputs and receptors (19). We do not know for certain whether changes in network dynamics at the injection site might lead to the changes observed in the far region. Our neurophysiological recordings were collected simultaneously and from upper cortical layers, where the majority of the output and lateral connections are located. However, we believe that neural network effects are less likely to contribute to the changes in the far region, because neurons in the near and

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*Fig. 4.* CBF responses elicited by local injection of ACh. (A) Activation maps of FCBF in primary visual cortex (monkey E04) in response to visual stimulation and pharmacological injection. (B and C) Time course of the CBF response for the two ROIs (seven experimental sessions). (B) CBF near shows an increase in the baseline CBF and a decrease in visually induced CBF. (C) In contrast, the visually induced modulation in the CBF far increased, while the baseline did not change. (D) The average visually induced modulation for CBF near and CBF far showed a decrease (~40%) and increase (~20%), respectively, in response to ACh injection. (E) The baseline CBF near increased ~30%, whereas the baseline CBF far did not show any changes following the injection.
far region are driven by the same sensory stimulus—that is, a full-field stimulus. The neuronal activity of nearby neurons (within <2 mm) is usually synchronized, suggesting a prominent contribution from horizontal cortical connections (57). However, at larger distances, the correlation drops, reflecting a rapid spatial decay of lateral connections (57, 58). Interestingly, in vitro studies in cortical slice preparations have shown that ACh suppresses the spread of cortical excitability within a distance of <2 mm (59).

Cholinergic terminals in the cortex not only support wired neurotransmission, but also volume transmission, suggesting that ACh can influence an area beyond its site of release (60–63). Therefore, the increase in LFP power further away from the injector could be related to the diffusion of ACh from the site of injection. However, concentration differences between both regions cannot explain the distinct operational modes observed in our results because lower concentrations of ACh in the far region relative to the site of the injection would have decreased neural activity (29). In addition, the diffusion of ACh over a larger area is usually limited by AChE, which is highly expressed in cortex (21, 64). Thus, the most likely explanation of our results in the region far from the injector is that these effects are mediated by the metabolites of ACh. ACh is metabolized into Ch and acetate (65). Interestingly, studies have suggested that Ch activates nicotinic receptors (nAChRs), particularly those expressing the alpha-7 unit (44, 45, 66, 67). The activation of nAChRs is known to increase neural activity as it enhances the thalamocortical postsynaptic potentials (32, 51, 68), which in monkey V1 leads to an
Neuromodulators

Much of what we know no. 51 changes can be measured by using the cali-
E12079 A limitation of our study is that the combined elec-
bands. This might be
vol. 115 of changes to the baseline further away from the injector also
33, 49). Although an increase in GABA would reduce neural ac-
to the continuous infusion of ACh during the injection period (31,
rodents, and their activation was shown to increase blood flow (73,
also expressed on vessels in monkey or human cortex (75). An-
39, 71). Given that the baseline neural activity did not change, such a vascular effect is
Type 5 mAChRs are expressed on the surface of vessels in
in neural activity, BOLD, and CBF in the presence and absence of
which the Ch-induced activation of nAChRs in the far region is the most likely scenario. However, further experiments are needed to better understand these mechanisms.

Cholinergically Induced BOLD and CBF Profiles. Neurmodulators affect the intrinsic dynamics of target neurons as well as the metabolic properties of neural circuits (8, 10, 11, 22, 70), and, in addition, they can have direct vascular effects. To better understand these different changes associated with ACh, we measured BOLD and fCBF, which reflect different aspects of the hemodynamic response (71). The BOLD signal is driven by changes in [dHb] and depends on the combined changes of CBF, CBV, and the CMRO2. The CBF-based MRI signal reflects protons in arterioles and capillaries and their exchange with tissue water. In agreement with our neurophysiology results, we found two distinct cholinergically induced profiles in the BOLD and CBF responses, which depended on distance to the injection site. Near the injector, we observed a decrease in the visually evoked BOLD and CBF responses and an increase in the baseline. The region further away from the injection site was characterized by increased visually evoked BOLD and CBF responses, without affecting the baseline of fMRI signals. These hemodynamic responses to visual modulation after ACh injection qualitatively reflect the changes in neural activity, suggesting that ACh and its metabolites do not cause large changes to the correspondence between fMRI signals and the neurophysiological responses.

The decrease in the stimulus-evoked BOLD and CBF responses in the near region indicate that the [dHb] increased during the ACh injection compared with the preinjection response. A decrease in BOLD and CBF modulation occurs when the O2 consumption stays the same or decreases by a smaller amount than the reduction in flow (72); the reduced neural activity suggests that there is also a decrease in metabolic demand. The increases in modulation of the hemodynamic and neural responses in the region far from the injector suggest an increased metabolic demand due to the increased neural activity, which might be due to the activation of nAChRs caused by Ch (45). The ACh- and Ch-induced changes to BOLD, CBF, and neural activity are in the same direction, suggesting qualitatively that neurovascular coupling is preserved. Further studies are needed to determine quantitative relationships between neural activity, BOLD, and CBF and to determine the impact of ACh and Ch on the neural population activity and fMRI responses in behaving animals.

The increase in the baseline of the BOLD and CBF near the injector may be due to an increase in blood flow due to vaso-
dilation as a direct vascular effect of ACh (39, 71). Given that the baseline neural activity did not change, such a vascular effect is highly likely. Type 5 mAChRs are expressed on the surface of vessels in rodents, and their activation was shown to increase blood flow (73–74). However, it is still unclear whether this subclass of mAChRs is also expressed on vessels in monkey or human cortex (75). Another possible explanation may be the increased concentration of extracellular GABA resulting from the activation of mAChRs due to the continuous infusion of ACh during the injection period (31, 33, 49). Although an increase in GABA would reduce neural activity and thereby fMRI responses, the increased GABA can also lead to enhanced production of nitric oxide and consequently vasodilation (76). Indeed, this has been demonstrated in opto-
genetic studies where selective activation of PV-ir interneurons caused vasodilation and increased the CBF (76–78). The absence of changes to the baseline further away from the injector also argues for a possible effect in this region, which is not known to have direct effects on the vasculature. Further studies are needed to elucidate the exact contributions of these mechanisms.

The reduction of stimulus-evoked neural activity, BOLD, and CBF nearby the injector suggest a reduction of energy use after stimulation as a result of the reduced neural activity. Similarly, the increase in stimulus-induced BOLD, CBF, and neural activity suggests an increase in energy use upon stimulation. Although these are relative changes, CBV changes can be quantified in absolute terms, and CMRO2 changes can be measured by using the cali-
45, 82) and to increase neural activity at different rates (44, 67).

How this affects behavior needs to be tested in awake animals.

Limitations. A limitation of our study is that the combined electrophysiology and BOLD data and the CBF and the ECoG data were collected in different experimental sessions/animals. This

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was due to the technical difficulty of the experiments and the long duration of the injection experiment. Hardware issues limited the techniques that could be combined; for instance, the transmit radiofrequency (RF) coil and phased array needed for CBF measurement was not compatible with simultaneous electrophysiological recording, and the presence of electrodes reduces image SNR and creates artifacts, precluding accurate CBF measurement. Also, the ECoG grid was not compatible with simultaneous MRI. Although average traces allow qualitative comparisons of neural activity and BOLD and CBF data, the data do not support direct quantitative comparison. The interindividual variation between animals (83) also limits the quantitative inferences that can be made, and further combined experiments would be needed to address such questions.

Another possible limitation might be the layer specificity of our neurophysiological recordings and pharmacological manipulations. The injections and recordings performed by using the μECoG arrays were confined to upper cortical layers, known to express mAChRs and to most accurately encode visual information (41). Although we cannot assign a specific laminar origin to the injection and recording sites in the concurrent neurophysiology and fMRI experiments, we believe that our recordings and injections were mostly performed in superficial layers (e.g., II/III) based on the following points: First, the electrode and the injector formed a single unit and were inserted by using an micromanipulator that allowed us to determine the depth of the electrode penetration. Additionally, electrode insertion was done slowly under visual and auditory guidance. The electrode has to traverse layers II/III, which are layers with strong stimulus activity (84). Given the difficulty of our experiments and the long duration of the injection trials, we usually recorded from a single site (one electrode penetration) per day.

We recorded all experiments under general anesthesia, and we do not foresee opportunities to perform similar manipulations in alert monkeys in the near future. However, our anesthesia protocol targets μ-opioid receptors which are highly expressed in basal ganglia and thalamus, especially in regions related to motor function and pain, but regions associated with cognition (e.g., the ventral tegmental area, substantia nigra, and frontal regions) have low densities of μ-opioid receptors (85). A study from our laboratory that compared results obtained in anesthetized monkeys with results from alert monkeys found that the neurovascular coupling was relatively unaffected by this anesthesia protocol (41, 42), and a subsequent study reported that the face-processing network was largely unchanged under this anesthesia regimen (86). Therefore, it is unlikely that the anesthetic regimen we used had a major influence on the profiles of neural and hemodynamic responses reported here. Anesthetized animals are, of course, not affected by changes in behavioral state, and although this poses an advantage by allowing us to assess effects of cholinergic neuromodulation without the need to take behavioral parameters or the effect of other neuromodulators into account, it is a limitation in the sense that it does not allow us to relate our results directly to behavior.

Conclusions
Our results provide a comprehensive account of the effects of locally applied ACh on neural and hemodynamic responses in the macaque visual cortex. Our results show that local manipulations of cholinergic neuromodulation elicit changes in neural and fMRI activity that differ depending on the distance from the injection and also from changes reported during systemic manipulation of ACh (22, 23, 74), where increases in fMRI and neural responses were observed. This illustrates the difference between local and global neuromodulatory effects of ACh on neurovascular coupling and shows that it is essential to understand the specific profiles of neural and metabolic activity in any circuit of interest. The combination of BOLD with CBF and/or CBV measurements as well as with electrophysiology can help resolve some of these complexities and can deepen our understanding of the physiological function and pathologies involving the cholinergic system. A better understanding of the effects of neuromodulators on the BOLD response may improve the interpretation of fMRI studies such as studies of reward and attention, etc., where neuromodulation plays a role.

Materials and Ethical Statement. We collected data from eight rhesus monkeys (Macaca mulatta, seven males and one female), 5–9 y old weighing 8–12 kg. All experimental procedures were carried out under anesthesia and were approved by the local authorities (Regierungsspräsidium, Tübingen, Germany, Project nos. KY01-07 and KY04-16). Experiments were in full compliance with the guidelines of the European Community for the care and use of laboratory animals (EUVD 86/609/EEC).

MRI Scanning. We acquired BOLD signals and fCBF using a vertical 4.7 Tesla Bruker BioSpec scanner with 40-cm-diameter bore (Bruker BioSpin GmbH). We performed 15 BOLD experiments and 7 CBF experiments. For the BOLD experiments, we used a custom-built RF surface coil with an inner diameter of 30 mm as transceiver, positioned around the recording chamber. For BOLD experiments we used an 8-shot Gradient Echo EPI with a field of view (FOV) of 35 x 35 mm, slice thickness of 1 mm, a voxel size of 0.25 mm×0.25 mm×0.25 mm, matrix 128 × 256, and receiver bandwidth (BW) 100 kHz. Echo time (TE) and repetition time (TR) were 18 and 500 ms, respectively, with a flip angle of 30°. Each experimental session consisted of 592 volumes. To ensure good homogeneity of the magnetic field, we used FASTMAP to shim over a volume of 12 mm×12 mm×12 mm (87) positioned in the area of the electrode tip. For fCBF measurements, we used a volume coil to transmit in combination with a custom-built two-channel receive phased array (88). For perfusion imaging, a flow-sensitive alternating inversion recovery (FAIR) sequence (89) was used with alternating slice-selective and nonslice-selective inversion pulses (13–15 ms hyperbolic secant pulse). After an inversion time (TI) of 1.300 ms, a single-slice, single-shot EPI image was collected. FOV was 64 × 64 mm², resolution 1 × 1 mm², slice thickness 3 mm, receiver BW 100 kHz, and TE/TR 11.53/500 ms. Anatomical reference scans were T₂-weighted scans (RARE) with a FOV of 64 × 65 mm², slice thickness 1 mm, resolution 0.1 × 0.1 mm², and TE/TR 46.8/ 4.800 ms.

Single-Electrode Neurophysiology. Single-electrode recordings were made simultaneously with BOLD fMRI. The electrodes were custom-built and consisted of glass-coated platinum-iridium wires. The tip of the wires was custom-ground with a 50° angle (3). The amplifiers for the single-electrode neurophysiology recordings were custom-built and consisted of an analog compensation mechanism for the noise induced by gradient switching and RF pulses of the scanner. The details of the methods for interference compensation and signal conditioning are given elsewhere (1, 4, 90). The interference-compensated signal was then read in by an analog–digital (AD) converter with 16-bit resolution (National Instruments). The AD converter was linked directly to a PC running a real-time operating system (QNX). Since both the gradient noise and the compensation is incomplete, additional software-based cleaning of scanner-induced interference was done with custom-written code based on principal component analysis in MATLAB (The MathWorks; for details, see refs. 1 and 91).

μECoG Arrays. We custom-designed a μECoG array to record field potentials from macaque visual cortex (NeuroNexus Technologies, Inc.). The array was microfabricated on a very thin polyimide film (20 μm) with electrode sites of 50-μm diameter spaced 500 μm apart on a 4 × 8 grid (i.e., 1.5 × 4.0 mm² rectangular grid). We acutely implanted the μECoG arrays on the surface of V1. For this, we first performed small skull trepanations (6 mm diameter) using stereotaxic coordinates in each monkey. Subsequently, we carefully dissected the meninges layer-wise under the microscope (Zeiss Opmi MDU/55) to expose the surface of V1 (Fig. 4A). The injector was attached to a manual micromanipulator (Narashige Group), which kept the probe in the desired position throughout the experiment. We measured the impedance of the electrode sites before and during the experiments, which ranged from 400 to 600 kΩ. The signals were amplified and filtered into a band of 1 Hz to 8 kHz using a multichannel–processor amplifier system (Alpha-Omega Engineering) and then digitized at 20,833 kHz with a 16-bit resolution ADC converter (National Instruments). LFPs were extracted by bandpass-filtering the signals and down-sampled in two steps: (i) using a sampling rate of 1.5 kHz by using a fourth-order Butterworth filter (500-Hz cutoff edge); and (ii) to 500 Hz by using a Kaiser window between 1 and 150 Hz, with a (1-Hz) transition band and stopband attenuation of 60 dB. This two-step procedure was computationally more efficient.
than a single filtering operation to the final sampling rate. The sharp second filter was used to eliminate phase shifts introduced by the filters. The boundaries for each frequency band were estimated by quantifying amplitude covariations across LFP frequencies. This procedure involved computing the signal and noise correlations to distinguish LFP frequencies sharing common neural properties (for details, see refs. 34 and 95).

To better visualize the power changes due to the pharmacological intervention, we computed the visually induced modulation for each frequency band in the same way as described in 

**Electrophysiology Data Analysis.** To analyze the electrophysiology data, we used a 1-s nonoverlapping window to calculate the PSD of different frequency bands. We extracted the LFPs and MUA by band-pass filtering the signals using custom-written MATLAB routines. Forward and backward filtering was used to eliminate phase shifts introduced by the filters. The boundaries for each frequency band were estimated by quantifying amplitude covariations across LFP frequencies. This procedure involved computing the signal and noise correlations to distinguish LFP frequencies sharing common neural properties (for details, see refs. 34 and 95).

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