Acetylcholine excites neocortical pyramidal neurons via nicotinic receptors

Abbreviated Title: Nicotinic responses of neocortical pyramidal neurons

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ABSTRACT
The neuromodulator acetylcholine shapes neocortical function during sensory perception, motor control, arousal, attention, learning and memory. Here we investigate the mechanisms by which ACh affects neocortical pyramidal neurons in adult mice. Stimulation of cholinergic axons activated muscarinic and nicotinic ACh receptors on pyramidal neurons in all cortical layers and in multiple cortical areas. Nicotinic receptor activation evoked short-latency, depolarizing postsynaptic potentials in many pyramidal neurons. Nicotinic receptor-mediated postsynaptic potentials promoted spiking of pyramidal neurons. The duration of the increase in spiking was membrane potential-dependent, with nicotinic receptor activation triggering persistent spiking lasting many seconds in neurons close to threshold. Persistent spiking was blocked by intracellular BAPTA, indicating that nAChR activation evoked persistent spiking via a long-lasting calcium-activated depolarizing current. We compared nicotinic PSPs in primary motor, prefrontal and visual cortices. The laminar pattern of nicotinic excitation was not uniform, but was broadly similar across areas, with stronger modulation in deep than superficial layers. Superimposed on this broad pattern were local differences, with nicotinic PSPs being particularly large and common in layer 5 of M1, but not layer 5 of PFC or V1. Hence, in addition to modulating the excitability of pyramidal neurons in all layers via muscarinic receptors, synaptically-released ACh preferentially increases the activity of deep-layer neocortical pyramidal neurons via nicotinic receptors, thereby adding laminar selectivity to the widespread enhancement of excitability mediated by muscarinic ACh receptors.
INTRODUCTION

The neuromodulator acetylcholine (ACh) shapes neocortical function during sensory perception (Metherate, 2004; Disney et al., 2007), motor control (Berg et al., 2005), arousal (Steriade, 2004; Jones, 2008), attention (Herrero, 2008; Parikh & Sarter, 2008), learning (Kilgard, 2003; Ramanathan et al., 2009) and memory (Winkler et al., 1995). A decline in neocortical ACh has been tied to conditions such as depression (Dislaver, 1986), schizophrenia (Raedler & Tandon, 2006), Alzheimer’s disease (Whitehouse et al., 1982) and Parkinson’s disease (Whitehouse et al., 1983).

Most cholinergic axons in neocortex arise from nucleus basalis and other basal forebrain nuclei such as substantia innominata (Wainer & Mesulam, 1990). The cholinergic projection from basal forebrain plays a central role in shaping neocortical components of arousal, attention, learning, memory, sensory perception and motor control. For example, stimulation of vibrissal M1 evokes whisker movements that are enhanced by activation of basal forebrain (Berg et al., 2005) and basal forebrain lesions impair motor control (Garbawie & Whishaw, 2003) and motor map rearrangement during motor learning (Conner et al., 2003).

ACh affects neocortical networks, in part by modulating the activity of pyramidal neurons. Pyramidal neurons express nicotinic and muscarinic ACh receptors (nAChRs and mAChRs) on their plasma membranes (van der Zee et al., 1992; Mrzljak et al., 1993), but ACh is thought to act on pyramidal neurons primarily via mAChRs. Activation of mAChRs evokes an initial hyperpolarization and subsequent slow depolarization of many cortical pyramidal neurons. The hyperpolarization results from activation of an SK-type potassium current, whereas the slow depolarization has been linked to a number of currents, including M-, AHP- and inward rectifier-type potassium currents and a non-
specific cation current (Krnjevic, 1971; McCormick & Prince, 1985, 1986; McCormick & Williamson, 1989; Haj-Dahmane & Andrade, 1996; Delmas & Brown, 2005; Gulledge & Stuart, 2005; Carr & Surmeier, 2007; Zhang & Séguéla, 2010). There are reports of ACh activating nAChRs on pyramidal neurons (Roerig et al., 1997; Chu et al., 2000; Kassam et al., 2008; Zolles et al., 2009; Guillem et al., 2011; Poorthuis et al., 2012; but see also Vidal & Changeux, 1993; Gil et al., 1997; Porter et al., 1999). Few authors have studied nicotinic postsynaptic currents in neocortical pyramidal neurons (Roerig et al., 1997; Chu et al., 2000). Hence the functional roles of nAChRs on pyramidal neurons remain obscure.

Here we studied how ACh affects pyramidal neurons, focusing on the role of nAChRs. To drive synaptic release of ACh, we expressed the light-activated protein channelrhodopsin-2 (ChR2) in cholinergic neurons in the basal forebrain, allowing us to selectively stimulate cholinergic axons in neocortex (Kalmbach et al., 2012). In contrast to many previous reports, we find that ACh excites pyramidal neurons via both mAChRs and nAChRs. Activation of nAChRs occurs with short latency, consistent with nAChRs being located at synapses between cholinergic axons and pyramidal neurons, and can evoke persistent spiking via a calcium-activated conductance. Direct nAChR-mediated effects occurred in pyramidal neurons in several neocortical areas and in all neocortical layers, indicating that direct excitation of pyramidal neurons via nAChRs can occur across neocortical layers and areas. However, laminar and regional differences in both the incidence and amplitude of the nAChR-mediated depolarization suggest regional differences in the modulation of neocortical networks by nAChRs.
METHODS

All experiments and procedures were approved by the Northwestern University Institutional Animal Care and Use Committee (IACUC).

Two approaches were employed to selectively express ChR2 in cholinergic neurons:
(1) stereotaxic injection of a floxed viral vector into the basal forebrain of ChAT-Cre mice, and (2) crossing ChAT-Cre and floxed ChR2 mouse lines.

For experiments using virally-delivered ChR2, we used Tg(ChAT-Cre)60Gsat mice (GENSAT), which express Cre-recombinase on a choline-acetyltransferase (ChAT) promoter, resulting in Cre expression in cholinergic neurons throughout the brain. Into this mouse we injected adeno-associated virus with a double-floxed inverse open reading frame (EF1a-DIO-hChR2(H134R)eYFP, Virus Vector Core, University of North Carolina), which drives expression of ChR2-yellow fluorescent protein (ChR2-YFP) in infected neurons containing Cre. 400 nl of virus was injected into the basal forebrain at postnatal day 21 using stereotaxic coordinates (0.2 mm A-P, 1.7 mm M-L, 4.5 mm D-V). Three weeks after injection, ChR2 is expressed almost exclusively in cholinergic neurons and their axons in neocortex, with no adverse effects (Porter et al., 1999).

In some experiments, selective expression of ChR2 in cholinergic neurons was achieved without the use of viral vectors by crossing ChAT-Cre (B6;129S6-Chat^mut(cre)Lou/J, Jax 006410) and floxed ChR2 (129S-Gt(ROSA)26Sor^tm32(CAG-4COP4*H134R/EYFP)Hze/J, Jax 012569, Ai32 (Madisen et al., 2012) mouse lines to produce ChAT-ChR2(Ai32) mice. Cre+ mice were crossed with ChR2+/+ mice to yield Cre+ ChR2+/− offspring. These offspring were crossed to generate Cre+ ChR2+/+ mice, which were used for experiments. Results from ChAT-ChR2(Ai32) mice were similar to those from viral infections and results from these two approaches were pooled, unless otherwise noted.
Somatic and axonal labeling with ChR2-YFP was examined in fixed sections. Tissue was fixed by transcardial perfusion with 4% paraformaldehyde in phosphate buffer and 100-200 µm thick coronal sections were cut using a vibrating microtome. In some sections, the YFP signal was enhanced with an anti-GFP primary (ab13970, 1:10,000, Abcam) and a fluorescent secondary antibody (613111, 1:750, Invitrogen). Images were acquired by widefield or 2-photon microscopy. Widefield images were acquired using a GFP filter set and a Hamamatsu Orca-285 camera. 2-photon images were acquired with 880 nm illumination from a Coherent Chameleon Ultra II Ti:sapphire laser and a 505-545 nm emission filter.

Whole-cell recordings were obtained from pyramidal neurons in 300 µm-thick acute parasagittal slices of primary motor cortex and coronal slices of prefrontal and visual cortices from postnatal day 33-61 mice of either sex. In virally-infected mice, recordings were obtained ~3 weeks after viral injection. Slices were prepared in ice-cold artificial cerebro-spinal fluid (ACSF; in mM): 125 NaCl, 2.5 KCl, 1.25 NaH2PO4, 20 NaHCO3, 5 HEPES, 25 Glucose, 2 CaCl2, 1 MgCl2, pH 7.3, oxygenated with 95% O2/5% CO2. Whole-cell recording pipettes were 4–8 MΩ when filled with intracellular solution: 135 mM K gluconate, 4 mM KCl, 10 mM HEPES, 10 mM Na2-phosphocreatine, 4 mM Mg-ATP, 0.3 mM Na2-GTP, 0.2% (w/v) biocytin, 10 µM alexa 594, pH 7.3.

Recordings were obtained at 35-37 °C with a feedback circuit and temperature controller (TC-324B; Warner Instruments, Hamden, CT, USA). In experiments using calcium-free ACSF, 2 mM CaCl2 was replaced with 4 mM MgCl2 to give a final MgCl2 concentration of 5 mM. ChR2 was activated by wide field illumination through a microscope objective (Olympus, x20/0.95 NA or x40/0.8 NA) using a blue light-emitting diode (LED; Thorlabs LEDC5 and LEDD1 or DC2100 driver). The maximum steady-state
intensity was 20 mW/mm². In some experiments (see figure 4E-G), illumination was restricted by closure or partial closure of the fluorescence field stop. The illumination area was measured offline by bleaching a thin, immobilized film of fluorescence on a microscope slide. Closure of the field stop had no effect on illumination intensity per unit area. In restricted illumination experiments, the soma was positioned in the center of the field of illumination unless noted otherwise.

The amplitudes of voltage responses were calculated by subtracting the average baseline membrane potential for ≥1 s before illumination from the peak of the response. Voltage responses which failed to exceed 3 standard deviations of the baseline membrane potential were assigned an amplitude of 0 mV. During brief bursts of nAChR PSPs, the peak amplitude was calculated by subtracting the pre-burst membrane potential from the most depolarized potential during the burst.

Local application of ACh was by pressure ejection of 100 µM ACh in ACSF from a glass pipette ~50 µm from the soma, 30 psi, Toohey Spritzer IIe (Toohey Company, Fairfield NJ).

Mecamylamine hydrochloride and atropine were obtained from Sigma-Aldrich. Dihydro-β-erythroidine hydrobromide (DHβE), methyllycaconitine citrate, galanthamine hydrobromide and physostigmine hemisulfate were obtained from Tocris Bioscience. NBQX disodium salt, (R)-CPP, gabazine (SR95531), CGP 52432, tetrodotoxin citrate and (R,S)-MCPG were obtained from Ascent Scientific. In some experiments, we used (2R)-amino-5-phosphonopentanoic acid (AP5) to block NMDA receptors, instead of CPP. AP5 was obtained from Tocris.

Statistical analyses were performed using Graphpad QuickCalcs online tool (http://www.graphpad.com/quickcalcs/) or in Graphpad Instat 3.06 (GraphPad...
Continuous data (such pharmacology of the nAChR PSP) were analyzed with a two-tailed t-test, Kruskal-Wallis test or Mann-Whitney test and categorical data with Fisher's exact test.

The kinetics and latency of nAChR PSPs were measured by fitting a sum of two exponentials to the mean of ten trials. In each trial the PSP was evoked with a single 2 ms (or occasionally 5 ms) blue light illumination. The signal-to-noise ratio of our recordings was not sufficient to permit accurate measurement of the timing of the initial rise of the membrane potential of a nAChR PSP, which is smaller and slower than a glutamatergic PSP. To accurately estimate the point of initial rise we therefore measured the time from the start of illumination to 5% of the peak amplitude of the PSP. We would expect this method of latency measurement to overestimate the latency of each PSP by approximately 1 ms and we therefore corrected the latency of each PSP, by back-extrapolating the fit to the resting membrane potential preceding the stimulus.

Pyramidal neurons were identified by their somatic shape and spiking pattern and by their large apical dendrites, which were visible by fluorescence microscopy once filled with indicator. Neurons were routinely filled with indicator and their dendrites examined by fluorescence microscopy. Neurons with truncated primary apical dendrites, lacking apical dendrites and with spiking patterns more typical of interneurons (narrow spikes, large after-hyperpolarization) were excluded from further analysis.

Pyramidal cells were classified according to the laminar locations of their somata. Laminar borders were based on the Allen Brain Atlas and consistent with laminar variation in opacity of slices, which was visible under brightfield illumination. Distances from the pial surface of the slice were measured perpendicular to the pia. In primary motor cortex, cortical layers were: layer 2/3 150-500 µm, layer 5A 600-750 µm, layer 5B...
800-1200 µm, layer 6 >1300 µm. In prefrontal cortex, cortical layers were: layer 2/3
100-300 µm, layer 5 330-550 µm, layer 6 >550-800 µm (Guillem et al., 2011). In visual
cortex, cortical layers were: layer 2/3 100-275 µm, layer 5 450-750 µm, layer 6 >750 µm
(Olivas et al., 2012; Petrof et al., 2012).

Pyramidal neurons projecting to defined postsynaptic target tissues were labeled
using fluorescent microspheres (RetroBeads, Lumafluor). Beads were injected into
dorsolateral striatum (0 mm A-P, -2 mm M-L, 2.5 mm D-V), cervical spinal cord
(between ~0.5 mm M-L and the midline, ~1 mm D-V, at the level of the C2 vertebra) or
ventral posteromedial thalamus (two sites: 1.6 mm A-P, 1.2 mm M-L, 3.25 mm D-V and
1.6 mm A-P, 1.7 mm M-L, 3.5 mm D-V). Recordings were obtained 5-11 days after bead
injection from M1 cortex contralateral to striatal and spinal injections and ipsilateral to
thalamic injections.
RESULTS

To stimulate cholinergic axons entering neocortex, we used the blue light-activated membrane protein channelrhodopsin-2 (ChR2; Nagel et al., 2003). We expressed ChR2 in cholinergic neurons in the basal forebrain (figure 1A-C), obtaining selectivity for cholinergic neurons with a ChAT-Cre mouse line and floxed virus or by crossing ChAT-Cre and floxed ChR2 mouse lines. We obtained whole-cell recordings from layer 5B pyramidal neurons in acute slices of primary motor cortex containing ChR2-labeled axons (figure 1D). Mean resting potential was -62 ± 1 mV (51 neurons). Widefield illumination of the slice with a blue LED evoked one or more of four voltage responses: a slow depolarization, a hyperpolarization, a medium depolarization, or a fast depolarization (figure 1F). All responses were absent in tetrodotoxin (500 nM TTX, 17 neurons) and after removal of extracellular calcium (10 neurons), indicating that all four voltage responses were evoked by spikes, leading to vesicular release (figure 2).

We used pharmacology to identify the receptors underlying each of the four voltage responses. The hyperpolarization and slow depolarization were mediated by muscarinic ACh receptors (mAChRs): both hyperpolarization and slow depolarization were eliminated by the mAChR antagonist atropine (1 µM, 140 of 141 neurons, figure 3), but not by the nAChR antagonist mecamylamine (100 µM, 11 of 13 neurons, figure 3) or by glutamate or GABA receptor antagonists (10 µM NBQX and 10 µM CPP; 1 µM gabazine and 3 µM CGP 52432; 14 of 16 neurons).

The medium depolarization was mediated by nicotinic ACh receptors (nAChRs). It was eliminated by mecamylamine (100 µM, 12 of 12 neurons, P < 0.05, paired two-tailed t-test), enhanced 73 ± 21% by the nAChR allosteric potentiator and cholinesterase antagonist galanthamine (1 µM, 23 neurons, P < 0.05, paired two-tailed t-test) and 27 ±
15% by the ACh esterase inhibitor physostigmine (0.5 µM, 4 neurons, \( P < 0.05 \), paired two-tailed t-test), and insensitive to antagonists of mAChRs, GluRs and GABA receptors (5 of 5, 7 of 7 and 8 of 8 neurons, respectively; figure 4). The medium depolarization was unaffected by the \( \alpha_7 \)-nAChR antagonist methyllycaconitine (MLA, 10 nM, 12 of 12 neurons) and eliminated by dihydro-\( \beta \)-erythroidine (DH\( \beta \)E, 10 µM, 16 of 16 neurons, \( P < 0.05 \), paired two-tailed t-test, figure 4), which has high affinity for \( \alpha_4 \beta_2 \)-containing nAChRs. Hence the medium depolarization is mediated by non-\( \alpha_7 \) nAChRs, probably \( \alpha_4 \beta_2 \)-containing nAChRs.

The fast depolarization was mediated by ionotropic glutamate receptors (GluRs): the fast depolarization was not inhibited by nAChR and mAChR nor by GABA receptor antagonists (each \( P < 0.05 \), paired two-tailed t-test), but was eliminated by ionotropic GluR antagonists (figure 5A). GluR-mediated fast depolarizations were observed only in a subset of ChAT-ChR2(Ai32) mice and not in virally-infected mice. Furthermore, for each ChAT-ChR2(Ai32) mouse we found that GluR-mediated fast depolarizations were obtained in either all or no neurons. We recorded from multiple neurons in slices from 25 mice. In 13 of these 25 mice, we observed GluR-mediated fast depolarizations in every neuron; 12 of 25 mice exhibited no GluR-mediated fast depolarization in any recording. Hence it is likely that the GluR-mediated fast depolarization results from expression of ChR2 in non-cholinergic neurons in a subset of ChAT-ChR2(Ai32) mice. To eliminate GluR-mediated responses, we included GluR receptor-antagonists in all remaining experiments with ChAT-ChR2(Ai32) mice. The presence of GluR-mediated responses in some ChAT-ChR2(Ai32) mice is the only difference we observed between genetic and viral methods of driving ChR2 expression in cholinergic neurons: in other respects, the two methods of driving ChR2 expression were equivalent (no difference in amplitudes of...
nAChR PSPs, \( P > 0.05 \), Matt-Whitney test, 37 and 22 neurons from virally-infected and ChAT-ChR2(Ai32) mice, respectively). We therefore pooled results from the two techniques.

All four postsynaptic responses could occur individually, but most pyramidal neurons exhibited a combination of responses mediated by two or more receptors (figure 5B). Hence synaptically-released ACh activates pyramidal neurons via nAChRs and mAChRs, and often via both types of ACh receptor in the same neuron.

**Activation of pyramidal neuron mAChRs by synaptically-released ACh**

The peak amplitude of the hyperpolarization, evoked by brief illumination (\( \leq 10 \times 5 \) ms at 20 Hz), was 1.1 ± 0.5 mV and the decay time constant was 774 ± 73 ms (10 neurons). The slow depolarization displayed a peak amplitude of 2.0 ± 0.6 mV (6 neurons) and lasted several seconds. At resting membrane potentials, the hyperpolarization and slow depolarization were rare, occurring in only 7% (8 of 112) and 19% (21 of 112) of layer 5B pyramidal neurons, respectively.

Many previous authors have reported activation of pyramidal neurons via mAChRs upon application of ACh to the soma, by pressure ejection from a nearby pipette (McCormick & Prince, 1985; McCormick & Williamson, 1989; Gulledge & Stuart, 2005; Gulledge et al., 2007), with relatively few authors reporting nAChR-mediated depolarization of pyramidal neurons (Roerig et al., 1997; Chu et al., 2000; Kassam et al., 2008; Zolles et al., 2009; Guillem et al., 2011; Poorthuis et al., 2013). Similar to many previous studies, we found that pressure ejection of ACh onto the soma evoked a mAChR-mediated hyperpolarization and slow depolarization (5 of 6 and 6 of 6 neurons, respectively; figure 6A), but no nAChR-mediated depolarization.
One possible interpretation of our results might be that synaptically-released ACh activates primarily nAChRs and usually fails to activate mAChRs, whereas pressure ejection onto the soma activates a different population of receptors, primarily mAChRs. mAChR activation modulates primarily potassium conductances (McCormick, 1992) and the reversal potential for potassium is ~-90 mV. mAChR activation may therefore exert little effect on the membrane potential at rest: both mAChR-mediated hyperpolarization and slow depolarization are larger when the neuron is depolarized (McCormick & Prince, 1986; Gulledge & Stuart, 2005). To maximize the effects of mAChR activation on the membrane potential and, therefore, the probability that we would observe activation of mAChRs, we depolarized neurons by somatic current injection (figure 6B). Brief stimuli (≤10 x 5 ms illumination at 20 Hz) evoked mAChR-mediated voltage responses in 79% of depolarized layer 5B pyramidal neurons (figure 6C), indicating that synaptically-released ACh activates mAChRs in the majority of layer 5B pyramidal neurons, but that the resulting hyperpolarization or depolarization from rest is often too small to be observed.

Previous authors have also reported that ACh, applied by pressure application, more readily evokes postsynaptic mAChR-mediated responses in pyramidal neurons in deep-than in superficial-layers of the neocortex (McCormick & Prince, 1986; McCormick & Williamson, 1989; Gulledge et al., 2007). Similarly, we found that synaptically-released ACh more readily excites pyramidal neurons via mAChRs in deep than superficial layers of M1 cortex, with the proportion of pyramidal neurons that exhibited a mAChR-mediated slow depolarization or hyperpolarization ranging from 53% in layer 2/3 to 91% in layer 6 pyramidal neurons (figure 6C).

We conclude that ACh released by a brief burst of cholinergic activity activates mAChRs on the majority of pyramidal neurons throughout primary motor cortex. In
comparison to pressure application of ACh, activation of cholinergic synapses with brief bursts of stimuli provides relatively weak activation of mAChRs which often fails to affect the somatic membrane potential at rest.

ACh release evokes a nicotinic PSP

Brief illumination (≤10 x 5 ms at 20 Hz) in mAChR, GluR and GABAR antagonists evoked a nAChR-mediated medium depolarization in almost all (82 of 90) layer 5B pyramidal neurons at rest. We stimulated cholinergic axons with brief bursts of stimuli at 20 Hz, a stimulus designed to mimic the spiking of cholinergic basal forebrain neurons during paradoxical sleep and awake states, when cholinergic basal forebrain neurons spike in bursts, each of ~4-6 spikes at ~25 Hz (Manns et al., 2000; Lee et al., 2005). During such bursts, summation occurred at stimulus frequencies greater than ~5 Hz. In our experiments summation may result, in part, from accumulation of calcium entering through ChR2 channels in the presynaptic terminal (Zhang & Oertner, 2007) and may therefore be an artifact of the optogenetic stimulus. Nonetheless, summation permitted us to evoke a substantial depolarization via nAChRs: a burst of stimuli at 20 Hz frequently evoked a peak depolarization of 5-10 mV (figure 7A,B).

We measured the latency and kinetics of the nAChR-mediated depolarization evoked by a single 2 ms illumination, in 1 µM atropine to inhibit mAChRs and GluR and GABAR antagonists to eliminate any indirect effects. In 15 of 15 neurons, 2 ms illumination evoked reproducible depolarizations (figure 7C), with a mean amplitude of 1.4 ± 0.2 mV (11 neurons). To the mean response we fit a sum of two exponentials (figure 7D) with mean rise and decay time constants of 28 ± 4 ms and 180 ± 23 ms (14 neurons). These relatively slow kinetics are comparable to those of PSPs mediated by α4β2-containing
nAChRs in interneurons (Bell et al., 2011), but slower than PSPs mediated by α7-nAChRs (Frazier et al., 1998; Fedorov et al., 2012). These kinetics suggest that the medium depolarization is a PSP mediated by non-α7 nAChRs, consistent with the pharmacology of the medium depolarization, presented above.

The latency of the nAChR PSP, measured from the start of illumination, was 5.9 ± 0.8 ms (12 neurons). Compared to electrical stimulation, ChR2 drives relatively slow depolarization of the neuronal membrane, typically with rise and decay time constants of at least several milliseconds (Lin et al., 2009; Yizhar et al., 2011), which may be further elongated by the time constant of the membrane. The latency of the fast depolarization was 5.3 ± 0.5 ms (3 neurons). Hence the latency of the nicotinic PSP was only ~0.5 ms longer than that of a monosynaptic glutamatergic PSP, consistent with the medium depolarization being a monosynaptic PSP generated at synapses between cholinergic axons and pyramidal neurons.

nAChRs appear to be distributed throughout the dendritic trees of cortical pyramidal neurons (van der Zee et al., 1992; Nakayama et al., 1995) but the locations of cholinergic synapses are unknown. To determine whether nAChR PSPs were evoked primarily by cholinergic synapses in the proximal or distal dendrites of layer 5B pyramidal neurons we measured nAChR PSPs during restricted illumination of the slice (figure 8A,B). Restricting illumination to a radius of less than ~300 µm around the soma was necessary to reduce the amplitude of the nAChR PSP and the amplitude was reduced by 50% when the radius of illumination was ~50 µm (figure 8C, 7 neurons). Illumination of the tuft dendrites failed to evoke a nAChR PSPs at the soma (figure 8B, 3 neurons). Hence the nAChRs which contribute to the somatic depolarization in our experiments are likely to
nAChR activation can evoke persistent spiking

We next addressed the functional consequences of nAChR activation. nAChR PSPs increased the spike rate of layer 5B pyramidal neurons in primary motor cortex, depolarized beyond threshold by somatic current injection (figure 9A). The increase was independent of initial spike rate (figure 9B), with 2-5 ms illumination increasing spike rate by $2.2 \pm 0.1$ Hz (6 neurons) and a brief burst (10 x 5 ms at 20 Hz) increasing spike rate by $3.1 \pm 0.2$ Hz (4 neurons). Hence, during spiking nAChR activation causes a linear, additive change in spike rate with no change in the slope of the input-output relationship. The elevated spike rate was maintained during repetitive stimulation and declined after cessation of the cholinergic stimulus with a decay time constant of $185 \pm 21$ ms (figure 9C; 2 or 5 ms illumination; 6 neurons), which matches the decay of the underlying nAChR PSP.

With the membrane depolarized from rest but subthreshold, cholinergic stimulation evoked persistent spiking. We evoked nAChR PSPs during long step current injections of increasing amplitude until the nAChR PSP evoked one or more spikes. A nAChR PSP reduced rheobase by $11.6 \pm 3.7$ pA (from $344 \pm 26$ pA to $337 \pm 26$ pA, 18 neurons, 5 ms illumination in the presence of atropine). However, under these conditions, a nAChR PSP typically evoked multiple spikes. The spike rate during the first second after stimulus onset was $3.0 \pm 0.2$ Hz (6 neurons) for a single 2-5 ms illumination and $7.1 \pm 1.2$ Hz for a brief burst (10 x 5 ms at 20 Hz, 7 neurons). Spike rate declined slowly after
the last nAChR PSP (figure 10A,B), but spiking typically continued until the holding
current was removed, which was up to 4 seconds after the initial spike (figure 10A,B).

We defined persistent spiking as spiking that continued for at least 500 ms after the
dead of the cholinergic stimulus. Using this definition, persistent spiking occurred in
every neuron (13 of 13 neurons), but in 6 of 13 neurons persistent spiking occurred on
some but not all trials. In trials in which persistent spiking failed to occur, the nAChR
PSP evoked only a single spike (mean 1.0 ± 0, 14 trials from 4 neurons, single 2-5 ms
illumination), whereas in trials in which persistent spiking occurred the nAChR PSP
evoked 7.5 ± 1.6 spikes (10 trials in the same 4 neurons with the same stimulus).

Comparing trials with and without persistent spiking, the resting membrane potential at
the start of the trial (-62.3 ± 1.9 and -63.2 ± 1.5 mV, respectively), the current injected to
depolarize the neuron (209 ± 20 and 268 ± 23 pA, respectively), and the membrane
potential immediately before the nAChR PSP (-48.0 ± 2.0 and -48.5 ± 1.8 mV,
respectively) and the threshold of the first spike (-35.2 ± 1.9 and -39.1 ± 1.9 mV,
respectively) were similar (18 and 14 trials respectively, each P > 0.05, paired 2-tailed t-
test). These measurements indicate that trial-to-trial variability in perisomatic
membrane potential, input resistance and spike threshold do not account for the
variability in persistent spiking, although they do not exclude a role for such changes in
the distal dendrite, as a result of ongoing synaptic activity, for example.

Persistent spiking required activation of nAChRs on pyramidal neurons, but not
glutamate or GABA receptors or mAChRs (figure 11). Persistent spiking occurred in the
presence of 10 μM NBQX, 10 μM CPP, 1 μM gabazine, 3 μM CGP and 1-10 μM atropine
(13 of 13 neurons). Subsequent addition of 100 μM mecamylamine (in the continued
presence of glutamate and GABA receptor antagonists and of atropine) blocked
persistent spiking (3 of 3 neurons, figure 11A) but 100 µM MCPG did not (3 of 3 neurons).

nAChR activation provides more than just the initial depolarization required to initiate persistent spiking since in the absence of cholinergic stimulation, brief depolarization failed to evoke persistent spiking (figure 11C). Furthermore, during persistent spiking evoked by cholinergic stimulation, brief hyperpolarization of the membrane inhibited persistent spiking, only for spiking to resume after the hyperpolarizing pulse (figure 11D). Presumably, an additional depolarizing conductance is activated (or hyperpolarizing conductance deactivated) by nAChR activation or by another receptor co-activated with nAChRs and this additional conductance remains active for several seconds, long after the decay of the nAChR PSP. The spike waveform changed little during persistent spiking (figure 12), suggesting that this additional current is unlikely to arise from one of the sodium or potassium conductances that shape the spike waveform.

Persistent spiking was eliminated by 10 mM intracellular BAPTA. With or without intracellular BAPTA, nAChR PSPs evoked 1 or more spikes (figure 11E), but in BAPTA spiking did not continue after the decay of the underlying PSP. Without BAPTA spiking continued until the holding current was removed, with the last spike 2155 ± 261 ms after the start of a single 2-5 ms illumination and 3357 ± 169 ms after a burst of stimuli (10 x 5 ms at 20 Hz; 13 neurons). With BAPTA, spiking ended 185 ± 38 ms after a single illumination and 445 ± 115 ms after a burst (3 neurons; single illumination and burst each P < 0.05, unpaired 2-tailed t-test). As a result, cholinergic stimuli evoked fewer spikes with BAPTA (single 5 ms illumination, 1.8 ± 0.1 spikes, 2 neurons; burst 3.3 ± 1.7 spikes, 3 neurons) than without BAPTA (single 5 ms illumination, 6.6 ± 0.8 spikes, 7
neurons; burst 21.7 ± 0.6 spikes, 7 neurons; single illumination and burst each P < 0.05, unpaired 2-tailed t-test). Hence activation of cholinergic axons evokes persistent spiking that requires activation of nAChRs and a calcium-activated current that does not affect the spike waveform. Our results indicate that ACh has both brief, additive and prolonged, non-linear effects on the spiking of layer 5B pyramidal neurons, with neurons being particularly sensitive to cholinergic activity when their membrane potentials are within ~10 mV of spike threshold, such that a nAChR-mediated increase in spiking can be short-lived or can be more dramatic, evoking spiking that persists for many seconds.

**Cholinergic responses by projection target**

In primary sensory neocortices, nAChRs are expressed by selected sub-populations of presynaptic terminals. For example, ACh can enhance the transmission of sensory information to neocortex via the activation of nAChRs on thalamocortical terminals in primary somatosensory and visual cortices (Metherate, 2004; Disney *et al.*, 2007; Gil *et al.*, 1997). Neuromodulators can also act selectively on different projection pathways out of neocortex (Gaspar *et al.*, 1995; Beique *et al.*, 2007; Sheets *et al.*, 2011; Avesar & Gulledge, 2012; Gee *et al.*, 2012; Seong & Carter, 2012). For example, in medial prefrontal cortex mAChR activation by ACh has a greater effect on the excitability of layer 5 pyramidal neurons that project to the pons than on neurons that project to contralateral cortex (Dembrow *et al.*, 2010) and nAChR activation evokes larger-amplitude currents from corticothalamic layer 6 pyramidal neurons than from layer 6 pyramidal neurons that do not project to thalamus (Kassam *et al.*, 2008). Might ACh differentially modulate the output of motor cortex via expression of nACh receptors in
pyramidal neurons that project to some sub-cortical targets, but not pyramidal neurons
that project to other target tissues?

In motor cortex, descending axons of layer 5 pyramidal neurons project into the
pyramidal tract or to the contralateral striatum and these two pathways are mutually
exclusive (Shepherd, 2013). Within layer 6, the primary subcortical output is to thalamus
and layer 6 pyramidal neurons may therefore be divided into corticothalamic and non-
corticothalamic, or intracortical, neurons. We compared the incidence of nAChR- and
mAChR-mediated potentials in each of these subpopulations in motor cortex after
retrograde labeling of pyramidal neurons by injection of fluorescent beads into spinal
cord, contralateral striatum or ipsilateral thalamus (figure 13A). In slices, we identified
neurons with different projection targets by the somatic accumulation of fluorescent
beads (figure 13B).

Synaptically-released ACh frequently evoked nAChR PSPs in all four subpopulations of
deep layer M1 pyramidal neurons: corticospinal layer 5 pyramidal neurons;
corticostriatal layer 5 pyramidal neurons; corticothalamic layer 6 pyramidal neurons;
non-corticothalamic (intracortical) layer 6 pyramidal neurons (figure 13C). The
incidence of nAChR PSPs was not different between the two populations of layer 5
neurons nor between the two populations of layer 6 neurons (nAChR PSPs in 5 of 10
corticospinal layer 5 pyramidal neurons, 8 of 11 corticostriatal layer 5 pyramidal
neurons, P > 0.05, Fisher's exact test; nAChR PSPs in 8 of 11 corticothalamic layer 6
pyramidal neurons, 6 of 12 non-corticothalamic layer 6 pyramidal neurons, P > 0.05,
Fisher's exact test). Hence our experiments revealed no evidence for different
probabilities of nAChR PSPs in sub-populations of deep-layer pyramidal neurons with
different projection targets. However, the amplitudes of nAChR PSPs were greater in
layer 6 pyramidal neurons that projected to thalamus (9.5 ± 2.6 mV, 7 neurons) than in layer 6 neurons that did not project to thalamus (6.0 ± 2.3 mV, 6 neurons; P < 0.05, paired 2-tailed t-test). nAChR-mediated currents are larger in corticothalamic than non-corticothalamic pyramidal neurons in prefrontal cortex (Kassam et al., 2008) and our results suggest that this enhancement of nAChR responses in corticothalamic layer 6 pyramidal neurons extends to primary motor cortex.

The mAChR-mediated slow depolarization was also common in neurons from all four projection-based populations of deep-layer pyramidal neurons (figure 13C; no difference in incidence of slow depolarization between layer 5 or layer 6 subpopulations, P > 0.05, Fisher's exact test). In contrast, the hyperpolarization displayed differential expression by projection target (figure 13C), occurring often in both layer 5 projection-based populations and in non-corticothalamic layer 6 pyramidal neurons (no difference in incidence of hyperpolarization between layer 5 subpopulations, P > 0.05, Fisher's exact test), but being completely absent from corticothalamic layer 6 pyramidal neurons (different incidence of slow depolarization between layer 6 subpopulations, P < 0.05, Fisher's exact test).

**nAChR PSPs responses across layers and cortical areas**

In primary motor cortex, nAChRs are expressed by pyramidal neurons throughout the layers of neocortex (van der Zee et al., 1992; Nakayama et al., 1995; Duffy et al., 2009) and cholinergic axons ramify through all layers (Wainer & Mesulam, 1990; Lysakowski et al., 1989). Hence nAChR PSPs might be expected in pyramidal neurons in all layers. To test this hypothesis, we determined the frequency with which synaptically-released ACh
evoked nAChR PSPs in pyramidal neurons in layers 2/3, 5, and 6 of primary motor, prefrontal, and visual cortices.

In primary motor cortical slices with abundant ChR2-labeled axons in all neocortical layers (figure 14A) and nAChR PSPs in layer 5B pyramidal neurons, cholinergic stimuli (10 x 5 ms at 20 Hz) rarely evoked nAChR PSPs in layer 2/3 pyramidal neurons (4 of 21 layer 2/3 neurons; figure 14B; lower probability in layer 2/3 than in layer 5A, layer 5B, or layer 6, $P < 0.05$ for each, Fisher's exact test). In layers 5A and 5B, nAChR PSPs were common (6 of 8 layer 5A neurons, 82 of 90 layer 5B neurons; figure 14B; no difference in probability between layers 5A and 5B, Fisher's exact test) and in layer 6, nAChR PSPs were evoked in approximately half of neurons (16 of 32 neurons; figure 14B; greater probability in layer 6 than in layer 2/3 and lower than in layer 5B, $P < 0.05$ for each, Fisher's exact test). Hence in primary motor cortex, nAChR PSPs occur almost exclusively in deep-layer pyramidal neurons.

In prefrontal and primary visual cortices (PFC and V1), the laminar pattern of nAChR PSPs was different from that in primary motor cortex. In prefrontal cortex, cholinergic stimuli (10 x 5 ms at 20 Hz) evoked nAChR PSPs in 2 of 6 layer 2/3 pyramidal neurons, 2 of 13 layer 5 pyramidal neurons and 5 of 8 layer 6 pyramidal neurons (figure 14B). Hence nAChR PSPs were less common in all three layers of PFC than in layer 5B neurons in M1 ($P < 0.05$ for each layer, Fisher's exact test). Within PFC, nAChR PSPs were more common in layer 6 than in more superficial layers ($P < 0.05$, Fisher's exact test). As expected from previous studies (Kassam et al., 2008; Poorthuis et al., 2012; Bailey et al., 2010), nAChR PSPs in layer 6 of prefrontal cortex arose from activation of non-α7 nAChRs, being unaffected by MLA and eliminated by DHβE (4 of 4 neurons). In visual cortex, cholinergic stimuli (10 x 5 ms at 20 Hz) commonly evoked nAChR PSPs in
pyramidal neurons in all cortical layers (5 of 6 layer 2/3 neurons, 9 of 11 layer 5 pyramidal neurons, 8 of 9 layer 6 pyramidal neurons; figure 14B; no differences in probability, Fisher's exact test).

The amplitudes of nAChR PSPs also differed across cortical layers and areas (figure 14C). The largest responses were observed in layer 5B of primary motor cortex (maximum 18.3 mV), but the mean nAChR PSP amplitude was greatest in layer 6 pyramidal neurons (M1 6.06 ± 1.21 mV, maximum 16.2 mV; PFC 5.99 ± 2.86 mV, maximum 15.9 mV; V1 2.76 ± 0.74 mV, maximum 5.8 mV; for M1, P < 0.05, Kruskal-Wallis test; L5A different from L5B, L5B different from L6, each P < 0.05, Mann-Whitney test). In all areas, mean peak amplitudes in layers 2-5 were between 1 and 2 mV, with the exception of motor cortex (figure 14C), where responses in layer 5B were larger than in layer 5A (P < 0.05, Mann-Whitney test) and larger than in layer 5 of prefrontal or visual cortices (L5B of M1 14.21 ± 1.14 mV; amplitude larger in M1 L5B than PFC L5 and V1 L5; P < 0.05, Kruskal-Wallis test).

To compare the effects of nAChR activation on pyramidal neurons in different layers and areas, we multiplied the probability and amplitude of nAChR PSPs for each layer (figure 14D). This analysis provides a measure of the overall effect of nAChR PSPs on laminar excitability and reveals that, in all three cortical areas, the effects of nAChR activation are greater in deep layers than in superficial layers. To summarize the average effect of ACh via nAChRs across cortical areas, we plot the mean effect by layer by averaging the effects in M1, PFC and V1 (figure 14E). Hence in these cortical areas there is a general pattern of increased effectiveness of nAChR activation in deep layers, on which is superimposed area-specific variations in laminar sensitivity to ACh.
Hence our results indicate that ACh, acting via nAChRs, can directly excite pyramidal neurons in many cortical areas and layers. Our experiments reveal differences in the nAChR-mediated responsiveness of pyramidal neurons between cortical areas and neocortical layers. However, these local variations appear to operate within a more general framework which is common to neocortical areas, in which ACh exerts greater nAChR-mediated effects on deep-layer pyramidal neurons.
DISCUSSION

nAChRs on neocortical pyramidal neurons have proven difficult to activate in brain slice preparations, limiting the study of nAChRs. We have overcome this barrier by expressing channelrhodopsin in cholinergic axons and evoking ACh release in neocortical slices. Our results indicate that ACh activates nAChRs on pyramidal neurons in multiple layers and three cortical regions, probably via synapses between cholinergic axons and pyramidal neurons. Hence cholinergic activation of pyramidal neurons via nAChRs is common across neocortex.

Effects of ACh on pyramidal neurons

Several authors have reported nAChR-mediated responses from pyramidal neurons (Roerig et al., 1997; Chu et al., 2000; Kassam et al., 2008; Zolles et al., 2009; Guillem et al., 2011; Poorthuis et al., 2012), but in other studies no such responses were observed (Vidal & Changeux, 1993; Gil et al., 1997; Porter et al., 1999) and in many the actions of ACh were mediated by mAChRs (Krnjevic, 1971; McCormick & Prince, 1986; Haj-Dahmane & Andrade, 1996; Gulledge & Stuart, 2005; Gulledge et al., 2007; McCormick, 1992; Schwindt et al., 1988; Giessel & Sabatini, 2010). The absence of nAChR responses is puzzling as nAChRs are expressed in the dendrites of pyramidal neurons (van der Zee et al., 1992; Nakayama et al., 1995; Duffy et al., 2009; Lubin et al., 1999; Levy & Aoki, 2002).

In most studies, ACh was applied by bulk perfusion or from a nearby pipette, which results in a relatively slow, widespread increase in concentration. nAChR desensitization during ACh application might be significant, reducing nAChR-mediated currents. Furthermore, many mAChRs are located extrasynaptically (Mrzljak et al., 1998;
Yamasaki et al., 2010) and might be more strongly activated by applied ACh than by ACh released from cholinergic axons. Our results suggest that ACh application favors mAChR- over nAChR-mediated currents in pyramidal neurons and this weighting may account for the paucity of nAChR-mediated responses in the literature.

ACh can act on interneurons in layer 1 of sensorimotor cortex via α7 nAChR-mediated synaptic and non-α7 nAChR-mediated diffuse mechanisms (Bennett et al., 2012) and there is a wider debate on whether ACh acts in neocortex primarily via synaptic contacts or volume transmission (Sarter et al., 2009; Arroyo et al., 2014). The short latency of nAChR-mediated responses in our experiments suggests that ACh forms cholinergic synapses with pyramidal neurons, but via non-α7 nAChRs. We found no evidence for an α7 nAChR-mediated effect or nAChR-mediated actions via volume transmission, but our results do not exclude such responses in pyramidal neurons.

Although our recordings were from somata, there are nAChRs throughout the dendritic trees of pyramidal neurons (van der Zee et al., 1992), and it therefore seems likely that there are additional effects of ACh on the dendrites of pyramidal neurons.

**Persistent spiking evoked by nAChRs**

Our results indicate that nAChR activation can evoke persistent spiking when paired with additional depolarization. Persistent spiking was prevented by intracellular BAPTA, suggesting that a rise in intracellular calcium concentration is also required. Calcium might enter through nAChRs or arise from a secondary source, such as voltage-activated calcium channels or a transmitter co-released by cholinergic axons. Cholinergic axons may co-release glutamate (Manns et al., 2001; Allen et al., 2006; Gritti et al., 2006; Henny & Jones, 2008), but in our experiments persistent spiking was unaffected by
glutamate receptor antagonists. Other potential co-transmitters in the basal forebrain include neurotensin, somatostatin, neuropeptide Y and galanin (Koliatsos et al., 1990).

nAChR-dependent persistent spiking has been reported in dopaminergic neurons in the substantia nigra pars compacta and subthalamic nucleus (Yamashita & Isa, 2003a, 2003b), but not cortical neurons. In entorhinal, perirhinal, cingulate and somatosensory cortices, persistent spiking can be evoked in excitatory neurons, but via mAChRs and a rise in intracellular calcium concentration (Zhang & Séguéla, 2010; Egorov et al., 2002; Navaroli et al., 2011; Rahman & Berger, 2011). Hence nAChR-dependent persistent spiking shares common mechanistic elements with mAChR-dependent persistent spiking, but is initiated by activation of nAChRs, not mAChRs.

In spiking neurons, nAChR PSPs evoked a brief and modest increase in spike rate. Why was the effect during ongoing spiking not more prolonged and why was the resulting spike rate typically lower than during nAChR-evoked persistent spiking? Presumably ongoing spiking suppresses the current that underlies persistent spiking or the resulting depolarization, perhaps by shunting the membrane.

Previous studies have revealed other mechanisms of prolonged spiking in pyramidal neurons, particularly deep-layer pyramidal neurons. For example, subthreshold DC current injection into the trunk of the apical dendrite can facilitate propagation of a dendritic spike from the distal apical dendrite to the soma, resulting in a burst of spikes (Larkum et al., 2001). Similarly, activation of glutamate receptors in the basal dendrites can evoke a burst of spikes (Milojkovic et al., 2004; Milojkovic et al., 2007). Presumably nAChR-persistent spiking and other mechanisms that can evoke prolonged spiking share some common mechanistic elements and differ important ways. Further experiments will be required to investigate these similarities and differences, but our results add...
nAChR activation to the collection of identified mechanisms that can evoke prolonged spiking from cortical pyramidal neurons.

In summary, nAChR activation increases the excitability of pyramidal neurons. The increase in spiking can be modest and transient or profound and persistent, depending on the membrane potential of the neuron. Hence ongoing synaptic drive to the pyramidal neuron determines the strength and duration of the increase in spiking evoked by ACh.

Laminar and regional variation in nAChR PSPs

α3, α4, α7 and β2 nAChR subunits are located on neocortical pyramidal neuron somata, dendrites and spines (Disney et al., 2007; Nakayama et al., 1995; Duffy et al., 2009; Lubin et al., 1999; Levy & Aoki, 2002; Wevers et al., 1994) and several studies describe responses mediated by α4β2-, α7- and α5-containing nAChRs, the latter probably in heteromeric assembly with α4 and β2 subunits (Kassam et al., 2008; Zolles et al., 2009; Poorthuis et al., 2012).

Our results are generally consistent with these previous studies, but there are contrasts. Layer 6 contains high expression of non-α7 nAChRs (Tribollet et al., 2004), including α5 (Wada et al., 1990; Proulx et al., 2013) and α4 (Lein et al., 2007) subunits, consistent with the high incidence of nAChR-mediated responses in layer 6 of PFC in previous studies (Kassam et al., 2008; Poorthuis et al., 2012; Mailey et al., 2010). We found that nAChR PSPs in layer 6 are common and of large amplitude in all three areas studied, suggesting that the presence of α4/α5-mediated PSPs is a feature of layer 6 pyramidal neurons across cortical regions.
In layer 5, we found that nAChR PSPs are common in M1 and V1 and rare in PFC. In M1, nAChR PSPs were mediated by non-α7 nAChRs. In contrast, Poorthuis et al., 2012 observed α7 nAChR-mediated responses in layer 5 pyramidal neurons in PFC. nAChR PSPs that we observed originated from nAChRs in the proximal dendrites. Hence one explanation for the lack of α7-mediated PSPs in our results might be that α7 nAChRs are located primarily in the distal dendrites and that α7-mediated depolarization of the distal dendrite failed to evoke depolarization at the soma in our experiments.

M1 is unusual in that the pyramidal neurons in layer 5B of M1 commonly display large-amplitude nAChR PSPs, unlike layer 5 pyramidal neurons in PFC and V1. The α5 subunit is not present in layer 5 (Wada et al., 1990). There is evidence for greater expression of α4 subunits in deep than in superficial layers (Lein et al., 2007), but see also (Tribollet et al., 2004), but this expression pattern is not unique to M1. Hence it is unclear which nAChR subunit(s) underlie the unusually large nAChR PSPs in layer 5 of M1, but α5 subunits are unlikely to be involved.

In superficial layers, we found that pyramidal neurons in M1 and PFC rarely displayed nAChR PSPs, consistent with results from PFC (Poorthuis et al., 2012), but that nAChR PSPs are common in superficial pyramidal neurons in V1. Layer 2/3 contains dense non-α7 labeling (Tribollet et al., 2004). Our results suggest that this dense labeling is from interneurons and perhaps in the dendrites of deep-layer pyramidal neurons.

Our results extend our knowledge of pyramidal neuron nAChRs from PFC into M1 and V1, revealing variation in laminar responsiveness to ACh between cortical regions; presumably the responsiveness of pyramidal neurons is tuned to the unique demands of each area. However, our results also reveal a general tendency for ACh to exert stronger effects in deep than superficial layers, suggesting that preferential modulation of deep
layer pyramidal neurons via nAChRs is a general property of the actions of ACh in neocortex.

**nAChR-mediated modulation of neocortical circuits**

How might the layer-selective effect of ACh influence the flow of excitation through neocortex? The principal ascending excitatory drive to cortex is thalamocortical axons, which contact pyramidal neurons primarily in layers 5B and 4 (5B and 3 in primary motor cortex, which lacks layer 4 (Shepherd, 2009; Hooks et al., 2013). From layer 4, information is passed by excitatory connections through layer 2/3 to layer 5 and to the sub-cortical projection targets of neocortex. Hence there are two primary excitatory pathways through neocortex: a short loop which connects thalamus with target structures through layer 5 and a longer loop which includes layers 4 and 2/3 (Armstrong-James et al., 1992; de Kock et al., 2007; Petreanu et al., 2009; Constantinople & Bruno, 2013).

ACh enhances activation of neocortical pyramidal neurons by ascending thalamic drive. This enhancement arises from mAChR-mediated depolarization of pyramidal neurons and enhanced glutamate release from thalamocortical terminals in layer 4 (Metherate, 2004; Disney et al., 2007; Gil et al., 1997). In addition, ACh activates non-fast spiking, non-parvalbumin-expressing interneurons in layers 1 and 2/3 via non-α7 nAChRs (Porter et al., 1999; Gulledge et al., 2007; Letzkus et al., 2011; Arroyo et al., 2012; Brombas et al., 2014). These interneurons inhibit parvalbumin- and somatostatin-expressing interneurons that target the somata and dendrites of pyramidal neurons. Hence nAChR-mediated inhibition of superficial interneurons reduces inhibition of superficial pyramidal neurons (Letzkus et al., 2011; Brombas et al., 2014). Our results
indicate that ACh, again acting at nAChRs, directly promotes the spiking of deep-layer pyramidal neurons. Hence ACh modulates cortical output by at least three different nAChR-dependent mechanisms which enhance the responsiveness of neocortex to incoming sensory drive: by increasing the release of glutamate in layer 4, by indirectly enhancing the excitability of superficial pyramidal neurons and by directly enhancing the excitability of deep-layer pyramidal neurons.

Why employ several mechanisms to modulate the excitability of pyramidal neurons in cortex? Multiple mechanisms may offer circuit specificity and semi-independent control. Multiple mechanisms of network modulation might allow ACh to independently modulate the excitability of deep and superficial layer pyramidal neurons, thereby gating the balance of sensory information processing in different cortical layers and controlling the balance of information flowing through the long and short loops from thalamus to the output structures of neocortex.
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Figure legends

Figure 1. Stimulation of cholinergic axons evokes four responses in pyramidal neurons

(A) Schematic of a coronal slice illustrating the expression of ChR2-YFP in cholinergic neurons in the basal forebrain and their axonal projections to neocortex. CP: caudate putamen, LV: lateral ventricle. (B) Image of ChR2-YFP labeled neurons in nucleus basalis in a ChAT-ChR2(Ai32) mouse. Maximum intensity projection from a 2-photon z-stack through a fixed section. (C) Image of ChR2-YFP labeled axons in primary motor cortex in a ChAT-ChR2(Ai32) mouse. Maximum intensity projection from a 2-photon z-stack through a fixed section. (D,E) Pyramidal neuron in layer 5B of primary motor cortex, 3 weeks after virus injection. Neuron was filled with Alexa 594 during whole-cell recording (D) and surrounding cholinergic axons expressing ChR2-YFP (E). Both images are maximum intensity projections from 2-photon z-stacks. (F) Examples of voltage recordings from four layer 5B pyramidal neurons at rest. Each voltage response evoked by stimulation of cholinergic axons by widefield illumination of the slice with a blue LED (stimulus: 10 x 5 ms at 20 Hz; blue bars). Example of slow depolarization includes a preceding hyperpolarization. Examples of slow depolarization and fast hyperpolarization were obtained from virus-injected mice. Examples of medium depolarization and fast depolarization were obtained from ChAT-ChR2(Ai32) mice.

Figure 2. Light-evoked responses are eliminated by TTX and by removal of extracellular calcium

Examples of the slow depolarization and hyperpolarization (A, neuron depolarized by DC current injection), medium depolarization (B, neuron at rest), and fast depolarization (C,
neuron at rest) from three neurons. All responses were reversibly eliminated by removal of external calcium, and by addition of 500 nM TTX. Stimulus: 10 x 5 ms illumination at 20 Hz. All examples were obtained from ChAT-ChR2(Ai32) mice.

**Figure 3. Pharmacology of the hyperpolarization and slow depolarization**

(A) Example of sequential addition of 100 µM mecamylamine and 1 µM atropine to a neuron displaying a light-evoked hyperpolarization. Stimulus: 10 x 5 ms illumination at 20 Hz. Neuron held close to threshold by DC current injection. Recording from a virus-injected mouse. (B) Example of sequential addition of 100 µM mecamylamine and 1 µM atropine to a neuron displaying a light-evoked slow depolarization. Stimulus: 10 x 5 ms illumination at 20 Hz. Neuron held close to threshold by DC current injection. Recording from a ChAT-ChR2(Ai32) mouse. (C) Probability of evoking a mAChR-mediated potential during constant depolarization by DC current injection. Control, 38 neurons; mec, 100 µM mecamylamine, 13 neurons; atr, 1 µM atropine, 141 neurons.

**Figure 4. Pharmacology of the medium depolarization**

Example recordings from six neurons. Stimulus: 2-3 x 5 ms illumination at 20 Hz. Physo, 0.5 µM physostigmine; atr, 1 µM atropine; mec, 100 µM mecamylamine; gal, 1 µM galanthamine; NBQX/CPP, 10 µM NBQX and 10 µM CPP; GBZ / CGP, 1 µM gabazine and 3 µM CGP 52432; MLA, 10 nM methyllycaconitine; DHβE, 10 µM dihydro-β-erythroidine hydrobromide. All examples at resting membrane potential and from virus-injected mice. Summary: mean ± SEM, normalized to pre-drug amplitude. TTX, 7 neurons; 0 Ca, 7 neurons; NBQX/CPP, 7 neurons; GBZ / CGP, 8 neurons; physostigmine, 4 neurons; atropine, 5 neurons; mecamylamine, 12 neurons;...
Figure 5. Pharmacology of the fast depolarization and pharmacological separation of multiple responses

(A) Example and summary of the pharmacology of the fast depolarization in a ChAT-ChR2(Ai32) mouse. Example: sequential addition of mec/atr, 100 µM mecamylamine and 1 µM atropine; GBZ/CGP, 1 µM gabazine and 3 µM CGP 52432; NBQX/CPP, 10 µM NBQX and 10 µM CPP. 2 ms illumination. Neuron at rest. Summary: mean ± SEM, normalized to pre-drug amplitude. 5, 3 and 3 neurons, for mec/atr, GBZ/CGP and NBQX/CPP, respectively. Antagonists were added sequentially in each recording. (B) Example of the effect of sequential application of drugs to a neuron from a ChAT-ChR2(Ai32) mouse with a combination of all four responses. Lower row: voltage responses at resting membrane potential; upper row: voltage responses during depolarizing somatic current injection.

Figure 6. mAChR-mediated hyperpolarization and slow depolarization

(A) Example of pressure application of ACh (100 µM, 10 ms, arrow heads) at rest and during constant depolarization in a neuron displaying a mAChR-mediated slow depolarization. NBQX, CPP, gabazine and CGP 52432 were present to prevent network effects. (B) Example of blue light stimulus at rest and during constant depolarization in a neuron from a virus-injected mouse displaying a mAChR-mediated hyperpolarization and slow depolarization. Stimulus: 10 x 5 ms illumination at 20Hz. NBQX, CPP, gabazine and CGP 52432 present throughout. (C) Incidence of light-evoked mAChR-
mediated responses in pyramidal neurons in different cortical layers at rest and during depolarization by somatic current injection. Probability was calculated as the number of neurons in which we observed mAChR responses divided by the total number of recordings. Laminar locations determined by distance from pia (see Methods section).

Depolarized: L2/3, 8 neurons; L5A, 8 neurons; L5B, 48 neurons; L6, 7 neurons. At rest: L2/3, 22 neurons; L5A, 8 neurons; L5B, 84 neurons; L6, 32 neurons.

Figure 7. Latency and kinetics of nAChR-mediated medium depolarization

(A) Example of summation of nAChR PSPs in a neuron from a virus-injected mouse. Six 2 ms illuminations at 1, 5 and 20 Hz in the presence of atropine, NBQX, CPP, gabazine and CGP 52432. Measured at rest. (B) Summary plot showing peak amplitudes following six 2 ms illuminations at up to 20 Hz. In each trial, peak amplitude was normalized to that of the first PSP. Points represent mean ± SEM. 0.033 Hz 11 neurons, 0.1 Hz 13 neurons, 0.5 Hz 14 neurons, 0.7 Hz 14 neurons, 1 Hz 14 neurons, 2 Hz 14 neurons, 3 Hz 2 neurons, 5 Hz 7 neurons, 10 Hz 11 neurons, 15 Hz 2 neurons, 20 Hz 14 neurons. (C) nAChR PSPs in a neuron from a virus-infected mouse, evoked by 2 ms illumination in atropine, NBQX, CPP, gabazine and CGP 52432. Measured at rest. (D) Mean response to 2 ms illumination, with fit (sum of two exponentials; grey). Rise and decay time constants 27.5 and 231 ms. Virus-infected mouse.

Figure 8. Fast depolarization evoked by activation of perisomatic nAChRs

(A) Examples of nAChR PSPs during localized illumination centered on the soma, with schematic illustrating approximate illumination areas. 10 x 5 ms illumination at 20 Hz. ChAT-ChR2(Ai32) mouse. Measured at rest. (B) Examples of nAChR PSPs during
localized illumination of different regions of the dendritic arbor, with schematic
illustrating approximate illumination areas. 2 x 5 ms illumination at 20Hz. Virus-
injected mouse. (C) Summary plot showing peak amplitude of depolarization with
illumination areas of different radii, centered on the soma, in 7 recordings. Points
represent mean ± SEM peak amplitude normalized to average amplitude with full field of
illumination. Illumination radii: 55 um, 7 neurons; 110-118 um, 7 neurons; 235 um, 2
neurons; 312.5 um, 7 neurons; 625 um, 2 neurons.

Figure 9. nAChR activation enhances spiking of layer 5 pyramidal neurons

(A) Example of effect of cholinergic stimulation on spike rate during ongoing spiking.
400 pA constant current injection at soma. 10 x 5 ms illumination at 20 Hz. NBQX, CPP,
gabazine, CGP 52432 and atropine present throughout. Virus-injected mouse. (B)
Summary of increase in spike rate during ongoing spiking. Each point represents one
trial. Lines best linear fit. Grey: single 2-5 ms illumination, increase in spike rate 2.2 ±
0.1 Hz, 6 neurons. Black: 10 x 5 ms illumination at 20 Hz, increase in spike rate 3.1 ± 0.2
Hz, 4 neurons. (C) Kinetics of change in spike rate with one 2 ms illumination and burst
of 10 x 5 ms at 20Hz. Each line represents a single trial.

Figure 10. Persistent spiking following nAChR activation

(A) Examples from virus-injected mice of persistent spiking in atropine, NBQX, CPP,
gabazine and CGP 52432. Left: single 5 ms illumination at resting membrane potential.
Inset: expanded view of same response, same time scale. Center: persistent spiking
following single 2 ms illumination, 350 pA constant current injection at soma. Right:
persistent spiking following 10 x 5 ms illumination at 20 Hz, 400 pA constant current
injection at soma. **(B)** Mean spike rate during persistent spiking. Stimulus starting at 0 ms. Left, single 2-5 ms illumination, 7 neurons. Right, 10 x 5 ms at 20 Hz, 7 neurons.

**Figure 11. Pharmacology of persistent spiking**

**(A)** Inhibition of persistent spiking by 100 µM mecamylamine (mec). 10 x 5 ms illumination at 20 Hz. Atropine, NBQX, CPP, gabazine and CGP 52432 present throughout. Measured near threshold. Virus-injected mouse. **(B)** Failure of 1 µM atropine (atr) to inhibit persistent spiking. 10 x 5 ms illumination at 20 Hz. NBQX, CPP, gabazine and CGP 52432 present throughout. ChAT-ChR2(Ai32) mouse. **(C)** Examples of failure to evoke persistent spiking using somatic current injection. 400 pA constant current injection to bring cell close to threshold. Left: baseline membrane potential -49 mV, additional 100 pA for 50 ms, resulting in two spikes. Right: baseline membrane potential -47 mV, additional 50 pA for 1 s. NBQX, CPP, gabazine, CGP 52432 and atropine present throughout. Virus-injected mouse. **(D)** Hyperpolarization failed to end persistent spiking. 10 x 5 ms illumination at 20 Hz, -300 pA hyperpolarizing current injection. Atropine, NBQX, CPP, gabazine and CGP 52432 present throughout. Virus-injected mouse. **(E)** Inhibition of persistent spiking by 10 mM intracellular BAPTA in a ChAT-ChR2(Ai32) mouse. Left: 5 ms illumination. Right: 10 x 5 ms illumination at 20 Hz. Atropine, NBQX, CPP, gabazine and CGP 52432 present throughout.

**Figure 12. Persistent spiking and intrinsic conductances.**

**(A)** Example of persistent spiking in atropine, NBQX, CPP, gabazine and CGP 52432. 400 pA constant current injection. Baseline membrane potential -47 mV. 10 x 5 ms illumination at 20 Hz. Dashed horizontal line denotes 0 mV. Virus-injected mouse. **(B-**
D) Spike threshold, half width (width at half amplitude), and amplitude (threshold to peak) during persistent spiking for the example in panel A. Each point represents one spike. (E) Summary of changes in spike threshold, amplitude, half width, 10-90% rise time and 10-90% decay time, expressed as mean ± SEM percentage change per spike throughout persistent spiking for the neuron in panel A.

Figure 13. nAChR-mediated PSPs by projection target

(A) Schematic illustrating stereotaxic injections of fluorescent beads into subcortical projection targets. Red lines indicate approximate axonal projections from primary motor cortex. (B) 2-photon maximum intensity projection acquired during recording from a L5B pyramidal neuron from a ChAT-ChR2(Ai32) mouse, 5 days after bead injection into contralateral striatum. Neuron was filled with Alexa 488 (green) during whole cell recording. Numerous beads (red) are located in the soma. (C) Relative incidence of nAChR PSPs and mAChR-mediated slow depolarization and hyperpolarization in pyramidal neuron subpopulations identified based upon somatic accumulation of fluorescent beads. C-striat: corticostriatal, 10 neurons; c-spinal: corticospinal, 12 neurons; c-thal: corticothalamic, 11 neurons; c-thal+: non-corticothalamic, 12 neurons. Asterisk denotes significant difference (P < 0.05, Fisher’s exact test). Probability of recording nAChR PSPs was determined at rest; probability of observing mAChR responses determined near threshold.

Figure 14. nAChR-mediated PSPs in motor, prefrontal and visual cortices.

(A) Images of layer 2/3 pyramidal neuron from a virus-injected mouse filled with biocytin during whole-cell recording and visualized with Alexa 594-linked streptavidin,
and ChR2-YFP axons in the same field of view. Maximum intensity projections from 2-
photon stacks. (B) Bar charts showing the probability of recording a nAChR PSP at rest
in pyramidal neurons from each layer of primary motor (M1), prefrontal (PFC) and
primary visual (V1) cortices. Asterisks indicate significant differences (P < 0.05, Fisher's
Exact test: M1 L2/3 different from L5A, from L5B and from L6; M1 L5B different from
L6; PFC L6 different from sum of layers 2/3 and L5). (C) Bar charts showing the mean
amplitudes of nAChR PSPs in pyramidal neurons in M1, PFC and V1. Each bar denotes
mean ± SEM. Asterisks indicate significant differences (M1: P < 0.05, Kruskal-Wallis
test; M1 L5A different from L5B, M1 L5B different from L6, each P < 0.05, Mann-
Whitney test). (D) Plot to illustrate the net effect of nAChR activation (product of
probability and amplitude of nAChR PSPs) on each layer of M1, PFC and V1. Results in
B, C and D are derived from 21, 8, 90 and 32 neurons from layer 2/3, 5A, 5B and 6 of M1;
6, 13 and 8 neurons from layer 2/3, 5 and 6 of PFC; and 6, 11 and 9 neurons from layer
2/3, 5 and 6 of V1. (E) Summary of the strength of nAChR modulation of pyramidal
neurons by cortical layer. Each bar shows the mean across M1, PFC and V1 (equally
weighted) of the product of probability and amplitude of the nAChR PSP.

Table 1. Amplitude and kinetics of cholinergic responses evoked in
pyramidal neurons in layer 5 of primary motor cortex. Values report mean ±
SEM. Measurements at resting membrane potential. For mAChR-mediated responses,
amplitude and decay time constant were measured following 10 x 5ms illuminations at
20 Hz. For the nAChR PSP, amplitudes were measured following 10 x 5ms illuminations
at 20 Hz and the decay time constant following a single 2-5 ms illumination.
<table>
<thead>
<tr>
<th></th>
<th>Amplitude</th>
<th>Decay time constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium Depolarization (nAChR)</td>
<td>4.2 ± 1.1 mV</td>
<td>180 ± 23 ms</td>
</tr>
<tr>
<td></td>
<td>(22 neurons)</td>
<td>(14 neurons)</td>
</tr>
<tr>
<td>Hyperpolarization (mAChR)</td>
<td>1.2 ± 0.5 mV</td>
<td>774 ± 73 ms</td>
</tr>
<tr>
<td></td>
<td>(10 neurons)</td>
<td>(10 neurons)</td>
</tr>
<tr>
<td>Slow Depolarization (mAChR)</td>
<td>2.0 ± 0.6 mV</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(6 neurons)</td>
<td></td>
</tr>
</tbody>
</table>
A 1mm ChR2-YFP cholinergic axons CP LV neocortex

B NB 50 µm

C M1 25 µm

D M1 25 µm

E M1 25 µm

F slow depolarization 1 mV 2 s

hyperpolarization 1 mV 0.2 s

medium depolarization 1 mV 0.2 s

fast depolarization 2 mV 0.2 s
Figure 2

A. 2 mM Ca$^{2+}$

B. 0 Ca$^{2+}$

C. 2 mM Ca$^{2+}$

TTX

0 mV

20 mV

5 mV

1 s

500 ms

250 ms
figure 3

A

B

C

probability

control  mec  atr

5 mV

2 s

20 mV
Figure 4

- Physo
- Atr
- NBQX/CP
- GBZ/CGP
- MLA DHβE

Data analysis:
- Normalized amplitude
- Graph showing effects of different compounds on normalized amplitude
- Key compounds: TTX, Ca^{2+}, physostigmine, atropine, mecamylamine, MLA DHβE

Graph legend:
- 0 Ca^{2+}/CPP
- * denotes significant difference
Figure 5

Normalized amplitude

A

mec atr ➔ GBZ CGP ➔ NBQX CPP

10 mV

100 ms

B

depolarized

resting $V_m$

mec ➔ atr

20 mV

1 s

5 mV

resting $V_m$
figure 6

A

B

C

- depolarized
- at rest

L2/3, L5A, L5B, L6

probability
Figure 7

A. 1 Hz
B. 5 Hz
C. 20 Hz

1 mV
1 s

D. 0.5 mV
250 ms

250 ms

Normalized amplitude

Frequency (Hz)
Figure 8

A

B

C

illumination radius (µm)

normalized amplitude
figure 10

A

B
Figure 13

A. Diagram showing the distribution of nAChR PSP, mAChR slow depol, and mAChR hyperpol in L5, L6, and the striatum and thalamus.

B. Image showing a 10 µm scale.

C. Bar graphs showing the probability of nAChR PSP, mAChR slow depol, and mAChR hyperpol in the striatum and thalamus.

* Indicates significance.
Figure 14

A. Alexa 594

B. M1, PFC, V1

C. Mean of M1, PFC, V1

D. Prob x Amp (mV)

E. Mean of M1, PFC, V1

ChR2-YFP