Title: “Cholinergic control of cortical circuit dynamics”

Abstract/Specific Aims:
The primary sensory cortices receive extensive cholinergic innervation, primarily originating in nuclei of the basal forebrain. These afferents target diverse cell types in multiple cortical layers. A growing body of evidence indicates that cortical cholinergic transmission plays a critical role in cognitive processes as varied as sensory processing, reward timing and fear learning. Defects in cortical cholinergic signaling due to degeneration or altered expression of cholinergic receptors have been shown to underlie psychological pathologies like schizophrenia. Elucidating the specific circuit elements mediating the cholinergic effects is of crucial importance in generating a complete picture of cortical function and dysfunction.

Although traditionally, cholinergic effects have been considered to be primarily non-synaptic and acting by slow, diffuse signaling, recent studies demonstrate the spatiotemporal accuracy with which endogenously released acetylcholine (ACh) influences cortical function. Cholinergic activity affects cognitive processes which require reliable control of cortical activity at precise timescales. Studies at both the cellular and population levels have shown the effects of cholinergic modulation to be diverse: they employ multiple receptor subtypes; they may excite or inhibit, facilitate transient modulation, or mediate long-term plasticity. However, establishing a mechanistic link between cholinergic effects at the neuronal and systems levels has proven to be difficult, since it is unclear what their effects are at the level of networks of inter-connected neurons, which form the basic information processing units in the neocortex. It is also unclear how distinct cell types and receptors interact to mediate cholinergic modulation of cortical circuits at various timescales.

Our long-term objective is to develop and characterize a model system that describes the precise cholinergic modulation of cortical network dynamics at both short (millisecond-second) and long (minute) timescales. To attain this goal, we have employed a slice model of mouse somatosensory cortex, to reliably evoke transient “recurrent” activity resulting from reciprocal connections between pyramidal cells. This activity may then be paired with precisely timed cholinergic activation by optically engaging light-responsive channel proteins expressed in cortical cholinergic afferents. This allows us to examine cholinergic control of ongoing cortical activity. Guided by our preliminary findings, we hypothesize that cholinergic transmission suppresses recurrent network activity in the primary somatosensory cortex by recruiting multiple cell types and receptors, and produces long-term changes in the processing of sensory input by cortical circuits. We propose the following Specific Aims:

Specific Aim 1: Examine whether temporally precise cholinergic activation suppresses recurrent activity in somatosensory cortex, and the contributions of cholinergic receptor subtypes. We hypothesize that cholinergic transmission limits the magnitude and spatial spread of recurrent excitation by engaging nicotinic and muscarinic receptors in a temporally specific fashion. We will employ slice electrophysiology, pharmacology and optical imaging of neuronal activity to address this question.

Specific Aim 2: Determine the contributions of different cellular subtypes in mediating cholinergic control of cortical activity. We predict distinct contributions of 5HT3aR+ interneurons and cortical pyramidal cells in mediating cholinergic control of network activity. Cholinergic receptors on these cells likely act by means of pre- and post-synaptic mechanisms to mediate the action of ACh on cortical networks. We will use a combination of slice electrophysiology, genetic manipulation and pharmacology to address this question.

Specific Aim 3: Elucidate the long-term effects of paired cholinergic and glutamatergic transmission to the cortex, and the cellular/circuit mechanisms thereof. We hypothesize that synaptically released cortical ACh produces long-term changes in the processing of sensory input by cortical circuits by increasing the efficacy of synaptic communication between pyramidal cells in a muscarinic receptor dependent manner. We will use similar techniques as in Aim 2.

Testing these hypotheses will help integrate diverse cellular and synaptic effects of cholinergic signaling into a comprehensive network model that is closer to behavioral observations. This study will also help form a mechanistic understanding of cholinergic modulation in an array of cortical processes. This is necessary to find novel means of treating pathologies which are often characterized by defects in that modulation.
Background:
Most regions of the cortex, including the somatosensory cortex, receive extensive long-range projections from cholinergic neurons located primarily within a group of nuclei ventromedial to the thalamus, called the basal forebrain (BF). BF cells send widespread projections to all six cortical layers in primary and secondary sensory cortices and higher association areas (Bigl et al, 1982). Although a mechanistic framework that details the various actions of ACh signaling has not been described, it is established that cortical ACh plays a crucial role in numerous cognitive processes. For instance, ACh application induces visual receptive field plasticity (Greuel et al, 1988) and blocking ACh signaling mitigates the effects of attentional modulation (Herrero et al, 2008). Furthermore, cholinergic synapses are thought to function by classical synaptic transmission (as opposed to volume transmission) and are thus capable of precise spatiotemporal signaling (Munoz and Rudy, 2014).

Cellular subtypes in the cortex: ACh afferents in the neocortex target numerous cell types that are interconnected in stereotypical patterns (Figure 2). In the primary somatosensory (S1) cortex, glutamatergic spiny stellate cells (SSCs) in cortical layer 4 are the major recipients of feed-forward sensory information from the thalamus (Feldmeyer, 2012). Layer 4 of rodent S1 cortex is functionally segregated into columnar sub-regions called “barrels”, such that SSCs in a given barrel receive sensory input only from a single whisker (Simons and Carvell, 1989, Petersen, 2007). SSCs then relay incoming sensory information up to glutamatergic pyramidal cells in layer 2/3. Layer 2/3 pyramidal cells share extensive reciprocal “recurrent” connections with one another, forming a self-exciting cortical network (Holmgren et al, 2003). Unlike layer 4, layer 2/3 pyramidal cell projections are not restricted to the same column, and recurrent excitation communicates sensory information to neighboring columns. Layer 2/3 pyramidal cells also project to pyramidal cells in layer 5. In addition to excitatory cells above, neocortex contains numerous inhibitory GABAergic neurons, belonging to three groups: (i) PV+ interneurons, that express the Ca2+-binding protein parvalbumin (Rudy et al, 2011), (ii) Sst+ interneurons, that express the neuropeptide somatostatin, and (iii) 5HT3AR+ interneurons, that express the ionotropic serotonin receptor 5HT3aR (Lee et al, 2010). PV+ interneurons project primarily onto pyramidal cells and also receive strong feed-forward sensory excitation from layer 4 (Helmstaedter et al, 2008). Thus, they temporally restrict the window of sensory excitation for pyramidal cells (Kawaguchi and Kubota, 1997) and preclude recurrent excitation for weak stimuli. Like PV+ cells, Sst+ interneurons receive input from pyramidal cells and strongly inhibit other pyramidal cells, along with other cell types (Pfeffer et al, 2013). 5HT3aR+ interneurons potentiate inhibit all other cell types.

Nicotinic receptor expression: Both types of ACh receptors, namely nicotinic and muscarinic receptors, display considerable heterogeneity in their expression patterns across cortical layers. The cation channels α7 and α4β2 nicotinic receptors (nAChR) are expressed by 5HT3aR+ interneurons in layers 1 and 2/3 (Arroyo et al, 2014, Brombas et al, 2014). Since 5HT3aR+ cells inhibit PV+ and Sst+ interneurons, which in turn inhibit pyramidal cells, nAChR-mediated 5HT3aR+ cell activation can disinhibit pyramidal neurons (Poorthuis et al, 2014). In vivo, there is evidence for an nAChR-mediated disinhibitory circuit that increases the excitability of layer 2/3 pyramidal cells, thereby mediating associative learning (Letzkus et al, 2011). Furthermore, activation of cholinergic afferents increases pyramidal cell responses to stimuli (Fu et al, 2014), and improves the effects of orientation-selective visual training (Kang et al, 2014). However, 5HT3aR+ cells also directly inhibit pyramidal cells and in vitro studies have yielded contradictory results: activating cortical ACh afferents in slice preparations causes disynaptic inhibition of pyramidal cells (Alitto and Dan, 2012, Arroyo et al, 2012). Thus it is not clear what effect nAChR activation has on cellular excitability or cortical network activity.

Muscarinic receptor expression: Five distinct subclasses of muscarinic ACh receptors (mAChR), which are G-protein coupled receptors, are expressed in neocortex, labeled as M1-M5 mAChRs (Munoz and Rudy, 2014). Signaling by mAChRs is prolonged, lasting several seconds (Jensen et al, 2009). M1-type (M1, M3 and M5) mAChRs may be expressed by Sst+ interneurons, which are depolarized by muscarinic agonists (Fanselow et al, 2008). M2-type (M2 and M4) mAChRs expressed pre-synaptically by pyramidal cells transiently decrease the efficacy of pyramidal-pyramidal cell synaptic communication (Kimura and Baughman, 1997), decreasing the ability of pyramidal cells to excite each other and possibly lowering network excitability. At the population level, mAChRs decrease correlation in neuronal firing: their activation decreases spatial integration areas in visual cortex (Roberts et al, 2005), reduces cortico-cortical coherence (Goard and Dan,
2009) and improves discriminability of population responses (Pinto et al., 2013). ACh has also been shown to excite a small proportion of pyramidal neurons (Alitto and Dan, 2012, Hedrick and Waters, 2015). It is unclear how these various receptors and cell types interact to mediate cholinergic control of cortical network activity.

Cholinergic-induced plasticity in cortex: On the temporal axis, mAChR-signaling mediates multiple forms of long-term plasticity. For instance, M2-type and M1-type mAChRs facilitate long-term potentiation and depression in layer 2/3, respectively (Origlia et al., 2006). ACh signaling may also underlie a form of reward timing in primary sensory cortices, since BF neurons are activated when a reward is received. When Ach agonists are puffed onto slices (to mimic reward receipt) at a certain delay following stimulus delivery, the spike trains evoked by the stimulus post-training increase in duration to reflect the delay between stimulus and reward (Chubykin et al., 2013). This plasticity is mAChR-dependent, and is thought to be produced by a long-term increase in the efficacy of transmission at recurrent synapses between pyramidal cells (Gavornik et al., 2009). What long-term changes are induced in cortical network activity by synaptically-released Ach and what role mAChR sub-types have in mediating those changes are not known.

Despite increasing evidence of ACh control over several cortical circuit elements, the effect of cholinergic activation on cortical network activity has not been explored. To address that question, we will employ slice electrophysiology and use recurrent activity in layer 2/3 as a model system for cortical network dynamics. Layer 2/3 recurrent activity can be reliably evoked by high-frequency electrical stimulation in layer 4 (Beierlein et al., 2002). Initiation of recurrent activity likely involves a transient increase in the excitation/inhibition ratio. This increase may be offset by Ach activation (which excites inhibitory interneurons and impedes excitatory synaptic transmission), thereby suppressing recurrent excitation. Indeed, ACh application limits the spread of excitation in visual cortex (Kimura et al., 1999). In our preliminary data, optical activation of cholinergic afferents produces a dramatic reduction in the degree of experimentally evoked recurrent activity (Figure 1). Our data also suggests that this is achieved by recruiting both nAChRs and mAChRs. Based on these observations, we hypothesize that cholinergic transmission suppresses recurrent network activity in the primary somatosensory cortex by recruiting multiple cell types and receptors, and produces long-term changes in the processing of sensory input by cortical circuits.

Research Significance: Deficits in cholinergic signaling cause numerous cognitive defects, including impaired attention, learning and memory. Expression patterns of cholinergic receptors are altered in various forms of schizophrenia (Terry, 2008): M1 and M4 mAChRs are downregulated in the neocortex (Zavitsanou et al., 2004), and schizophrenic brains show postmortem deficits in nAChR expression (Guan et al., 1999). Drawing links between pathological cognitive defects and their underlying alterations in cellular cholinergic signaling (in order to discover effective treatment strategies) will require a thorough understanding of the network effects of ACh. Although several cortical cell types are open to robust cholinergic control, a cogent framework that describes cholinergic modulation at the network level is lacking, especially since studies examining the effects of cortical ACh have been particularly disparate. A significant hurdle is the absence of a large body of information bridging phenomena at the cellular and systems levels. Therefore, it is essential to understand how ACh modulates network activity in well-defined canonical microcircuits, which form the most essential unit of information processing in the neocortex (Feldmeyer et al., 2013).

Research Plan:
Transgenic mice: Transgenic lines will be bred onto a C57BL/6 background. Aims 1, 2 & 3: In order to precisely induce endogenous synaptic ACh release, we will use male and female bacterial artificial chromosome (BAC)-transgenic mice expressing channelrhodopsin-2 (ChR2) under the control of the promoter for choline acetyltransferase (ChAT), which synthesizes acetylcholine (ChAT–ChR2–EYFP). In these mice,
ChR2 expression is restricted to cholinergic neurons (Zhao et al., 2011). **Aims 2 & 3:** Mice expressing Cre recombinase under the control of the calcium/calmodulin-dependent protein kinase II α promoter (Camk2a-Cre; Cre recombinase expression in pyramidal cells) are commercially available and will be obtained from The Jackson Laboratory. Mice expressing Cre recombinase under the control of the 5-HT3aR promoter (5Htr3a-Cre) will be obtained from the laboratory of Chris McBain, National Institutes of Health, Bethesda. These mice will be crossed with ChAT-ChR2-EYFP mice to obtain ChAT–ChR2;Camk2a-Cre and ChAT–ChR2;5Htr3a-Cre bi-transgenic mice (Chen, 2013). To ensure Cre expression specifically in target sub-populations, the bi-transgenic mice will be crossed with R26-CAG-LoxP-mTFP1 reporter mice (Imayoshi et al., 2012). Finally, we will obtain transgenic mice carrying the following loxP-flanked sequences: (1) loxP-flanked fourth exon of the α7 nAChR gene (Chrna7) (Hernandez et al., 2014); (2) loxP-flanked fifth exon of the α4 nAChR subunit (McGranahan et al., 2011); (3) loxP-flanked M1 mAChR gene (Kamsler et al., 2010); (4) loxP-flanked M2 mAChR gene (Wess, 2009); and (5) loxP-flanked M4 mAChR gene (Wess, 2009). These mice will be crossed with Cre-line mice to achieve cell-type specific knockdowns of cholinergic receptor subtypes.

### Table 1: Transgenic mouse lines and loxP-flanked sequences to be used in the study

<table>
<thead>
<tr>
<th>Transgenic Mouse Line (Expression): Specific Aim (SA)</th>
<th>loxP-flanked Sequences: (SA)</th>
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<tbody>
<tr>
<td>ChAT–ChR2–EYFP (ChR2 in cholinergic neurons): SA 1,2,3</td>
<td>Fourth exon of α7 nAChR gene: SA 2.1</td>
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<tr>
<td>5Htr3a-Cre (Cre recombinase in 5HT3aR+ interneurons): SA 2.1</td>
<td>Fifth exon of α4 nAChR gene: SA 2.1</td>
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<tr>
<td>Camk2a-Cre (Cre recombinase in pyramidal neurons): SA 2.2, 3</td>
<td>M1 mAChR gene: SA 3</td>
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<td></td>
<td>M2 or M4 mAChR gene: SA 2.2, 3</td>
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**Brain Slicing:** Thalamocortical brain slices (400 µm) will be obtained from male and female young (P12-P16) transgenic mice. Animals will be anesthetized with isoflurane and decapitated following procedures in accordance with National Institutes of Health guidelines and approved by the University of Texas Health Science Center at Houston animal welfare committee. Acute brain slices will be prepared as previously described (Pita-Almenar et al., 2014) and then stored in artificial cerebrospinal fluid (ACSF).

**Electrophysiology and pharmacology:** Recordings from layers 1 and 2/3 in S1 of thalamocortical slices will be made using glass pipettes (2-4 MΩ) filled with a Cs+-containing internal solution for whole-cell voltage-clamp configuration, and with ACSF for cell-attached recordings. Pyramidal cells in layer 2/3 will be identified morphologically by labeling with Alexa Fluor 594 biocytin. Pharmacological blocking of α4β2 nAChRs, α7 nAChRs and mAChRs will be achieved by washing in 5nM methyllycaconitine (MLA), 500nM DHβE or 10µM atropine into the recording chamber, respectively (Arroyo et al., 2012).

**Electrical stimulation/recurrent network activity:** Cortical network activity will be evoked by conducting extracellular electrical stimulation using glass electrodes filled with ACSF and embedded in layer 4 barrels in S1. This will activate layer 4 glutamatergic afferents and imitate information flow from layer 4 to layer 2/3 during sensory perception (**Figure 2**). Recurrent activity in layer 2/3 will be induced by delivering electrical stimulus trains of 4 low-intensity (2-16 µA) pulses at 40 Hz (Beierlein et al., 2002). Recurrent excitation will be quantified as either number of spikes in cell-attached mode or the area under the curve below baseline (representing charge transferred to the layer 2/3 cell) in whole-cell voltage-clamp mode.

**Figure 2.** Schematic of somatosensory (S1) cortical circuit under study. Recordings will be made from layer 2/3 neurons in whole-cell or cell-attached mode. Recurrent activity evoked by electrically stimulating glutamatergic afferents will be paired with optogenetic activation of cholinergic inputs.
Cholinergic activation: Endogenous synaptic ACh release will be induced by activating cholinergic afferents from BF neurons. This will be achieved by delivering a 5 millisecond (ms) LED pulse (473nm) in slices obtained from ChAT–ChR2–EYFP transgenic mice. Unless otherwise noted, in trials where paired (cholinergic and glutamatergic) stimuli are to be delivered, cholinergic afferents will be activated 15 ms prior to delivering electrical stimulus train (to initiate recurrent activity). In Aims 1 & 2, trials in which paired (cholinergic + glutamatergic) and unpaired (glutamatergic only) stimuli are delivered will be interleaved. Based on power analysis (using Type 1 error probability 0.05 and 80% power), each data-set will include a minimum of 5 cells.

Optical Imaging: Slices will be loaded with the Ca²⁺ indicator fura-2 AM dissolved in DMSO to a final concentration of 20 µM. Changes in intracellular Ca²⁺ will be visualized using upright fluorescence microscopy.

Specific Aim 1: Examine whether temporally precise cholinergic activation suppresses recurrent activity in somatosensory cortex, and the contributions of cholinergic receptor subtypes

Rationale: Cholinergic innervation from the basal forebrain modulates cortical function and plasticity (Bear and Singer, 1986, Greuel et al, 1988) by engaging nicotinic and muscarinic receptors (Gil et al, 1997, Herrero et al, 2008). Evoked synaptic release of cortical ACh, made possible by the advent of optogenetics, has been shown to define neuronal excitability at the cellular level (Letzkus et al, 2011, Arroyo et al, 2012), and firing synchrony at the population level (Goard and Dan, 2009, Pinto et al, 2013). However, local canonical microcircuits form the essential information processing units in the neocortex, determining its properties and activity (Feldmeyer et al, 2013, Hirabayashi and Miyashita, 2014). To fully understand cortical function, we must elucidate the action of endogenous ACh on cortical activity at the level of these circuits, and the relative contributions of nAChRs and mAChRs. Our preliminary data indicate an ACh-induced suppression of cortical network activity.

Experimental Methods: Thalamocortical brain slices from young ChAT–ChR2–EYFP mice will be obtained. Recurrent activity in layer 2/3 (evoked by delivering electrical stimulus train in the layer 4 barrel) will be paired with optical cholinergic (ACh) activation. Recordings will be made in whole-cell voltage-clamp or cell-attached patch clamp configurations from layer 2/3 pyramidal neurons in the same column. Paired and unpaired stimulus conditions will be compared using Student’s t-test. To examine the temporal dynamics of cholinergic modulation of cortical network activity, we will vary the temporal delay between stimuli, so that cholinergic activation precedes glutamatergic excitation by various delays, ranging between 15 ms and 10 seconds (s). To gauge the effect of synaptic ACh on the spatial spread of recurrent excitation within and across columns, patch clamp recording data will be complemented with fura-2-assisted Ca²⁺-imaging experiments to visualize neuronal activity. To determine the relative contributions of ACh receptor subtypes in mediating cholinergic control of network activity, specific pharmacological blockers for α7 nicotinic (DHβE), α4β2 nicotinic (MLA) and muscarinic receptors (atropine) will be washed into the bath while paired stimuli at various temporal delays are delivered. Paired and unpaired stimulus conditions before and after drug application will be compared.

Expected Results: Optimal stimulation with LED pulse will specifically activate cholinergic afferents in all cortical layers. Based on our preliminary observations, we expect paired cholinergic activation to cause a reduction in both the duration and charge transferred by recurrent network activity. ACh should also decrease the spatial spread of recurrent activation, both across layers and across columns. We expect this modulation to be prolonged, so that cholinergic input that precedes glutamatergic input by >5 s should also achieve some suppression of recurrent activity. However, cholinergic modulation will correlate negatively with temporal delay. Since both nAChR and mAChR signaling likely contribute to cholinergic modulation, we expect a mitigation of cholinergic suppression of recurrent activity when each receptor subtype is blocked. At smaller temporal delays between cholinergic and glutamatergic activation, both receptor subtypes should play a role, while at greater delays, atropine alone should eliminate cholinergic modulation.

Limitations and Alternative Strategies: Some cortical interneurons have been found to also express ChAT, and are therefore likely to be activated by optical stimulation in ChAT–ChR2–EYFP mice (von Engelhardt et al, 2007). Cholinergic antagonists will have to be bath applied to ensure all observed modulation is mediated strictly by cholinergic transmission. Secondly, it may be necessary to validate all observations in older animals, since some further developmental changes may occur later than the ages of animals tested here (Villalobos et al, 2001). Similar experiments can be carried out in brain slices obtained from P30 mice by cardiac perfusion.
Specific Aim 2: Determine the contributions of different cellular subtypes in mediating cholinergic control of cortical activity.

Rationale: It is unclear how the numerous cortical cell types that receive cholinergic innervation contribute to cholinergic control of network activity. 5HT3αR+ interneurons express α7 and α4β2 nAChRs, which produce excitatory post-synaptic currents (EPSC) upon Ach-activation. 5HT3αR+ cells are strongly driven to fire action potentials by α4β2 nAChR transmission (Arroyo et al, 2012). Although disinhibition of pyramidal cells by 5HT3αR+ cells has been widely reported (Letzkus et al, 2011, Pfeffer et al, 2013, Fu et al, 2014), in vitro, nAChR-mediated 5HT3αR+ cell activation leads to inhibition of pyramidal cells (Altito and Dan, 2012). This is likely a major contributor to ACh-mediated suppression of recurrent activity observed in our preliminary data. Also, pyramidal cells pre-synaptically express M2-type mAChRs, which decrease the efficacy of recurrent synaptic transmission (Kimura and Baughman, 1997), thus possibly decreasing network excitability. The relative contributions of these cell types in mediating ACh suppression of cortical network activity are unknown.

Experimental Methods: **Aim 2.1: To determine whether 5HT3αR+ interneurons contribute to cholinergic control of cortical circuits.** ACh-induced responses will be recorded in whole-cell and cell-attached mode from layer 1 5HT3αR+ cells in slices obtained from ChAT-ChR2-EYFP mice. ChAT–ChR2;5Htr3a-Cre mice will be crossed with mice carrying either loxP-flanked α4 nAChR or loxP-flanked α7 nAChR gene sequences. This will result in knockout of either nAChR subtype specifically in 5HT3αR+ interneurons. In the resultant ChAT–ChR2;5Htr3a-Creα4/– and ChAT–ChR2;5Htr3a-Creα7/– animals, loss of nAChR signaling will be confirmed by recording 5HT3αR+ cell responses to ACh-activation. Recordings will be made from layer 2/3 pyramidal cells while paired or unpaired stimuli are delivered, as in SA 1. Cholinergic modulation of recurrent activity (observed by delivering paired and unpaired stimuli) in these animals will be compared with the same in ChAT–ChR2;5Htr3a-Cre mice, and significance of any difference will be tested using 2 way repeated measures ANOVA. To assess the proportion of nAChR-mediated modulation specifically that is facilitated by 5HT3αR+ cells, we will conduct the same experiments while mAChRs are blocked by washing in atropine.

**Aim 2.2: To determine whether pyramidal neurons contribute to cholinergic control of cortical circuits.** We will perform dual whole-cell recordings from nearby pyramidal cells in layer 2/3, and record EPSCs produced at recurrent synapses in one cell by action potentials evoked in the other by current injection. This will be done either unpaired or with optical ACh-activation 100 ms prior to current injection. ChAT–ChR2;CamK2a-Cre mice will be crossed with mice carrying loxP-flanked M2 mAChR or loxP-flanked M4 mAChR gene to knock out either M2-type mAChR specifically in pyramidal cells. In the resultant ChAT–ChR2;CamK2a-CreM2/- and ChAT–ChR2;CamK2a-CreM4/- mice, dual recordings from nearby pyramidal cells will be performed and EPSCs at recurrent synapses with or without ACh-activation will be recorded, as above, to confirm loss of mAChR signaling in pyramidal cells. ACh modulation of recurrent activity will be recorded by delivering paired and unpaired stimuli, and compared to the same in ChAT–ChR2;CamK2a-Cre mice.

Expected Results: We expect distinct contributions of cellular sub-types to cholinergic control of cortical activity. **Aim 2.1:** With the loss of α4β2 nAChR expression, 5HT3αR+ interneurons should no longer be driven to spike by ACh-activation. As a result, pyramidal neurons should be less inhibited. Since we expect that inhibition of pyramidal cells by 5HT3αR+ cells is the primary mechanism by which nAChRs suppress recurrent activity, loss of that inhibition should result in a greater degree of recurrent excitation with paired stimuli, compared with ChAT–ChR2;5Htr3a-Cre animals. Atropine application should recover recurrent activity to control (unpaired) levels. **Aim 2.2:** Signaling by M2-type mAChRs decreases transmission efficacy at recurrent synapses. Thus, paired ACh-activation prior to current injection will decrease evoked EPSC magnitudes at recurrent synapses between pyramidal cells in dual recordings, compared to unpaired trials. In mice lacking mAChR-signaling in pyramidal cells, recurrent EPSC magnitude should be unchanged with or without ACh-activation. We expect that the mAChR-mediated reduction in recurrent EPSC magnitude is a major contributor to ACh suppression of recurrent activity. Thus, mAChR knockout in pyramidal cells should result in a greater degree of recurrent excitation with paired stimuli, compared with ChAT–ChR2;CamK2a-Cre mice.

Limitations and Alternative Strategies: In Aim 2.1, reduced 5HT3αR+ cell activity could mean that Sst+ cells (which are excited by mAChRs and also inhibit pyramidal cells) will be less inhibited. This could result in
increased inhibition and reduced recurrent activity in knockout animals, yielding misleading results. Conducting experiments in the presence of atropine will allow us to accurately assess the contribution of 5HT3aR+ cells. The contribution of Sst+ cells to ACh modulation will not be examined here, since which mAChR sub-types they express is not well-characterized. Secondly, Cre recombinase is constitutively expressed in 5Htr3a-Cre and CamK2a-Cre mice, and knockout animals used here may show some functional compensation. As an alternative strategy, ChAT–ChR2 mice can be crossed with mice carrying loxP-flanked genes, and then viral vectors with 5Htr3a-Cre or CamK2a-Cre constructs can be injected into somatosensory cortex of the progeny.

Specific Aim 3: Elucidate the long-term effects of paired cholinergic and glutamatergic transmission to the cortex, and the cellular/circuit mechanisms thereof.

Rationale: Cholinergic signaling in the cortex underlies multiple forms of learning and plasticity (Greuel et al., 1988). Signaling by mAChRs facilitates long-term depression or potentiation in layer 2/3 (Origlia et al., 2006). Puff application of ACh agonists at defined temporal delays following stimulus-evoked network activity in primary sensory cortices induces long-term increases in spike-time duration in response to stimulus, in an mAChR-dependent manner (Chubykin et al., 2013). Post-training spike-time durations reflect the delay between stimulus and ACh activation during training. Computational modeling data suggests this plasticity involves an increase in the efficacy of communication at recurrent synapses between pyramidal cells (Gavornik et al., 2009). Consistent with this notion, pyramidal cells express M1 mAChRs post-synaptically (Yamasaki et al., 2010) and M2 mAChRs pre-synaptically (Alger et al., 2014). It is unclear how synaptically-released ACh recruits mAChRs on pyramidal cells to induce long-term changes in sensory input processing by cortical networks.

Experimental Methods: Aim 3.1: To determine whether ACh-activation following recurrent activity increases duration of recurrent excitation. We will record in whole-cell and cell-attached mode from layer 2/3 pyramidal cells in slices obtained from young ChAT–ChR2–EYFP mice. In pre-conditioning trials, unpaired recurrent activity (evoked as before) will be recorded. This will be followed by conditioning trials, in which each electrical stimulus train will be followed by optical ACh activation at temporal delays ranging between 0.5 and 2 seconds. 30 minutes after conditioning, post-conditioning state will be evaluated by delivering unpaired electrical stimulus. Charge transferred and pyramidal cell spike-time durations due to recurrent excitation will be compared in pre- and post-conditioning trials, and significance of any difference will be tested using Student’s t-test. Experiments will be repeated in the presence of atropine and in slices obtained from mice resulting from crossing CamK2a-Cre;ChAT-ChR2 mice with mice carrying either loxP-flanked M1 mAChR gene or loxP-flanked M2 mAChR gene (mAChR knockout in pyramidal cells, see Aim 2.2).

Aim 3.2: To determine whether ACh-activation following recurrent activity increases efficacy of communication between pyramidal cells. We will perform dual whole-cell recordings from nearby pyramidal cells in layer 2/3, and record EPSCs produced in one cell by stimulation of the other. Training trials where ACh activation follows recurrent activity at various delays will be conducted (as in Aim 3.1). Pyramidal-pyramidal cell EPSCs will be recorded post-training and compared to pre-training conditions. Experiments will be repeated in the presence of atropine and in slices obtained from animals with mAChR knockout in pyramidal cells.

Expected Results: We expect robust ACh-mediated plasticity in recurrent excitation and pyramidal-pyramidal cell communication. Aim 3.1: ACh activation at various temporal delays following recurrent network activity during training will lead to a long-lasting increase in the duration of evoked recurrent activity post-training, with recurrent excitation subsiding at the trained delay between electrical and cholinergic stimuli. We expect this plasticity to be mAChR-mediated, so that conditioning in the presence of atropine should preclude it. Pre and post-training conditions will not be significantly different in mAChR knockout animals. Aim 3.2: We predict long-term potentiation in pyramidal-pyramidal cell recurrent synapses as a result of training. We expect that this plasticity will be eliminated in the presence of atropine and will be absent in mAChR knockout animals.

Limitations and Alternative Strategies: mAChRs expressed on pyramidal cells may be sufficient but not necessary for reward-timing plasticity, i.e. some plastic changes may be observed even in the mAChR-knockout animals used here. It may be necessary to block mAChR-signaling in non-pyramidal cells. Secondly, synapses in mice during the ages tested here are somewhat more plastic than in adults (Jiang et al., 2007), and any long term plasticity observed may be age-specific. Results will have to be validated in older animals.
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