

Cholinergic excitation in mouse primary vs. associative cortex: region-specific magnitude and receptor balance

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Abstract

Cholinergic stimulation of the cerebral cortex is essential for tasks requiring attention; however, there is still some debate over which cortical regions are required for such tasks. There is extensive cholinergic innervation of both primary and associative cortices, and transient release of acetylcholine (ACh) is detected in deep layers of the relevant primary and/or associative cortex, depending on the nature of the attention task. Here, we investigated the electrophysiological effects of ACh in layer VI, the deepest layer, of the primary somatosensory cortex, the primary motor cortex, and the associative medial prefrontal cortex. Layer VI pyramidal neurons are a major source of top-down modulation of attention, and we found that the strength and homogeneity of their direct cholinergic excitation was region-specific. On average, neurons in the primary cortical regions showed weaker responses to ACh, mediated by a balance of contributions from both nicotinic and muscarinic ACh receptors. Conversely, neurons in the associative medial prefrontal cortex showed significantly stronger excitation by ACh, mediated predominantly by nicotinic receptors. The greatest diversity of responses to ACh was found in the primary somatosensory cortex, with only a subset of neurons showing nicotinic excitation. In a mouse model with attention deficits only under demanding conditions, cholinergic excitation was preserved in primary cortical regions but not in the associative medial prefrontal cortex. These findings demonstrate that the effect of ACh is not uniform throughout the cortex, and suggest that its ability to enhance attention performance may involve different cellular mechanisms across cortical regions.

Keywords

acetylcholine; attention; Chrna5; mouse; muscarinic; nicotinic

Introduction

Attention is a complex cognitive function that focuses the mind on relevant information in the presence of distraction (Crick, 1984). The cholinergic system is considered to be essential for attention (Robbins *et al.*, 1989; Dunnett *et al.*, 1991; Muir *et al.*, 1992; Klinkenberg *et al.*, 2011). In support of this model, cue detection during attention tasks triggers transient increases in acetylcholine (ACh) release in the deep layers of several different cortical regions (Passetti *et al.*, 2000; Fournier *et al.*, 2004; Kozak *et al.*, 2006; Parikh *et al.*, 2007; Parikh & Sarter, 2008). Such release fits with the relatively uniform innervation of cortical regions by cholinergic fibers from the basal forebrain (Mesulam *et al.*, 1983; Mechawar & Descarries, 2001), and raises questions in view of the ongoing debate about which cortical regions are essential for attention (Meyer, 2011). Whereas it is acknowledged that activity in the associative prefrontal cortex is important for particularly challenging attention tasks (Marti *et al.*, 2012), it appears not to be central under conditions of lower demand (Smucny *et al.*, 2013). In fact, it has been suggested that primary cortical regions may carry out certain types of attentional processing on their own (Meyer, 2011). For example, alpha-band activity in the primary somatosensory cortex (SSC) strongly reflects somatosensory attention (Pfurtscheller *et al.*, 1996; Jones *et al.*, 2010), and such oscillations in the primary cortex are powerfully modulated by cholinergic stimulation (Bauer *et al.*, 2012). Consistent with the hypothesis that attention relies on cholinergic modulation of primary as well as associative cortical regions, both primary and associative cortical regions send top-down projections that can control the thalamic and thalamocortical circuitry involved in attention (Guillery, 1995; Sherman, 2005; Zikopoulos & Barbas, 2006).

In particular, layer VI of the cerebral cortex sends and receives projections to thalamic nuclei, forming a corticothalamic feedback loop that is important for top-down control of normal attentional function (Bourassa *et al.*, 1995; Guillery & Sherman, 2002; Gabbott *et al.*, 2005; Parikh *et al.*, 2010; Thomson, 2010). However, layer VI is heterogeneous (Andjelic *et al.*, 2009; Briggs, 2010; Thomson, 2010), and includes corticocortical neurons (Mercer *et al.*, 2005; Kumar & Ohana, 2008; Petrof *et al.*, 2012), which may also be involved in attentional processing. For example, recent work has demonstrated a local circuit within the primary cortex whereby layer VI pyramidal neurons control cortical gain modulation (Olsen *et al.*, 2012).

Layer VI is strongly innervated by cholinergic fibers (Mechawar & Descarries, 2001), and its neurons express ACh receptors of both the nicotinic and muscarinic subtypes (Buckley *et al.*, 1988; Tribollet *et al.*, 2004). However, responses to cholinergic stimulation have only been assessed previously in layer VI of the prelimbic cortex (Tian *et al.*, 2011). The relative consequences of cholinergic stimulation on layer VI pyramidal neurons across primary and associative regions of the cortex are unknown.

Here, we investigated whether ACh exerts similar electrophysiological effects on layer VI neurons in primary and associative cortical regions. Then, we studied the underlying receptor mechanisms involved by using pharmacological manipulations. Heterogeneous responses observed in the SSC were examined further. Finally, we investigated regional

variations in cholinergic excitation of layer VI neurons in a population of mice known to have attention deficits under demanding circumstances.

Materials and methods

Animals

Electrophysiological recordings were performed on brain slices from adult male mice (age range, postnatal day 50 to postnatal day 130; mean, 80 ± 6 days; $n = 42$ mice). To facilitate recording from layer VI neurons in the adult cortex, we employed BAC transgenic mice with extensive labeling of layer VI neurons by enhanced green fluorescent protein (eGFP) expression driven by the synaptotagmin 6 promoter (Syt6-eGFP; MMRRC) on a Swiss Webster background. Labeled neurons have pyramidal somata and apical dendrites that project towards the pial surface. None of the eGFP-positive neurons recorded exhibited electrophysiological characteristics of fast-spiking interneurons. Conversely, fast-spiking interneurons that were visually identified by infrared differential interference contrast microscopy and confirmed electrophysiologically ($n = 10$ across regions) were eGFP-negative. A subset of experiments were performed in C57BL/6 adult male mice with deletion of the nicotinic receptor $\alpha 5$ subunit ($\alpha 5^{-/-}$) (Salas *et al.*, 2003) and their matched wild-type (WT) controls. Mice were deeply anesthetized with chloral hydrate (400 mg/kg) and killed by decapitation. All animal care and experimental protocols were performed in accordance with the guidelines of the Canadian Council on Animal Care, and were approved by the University of Toronto Animal Care Committee.

Brain slice preparation

Brains were rapidly excised and chilled in 4 °C oxygenated sucrose artificial cerebrospinal fluid (ACSF) (254 mM sucrose, 10 mM D-glucose, 24 mM NaHCO₃, 2 mM CaCl₂, 2 mM MgSO₄, 3 mM KCl, 1.25 mM NaH₂PO₄; pH 7.4). Coronal slices (thickness, 400 μ m; 2.34–0.74 mm from Bregma) were cut on a Dosaka Linear Slicer (SciMedia, Costa Mesa, CA, USA), and allowed to recover in 30°C oxygenated ACSF (128 mM NaCl, 10 mM D-glucose, 26 mM NaHCO₃, 2 mM CaCl₂, 2 mM MgSO₄, 3 mM KCl, 1.25 mM NaH₂PO₄; pH 7.4) for at least 2 h.

Electrophysiology

Recovered slices were transferred to a superfusion chamber on the stage of a BX50WI microscope (Olympus, Richmond Hill, ON, Canada). ACSF was bubbled (95% O₂ and 5% CO₂ at room temperature) and perfused through the chamber at a rate of 3–4 mL/min. As shown in Fig. 1(A), layer VI was landmarked with fluorescently identified eGFP-positive neurons (X-cite Series 120; Lumen Dynamics, Mississauga, ON, Canada) in Syt6-eGFP mice. Recording electrodes (2–4 M Ω) containing 120 mM potassium gluconate, 5 mM KCl, 2 mM MgCl₂, 4 mM K₂-ATP, 0.4 mM Na₂-GTP, 10 mM Na₂-phosphocreatine and 10 mM Hepes buffer (adjusted to pH 7.3 with KOH) were used to patch layer VI pyramidal neurons in acute coronal prefrontal slices. Layer VI neurons in the SSC, primary motor cortex (M1) and associative medial prefrontal cortex (mPFC; prelimbic region) were specifically targeted on the basis of local landmarks, as previously described (Paxinos & Franklin, 2001; Fig. 1B). Currents and membrane potential were recorded in voltage clamp mode (at a holding

potential of -75 mV) and in current clamp mode with an EPC10 (HEKA Electronics, Chester, NS, Canada). All data were acquired at 20 kHz and low-pass filtered at 3 kHz with PCLAMP software (Molecular Devices, Sunnyvale, CA, USA).

Electrophysiological properties of the layer VI neurons (resting membrane potential, input resistance, and spike amplitude) are shown in Table 1. Input resistance was calculated by measuring the steady-state deflections in membrane potential in response to injections of hyperpolarizing current (50–100 pA, 500 ms). Resting membrane potential was corrected for the liquid junction potential.

Pharmacology

Cholinergic responses were probed with the addition of ACh (100 μ M or 1 mM, 15 s) in standard ACSF to the bath perfusion after a baseline recording period. ACh applications were timer-controlled. Note that the high level of acetylcholinesterase in cortical slices means that only a fraction of the bath-applied ACh is likely to reach the cholinergic receptors (Bailey *et al.*, 2010). A subset of experiments were performed after blockade of glutamatergic and GABAergic synaptic transmission with a combination of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 20 μ M), D-(–)-2-amino-5-phosphonopentanoic acid (APV; 50 μ M) and bicuculline (10 μ M) added to the bath perfusion. Nicotinic and muscarinic effects were examined individually by applying ACh in the presence of the muscarinic antagonist atropine (200 nM) or the nicotinic antagonist dihydro- β -erythroidine hydrobromide (DH β E; 3 μ M). All compounds were obtained from Sigma or Tocris Bioscience, and stored in stock solutions at -20 °C.

Statistical analysis

Current responses and changes in membrane potential resulting from bath-applied ACh were measured with CLAMPFIT software (Molecular Devices) and assessed with one-way ANOVAs and *post hoc t*-tests. For illustrative purposes, averaged responses to ACh in different groups were calculated by the use of data points extracted from all individual recordings. Comparisons between cholinergic responses and kinetics in SSC neurons were performed with unpaired *t*-tests. Comparisons between genotype and the regional effects of ACh in $\alpha 5^{-/-}$ and WT mice were performed with two-way ANOVAs. Regional and genotype differences in the proportion of neurons depolarized to threshold by ACh were compared by the use of Fisher's exact tests.

Results

ACh exerts unique effects on layer VI neurons in primary and associative cortical regions

To determine the whole-cell effects of cholinergic stimulation on SSC, M1 and mPFC layer VI pyramidal neurons, we bath-applied ACh (1 mM, 15 s) and quantified the magnitude of the ACh-elicited inward currents in voltage clamp mode, holding neurons at -75 mV in the absence of any pharmacological blockers. There were significant regional differences ($F_{2,86} = 10.5$, $P < 0.0001$, one-way ANOVA; Fig. 2A). In particular, ACh-elicited inward currents were significantly smaller in SSC layer VI neurons (38.2 ± 4.5 pA, $n = 39$ neurons, $t_{67} = 4.3$, $P < 0.0001$, unpaired two-tailed *t*-test) and M1 layer VI neurons (46.6 ± 4.9 pA, $n = 20$

neurons, $t_{48} = 2.8$, $P = 0.008$, unpaired two-tailed t -test) than in mPFC layer VI neurons (69.5 ± 5.9 pA, $n = 30$ neurons). The responses to cholinergic stimulation were relatively homogenous within the M1 and mPFC, whereas there appeared to be greater diversity in cholinergic responses within the SSC (two examples are shown in Fig. 2B).

As a majority of corticothalamic layer VI neurons are typically silent under resting conditions (Sirota *et al.*, 2005; Zhou *et al.*, 2010), we next investigated the ability of cholinergic stimulation to depolarize and elicit action potentials in these neurons in current clamp mode. We found significant regional differences in the degree of ACh-elicited excitation in layer VI neurons ($F_{2,83} = 19.2$, $P < 0.0001$, one-way ANOVA; Fig. 2D). Average depolarization in SSC neurons (10.0 ± 1.3 mV, $n = 37$ neurons) was significantly smaller than that in M1 neurons (14.9 ± 1.6 mV, $n = 20$ neurons, $t_{55} = 2.3$, $P = 0.02$, unpaired two-tailed t -test) and mPFC neurons (21.2 ± 1.2 mV, $n = 29$ neurons, $t_{64} = 6.2$, $P < 0.0001$, unpaired two-tailed t -test). The mPFC neurons showed the strongest excitation by ACh, having significantly larger depolarization than M1 neurons ($t_{47} = 3.1$, $P = 0.003$, unpaired two-tailed t -test). Moreover, the depolarization elicited by ACh within layer VI neurons resulted in action potential firing in some neurons (Fig. 2E). In this regard, the region-specific consequences of cholinergic stimulation also differed between SSC, M1 and mPFC layer VI neurons. SSC neurons were less likely to depolarize to threshold in response to ACh (8/38 neurons, 21%) than M1 neurons (10/20 neurons, 50%, $P < 0.001$, Fisher's exact test) or mPFC neurons (30/30 neurons, 100%, $P < 0.0001$, Fisher's exact test). mPFC layer VI neurons were unique, in that every neuron depolarized to threshold in response to ACh; this is a significantly larger proportion than found in M1 ($P < 0.0001$, Fisher's exact test).

This pattern of strong regional difference in the layer VI cholinergic response was also observed with a lower concentration of ACh (100 μ M; $F_{2,35} = 3.8$, $P < 0.03$, one-way ANOVA). ACh elicited significantly smaller average inward currents in SSC neurons (15.3 ± 3.3 pA, $n = 11$ neurons, $t_{23} = 2.3$, $P = 0.03$, unpaired two-tailed t -test) and M1 neurons (14.2 ± 3.9 pA, $n = 13$ neurons, $t_{25} = 2.4$, $P = 0.02$, unpaired two-tailed t -test) than in mPFC neurons (26.5 ± 3.4 pA, $n = 14$ neurons). Significant regional differences were also observed in ACh-elicited depolarization (100 μ M; $F_{2,26} = 10.1$, $P = 0.0006$, one-way ANOVA). ACh elicited significantly smaller average depolarization in SSC neurons (6.1 ± 1.5 mV, $n = 9$ neurons, $t_{18} = 4.8$, $P = 0.0001$, unpaired two-tailed t -test) and M1 neurons (8.8 ± 2.5 mV, $n = 9$ neurons, $t_{18} = 3.0$, $P = 0.008$, unpaired two-tailed t -test) than in mPFC neurons (17.8 ± 1.8 mV, $n = 11$ neurons). It is of note that this milder cholinergic stimulus was sufficient to elicit action potentials in the majority of mPFC neurons.

Region-specific effects of ACh on layer VI neurons appear to be postsynaptic in nature

As ACh can affect both presynaptic and postsynaptic aspects of the cortical circuitry (Proulx *et al.*, 2013; Bloem *et al.*, 2014), we examined the effects of ACh in the presence of antagonists of synaptic transmission (20 μ M CNQX, 50 μ M APV, 10 μ M bicuculline; in bath). It is of note that the inward currents elicited by bath application of 1 mM ACh persisted during treatment with glutamatergic and GABAergic antagonists (examples from each region are shown in Fig. 3A). Furthermore, significant regional differences were seen

when synaptic transmission was blocked ($F_{2,16} = 10.9$, $P = 0.001$, one-way ANOVA; Fig. 3B). Under these conditions, ACh also elicited smaller responses in SSC neurons (17.2 ± 6.5 pA, $n = 7$ neurons, $t_{11} = 4.2$, $P = 0.001$, unpaired two-tailed t -test) and M1 neurons (43.6 ± 6.4 pA, $n = 6$ neurons, $t_{10} = 2.2$, $P = 0.05$, unpaired two-tailed t -test) than in mPFC neurons (75.9 ± 13.2 pA, $n = 6$ neurons). Likewise, depolarization by ACh in the presence of glutamatergic and GABAergic antagonists was also significantly different across primary and associative cortical regions ($F_{2,16} = 8.2$, $P = 0.003$, one-way ANOVA; Fig. 3C). SSC neurons (5.7 ± 2.6 mV, $n = 7$ neurons) were significantly less depolarized by ACh than M1 neurons (16.5 ± 3.2 mV, $n = 6$ neurons, $t_{11} = 2.7$, $P = 0.02$, unpaired two-tailed t -test) and mPFC neurons (23.6 ± 3.9 mV, $n = 6$ neurons, $t_{11} = 3.9$, $P = 0.002$, unpaired two-tailed t -test). These results suggest that postsynaptic cholinergic receptors are predominantly responsible for the large excitatory responses that we observed in layer VI neurons of primary and associative cortical regions.

The balance of nicotinic and muscarinic receptor contributions depends on cortical region

To determine the respective contributions of nicotinic and muscarinic ACh receptors to the regional dependence of the ACh responses in layer VI, we tested the effects of ACh in the continued presence of either the non-specific muscarinic antagonist atropine (200 nM) or the selective nicotinic antagonist DH β E (3 μ M). In a subset of experiments, a combination of these antagonists blocked all responses to ACh in layer VI pyramidal neurons of all three cortical regions examined (data not shown).

Nicotinic receptor-mediated inward currents elicited by ACh in the presence of atropine differed significantly across the cortical regions ($F_{2,48} = 13.3$, $P < 0.0001$, one-way ANOVA; Fig. 4A). Nicotinic stimulation of layer VI neurons resulted in smaller inward currents, on average, in SSC neurons (21.5 ± 4.6 pA, $n = 15$ neurons) than in M1 neurons (49.1 ± 4.5 pA, $n = 13$ neurons, $t_{26} = 4.2$, $P = 0.0002$, unpaired two-tailed t -test) and mPFC neurons (66.9 ± 7.2 pA, $n = 23$ neurons, $t_{36} = 4.7$, $P < 0.0001$, unpaired two-tailed t -test). Again, there was greater heterogeneity in the responses in the SSC than in the other two regions (two examples are shown in Fig. 4B).

Depolarization by ACh in the presence of atropine was significantly different across regions ($F_{2,42} = 32.9$, $P < 0.0001$, one-way ANOVA; Fig. 4D). Significantly smaller average depolarization was observed in SSC neurons (6.2 ± 1.2 mV, $n = 15$ neurons) than in M1 neurons (15.7 ± 2.0 mV, $n = 12$ neurons, $t_{25} = 4.2$, $P = 0.0003$, unpaired two-tailed t -test) and mPFC neurons (22.3 ± 1.3 mV, $n = 19$ neurons, $t_{32} = 8.8$, $P = 0.0001$, unpaired two-tailed t -test). Consistently, mPFC layer VI neurons were also more likely to depolarize to threshold (15/19 neurons, 78%) than M1 neurons (3/12 neurons, 25%, $P = 0.008$, Fisher's exact test) and SSC neurons (1/15 neurons, 7%, $P < 0.0001$, Fisher's exact test).

Next, we investigated muscarinic stimulation of layer VI neurons by ACh in the presence of the nicotinic receptor antagonist DH β E. The response to muscarinic stimulation was region-specific ($F_{2,38} = 5.6$, $P = 0.007$, one-way ANOVA; Fig. 5A). The largest average currents were observed in SSC layer VI neurons (12.5 ± 1.3 mV, $n = 12$ neurons), and they were significantly larger than those in mPFC neurons (6.3 ± 1.1 mV, $n = 19$ neurons, $t_{29} = 3.3$, $P = 0.003$, unpaired two-tailed t -test); M1 neurons showed intermediate responses (9.8 ± 1.5 mV,

$n = 10$ neurons). Unlike the ACh and nicotinic responses, muscarinic responses were relatively homogeneous within the SSC (Fig. 5B).

Despite our observation that muscarinic receptor-mediated inward currents were significantly different across the three regions, depolarization by ACh in the presence of DH β E did not differ across regions ($F_{2,39} = 1.6$, $P = 0.2$, one-way ANOVA; Fig. 5D). Therefore, there are no regional differences in the ability of ACh to depolarize neurons to threshold by muscarinic stimulation (SSC, 1/19 neurons, 5%; M1, 0/10 neurons, 0%; mPFC, 1/13 neurons, 8%; Fig. 5E). These paradoxical findings may be explained by the significantly higher average input resistance of mPFC layer VI neurons than M1 and SSC neurons, as shown in Table 1.

On the basis of our pharmacological dissection of the cholinergic response in layer VI neurons across primary and associative cortical regions, there appear to be significant differences in the respective contributions of nicotinic and muscarinic receptors to the overall cholinergic response between regions. These differences are illustrated in Fig. 6, and are calculated on the basis of the mean magnitudes of ACh-elicited inward currents in the presence of either atropine or DH β E. On average, there appears to be a shift from a combination of moderate nicotinic and muscarinic responses in SSC layer VI neurons (nicotinic ACh receptors, 63%; muscarinic ACh receptors, 37%) to larger and more dominant nicotinic responses in M1 neurons (nicotinic ACh receptors, 83%; muscarinic ACh receptors, 17%; $P = 0.002$, Fisher's exact test), and the strongest and most dominant nicotinic responses in mPFC neurons (nicotinic ACh receptors, 91%; muscarinic ACh receptors, 9%; $P < 0.0001$, Fisher's exact test).

Characterization of two distinct populations of cholinergic responses among SSC neurons

We observed greater heterogeneity in the SSC than in the M1 and mPFC for responses to cholinergic (Fig. 2B and E) and nicotinic (Fig. 4B and E) stimulation, but not for responses to muscarinic stimulation (Fig. 5B and E). On the basis of these results, we hypothesized that SSC layer VI neurons could be subdivided into two subtypes according to their responses to ACh. As summarized in Fig. 7, 'type 1' neurons showed prominent inward currents (57%, 22 of 39 SSC neurons) and 'type 2' neurons showed more subtle and complex responses (43%, 17 of 39 SSC neurons). The averaged ACh-elicited currents of type 1 and type 2 neurons are shown in Fig. 7(A) and compared with the overall average response in the SSC. Interestingly, some evidence indicates that these subgroups may reflect distinct subclasses of layer VI neurons, as characterized by their responses to depolarizing current injections. Previous work has suggested that layer VI corticothalamic neurons have regular-spiking firing patterns, whereas corticocortical neurons have initial-doublet firing patterns (Mercer *et al.*, 2005; Kumar & Ohana, 2008; Petrof *et al.*, 2012). Here, we found a highly significant difference in the prevalence of these different firing patterns in type 1 and type 2 neurons (Fisher's exact test, $P < 0.0001$), with the majority of regular-firing cells (22 of 27 neurons) yielding a type 1 response to ACh, and all initial-doublet neurons yielding a type 2 response (12 of 12 neurons).

Acetylcholine-elicited inward currents were significantly larger in type 1 neurons (type 1, 58.4 ± 4.4 pA, $n = 22$ neurons; type 2, 11.9 ± 1.7 pA, $n = 17$ neurons; $t_{37} = 8.9$, $P < 0.0001$,

unpaired two-tailed t -test; Fig. 7C and D). These inward currents also had different kinetics in terms of speed of onset (type 1, 10.8 ± 0.6 s, $n = 23$ neurons; type 2, 31.8 ± 4.6 s, $n = 13$ neurons; $t_{34} = 6.0$, $P < 0.0001$, unpaired two-tailed t -test; Fig. 7F) and duration of response (type 1, 42.7 ± 5.3 s, $n = 23$ neurons; type 2, 149.2 ± 20.4 s, $n = 13$ neurons; $t_{34} = 6.4$, $P < 0.0001$, unpaired two-tailed t -test; Fig. 7G). In the presence of the muscarinic antagonist atropine, fast inward currents were found in 63% (10/16) of SSC neurons, whereas 37% (6/16) showed a minimal or no response. It is of note that the type 2 SSC neurons consistently showed a transient outward current at the beginning of the ACh response, but this was only infrequently observed in type 1 SSC neurons (Fig. 7C). Such outward currents were also prominent among the muscarinic responses in all regions observed in the presence of the nicotinic antagonist DH β E. On the basis of the heterogeneity in the ACh and nicotinic responses and the homogeneity in the muscarinic responses in the SSC, we speculate that type 1 responses are composed of a mixture of nicotinic and muscarinic components, whereas type 2 responses are primarily muscarinic in nature. This heterogeneity of responses to ACh in the SSC contrasts with the relative homogeneity of the responses in the M1 and mPFC.

The mouse attention deficit model has disrupted associative and preserved primary responses

Associative cortical regions such as the mPFC are thought to be necessary for attention tasks only under conditions of high demand (Marti *et al.*, 2012). Here, we examined ACh responses in layer VI pyramidal neurons across primary and associative regions in a mouse model that shows deficient attention only under demanding conditions (Bailey *et al.*, 2010). This mouse is deleted for the *Chrna5* gene, which encodes the $\alpha 5$ nicotinic ACh receptor subunit expressed in layer VI across the cerebral cortex (Wada *et al.*, 1990; Salas *et al.*, 2003; Winzer-Serhan & Leslie, 2005). Recorded ACh responses from $\alpha 5^{-/-}$ mice and their WT controls showed a significant interaction between genotype and cortical region ($F_{2,63} = 3.9$, $P = 0.03$, two-way ANOVA; Fig. 8A and B). This interaction arises not from changes to ACh responses within the primary cortex, but from a significant reduction in the responses within the associative mPFC ($t = 3.9$, $P < 0.001$, Bonferroni *post hoc* test).

A similar interaction was found between genotype and cortical region on ACh-elicited depolarization ($F_{2,57} = 7.8$, $P = 0.01$, two-way ANOVA; Fig. 8C and D). Again, this interaction arises from a significant decrease in ACh-elicited depolarization only within the mPFC ($t = 3.5$, $P < 0.01$, Bonferroni *post hoc* test). Likewise, mPFC layer VI neurons in $\alpha 5^{-/-}$ mice appeared to be less likely to depolarize to threshold than those in WT controls (WT, 12/15 neurons, 80%; $\alpha 5^{-/-}$, 3/8 neurons; $P = 0.07$, Fisher's exact test). This decrease in cholinergic excitation was not observed in layer VI M1 or SSC neurons. These data show that a manipulation that only interferes with the performance of attention tasks under conditions of high demand (Bailey *et al.*, 2010) has selective effects on ACh-elicited responses in the associative cortex but not on those in primary cortical regions.

Discussion

There are prominent regional differences in the strength and nature of cholinergic responses in layer VI pyramidal neurons. These responses are primarily mediated by postsynaptic mechanisms, and are not a consequence of ACh-elicited changes in synaptic transmission. Pharmacological dissection of the cholinergic responses reveals that the balance between nicotinic and muscarinic ACh receptor-mediated responses is region-specific. SSC layer VI neurons showed the weakest average response to cholinergic stimulation. Their responses were not homogeneous: slightly more than half of the neurons responded to both nicotinic and muscarinic receptors; however, a large minority showed only muscarinic responses. In contrast, M1 neurons responded more strongly and homogeneously to ACh, with both nicotinic and muscarinic receptors contributing to cholinergic excitation. Finally, mPFC layer VI neurons were the most powerfully excited by cholinergic stimulation, an effect driven predominantly by large nicotinic responses that showed region-specific dependence on the nicotinic receptor $\alpha 5$ subunit. The physiological importance of these responses likely corresponds to their role in modulating normal attention, depending on the modality and demand of the attention task.

Layer VI neurons, attention, and the importance of cholinergic neurotransmission

We chose to target our recordings to layer VI neurons because they constitute a major source of top-down feedback to the thalamus, innervating both primary and higher-order thalamic nuclei (Bourassa *et al.*, 1995; Guillery & Sherman, 2002; Gabbott *et al.*, 2005). Coupled with thalamocortical inputs, they form a loop between the cortex and thalamus that is considered to be essential for normal attentional function (Parikh *et al.*, 2010; Thomson, 2010). The strong cholinergic stimulation that we observed is consistent with the presence of both nicotinic and muscarinic receptors within neurons of deep cortical layers (Buckley *et al.*, 1988; Tribollet *et al.*, 2004). These receptors are likely involved in mediating *in vivo* responses to local release of ACh upon cue detection during attention tasks, as observed in both primary (Fournier *et al.*, 2004; Parikh & Sarter, 2008) and associative (Parikh *et al.*, 2007; Rasmusson *et al.*, 2007) cortical regions. Within the SSC, ACh release has been extensively linked to the shifting of attentional focus in response to visual-sensory stimulation (Fournier *et al.*, 2004; Haegens *et al.*, 2011; Bauer *et al.*, 2012). In particular, top-down modulated alpha-band activity in the SSC reflects an anticipatory state that predicts the direction of attentional focus, as well as attentional performance (Jones *et al.*, 2010; Haegens *et al.*, 2011). In the M1, evoked tonic release of ACh through periods of attentional demand has been linked to performance in attention tasks (Parikh & Sarter, 2008), and functionally correlated with motor planning or the execution of necessary movements that contribute to normal performance in these tasks (Sirota *et al.*, 2005; Conner *et al.*, 2010). On the other hand, ACh release within the mPFC is observed just before cue detection, and, in fact, predicts the success of such detection (Parikh *et al.*, 2007; Howe *et al.*, 2013).

Within primary regions, cholinergic activity triggered by stimuli is affected by, but not dependent on, the presence of distractors (Kelly *et al.*, 2006; Sauseng *et al.*, 2009). However, cholinergic activity within the mPFC is not necessarily present across all attention tasks.

Some evidence suggests that it may not be central under conditions of lower attentional demand (Smucny *et al.*, 2013), and that it may be employed only in particularly challenging conditions (Marti *et al.*, 2012). Supporting this theory is the observation that the influence of cholinergic neuromodulation is highest when attention is taxed by distractors or prolonged trials (Kozak *et al.*, 2006; Sarter *et al.*, 2006; St Peters *et al.*, 2011).

The distinct regional responses that we report here show that layer VI neurons across the cortex do not respond uniformly to ACh. The heightened influence of muscarinic receptor-mediated responses to ACh in the primary regions suggests they may have a greater role in the sensorimotor components of attention, as seen in pharmacological studies involving behaving rodents and primates (Robbins *et al.*, 1998; Davidson & Marrocco, 2000). In particular, we found two distinct populations of neurons within layer VI of the SSC on the basis of their cholinergic response, which may reflect differences in this response among neurons within the SSC with different projection targets (Mercer *et al.*, 2005; Kumar & Ohana, 2008). The known heterogeneity of neurons within layer VI (Andjelic *et al.*, 2009; Briggs, 2010; Thomson, 2010) raises questions about their potentially distinct contributions, in terms of timing and direction of response, to the performance of somatosensory attention tasks. In addition to the postsynaptic effects of ACh reported here, cholinergic stimulation can elicit changes at presynaptic sites in the cerebral cortex (Bloem *et al.*, 2014). Although ACh can elicit strong postsynaptic responses in the presence of antagonists of synaptic transmission, ACh release *in vivo* likely plays an important role in the fine-tuning of activity within the local circuitry that can affect attention. More experiments, including the characterization of the two neuronal subtypes in the SSC, are necessary for a better understanding of the general circuitry within this region and how their unique responses to ACh can modulate attentional behavior.

The ACh response in the associative cortex and the nicotinic receptor $\alpha 5$ subunit

The larger overall responses to cholinergic stimulation and the dominant nature of the nicotinic receptor-mediated component sets layer VI neurons of the associative mPFC apart from the primary cortical regions. Our results suggest that the nicotinic receptor $\alpha 5$ subunit is required to maintain these large cholinergic responses within layer VI of the mPFC in adulthood, but does not play a role in the ACh responses in the primary cortical regions examined. These results are broadly consistent with studies suggesting that the $\alpha 5$ subunit shows not only laminar specificity in its expression (Birtsch *et al.*, 1997; Salas *et al.*, 2003; Winzer-Serhan & Leslie, 2005), but also some degree of regional specificity within the cortex (Wada *et al.*, 1990). Although incorporation of the $\alpha 5$ subunit into $\alpha 4\beta 2^*$ receptors can modulate nicotinic responses directly by increasing the channel Ca^{2+} permeability (Kuryatov *et al.*, 1997), our experiments do not conclusively show a direct effect of this subunit. The constitutive deletion of the $\alpha 5$ subunit in our mice does not preclude the potential for developmental effects of $\alpha 5$ subunits. Indeed, the nicotinic response is strongest during the juvenile period in the rodent mPFC, peaking during the second and third weeks of postnatal life (Kassam *et al.*, 2008; Bailey *et al.*, 2012, 2014). Therefore, cholinergic signaling mediated by $\alpha 5$ -containing receptors, either directly within mPFC layer VI neurons or within other regions during development, may have long-lasting consequences for the strength of cholinergic responses in adulthood.

Conclusions

These findings provide concrete functional data that demonstrate how layer VI neurons respond to cholinergic stimulation across several regions that are important in attentional performance. There is clear regional specificity in the direct effect of ACh on layer VI neurons of primary and associative cortical regions. Such neurons in M1 and the SSC have smaller responses to ACh, with a substantial muscarinic component, whereas the SSC is unusual for having two populations of layer VI neurons that show distinct responses to ACh. In contrast, mPFC layer VI neurons show dramatically greater cholinergic excitation, driven predominantly by nicotinic receptors. This region-specific pattern appears to be dependent on the presence of the nicotinic $\alpha 5$ subunit, and is lost in a mouse model of attention deficits under demanding conditions.

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Abbreviations

ACh	acetylcholine
ACSF	artificial cerebrospinal fluid
APV	D-(–)-2-amino-5-phosphonopentanoic acid
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
DHβE	dihydro- β -erythroidine hydrobromide
eGFP	enhanced green fluorescent protein
M1	primary motor cortex
mPFC	medial prefrontal cortex
SSC	primary somatosensory cortex
Syt6	synaptotagmin 6
WT	wild-type

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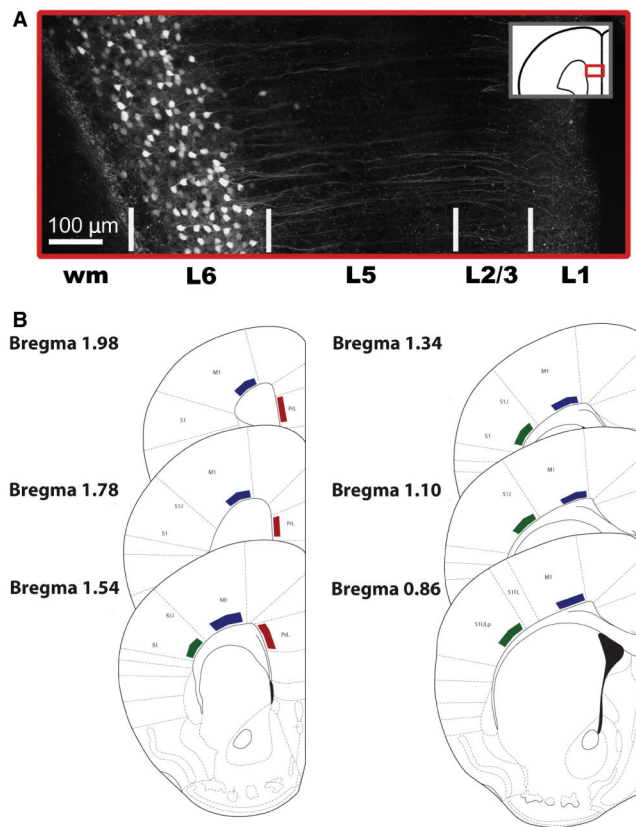


Fig. 1. Layer VI in primary and associative cortical regions is identified in transgenic eGFP-expressing mice. (A) A z-stack projection shows layer VI-specific expression of eGFP in neurons of Syt6-eGFP mice. This laminar specificity is conserved across primary and associative cortical regions. (B) Schematics showing regions of primary and associative cortices targeted for electrophysiological recordings. Bregma coordinates are indicated as described previously (Paxinos & Franklin, 2001). Red: mPFC. Blue: M1. Green: SSC. wm, white matter.

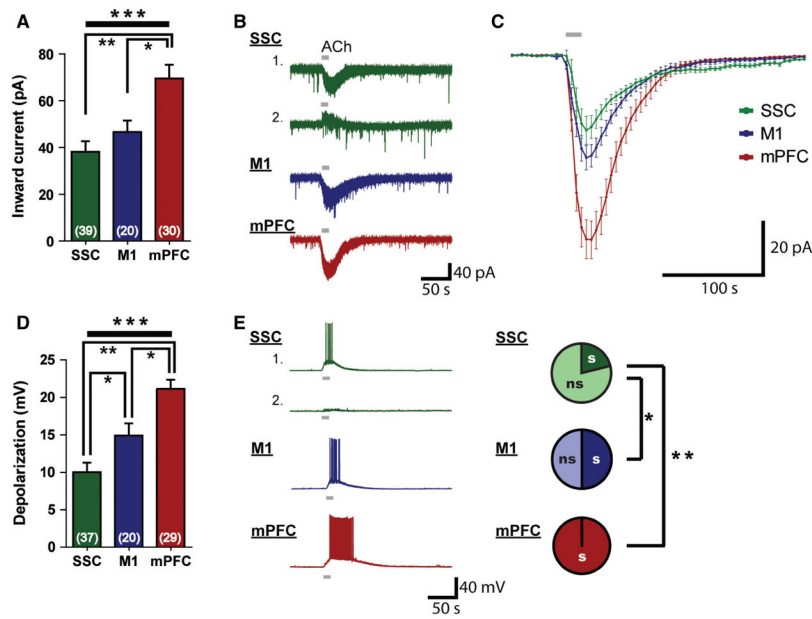


Fig. 2.

There is regional specificity in the cholinergic excitation of layer VI neurons in primary and associative cortical regions. (A) The average inward current elicited by ACh differs in layer VI of the SSC, M1, and mPFC ($F_{2,86} = 10.5$, $***P < 0.0001$, one-way ANOVA). SSC neurons ($**P < 0.0001$, unpaired two-tailed t -test) and M1 neurons ($*P = 0.008$, unpaired two-tailed t -test) have smaller ACh-elicited currents than mPFC neurons. (B) Representative examples of ACh-elicited currents in the SSC, M1, and mPFC. Two examples are provided to show the greater heterogeneity within the SSC. (C) Averaged plot of all recorded ACh-elicited currents in SSC, M1 and mPFC neurons. (D) Excitation from rest elicited by ACh differs significantly in layer VI of the SSC, M1, and mPFC ($F_{2,83} = 19.2$, $***P < 0.0001$, one-way ANOVA). Depolarization of layer VI neurons by cholinergic stimulation is smallest in the SSC (vs. M1, $*P = 0.02$, unpaired two-tailed t -test; vs. mPFC, $**P < 0.0001$, unpaired two-tailed t -test), larger in the M1 (vs. mPFC, $*P = 0.003$, unpaired two-tailed t -test), and largest in the mPFC. (E) Representative examples of ACh-elicited depolarization in the SSC, M1, and mPFC. SSC layer VI neurons are less likely to depolarize to threshold in response to cholinergic stimulation than M1 neurons ($*P < 0.001$, Fisher's exact test) and mPFC neurons ($**P < 0.0001$, Fisher's exact test). s, spiking; ns, non-spiking (in response to ACh).

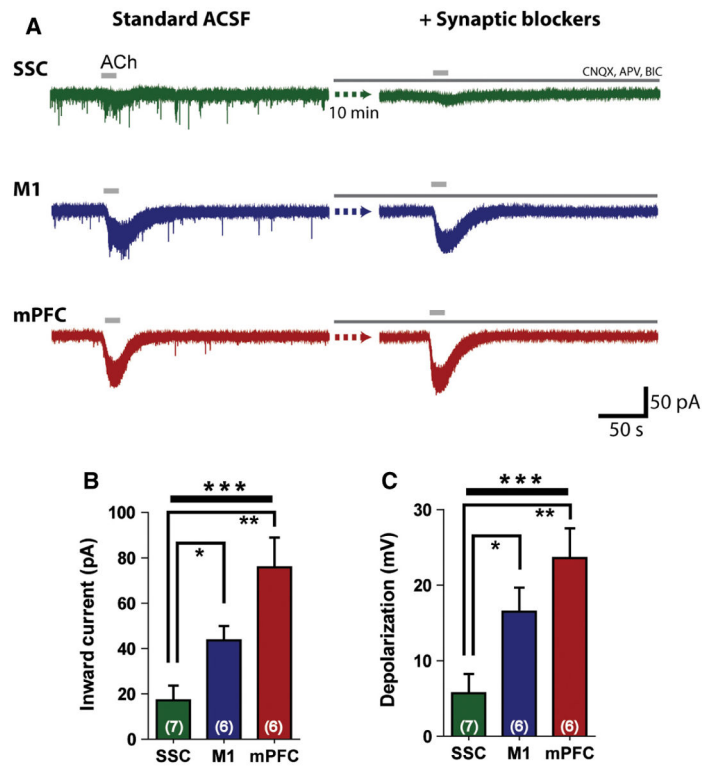


Fig. 3.

Region-dependent effects of ACh are preserved in the presence of antagonists of synaptic transmission. (A) Example traces from layer VI neurons in primary and associative cortical regions of inward currents elicited by ACh. Traces on the left show responses to ACh in standard ACSF. Traces on the right show responses to ACh in the same neuron in the presence of glutamatergic and GABAergic receptor antagonists [20 μ M CNQX; 50 μ M APV; 10 μ M bicuculline (BIC)] after a 10-min wash-in period. (B) Prominent regional differences in the inward currents elicited by ACh are seen in the presence of synaptic blockers ($F_{2,16} = 10.9$, $***P = 0.001$, one-way ANOVA). Currents are smallest in SSC neurons (vs. M1, $*P = 0.01$, unpaired two-tailed t -test; vs. mPFC, $**P = 0.001$, unpaired two-tailed t -test), larger in M1 neurons (vs. mPFC, $P = 0.05$, unpaired two-tailed t -test), and largest in mPFC neurons. (C) Depolarization by ACh in the presence of synaptic blockers is significantly different across the SSC, M1, and mPFC ($F_{2,16} = 8.2$, $***P = 0.003$, one-way ANOVA). Depolarization in SSC neurons is significantly smaller than in M1 neurons ($*P = 0.02$, unpaired two-tailed t -test) and mPFC neurons ($**P = 0.002$, unpaired two-tailed t -test).

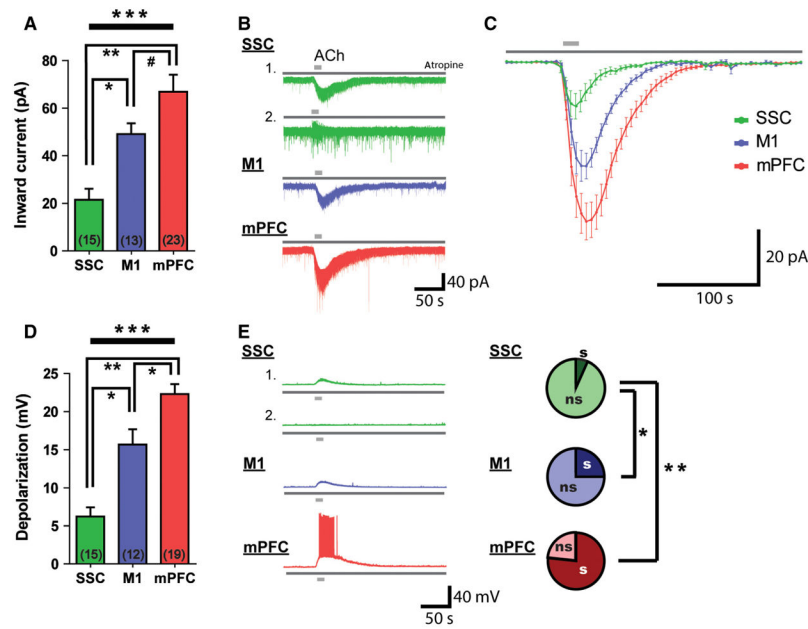
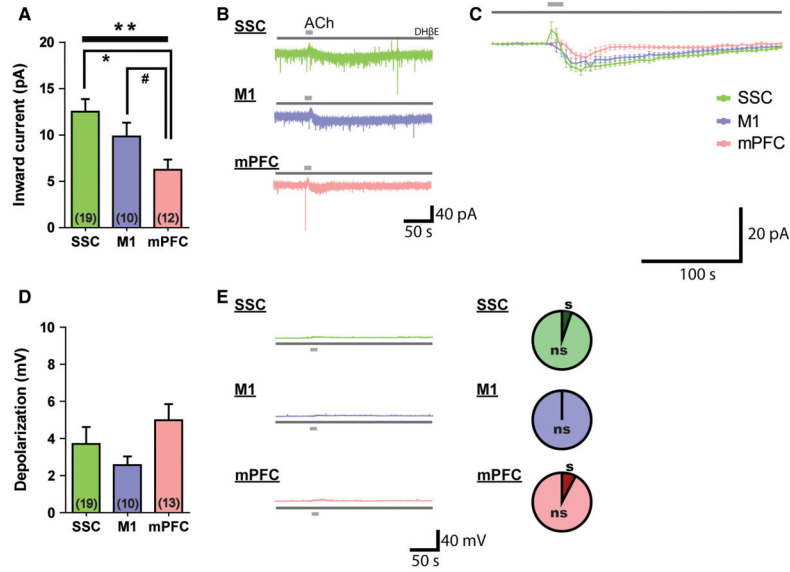


Fig. 4.

There is regional specificity in the nicotinic receptor-mediated responses to ACh in layer VI neurons of primary and associative cortical regions. (A) The average inward current elicited by nicotinic stimulation differs among the SSC, M1, and mPFC ($F_{2,48} = 13.3$, $***P < 0.0001$, one-way ANOVA). Nicotinic receptor-evoked inward currents are smallest in the SSC (vs. M1, $*P = 0.0002$, unpaired two-tailed t -test; vs. mPFC, $**P < 0.0001$, unpaired two-tailed t -test), and larger in the M1 (vs. mPFC, $\#P = 0.09$, unpaired two-tailed t -test) and mPFC. (B) Representative examples of nicotinic currents in the SSC, M1, and mPFC. (C) Averaged plot of all recorded nicotinic responses in SSC, M1 and mPFC neurons. (D) Depolarization by nicotinic stimulation differs significantly between layer VI neurons of primary and associative cortical regions ($F_{2,42} = 32.9$, $***P < 0.0001$, one-way ANOVA). This depolarization is smallest in SSC neurons (vs. M1, $*P = 0.0003$, unpaired two-tailed t -test; vs. mPFC, $**P = 0.0001$, unpaired two-tailed t -test), larger in M1 neurons (vs. mPFC, $*P = 0.007$, unpaired two-tailed t -test), and largest in mPFC neurons. (E) Representative examples of nicotinic receptor-mediated depolarization in the SSC, M1, and mPFC. SSC layer VI neurons are less likely to depolarize to threshold in response to nicotinic stimulation than M1 neurons ($*P = 0.008$, Fisher's exact test) and mPFC neurons ($**P < 0.0001$, Fisher's exact test). s, spiking; ns, non-spiking.

**Fig. 5.**

There is some regional specificity, albeit less pronounced, in muscarinic receptor-mediated responses to ACh in layer VI of primary and associative cortical regions. (A) Inward currents elicited by muscarinic stimulation differ among the SSC, M1, and mPFC ($F_{2,38} = 5.6$, $**P = 0.007$, one-way ANOVA). Muscarinic receptor-mediated currents are largest in SSC and M1 neurons and smallest in mPFC neurons (vs. SSC, $*P = 0.003$, unpaired two-tailed t -test; vs. M1, $\#P = 0.06$, unpaired two-tailed t -test). (B) Representative sample traces of muscarinic receptor-mediated currents in the SSC, M1, and mPFC. (C) Averaged plot of all recorded muscarinic currents in SSC, M1 and mPFC neurons. (D) Depolarization from rest by muscarinic stimulation does not differ significantly between SSC, M1 and mPFC layer VI neurons ($F_{2,39} = 1.6$, $P = 0.2$, one-way ANOVA). (E) Examples of representative responses to muscarinic stimulation. There are no differences in the proportions of neurons reaching threshold in response to muscarinic receptor-mediated excitation. s, spiking; ns, non-spiking.

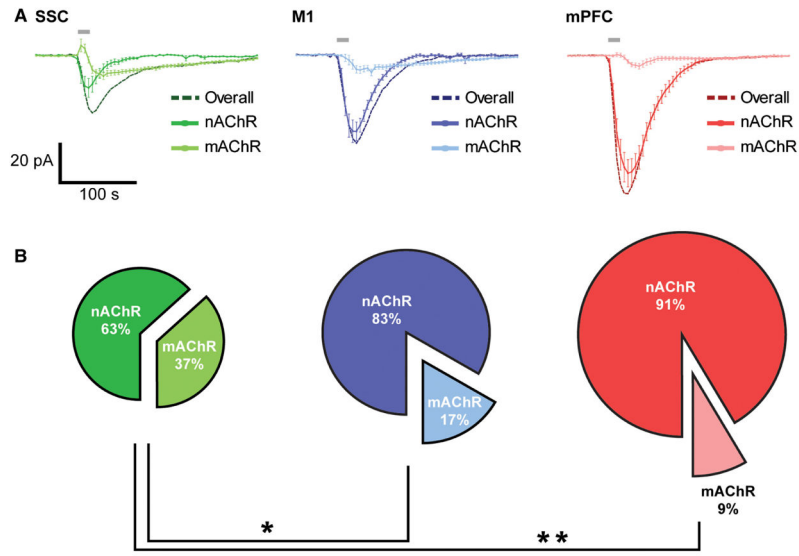
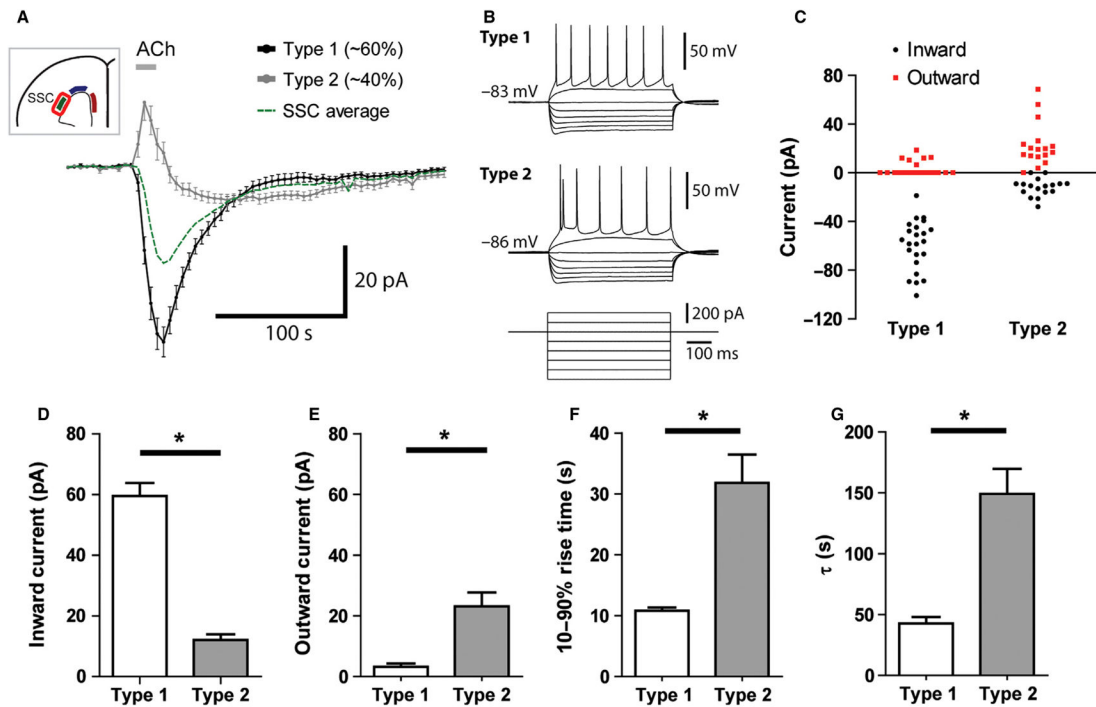
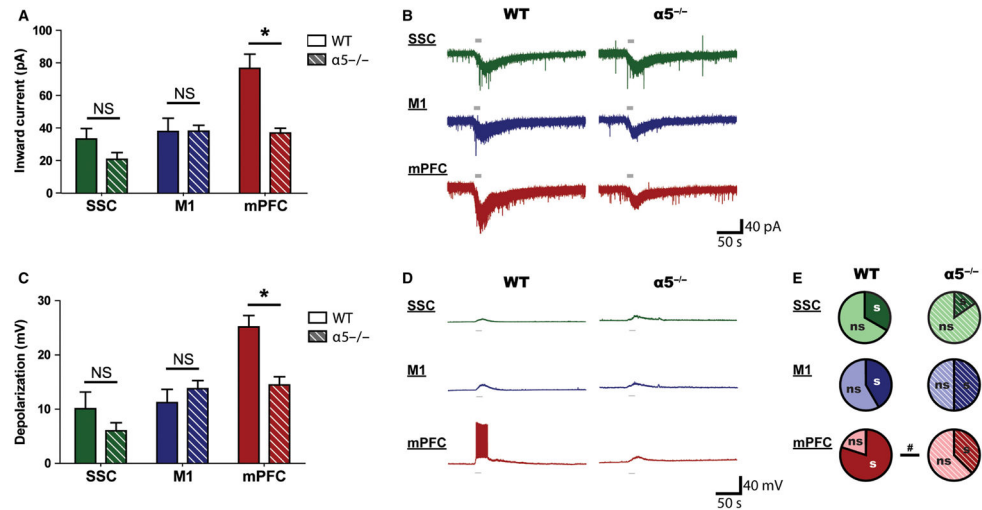


Fig. 6. Cortical region determines the overall cholinergic excitation of layer VI neurons and the degree of nicotinic and muscarinic contributions to the average response. (A) Superimposed average plots of all nicotinic [nicotinic ACh receptor (nAChR)], muscarinic [muscarinic ACh receptor (mAChR)] and overall responses to ACh in SSC, M1 and mPFC layer VI neurons. (B) Pie charts represent the relative contributions of nicotinic receptor-mediated and muscarinic receptor-mediated responses to ACh in SSC, M1 and mPFC neurons. Layer VI neurons are more dependent on nicotinic stimulation in the M1 ($*P = 0.002$, Fisher's exact test) and mPFC ($**P < 0.0001$, Fisher's exact test) than in the SSC.

**Fig. 7.**

Further characterization of the two distinct cholinergic responses observed in SSC layer VI neurons. (A) Averaged plots of ACh-elicited currents in these ‘type 1’ and ‘type 2’ SSC neurons. (B) These different responses to ACh may arise from two distinct subclasses of layer VI neurons, as illustrated by their typical spiking patterns in response to injection of depolarizing current. (C) Scatter plot showing uni-directional and bi-directional responses to ACh (outward current shown in red; inward current shown in black) in both populations of SSC neurons. Neurons with either no inward or no outward currents are plotted as zeroes. (D and E) Average inward and outward currents in type 1 and type 2 neurons. Type 1 neurons have significantly larger inward currents ($*P < 0.0001$, unpaired two-tailed t -test), but smaller outward currents on average ($*P < 0.0001$, unpaired two-tailed t -test). (F and G) Type 2 neurons have slower rise time ($*P < 0.0001$, unpaired two-tailed t -test) and longer response duration ($*P < 0.0001$, unpaired two-tailed t -test) following cholinergic stimulation.

**Fig. 8.**

The regional specificity of cholinergic responses is attenuated in the absence of the nicotinic receptor $\alpha 5$ subunit. (A) Inward currents elicited by ACh in primary and associative cortical regions of $\alpha 5^{-/-}$ mice and WT controls. There is a significant interaction between genotype and cortical region on cholinergic responses ($F_{2,63} = 3.9$, $P = 0.03$, two-way ANOVA). ACh-elicited inward currents in mPFC layer VI neurons are significantly smaller in $\alpha 5^{-/-}$ mice than in WT controls ($*P < 0.001$, Bonferroni *post hoc* tests), but a genotype difference is not observed in primary cortical regions. (B) Representative examples of cholinergic responses across primary and associative cortical regions in WT and $\alpha 5^{-/-}$ mice. (C) Depolarization by ACh in SSC, M1 and mPFC layer VI neurons of $\alpha 5^{-/-}$ mice and WT controls. A significant interaction exists between genotype and cortical region on ACh-elicited depolarization ($F_{2,57} = 7.8$, $P = 0.01$, two-way ANOVA). Depolarization by ACh in mPFC layer VI neurons, but not in those of primary cortical regions, is significantly smaller in $\alpha 5^{-/-}$ mice than in WT controls ($*P < 0.01$, Bonferroni *post hoc* tests). (D) Representative recordings of depolarization by cholinergic stimulation in the SSC, M1, and mPFC. (E) Proportions of neurons depolarized to threshold across primary and associative cortical regions in WT and $\alpha 5^{-/-}$ mice. mPFC layer VI neurons in $\alpha 5^{-/-}$ mice tend to be less likely to depolarize to threshold than those of WT controls ($\#P = 0.07$, Fisher's exact test). s, spiking; ns, non-spiking; NS, not significant.

Table 1

Electrophysiological parameters of SSC, M1 and mPFC layer VI neurons

	SSC (<i>n</i> = 73)	M1 (<i>n</i> = 43)	mPFC (<i>n</i> = 65)	<i>P</i> -value (one-way ANOVA)
Resting membrane potential (mV)	-83.5 ± 0.6*	-80.8 ± 0.7	-81.6 ± 0.7	0.01
Spike amplitude (mV)	90.7 ± 0.8	90.3 ± 0.9	90.3 ± 1.0	0.9
Input resistance (MΩ)	159.5 ± 7.7	179.3 ± 9.7	320.2 ± 14.2**	0.0001

Across these regions of the cerebral cortex, there is a small difference in resting membrane potential ($F_{2,178} = 4.4$, $P = 0.01$), and a larger difference in input resistance ($F_{2,178} = 66.2$, $P < 0.0001$). Values are shown as mean ± standard error of the mean. mPFC, medial prefrontal cortex; M1, primary motor cortex; SSC, somatosensory cortex.

* $P < 0.05$ for SSC vs. M1 or mPFC;

** $P < 0.05$ for mPFC vs. SSC or M1.