Controlling Prefrontal Attention Circuits: Neuromodulation of Cortical Layer 6 and its Local Outputs

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The prefrontal cortex is critical for mediating attention. Neuromodulation by acetylcholine and serotonin exerts opposing effects on attention. Layer 6 of prefrontal cortex is an important source of cortico-thalamic and cortico-cortical output, and this layer expresses both cholinergic and serotonergic receptors. The capacity of prefrontal layer 6 to influence attention through its cortical and thalamic connections highlights the necessity to understand its neuromodulation and the underlying cellular mechanisms involved. This thesis examines the modulation of prefrontal layer 6 by acetylcholine and serotonin, and the consequences of layer 6 activity on its downstream cortical targets.

Is the cholinergic modulation of layer 6 in medial prefrontal cortex distinct? Contrasting layer 6 neurons of associative and primary regions of cortex, I find significant differences in the receptor composition and strength of cholinergic responses. The stronger cholinergic response in medial prefrontal layer 6 appears driven by high affinity nicotinic receptors of the α4β2α5 subtype with a modest contribution by muscarinic receptors.

Functional disruptions in specific nicotinic receptor subunits are linked to attentional disruption, but what are their consequences for cholinergic modulation of layer 6? In mice with genetic deletion of key nicotinic receptor subunits, I demonstrate a compensatory upregulation in
the muscarinic response, inversely proportional to the impairment of nicotinic receptor function. This compensatory plasticity partially rescues the excitatory response to cholinergic stimulation at near-threshold membrane potentials.

Unlike acetylcholine, serotonin impairs attention, yet the underlying mechanisms are unclear. In medial prefrontal cortex, I show, for the first time, that serotonin suppresses of layer 6 excitability through 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors. Using transgenic mice that allow for light-mediated activation of layer 6, I identify a direct, excitatory, local connection between layer 6 and layer 5 interneurons of medial prefrontal cortex. Finally, I show that suppression of layer 6 activity by serotonin inhibits the activation of downstream layer 5 interneurons.

The findings in this thesis probe the receptor mechanisms underlying the modulation of prefrontal layer 6 by acetylcholine and serotonin. Furthermore, new insight is provided into the consequences of modulating layer 6 activity on its downstream cortical targets.
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<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine, serotonin</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td>ACSF</td>
<td>Artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>APV</td>
<td>D-(-)-2-amino-5-phosphonopentanoic acid</td>
</tr>
<tr>
<td>BF</td>
<td>Basal forebrain</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>ChAT</td>
<td>Choline acetyltransferase</td>
</tr>
<tr>
<td>ChR2</td>
<td>Channelrhodopsin-2</td>
</tr>
<tr>
<td>CHT</td>
<td>Choline transporter</td>
</tr>
<tr>
<td>CNQX</td>
<td>6-cyano-7-nitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DHβE</td>
<td>Dihydro-β-erythroidine</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>Epyc</td>
<td>Epiphycan</td>
</tr>
<tr>
<td>FS</td>
<td>Fast spiking</td>
</tr>
<tr>
<td>GABA</td>
<td>(\Gamma)-aminobutyric acid</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IR-DIC</td>
<td>Infrared Differential Interference Contrast microscopy</td>
</tr>
<tr>
<td>L</td>
<td>Layer</td>
</tr>
<tr>
<td>LED</td>
<td>Light emitting diode</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>MI</td>
<td>Primary motor cortex</td>
</tr>
<tr>
<td>mAChR</td>
<td>Muscarinic acetylcholine receptor</td>
</tr>
<tr>
<td>MDL</td>
<td>MDL 100,907</td>
</tr>
<tr>
<td>mPFC</td>
<td>Medial prefrontal cortex</td>
</tr>
<tr>
<td>nAChR</td>
<td>Nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>nFS</td>
<td>Non-fast spiking</td>
</tr>
<tr>
<td>P</td>
<td>Postnatal Day</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal cortex</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PrL</td>
<td>Prelimbic cortex</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RMP</td>
<td>Resting membrane potential</td>
</tr>
<tr>
<td>RN</td>
<td>Raphe nuclei</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SSC</td>
<td>Somatosensory cortex</td>
</tr>
<tr>
<td>Syt6</td>
<td>Synaptotagmin 6</td>
</tr>
<tr>
<td>TRAP</td>
<td>Translating ribosome affinity purification</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>VACht</td>
<td>Vesicular acetylcholine transporter</td>
</tr>
<tr>
<td>WAY</td>
<td>WAY 100,635</td>
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<tr>
<td>WT</td>
<td>Wildtype</td>
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1 General Introduction

1.1 The prefrontal cortex and attention

The prefrontal cortex (PFC) is an important site for executive control (Kolb, 1984; Goldman-Rakic, 1988; Baddeley, 1992; Funahashi and Kubota, 1994; Miller et al., 2002; Fuster, 2015). Its function has been likened to acting as a “central executive” that directs and coordinates multiple systems operating simultaneously towards a mentally specified goal against distracting alternatives (Baddeley and Della Sala, 1996; Perner and Lang, 1999). While the term “executive control” spans a broad range of high-level functions, the control of attention is widely accepted as a core component (Pineda et al., 1998; Smith and Jonides, 1999; Funahashi and Andreau, 2013). The following sections will examine the functional mechanisms and anatomical basis for the control of attention by the PFC.

1.1.1 The anatomy of the prefrontal cortex

In humans, the PFC comprises numerous Brodmann areas, initially based on cytoarchitectonic features (Brodmann, 1909). Furthermore, the region exhibits dense reciprocal projections with the mediodorsal nucleus of the thalamus (Rose and Woolsey, 1948; Leonard, 1969; Goldman-Rakic and Porrino, 1985; Uylings and van Eden, 1990; Kuroda et al., 1998; Fuster, 2001). The pattern and density of these connections are conserved across many mammalian species (Uylings and van Eden, 1990; Wise, 2008). Similarly, rodent PFC demonstrates analogous connectivity and function to humans and primates (Uylings et al., 2003). The PFC is an agranular cortical region, consisting of five layers but lacking a distinct layer 4 (Groenewegen and Uylings, 2000; Wise, 2008). These cortical layers mature in an “inside-out” manner, forming first the deep, then the upper layers (Rakic, 1974). Layer 1 comprises of
primarily interneurons, and incoming axons and apical dendrites from other cortical layers (Gabbott et al., 1997). Layer 2/3 consists of many cortico-cortical projecting pyramidal neurons that provide both short- and long-range connectivity within the cortex (Gabbott et al., 2003; Otsuka and Kawaguchi, 2008). Layer 5 is a primary output layer that is composed of heterogeneous groups of neurons with efferent projections to numerous regions, in particular the striatum and hypothalamus (Gabbott et al., 2005). Lastly, layer 6 is a major source of input into the cortex, with heterogeneous populations of pyramidal neurons that receive a number of afferent projections from primarily the mediodorsal thalamic nuclei (Gabbott et al., 2005), but also various cross-columnar cortical sources (Zarrinpar and Callaway, 2006; Thomson, 2010). In addition to the reciprocal connectivity between layer 6 and mediodorsal thalamic nuclei, layer 6 sends substantial, but less-studied cortico-cortical projections within cortex (Thomson, 2010). In somatosensory cortex, layer 6 innervation of local interneurons has been demonstrated (West et al., 2006), but this connection has not yet been identified in PFC. A simplified overview of these layers and their outgoing connections are illustrated in Figure 1.1.

The PFC receives subcortical afferents from the thalamus, amygdala, basal forebrain, and brainstem (Öngür and Price, 2000; Hoover and Vertes, 2007), and send efferent projections to the striatum, thalamus, hypothalamus, amygdala, and brainstem (Uylings and van Eden, 1990; Öngür and Price, 2000; Vertes, 2004; Gabbott et al., 2005). Notably, reciprocal connections are made between the PFC and cholinergic cells of the basal forebrain (Gaykema et al., 1991; Zaborszky et al., 1997; Ghashghai and Barbas, 2001), the serotonergic cells within the dorsal raphe (Aghajanian and Wang, 1977), as well as numerous other neuromodulatory centers such as the locus coeuleus (Cedarbaum and Aghajanian, 1978; Luppi et al., 1995) and the ventral tegmental area (Geisler and Zahm, 2005). Furthermore, there is dense reciprocal cortico-cortical
connectivity between the PFC and other associative and primary cortical regions, as well as within the PFC itself (Goldman-Rakic, 1988; Uylings and van Eden, 1990; Pandya and Yeterian, 1996; Barbas, 2000; Öngür and Price, 2000; Uylings et al., 2003). As such, these gross anatomical features of the PFC places it at an impactful location within the brain where it can influence attention, executive function, and the brain as a whole.

Figure 1.1 Simplified overview of the laminar distribution of pyramidal neurons in prefrontal cortex and their connectivity. Primary projection targets of layers 5 and 6 are labelled. Of note, layer 6 neurons send both cortico-thalamic and cortico-cortical projections. The nature of the cortico-cortical connectivity of layer 6 in PFC is unknown. Blue arrows represent axonal projections. Black projections represent apical dendrites. Smaller grey neurons represent interneurons.
1.1.2 The general function of the prefrontal cortex

The PFC is an essential and important controller of attention and executive function (Kolb and Whishaw, 1996; Fuster, 2015). Control of these cognitive functions would require the PFC to continually supervise and regulate multiple neuronal systems across various cortical and subcortical areas (Funahashi, 2001). As discussed above, the PFC is well positioned to provide these “top-down” signals by receiving integrated signals from subcortical sources and modulating the responses both in cortical and subcortical regions. The dense interconnectivity between PFC and other cortical and subcortical areas provide further evidence that it acts as a hub for processing information critical for executive function (Buckner et al., 2009; Sporns, 2014). Precise mechanisms underlying how the PFC mediates attention among the host of other cognitive function remains unclear, and it remains controversial whether specialization towards specific functions exists between subregions of the PFC (Robbins, 1996; Duncan and Owen, 2000; Duncan, 2001; Uylings et al., 2003; Wilson et al., 2010; Kesner and Churchwell, 2011; Dembrow and Johnston, 2014; Szczepanski and Knight, 2014). Additionally, neuroimaging studies show that distributed activation of PFC is associated with a wide range of cognitive demands (Duncan and Owen, 2000; Ikkai and Curtis, 2011; Cole et al., 2013), supporting the notion that networks within the PFC underlie the top-down control of executive function (Goldman-Rakic, 1988; Mesulam, 1990; Cole et al., 2013). In humans, damage to the PFC can elicit numerous cognitive deficits including impairments in attention, working memory, cognitive control, and planning (Szczepanski and Knight, 2014). Similarly, lesions of PFC in animal studies also lead to the same disruptions in executive function (Uylings et al., 2003; Dalley et al., 2004; Kesner and Churchwell, 2011). Emerging research has only relatively recently begun to allow us to better understand the cellular and connective mechanisms that underlie PFC control of executive function. The remainder of this section will focus on the role
of the PFC on attention in particular, and our growing appreciation of the medial PFC in this network.

1.1.3 Attention and the prefrontal cortex

Attention plays an important role in the organization of thoughts and actions in behaviour, and is thus considered to be a critical component to executive functions carried out by the PFC (Miller and Cohen, 2001). The ability to pay attention involves biasing the processing of certain pieces of neuronal information at the expense of others, thus amplifying relevant information while suppressing distracting or irrelevant information (Noudoost et al., 2010). The PFC in particular is noted for its direct influences on attention processing (Baluch and Itti, 2011; Miller and Buschman, 2013). While the neurophysiology and the cellular and computational mechanisms underlying the PFC-mediated control of attention have not been firmly established, a body of literature has emerged in the past two decades that sheds more light on this critical region. The earliest descriptions of the PFC’s role in attention arose from interpretations of deficits following frontal lobe damage (Bianchi, 1922; Duncan, 1986). Subsequent experiments looking at attentional function in human subjects further cemented PFC activity as being linked to attentional control (Mesulam, 1981; Corbetta et al., 1991; Pardo et al., 1991). In rodents, specific lesions to the medial part of PFC (mPFC), which roughly corresponds to the dorsolateral PFC in humans and other primates (Uylings et al., 2003; Vertes, 2004), result in attentional deficits (Muir et al., 1996; Passetti et al., 2003; Kahn et al., 2012). Furthermore, both neuroimaging and electrophysiological studies have linked the mPFC with sustained attention in behavioural tasks (Gill et al., 2000; Totah et al., 2009; Bentley et al., 2011; Guillem et al., 2011). In particular, activity within the deep layers of mPFC plays is necessary to facilitate normal, sustained attention (Guillem et al., 2011). While this evidence points to an important role of the
medial prefrontal cortex in challenging attention tasks, it may not be critical under conditions of lower demand (Smucny et al., 2013). There is evidence suggesting that the primary cortices may mediate certain types of attentional processing (Meyer, 2011). One such example lies in the primary somatosensory cortex, where alpha-band activity correlates with somatosensory attention (Pfurtscheller et al., 1996; Jones et al., 2010).

1.2 Neuromodulation of medial prefrontal cortex

The mPFC receives numerous afferents from the thalamus, amygdala, basal forebrain, and brainstem (Öngür and Price, 2000; Hoover and Vertes, 2007). Of these, reciprocal connections are made between the PFC and cholinergic cells of the basal forebrain (Gaykema et al., 1991; Zaborszky et al., 1997; Ghashghaei and Barbas, 2001), as well as the serotonergic cells within the dorsal raphe (Aghajanian and Wang, 1977) (See Figure 1.2). Several other neuromodulatory centers such as the locus coeuleus (Cedarbaum and Aghajanian, 1978; Luppi et al., 1995) and the ventral tegmental area (Geisler and Zahm, 2005) send connections to the mPFC. This thesis will focus on the cholinergic inputs into mPFC, which have been linked to normal attention (Passetti et al., 2003; Parikh et al., 2007; Hasselmo and Sarter, 2011). Additionally, serotonergic input will also be examined based on the strength of the inputs into mPFC, a potentially opposing signal compared to the cholinergic system, as well as experimental evidence of serotonin potentially affecting normal attention in human studies (Schmitt et al., 2000; Gallagher et al., 2003; Booij et al., 2005; Wingen et al., 2007). The following sections will provide a comprehensive examination of these two neuromodulatory systems in mPFC, and their relationship to attentional performance.
1.2.1 Cholinergic modulation of deep layers of medial prefrontal cortex

1.2.1.1 Cholinergic innervation into medial prefrontal cortex and the establishment of acetylcholine’s role in attention

The mPFC receives dense cholinergic innervation, with pronounced cholinergic afferents terminating in deep cortical layers (Mesulam et al., 1983; Mechawar and Descarries, 2001; Bloem et al., 2014). These projections arise from the basal forebrain, a region which contains numerous cholinergic nuclei such as the nucleus basalis, the septum, the diagonal band of Broca, and the substantia innominata (Mesulam, 1995; Zaborszky et al., 1999; Woolf and Butcher, 2011), and are a part of a wide network of cholinergic innervation across many brain regions (Fournier et al., 2004; Chandler et al., 2012; Bloem et al., 2014). Acetylcholine (ACh) production and release from within cholinergic neurons is highly regulated in a multi-step
process: the precursor choline is taken up by choline-transporter CHT1, synthesized locally in the cytoplasm by choline acetyltransferase (ChAT), loaded into synaptic vesicles by vesicular ACh transporter (VACHT). Following release of ACh from these terminals, it is rapidly degraded into acetate and choline by the enzyme acetylcholinesterase (AChE) (Prado et al., 2002; Prado et al., 2013). This pathway is summarized in Figure 1.3.

Over the past two decades, the role of the cholinergic system in attention has been extensively studied with an ever-increasing arsenal of techniques including novel behavioural tests, selective lesions, and microdialysis measurement of ACh release. Early experiments with 192-IgG-saporin used to selectively lesion the basal forebrain cholinergic neurons (Wiley, 1992) were effective in substantially reducing global cholinergic tone. Lesions in the PFC showed decreases in attentional performance (Gill et al., 2000; Chudasama et al., 2004; Dalley et al., 2004; Newman and McGaughy, 2008), suggesting a link between ACh’s activity within the PFC and attention. This pathway was further elucidated through the development of tools to measure ACh release in vivo, where global tonic increases (Sarter et al., 1996), as well as local phasic increases within the mPFC (Passetti et al., 2000; Dalley et al., 2001; Parikh et al., 2007; Parikh and Sarter, 2008) are observed with performance in sustained attention tasks. These transient increases in ACh within the mPFC correspond to the degree of attentional demand (Kozak et al., 2006). Human data parallels those found in rodents, where fMRI studies have shown involvement of prefrontal cholinergic activity in attention. However, due to the low temporal resolution of these measurements, there is still controversy between the role of tonic versus phasic release of ACh on attention (Parikh and Sarter, 2008; Sarter et al., 2009; Klinkenberg et al., 2011), though both forms are likely present (Parikh et al., 2007; Bennett et al., 2012).
Figure 1.3 Important components in the release and action of acetylcholine from cholinergic terminals in the prefrontal cortex. (1) Acetylcholine (ACh) is synthesized in cholinergic neurons from choline and acetyl-CoA by choline acetyltransferase (ChAT). (2) Synthesized ACh is transported into vesicles by vesicular acetylcholine transferase (VACHT). (3) Upon release, ACh can bind to postsynaptic nicotinic acetylcholine receptors (nAChR) or muscarinic acetylcholine receptors (mAChR). (4) ACh is quickly broken down to choline by acetylcholinesterase (AChE), and (5) brought back into the cholinergic neuron by choline transferase (CHT).

While the precise nature of ACh action on attentional performance remains unresolved due to the complexity of the behaviour, studies have discerned the unique and important roles of the nicotinic (nAChR) and muscarinic (mAChR) acetylcholine receptors in attention by local infusion of pharmacological agents into the mPFC (Robbins, 2002; Hahn et al., 2003b;
Chudasama et al., 2004). Both types of receptors directly influence the electrical activity of the cells they’re expressed on, and can have large effects on circuit dynamics through complex signaling cascades (Dajas-Bailador and Wonnacott, 2004; Gulledge and Stuart, 2005; Thiele, 2013; Yakel, 2013). Of particular interest, layers 5 and 6 of mPFC express both families of acetylcholine receptors (Kassam et al., 2008; Gulledge et al., 2009; Poorthuis et al., 2013; Proulx et al., 2014). Concurrently, these deep layers are innervated by cholinergic fibers (Ghashghaei and Barbas, 2001; Bloem et al., 2014). The following sections will discuss in more detail the functions of nAChR and mAChR in these layers of the mPFC, and their correlates to attention.

1.2.1.2 Nicotinic acetylcholine receptors

Nicotinic receptor activation by its ligand nicotine has been linked to enhancements in attention (Mumenthaler et al., 2003; Newhouse et al., 2004). This locus of this positive effect on cognition is thought to be within the prefrontal cortex (Levin, 1992; Granon et al., 1995; Levin et al., 2006; McGehee, 2007). Nicotinic acetylcholine receptors are ligand-gated cation channels that are permeable to Na\(^+\), K\(^+\), and Ca\(^{2+}\) ions (Fucile, 2004; Albuquerque et al., 2009; Gotti et al., 2009). A total of 12 identified neuronal subunits are divided into two families: \(\alpha\) subunits (\(\alpha_2 - \alpha_{10}\)), and \(\beta\) subunits (\(\beta_2 – \beta_4\)) (Gotti and Clementi, 2004), which form a myriad of functional pentameric receptors (Gotti et al., 2006). These different receptor subtypes arising from different subunit composition and stoichiometry give rise to substantial differences in ionic conductance (Fucile, 2004), and as such, have great influence on their functional properties (Moroni et al., 2006; Tapia et al., 2007).

The most commonly expressed nAChR in the brain are \(\alpha_4\beta_2\)-containing receptors (\(\alpha_4\beta_2^*\)) (Léna and Changeux, 1999; Gotti et al., 2009), and are highly expressed in cortex (Wada et al., 1989; Nakayama et al., 1995; Gotti and Clementi, 2004). The \(\alpha_4\beta_2^*\) nicotinic receptors
can assume various different stoichiometries, consisting of at least two of each \( \alpha 4 \) and \( \beta 2 \) subunit, with the last subunit being either an additional \( \alpha 4 \), \( \beta 2 \), or an accessory subunit such as \( \alpha 5 \) (Wada et al., 1990; Ramirez-Latorre et al., 1996; Kuryatov et al., 2008; Albuquerque et al., 2009; Gotti et al., 2009). The additional \( \beta 2 \) subunit confers higher sensitivity to ACh, slower desensitization, but lower \( \text{Ca}^{2+} \) permeability to the receptor (Nelson et al., 2003; Kuryatov et al., 2005; Tapia et al., 2007). As such, \( (\alpha 4)_3(\beta 2)_2 \) receptors are considered low-sensitivity \( \alpha 4\beta 2 \) receptors, whereas \( (\alpha 4)_2(\beta 2)_3 \) receptors are high-sensitivity \( \alpha 4\beta 2 \) receptors. Intriguingly, the possibility of an \( \alpha 5 \) subunit occupying the accessory slot has attracted significantly more interest in recent years due to its ability to substantially alter the functional properties of \( \alpha 4\beta 2^* \) receptors upon inclusion. While the \( \alpha 5 \) subunit cannot form functional channels by themselves, its incorporation into \( \alpha 4\beta 2^* \) receptors to form \( (\alpha 4)_2(\beta 2)_2\alpha 5 \) receptors enhances receptor expression, and can make up between 11 and 37% of \( \alpha 4\beta 2^* \) nAChRs in the brain (Ramirez-Latorre et al., 1996; Gotti and Clementi, 2004; Gotti et al., 2007; Mao et al., 2008). The presence of the \( \alpha 5 \) accessory subunit also increases receptor sensitivity to ACh (Moroni et al., 2006; Kuryatov et al., 2008; Gotti et al., 2009; Bailey et al., 2010), and increases \( \text{Ca}^{2+} \) permeability (Kuryatov et al., 2008). Interestingly, expression of the \( \alpha 5 \) subunit shows not only laminar specificity towards deep layers of cortex (Birtsch et al., 1997; Salas et al., 2003; Winzer-Serhan and Leslie, 2005; Alves et al., 2010) but selectivity towards mPFC (Wada et al., 1990). Indeed, electrophysiological studies suggest that \( \alpha 4\beta 2\alpha 5 \) receptors are found in abundance in layer 6 pyramidal neurons of mPFC (Bailey et al., 2010). These findings are summarized in Figure 1.4.
Figure 1.4 Common nicotinic acetylcholine receptor subtypes in layer 6 neurons of medial prefrontal cortex. A) \((\alpha 4)_2(\beta 2)_2^*\) receptors are pentameric ligand-gated nonselective cation channels. The fifth subunit may be an additional \(\alpha 4\) or \(\beta 2\) subunit, with either configuration providing differing sensitivities to acetylcholine. Additionally, an accessory \(\alpha 5\) subunit may occupy the final slot, which confers enhanced calcium permeability to the receptor. B) Left: expression of the nicotinic receptor \(\alpha 4\) subunit in medial prefrontal cortex (adapted from Alves et al., 2010). Note the band of expression in layer 6. Right: expression of the nicotinic receptor \(\alpha 5\) subunit mRNA in layer 6 neurons of medial prefrontal cortex by in situ hybridization (adapted from Wada et al., 1990). Figure adapted from Proulx et al., 2014.
Binding of ACh to α4β2* receptors elicits excitatory responses in layer 6 neurons of mPFC (Kassam et al., 2008; Bailey et al., 2010). This excitation is driven by flow of Na+, K+, and Ca²⁺ through the opened pore (Albuquerque et al., 2009; Gotti et al., 2009), and can generate action potentials upon depolarization to threshold. This excitatory response is disrupted in knockout mice lacking either the α5 subunit (Salas et al., 2003; Bailey et al., 2010) or the β2 subunit (Picciotto et al., 1998). As a result, these knockout mice exhibit aberrant attention performance in tasks with increased attentional demand (Bailey et al., 2010; Guillem et al., 2011), whereas rescuing the function of these receptors in the mPFC will rescue normal attentional performance (Guillem et al., 2011). These genetic disruptions are not accompanied by compensatory changes in expression of other nicotinic receptor subunit mRNA (Zoli et al., 1998; Salas et al., 2003), nor do they affect binding to other functional nicotinic receptors (Baddick and Marks, 2011). Likewise, both systemic and central injections of pharmacological agents targeting nicotinic receptors into the prelimbic region of mPFC showed marked effects on attention in rodents (Hahn et al., 2003b). Low doses of nicotine improved the response accuracy, reduced response latency, and reduced omissions in attention tasks (Hahn et al., 2003a), whereas injection of antagonists produced impairments in attention (Granon et al., 1995).

1.2.1.3 Muscarinic acetylcholine receptors

Muscarinic acetylcholine receptors (mAChR) are metabotropic 7-transmembrane-spanning G-protein coupled receptors (Felder, 1995; Bubser et al., 2012). Five defined subtypes of mAChR are grouped based on the coupled Gα-protein they’re bound to: the excitatory Gαq-coupled receptors or the inhibitory Gai/o coupled receptors (Brown, 2010; Bubser et al., 2012) Muscarinic M1, M3, and M5 receptors are Gαq-coupled, which lead to activation of phospholipase C and Ca²⁺ release from intracellular stores. Muscarinic M2 and M4 receptors on
the other hand are Gαi/o-coupled and lead to the inhibition of adenylyl cyclase activity, and decreasing intracellular cAMP production (Felder, 1995; Caulfield and Birdsall, 1998).

Muscarinic M1 receptors are highly expressed in the mPFC and cortex in general (Buckley et al., 1988; Levey et al., 1991). M1 receptors are expressed on perisomatic and dendritic compartments of pyramidal cells, and modulate the activity of layer 5 pyramidal neurons of mPFC (Mrzljak et al., 1993; Guldledge et al., 2009; Yamasaki et al., 2010; Goodfellow et al., 2012). In general, activation of M1 receptors can produce both excitation and/or inhibition via a Gαq-pathway (McCormick and Prince, 1986; Haj-Dahmane and Andrade, 1996; Guldledge and Stuart, 2005; Guldledge et al., 2009; Santini et al., 2012) (Figure 1.5). The mechanisms underlying the M1 receptor-mediated response remains controversial. Intracellular release of Ca²⁺ has been linked to subsequent neuronal inhibition by Ca²⁺-activated K⁺ channels (Guldledge and Stuart, 2005). On the other hand, the slower excitatory responses were traditionally thought to result from a closure of K⁺ channels following M1 activation (Krnjevic et al., 1971; McCormick and Prince, 1986). Examples include M1-mediated inhibition of constitutively active inwardly rectifying K⁺ channels (Carr and Surmeier, 2007) or the KCNQ-family of K⁺ channels (constituents of the “M-current”) (Zhang et al., 2003). Closing of inhibitory leak or sub-threshold K⁺ channels would result in neuronal excitation or depolarization. Alternatively, some evidence supports an M1-mediated activation of voltage-dependent non-selective cation channels (Haj-Dahmane and Andrade, 1996; Shalinsky et al., 2002; Yan et al., 2009), or increased activity of NCX and NCKX families of Na⁺-Ca²⁺ exchangers following a rise in intracellular Ca²⁺ (Proulx et al., 2014) as possible mechanisms underlying neuronal depolarization. Recent optogenetic studies have revealed a dissociation between neuronal muscarinic responses triggered by traditional bath or puff application of acetylcholine onto slice versus light-induced acetylcholine
release from endogenous cholinergic terminals (Hedrick and Waters, 2015), suggesting further complexities in the effects of muscarinic activation in vivo. However, the existing body of literature focuses heavily on the muscarinic responses in layer 5, but little is known about the responses in layer 6 despite the presence of M1 receptors in this layer (Buckley et al., 1988).

Manipulations of muscarinic receptor function have been associated with changes in attentional performance. Central administration of the M1 antagonist scopolamine showed reduced accuracy and an increased response latency in the 5-choice serial reaction timed task (Robbins et al., 1998). Moreover, infusion of scopolamine both centrally (Herrero et al., 2008) and into the PFC (Davidson and Marrocco, 2000) of rhesus macaques also reduced accuracy and response latency in attention tasks. Similarly, further boosting the excitatory effects of M1 activation by inhibition of SK also has beneficial effects to executive function (Brennan et al., 2008).
Figure 1.5 Muscarinic acetylcholine receptors and their signaling pathways. Muscarinic acetylcholine receptors are G-protein coupled receptors found ubiquitously across cortex. M1, M3, and M5 muscarinic acetylcholine receptors are coupled to Gaq and activate phospholipase C (PLC) to trigger various downstream signaling cascades including Ca$^{2+}$ release from intracellular stores, and activation of protein kinase C (PKC) which can modulate downstream targets. M2 and M4 muscarinic receptors are coupled to Gai/o, which leads to an inhibition of adenylyl cyclase, decreasing cAMP synthesis, and affecting downstream signaling cascades.

1.2.2 Serotonergic modulation of deep layers of medial prefrontal cortex

1.2.2.1 Anatomy of serotonergic innervation into medial prefrontal cortex and its role in attention

Serotonergic fibers originating from the dorsal and median raphe nuclei innervate the forebrain (Azmitia and Segal, 1978; Kosofsky and Molliver, 1987). This innervation develops
from an early developmental time point and matures throughout an animal’s lifespan (Dori et al., 1996). The prefrontal cortex receives innervation from the fine branch fibers originating from the dorsal raphe nuclei, and relatively fewer afferents from the median raphe nuclei (Lidov et al., 1980; Van Bockstaele et al., 1993). These innervations do not show laminar specificity, with fibers traversing through all cortical layers in sensory and associative regions (Crino et al., 1990; Wilson and Molliver, 1991; Dori et al., 1996; Linley et al., 2013; Muzerelle et al., 2016).

Electron microscopy experiments have identified a lack of membranous appositions and specializations, an indicator of synaptic junctions, in the vast majority of serotonergic fiber varicosities (Séguéla et al., 1989; Descarries et al., 1990). This absence of synaptic junctions suggest that serotonin release within the cortex occurs through volume transmission (Beaudet and Descarries, 1978; Descarries et al., 1990; Descarries and Mechawar, 2000), thus providing the serotonergic system a wide range of downstream neuronal targets to act on upon release.

With respect to attention, serotonin is known to shape and bias attention in both human and nonhuman primates. Changes in brain serotonin levels are known to influence attentional performance. Reduction of brain serotonin levels by tryptophan depletion enhances attention (Schmitt et al., 2000; Gallagher et al., 2003; Booij et al., 2005), whereas an elevation of brain serotonin by way of selective serotonin reuptake inhibitors (SSRIs) impairs attention task performance (Riedel et al., 2005; Oranje et al., 2008; Watson et al., 2015). The precise mechanisms by which serotonin can modulate normal attention is likely complex based on the diverse family of receptors and their widespread expression (Hoyer et al., 1994; Hoyer et al., 2002; Hannon and Hoyer, 2008), and is not well understood. Some existing hypotheses revolve around a modulatory influence on the effects of other neurotransmitter systems which are implicated in attention. One such example is the dopaminergic system associated with vigilance
and arousal (Koelega, 1993; Robbins, 1997), where serotonin can inhibit dopaminergic activity leading to impairments in attention (Spoont, 1992; Schmitt et al., 2002). Whether serotonin can have similar effects on other neurotransmitter systems important to attention is still not determined.

1.2.2.2 The serotonin receptor family and relevant receptors in medial prefrontal cortex

The serotonin (5-HT) receptor family is comprised of at least 14 different receptor subtypes (Hoyer et al., 1994; Barnes and Sharp, 1999; Hannon and Hoyer, 2008). With the exception of 5-HT₃ receptors, which are ionotropic, all other 5-HT receptors are metabotropic 7-transmembrane receptors are G-protein coupled receptors. These metabotropic receptors can be further subdivided into groups depending on their coupling to specific G-proteins: the Gᵢ/o-coupled 5-HT₁ receptor family, the G₉-coupled 5-HT₂ receptor family, and the Gₛ-coupled 5-HT receptor family (Table 1.1) (Barnes and Sharp, 1999).

<table>
<thead>
<tr>
<th>Receptor family</th>
<th>Coupled G-protein</th>
<th>Net effect</th>
<th>Downstream effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT₁</td>
<td>Gᵢ/o</td>
<td>Inhibitory</td>
<td>Opening inward-rectifying K⁺ channels</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Suppressing voltage-gated Ca²⁺ channels</td>
</tr>
<tr>
<td>5-HT₂</td>
<td>G₉</td>
<td>Excitatory</td>
<td>Closing leak K⁺ channels</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Opening non-selective cation channels</td>
</tr>
<tr>
<td>5-HT₃</td>
<td>n/a</td>
<td>Excitatory</td>
<td>Direct cation flux</td>
</tr>
<tr>
<td>5-HT₄,6,7</td>
<td>Gₛ</td>
<td>Excitatory</td>
<td>Closing K⁺ channels (leak)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Opening non-selective cation channels</td>
</tr>
<tr>
<td>5-HT₅</td>
<td>Gᵢ/o</td>
<td>Inhibitory</td>
<td>Opening inward-rectifying K⁺ channels</td>
</tr>
</tbody>
</table>

Table 1.1 Function and mechanisms of 5-HT various 5-HT receptor families. Information adapted from Hoyer et al., 1994; Barnes and Sharp, 1999; Hannon and Hoyer, 2008; Goodfellow et al., 2012.
The 5-HT$_1$ receptor family preferentially couple to $G_{i/o}$ proteins (Clarke et al., 1987) and elicit inhibitory effects on neurons expressing these receptors by way of activating various potassium channels, in particular inward-rectifying potassium channels (Andrade and Nicoll, 1987; Beck and Choi, 1991; Penington et al., 1993; Okuhara and Beck, 1994). The 5-HT$_1$ receptor family comprises of five receptors: 5-HT$_1$A, 5-HT$_1$B, 5-HT$_1$D, 5-HT$_1$E, and 5-HT$_1$F, each with unique patterns of distribution across the CNS. In mPFC, the 5-HT$_1$A receptor has been the center of much research in part due to its high levels expression, particularly in the upper cortical layers (Puig and Gulledge, 2011). However, a substantial proportion of layer 6 neurons in mPFC express 5-HT$_1$A receptors (Chalmers and Watson, 1991; Pompeiano et al., 1992, 1994; Corneahébert et al., 1999; Amargós-Bosch et al., 2004), similar to their expression in prefrontal layer 6 of human and nonhuman primates (de Almeida and Mengod, 2007; de Almeida and Mengod, 2008; Mengod et al., 2015). Expression of 5-HT$_1$A receptors is localized strictly to somatodendritic sites (Pompeiano et al., 1992), whereas distribution of 5-HT$_1$B and 5-HT$_1$D receptors are specific to presynaptic terminals (Bruinvels et al., 1994).

The 5-HT$_2$ receptor family preferentially couple to $G_{aq}$ proteins, and has been shown to typically produce excitation in its host neuron through activation of nonselective cation channels and closure of potassium channels (Zhang, 2003). Three receptors: the 5-HT$_2$A, 5-HT$_2$B, and 5-HT$_2$C receptors make up the 5-HT$_2$ receptor family. In the mPFC, 5-HT$_2$A and 5-HT$_2$C receptors are expressed in a variety of neurons. Layer 6 neurons express both receptors (Amargós-Bosch et al., 2004). In particular, co-expression of both 5-HT$_1$A and 5-HT$_2$A receptors is observed in a large proportion of layer 6 pyramidal neurons of mouse mPFC (Amargós-Bosch et al., 2004). Of note, an additional subset of these neurons can also express the 5-HT$_2$C receptor (Nocjar et al.,
Expression of the 5-HT2A receptor has been shown to be strictly somatodendritic in postsynaptic neurons of cortex by immunolabeling (Cornea-Hébert et al., 1999).

Despite the expression data, much of the behavioural research has not conclusively linked functional neuronal mechanisms to serotonin’s effects on attention, especially with regards to prefrontal layer 6. An interesting correlate can be made, however, between stress and disruptions in attention. Stress is well known to raise prefrontal serotonin levels (Adell et al., 1997; Fujino et al., 2002; Bland et al., 2004), whereas similar disruptions in attention are observed under conditions of stress (Minor et al., 1984; Sänger et al., 2014). More targeted manipulations involving the elevation of cortical 5-HT also elicits attention deficits and increases in impulsivity (Puumala and Sirviö, 1998; Dalley et al., 2002). Likewise, activation of 5-HT1A and 5-HT2A receptors produce similar attention deficits (Carli and Samanin, 2000; Koskinen et al., 2000) whereas antagonists to these receptors in the mPFC can improve attention in rodents (Passetti et al., 2003; Winstanley et al., 2003). However, a gap in knowledge persists in the neuronal mechanisms and circuitry that underlie these links between serotonin, mPFC, and attention.

1.3 Thesis perspective and scope
1.3.1 Summary of literature and unanswered questions

As outlined above, the existing compendium of data has demonstrated the importance of the PFC in attentional control. The cholinergic input into the mPFC is critical for normal attention, as it is actively released and potent in modulating neuronal activity within the mPFC during attention tasks. In particular, evidence points to layer 6 of the mPFC as an important source of top-down control during attention given its position within the attention network, and the expression of cholinergic receptors. While the general role of the mPFC in attention is
accepted, there remain gaps in our understanding of the precise functional map of circuitry
within the mPFC, and how each component can perform, or can be modulated during attention.

Cholinergic innervation into the mPFC can elicit neuronal responses across the cortical
layers. Likewise, transient increases in mPFC acetylcholine levels are observed following
detection of visual cues. Furthermore, pharmacological or structural lesions of cholinergic
receptors or cortical regions have resulted in deficits in attention. While these techniques
provided a good starting point to answering the question of how the cholinergic system mediates
attention, other questions of the mechanisms and circuitry that underlie attention remains
unanswered. In addition to the questions underlying cholinergic neurotransmission in this
region, numerous other inputs are received by the mPFC. It is known through expression studies
that receptors for these other inputs are also found in neurons of mPFC. One such
neurotransmitter, serotonin, has been implicated in attentional disruptions, but with little
understanding in terms of mechanism of action.

In order to further answer these pressing questions on the mechanistic effects of
cholinergic innervation into mPFC and how it contributes to attentional microcircuits, techniques
with much higher temporal and functional resolution are required to dissect these attention
circuits within this region. In the experimental chapters of this thesis, I will aim to address some
of these emerging questions on layer 6 of the mPFC as an important controller of attention
circuitry using techniques that provide more temporal and spatial resolution.

1.3.2 Brief overview of experimental chapter aims

Over the course of the experimental chapters of this thesis, I will use
electrophysiological, imaging, and optogenetic techniques to examine neurons in layer 6 across
several cortical regions for their responsiveness to acetylcholine, the plasticity of the cholinergic
response, as well as the opposing effects of serotonin, and finally the identification, characterization, and modulation of a local circuit important to attention.

In Chapter 3, I examine the distribution of cholinergic responses across several associative and primary regions of PFC. It is well understood that cholinergic afferents innervate many layers of cortex, and the three regions examined: medial prefrontal cortex, primary motor cortex, and primary somatosensory cortex, all receive large numbers of cholinergic terminals (Mesulam et al., 1983; Mechawar and Descarries, 2001). These findings support models of individual forms of attention being carried out by both primary and associative regions (Meyer, 2011; Smucny et al., 2013). Likewise, acetylcholine is released in these regions during attention (Fournier et al., 2004; Parikh et al., 2007), yet we have little understanding of how neurons within these regions respond to cholinergic stimulation. I aim to elucidate the region-specific responses to acetylcholine with an aim to understand whether different types of cholinergic responses can correlate to different forms of functional attention as described previously in literature.

In Chapter 4, I investigate the plasticity of nicotinic and muscarinic acetylcholine receptor-mediated responses in layer 6 neurons of mPFC. In layer 6 neurons of mPFC, nicotinic receptor-mediated responses to acetylcholine have been identified (Kassam et al., 2008; Bailey et al., 2010). Intriguingly, disruptions of these nicotinic receptors elicit significant, but relatively mild and inconsistent disruptions in attention (Bailey et al., 2010; Guillem et al., 2011). However, the presence of muscarinic acetylcholine receptors in neurons of this region (Buckley et al., 1988; Tribollet et al., 2004) raises the question of whether there is additional complexity to the cholinergic response. In particular with respect to layer 6 neurons, muscarinic responses to acetylcholine have been unexplored, despite a shared importance of these neurons and
muscarinic activity in mPFC to normal attention (Bailey et al., 2010; Klinkenberg et al., 2011). To address the question of how nicotinic disruptions affect functional responses to acetylcholine in layer 6 neurons of mPFC, I use transgenic mice lacking certain nicotinic receptor subunits which either disrupt or fully knock-out receptor expression. Additionally, muscarinic receptor-mediated responses to acetylcholine will be monitored using acetylcholine in the presence of nicotinic antagonists.

In Chapter 5, I investigate how serotonin can modulate layer 6 neurons of mPFC in an opposing manner to acetylcholine. Additionally, I characterize a local circuit between layer 6 pyramidal neurons and layer 5 interneurons which is important to normal attention (Kim et al., 2016), and examine the effects of serotonin on the activity of this circuit. Serotonin has been shown to shape and bias attention in both humans and non-human primates (Schmitt et al., 2000; Gallagher et al., 2003; Booij et al., 2005; Watson et al., 2015). While its functional effects in other layers of cortex have been extensively studied, we do not yet understand how serotonin interacts with layer 6 of mPFC despite expression of 5-HT receptors in these neurons (Amargós-Bosch et al., 2004). Since the inhibitory 5-HT$_{1A}$ receptors are prominently found in layer 6 neurons, I chose to examine the effects of serotonin as a counter-balance to the excitatory effects of acetylcholine on attention. Indeed, elevated serotonin can exert an opposing effect on attention (Riedel et al., 2005; Oranje et al., 2008; Watson et al., 2015). Using pharmacological methods, I examined the functional effects of serotonin on layer 6 neurons of mPFC, as well as elucidate the receptors responsible for the response. Furthermore, I investigate a circuit between layer 4 and layer 5 interneurons which has been shown to be important to normal attention (Kim et al., 2016). Using transgenic mice expressing channelrhodopsin in layer 6 neurons, I will characterize
the consequences of layer 6 activation on interneurons of layer 5, as well as examine the effects of serotonin on this circuit.
2 Materials and Methods

2.1 Animals

Different mouse lines and their wildtype controls used for the experiments in this thesis. Details of the mouse lines used are summarized in Table 2.1. In brief, Syt6-eGFP and Chrna5 mice were used to investigate the cholinergic response in L6 neurons of associative and primary cortical regions (Chapter 3). Both Chrna5 and Chrnb2 mice were used to study the plasticity of muscarinic receptors in L6 neurons of medial prefrontal cortex (Chapter 4). Finally, Epyc-cre;Ai:32 mice were generated by crossing Epyc-cre and Ai:32 mice for use in interrogating the serotonergic suppression of L6-L5 circuits in medial prefrontal cortex (Chapter 5). All mouse colonies were housed and maintained by the Department of Comparative Medicine facility in the Medical Sciences Building at the University of Toronto. All animal care and experimental protocols were performed in accordance to the guidelines of the Canadian Council on Animal Care and approved by the University of Toronto Animal Care Committee.

In Chapter 3, BAC transgenic mice on a Swiss Webster background expressing eGFP driven by the synaptotagmin 6 (Syt6) promoter were used to identify a subpopulation of layer 6 neurons in medial prefrontal, primary motor, and primary somatosensory cortex. Mice were obtained from MMRRC (Tg(Syt6-eGFP)EL71Gsat/Mmucd, stock #010557-UCD) and maintained as heterozygous litters with one heterozygous and one wildtype parent. Offspring were genotyped and selected for experiments by expression of eGFP. Additionally, mice genetically deleted for the Chrna5 gene encoding the nicotinic receptor α5 subunit (α5-/-, Salas et al., 2003) were used to investigate the different properties of nicotinic receptor-mediated responses to acetylcholine in the cortex. Congenic α5-/- mice were backcrossed into a C57BL/6J background for > 10 generations. The resulting heterozygous parents were used to generate
homozygous α5-/- offspring and their wildtype α5+/+ controls for subsequent breeding in separate homozygous lines.

In Chapter 4, mice with genetic deletion of the Chrna5 gene which encodes the nicotinic receptor α5 subunit (Salas et al., 2003) and mice with genetic deletion of the Chrnb2 gene encoding the nicotinic receptor β2 subunit (Picciotto et al., 1995) were backcrossed onto a C57BL/6J background. Heterozygous parents were used to generate homozygous α5 subunit knockout (α5-/-) and β2 subunit knockout (β2-/-) mice that were bred in separate homozygous lines for experiments to investigate changes in cholinergic responsiveness to acetylcholine upon disruption of normal nicotinic receptor function.

In Chapter 5, initial experiments used Syt6-eGFP and wildtype C57BL/6 mice to examine 5-HT responses in layer 6 neurons of medial prefrontal cortex. For optogenetic experiments, we obtained BAC transgenic mice expressing cre-recombinase driven by the epiphycan (Epyc) promoter as a gift from Dr. Nathaniel Heintz and Dr. Eric Schmidt at Rockefeller University (also obtainable from MMRRC, Tg(Epyc-cre)KR363Gsat). These heterozygous mice were crossed with Ai:32 mice, expressing floxed eGFP-channelrhodopsin-2, obtained from The Jackson Laboratories (B6;129S-Gt(ROSA)26SorIm32(CAG-COP4*H134R/EYFP)Hze/J, stock #012569). Offspring (Epyc-cre;Ai:32) were genotyped by PCR, and those positive for Epyc-cre expressed eGFP and channelrhodopsin-2 in Epyc positive neurons.
<table>
<thead>
<tr>
<th>Mouse line</th>
<th>Background</th>
<th>Description</th>
<th>Source</th>
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<tbody>
<tr>
<td>Syt6-eGFP</td>
<td>Swiss Webster</td>
<td>BAC transgenic mice expressing eGFP via the synaptotagmin 6 (Syt6) promoter, localized to layer 6 pyramidal neurons in cortex</td>
<td>MMRRC Tg(Syt6-eGFP)EL71Gsat/Mmucd</td>
</tr>
<tr>
<td>Chrna5 (α5+/+, α5-/-)</td>
<td>C57BL/6</td>
<td>Transgenic mice knocked out for nicotinic acetylcholine receptor α5 subunit</td>
<td>Dr. Mariella de Biasi (Salas et al., 2003)</td>
</tr>
<tr>
<td>Chrnb2 (β2+/+, β2-/-)</td>
<td>C57BL/6</td>
<td>Transgenic mice knocked out for nicotinic acetylcholine receptor β2 subunit</td>
<td>Dr. Marina Picciotto (Picciotto et al., 1995)</td>
</tr>
<tr>
<td>Epyc-cre</td>
<td>C57BL/6</td>
<td>BAC transgenic mice expressing Cre recombinase driven by the epiphycan (Epyc) promoter, localized to layer 6 neurons in cortex</td>
<td>Dr. Nathaniel Heintz at Rockefeller University</td>
</tr>
</tbody>
</table>
| Ai:32                   | C57BL/6    | Homozygous mice expressing floxed ChR2(H134R)-EYFP fusion gene              | The Jackson Laboratory B6;129S-Gt(ROSA)26So
| Epyc-cre;Ai:32          | C57BL/6    | Bred from crossing Epyc-cre and Ai:32 mice                                 | Bred in-house                                |

Table 2.1 List of mouse lines used in the thesis.
2.2 Brain slice preparation

Mice were deeply anaesthetized with chloral hydrate (400 mg/kg) and sacrificed by decapitation. 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 of the medial prefrontal cortex (400 µm thick, 2.34 – 0.74 mm from Bregma) were cut on a Dosaka Linear Slicer (SciMedia, Costa Mesa CA) and recovered 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 hours prior to experiments.

2.3 Electrophysiological recordings

Brain slices were transferred to a perfusion chamber atop the stage of a BX50W1 microscope (Olympus) after a minimum of 2 hours of recovery post-extraction. ACSF was bubbled at room temperature using 95/5% O₂:CO₂, and perfused into the recording chamber at a rate of 3-4 ml/min. Target neurons are visually identified through only IR-DIC or a combination of IR-DIC and fluorescence from reporter-expressing neurons in Syt6-eGFP mice (X-cite Series 120; Lumen Dynamics). Recording micropipettes were pulled using a Sutter Instrument micropipette puller with tip resistances between 2-4 MΩ. Recording pipettes were filled with solution 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). Target neurons were patched under the whole-cell configuration, and tested for electrophysiological properties by step protocol prior to further experimentation. In Chapter 5, interneurons are identified visually based on their small, circular somatic morphology in IR-DIC.
A subset of interneurons are filled with the fluorophore Alexa-594 (20 µM) or Texas red dextran (0.15%) added into the regular patch solution. Recordings in current clamp and voltage clamp are made with a HEKA EPC10 (HEKA Electroniks), and corrected for the liquid junction potential (14 mV). All data was acquired at 20 kHz and low-pass filtered at 3 kHz.

### 2.4 Visual identification of target neurons

Neurons in the slice are visually identified for whole-cell patch clamp recordings via IR-DIC. In the mPFC, layer 6 is located directly adjacent to white matter, and extends approximately 200 µm towards the pial surface. Pyramidal neurons in layer 6 are visually distinct from their layer 5 counterparts in that their visible somata are smaller in diameter and have a less pronounced apical dendrite in IR-DIC. Upon patching, these properties are reflected in layer 6 pyramidal neurons having higher input resistance than their layer 5 counterparts. To further facilitate targeting of layer 6 pyramidal neurons, Syt6-eGFP mice are used in Chapter 3 and Chapter 5. As previously discussed, Syt6-driven expression of eGFP in Syt6-eGFP mice is localized to postsynaptic layer 6 pyramidal neurons of prefrontal cortex. These neurons represent the same corticothalamic population of layer 6 in medial prefrontal cortex as previously described by Kassam et al., 2008. Taken together, a combination of distinct morphological traits, electrophysiological properties and fluorescent reporter genes allows me to record from a consistent population of layer 6 neurons in prefrontal cortex.

In Chapter 5, layer 5 interneurons are identified in IR-DIC by their distinct morphology compared to their larger pyramidal counterparts. Interneurons are targeted based on their small, circular somata, and confirmed by their distinct electrical properties and firing patterns.
2.5 Two-photon imaging

Multiphoton images in Chapter 3 and Chapter 5 are acquired with a Mai Tai Ti:sapphire laser (Spectra Physics) using an Olympus Fluoview FV1000 microscope and either an Olympus XLPlan N 25x water-immersion objective (Chapter 3 and Chapter 5) permitting for concurrent electrophysiology. Green fluorescence in Syt6-eGFP mice is visualized with the laser tuned to wavelength of 780 nm, while red fluorescence in filled neurons was visualized at 800 nm. Images were obtained via line-scan using Olympus Fluoview software, and captured in 500 μm x 500 μm xy stacks of varying z-axis depth. Captured stacks are stitched together post hoc using the MosaicJ plug-in in ImageJ software.

2.6 Optogenetics

Patched neurons in channelrhodopsin-2-expressing mice are stimulated by blue LED light (473 nm) delivered by optic fiber (Thorlabs) mounted on a mechanical precision micromanipulator (Narishige). The tip of the optic fiber is carefully aligned to just above the level of the brain slice such that the optical output is primarily focused on layer 6 of medial prefrontal cortex. Control of LED output is driven by a Thorlabs DC2100 LED driver triggered by software control (PatchMaster) via a TTL output from a HEKA EPC10 amplifier. Stimulation of layer 6 was carried out in brief light pulses (2-5 ms duration each) delivered at 20 Hz for a period of one second.
3 Cholinergic excitation in mouse primary versus associative cortex: Region-specific magnitude and receptor balance

Acknowledgements and Contributions

This work has been published in the *European Journal of Neuroscience*: Tian MK, Bailey CD, Lambe EK (2014) Cholinergic excitation in mouse primary versus associative cortex: Region-specific magnitude and receptor balance. *Eur J Neurosci* 40(4): 2608-2018. Michael Tian designed, performed, and analyzed the electrophysiology and imaging experiments for this study and wrote the original manuscript under the supervision of Dr. Evelyn Lambe at the University of Toronto. Michael Tian, Craig Bailey and Evelyn Lambe were all involved in experimental design and writing the final manuscript. Craig Bailey designed the genotyping and maintained the mouse colony of the initial cohorts of $\alpha_5$-/- and $\text{Syt6-eGFP}$ mice. We thank Dr. Mariella de Biasi of Baylor College of Medicine for the original gift of the $\alpha_5$-/- mice. We thank Dr. Zhong-Ping Feng and Dr. Beverley Orser for their insightful feedback and discussion. This work was supported by the grants from the following programs: Canadian Institutes of Health Research (MOP 89825) and the Canada Research Chairs Program (Evelyn Lambe), Banting and Best Canada Graduate Scholarship (Michael Tian), NSERC Discovery Grant (Craig Bailey).
3.1 Abstract

Cholinergic stimulation of 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 is detected in deep layers of the relevant primary and/or associative cortex depending on the nature of the attention task. Here, we investigate the electrophysiological effects of acetylcholine in layer 6, the deepest layer, of primary somatosensory cortex, primary motor cortex, and associative medial prefrontal cortex. Layer 6 pyramidal neurons are a major source of “top-down” modulation of attention, and we find that the strength and homogeneity of their direct cholinergic excitation is region specific. On average, neurons in the primary cortical regions show weaker responses to acetylcholine, which are under a balance of contributions from both nicotinic and muscarinic acetylcholine receptors. Conversely, neurons in associative cortex show significantly stronger excitation by acetylcholine, mediated predominantly by nicotinic receptors. The greatest diversity of responses to acetylcholine is found in 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 is preserved in primary cortical regions but not in associative cortex. These findings demonstrate that the effect of acetylcholine is not uniform throughout cortex and suggest its ability to enhance attention performance may tap into different cellular mechanisms across cortical regions.
3.2 Introduction

Attention is a complex cognitive function that focuses the mind on relevant information in the presence of distractions (Crick, 1984). The cholinergic system is considered 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 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 and 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 and Descarries, 2001) and raises questions in view of ongoing debate about which cortical regions are essential for attention (Meyer, 2011). While 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 cortices may carry out certain types of attentional processing on their own (Meyer, 2011). For example, alpha-band activity in primary somatosensory cortex reflects somatosensory attention (Pfurtscheller et al., 1996; Jones et al., 2010), and such oscillations in 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 thalamic and thalamocortical circuitry involved in attention (Guillery, 1995; Sherman, 2005; Zikopoulos and Barbas, 2006).

In particular, layer 6 of cerebral cortex sends and receives projections to thalamic nuclei, forming a corticothalamic feedback loop important to top-down control of normal attentional
function (Bourassa et al., 1995; Guillery and Sherman, 2002; Gabbott et al., 2005; Parikh et al., 2010; Thomson, 2010). Yet, layer 6 is heterogeneous (Andjelic et al., 2009; Briggs, 2010; Thomson, 2010) and includes corticocortical neurons (Mercer et al., 2005; Kumar and Ohana, 2008; Petrof et al., 2012), which may also be involved in attentional processing. For example, recent work has demonstrated a local circuit within primary cortex whereby layer 6 pyramidal neurons control cortical gain modulation (Olsen et al., 2012). Layer 6 is innervated by cholinergic fibers (Mechawar and Descarries, 2001) and its neurons possess acetylcholine receptors of both the nicotinic and muscarinic subtypes (Buckley et al., 1988; Tribollet et al., 2004). However, only nicotinic receptor-mediated responses in layer 6 neurons have been previously assessed in prelimbic cortex (Kassam et al., 2008; Bailey et al., 2010), whereas the relative consequences of cholinergic stimulation on layer 6 pyramidal neurons across primary and associative regions of cortex are unknown.

Here, we ask whether acetylcholine exerts similar electrophysiological effects on layer 6 neurons in primary and associative cortical regions. Next, we probe the underlying receptor mechanisms involved using pharmacological manipulations. Heterogeneous responses observed in primary somatosensory cortex were further examined. Finally, we investigate regional variation in cholinergic excitation of layer 6 neurons in a population of mice known to have attention deficits under demanding circumstances.

### 3.3 Methods

#### 3.3.1 Animals

Electrophysiological recordings were performed on brain slices from adult male mice (range: P50 to 130 days; mean: 80 ± 6 days; n = 42 mice). To facilitate the recording from layer 6 neurons in adult cortex, we employed BAC transgenic mice with extensive labelling of layer 6
neurons by eGFP expression driven by the synaptotagmin 6 promoter (Syt6-eGFP; MMRRC) on a Swiss Webster background. Labelled neurons have pyramidal somata and apical dendrites that project toward the pial surface. None of the eGFP positive neurons recorded displayed electrophysiological characteristics of fast-spiking interneurons. Conversely, fast-spiking interneurons that were visually identified under IR-DIC and confirmed electrophysiologically (n = 10 across regions) were eGFP negative. A subset of experiments were performed in C57BL/6 adult male mice deleted for the nicotinic receptor α5 subunit (α5−/−, Salas et al., 2003) and their matched wild-type (WT) controls. Mice were deeply anaesthetized with chloral hydrate (400 mg/kg) and sacrificed by decapitation. All animal care and experimental protocols were performed in accordance to the guidelines of the Canadian Council on Animal Care and approved by the University of Toronto Animal Care Committee.

3.3.2 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 (400 μm thick, 2.34 – 0.74 mm from Bregma) were cut on a Dosaka Linear Slicer (SciMedia, Costa Mesa CA) and recovered 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 hours.

3.3.3 Electrophysiology

Recovered slices were transferred to a superfusion chamber on the stage of a BX50W1 microscope (Olympus, Richmond Hill ON). ACSF was bubbled (95% O2, 5% CO2 at room
temperature) and perfused the chamber at a rate of 3-4 ml/min. As illustrated in Figure 3.1A, layer 6 was landmarked with fluorescently-identified eGFP-positive neurons (X-cite Series 120; Lumen Dynamics, Mississauga ON) in Syt6-eGFP mice. Recording electrodes (2-4 MΩ) 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 6 pyramidal neurons in acute coronal prefrontal slices. Layer 6 neurons in primary somatosensory cortex (SSC), primary motor cortex (M1), and associative prefrontal cortex (mPFC; prelimbic region) were specifically targeted based on local landmarks as previously described (Paxinos and Watson, 2001; Figure 3.1B). Currents and membrane potential were recorded in voltage clamp (at a holding potential of -75 mV) and current clamp using an EPC10 (HEKA Electroniks, Chester NS). All data were acquired at 20 kHz and low-pass filtered at 3 kHz with pClamp software (Molecular Devices, Sunnyvale CA).

Electrophysiological properties of the layer 6 neurons (resting membrane potential, input resistance, and spike amplitude) are shown in Table 3.1. Input resistance was calculated by measuring the steady state deflections in membrane potential to injections of hyperpolarizing current (50-100pA, 500 ms). Resting membrane potential was corrected for the liquid junction potential.
Table 3.1 Electrophysiological parameters of layer 6 neurons in SSC, M1, and mPFC.
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. *P < 0.05 for SSC vs. M1 or mPFC; **P < 0.0001 for mPFC vs. SSC or M1.

<table>
<thead>
<tr>
<th></th>
<th>SSC (n = 73)</th>
<th>M1 (n = 43)</th>
<th>mPFC (n = 65)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMP (mV)</td>
<td>-83.5 ± 0.6*</td>
<td>-80.8 ± 0.7</td>
<td>-81.6 ± 0.7</td>
<td>0.01</td>
</tr>
<tr>
<td>Spike amplitude (mV)</td>
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<td>90.3 ± 0.9</td>
<td>90.3 ± 1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>Input resistance (MΩ)</td>
<td>159.5 ± 7.7</td>
<td>179.3 ± 9.7</td>
<td>320.2 ± 14.2**</td>
<td>0.0001</td>
</tr>
</tbody>
</table>
Figure 3.1 Layer 6 in primary and associative cortical regions is identified in transgenic eGFP-expressing mice. A) A z-stack projection shows layer 6-specific expression of eGFP in neurons of Syt6-eGFP mice. This laminar specificity is conserved across primary and associative regions of cortex. B) Schematics showing targeted regions of primary and associative cortices for electrophysiological recordings. Bregma coordinates are indicated as described previously (Paxinos and Franklin, 2001). Red: medial prefrontal cortex (mPFC), Blue: primary motor cortex (M1), Green: primary somatosensory cortex (SSC), wm, white matter.
3.3.4 Pharmacology

Cholinergic responses were probed with the addition of acetylcholine (100 µM or 1 mM, 15 sec) in standard ACSF to the bath perfusion after a baseline recording period. Acetylcholine applications were timer-controlled. Note that the high level of acetylcholinesterase in cortical slices means that only a fraction of the bath-applied acetylcholine likely reaches 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 µM), D-(-)-2-amino-5-phosphonopentanoic acid (APV, 50 µM), and bicuculline (10 µM) added to the bath perfusion. Nicotinic and muscarinic effects were examined individually by applying acetylcholine in the presence of the muscarinic receptor antagonist atropine (200 nM) or the nicotinic receptor antagonist dihydro-β-erythroidine hydrobromide (DHβE, 3 µM). All compounds were obtained from Sigma or Tocris Bioscience and stored in stock solutions at -20°C.

3.3.5 Statistical analysis

Current responses and changes in membrane potential as a result of bath-applied acetylcholine were measured using Clampfit software (Molecular Devices) and assessed with one-way ANOVAs and post hoc t-tests. For illustrative purposes, averaged responses to acetylcholine in different groups were calculated using data points extracted from all individual recordings. Comparisons between cholinergic responses and kinetics in SSC neurons were made using unpaired t-tests. Comparisons between genotype and regional effects of acetylcholine in α5−/− and wildtype mice were made using two-way ANOVA. Regional and genotype differences
in the proportion of neurons depolarized to threshold by acetylcholine were compared by Fisher’s exact tests.

3.4 Results

3.4.1 Acetylcholine exerts unique effects on layer 6 neurons in primary and associative cortices

To ascertain the whole-cell effects of cholinergic stimulation on layer 6 pyramidal neurons in primary somatosensory cortex (SSC), primary motor cortex (M1), and the associative medial prefrontal cortex (mPFC), we bath applied acetylcholine (1 mM, 15 sec) and quantified the magnitude of the acetylcholine-elicited inward currents in voltage clamp, 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; Figure 3.2A). In particular, acetylcholine-elicited inward currents were significantly smaller in layer 6 neurons of SSC (38.2 ± 4.5 pA, n = 39 neurons, t_{67} = 4.3, \( P < 0.0001 \), unpaired two-tailed t-test) and M1 (46.6 ± 4.9 pA, n = 20 neurons, t_{48} = 2.8, \( P = 0.008 \), unpaired two-tailed t-test) compared to mPFC (69.5 ± 5.9 pA, n = 30 neurons). The responses to cholinergic stimulation were relatively homogenous within M1 and mPFC, whereas there appeared to be a greater diversity in cholinergic responses within SSC (two examples are shown in Figure 3.2B). Electrophysiological properties between neurons of these regions were quantified and reported in Table 3.1. Between the three regions, there is a small difference in resting membrane potential (F_{2,178} = 4.4, \( P = 0.01 \)), where SSC neurons had significantly more hyperpolarized resting potentials compared to M1 and mPFC (\( P < 0.05 \)). A larger difference existed between the regions in neuronal input resistance (F_{2,178} = 66.2, \( P < 0.0001 \)), where layer 6 mPFC neurons had significantly higher input resistance compared to those of M1 and SSC (\( P < 0.0001 \)).
Since a majority of corticothalamic layer 6 neurons are typically silent at 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. We found significant regional differences in the degree of acetylcholine-elicited excitation in layer 6 neurons ($F_{2,83} = 19.2, P < 0.0001$, one-way ANOVA; Figure 3.2D). Average depolarization in SSC ($10.0 \pm 1.3 \text{ mV, } n = 37 \text{ neurons}$) was significantly smaller than that in M1 ($14.9 \pm 1.6 \text{ mV, } n = 20 \text{ neurons, } t_{55} = 2.3, P = 0.02$, unpaired two-tailed $t$-test) and in mPFC ($21.2 \pm 1.2 \text{ mV, } n = 29 \text{ neurons, } t_{64} = 6.2, P < 0.0001$, unpaired two-tailed $t$-test). The mPFC neurons showed the strongest excitation by acetylcholine, having significantly larger depolarization than that seen in M1 ($t_{47} = 3.1, P = 0.003$, unpaired two-tailed $t$-test). Moreover, the depolarization elicited by acetylcholine within layer 6 neurons resulted in action potential firing in some neurons (Figure 3.2E). In this regard, the region-specific consequences of cholinergic stimulation also differed between layer 6 neurons of SSC, M1, and mPFC. Neurons of SSC were less likely to depolarize to threshold by acetylcholine (8/38 neurons, 21%) compared to neurons of M1 (10/20 neurons, 50%, $P < 0.001$, Fisher’s exact test) or mPFC (30/30 neurons, 100%, $P < 0.0001$, Fisher’s exact test). Layer 6 neurons of mPFC were unique in that they were all able to depolarize to threshold by acetylcholine, also a significantly larger proportion than found in M1 ($P < 0.0001$, Fisher’s exact test).

This pattern of regional difference in the layer 6 cholinergic response was also observed in response to a lower concentration of acetylcholine ($100 \mu\text{M}; F_{2,35} = 3.8, P < 0.03$, one-way ANOVA). Acetylcholine elicited significantly smaller average inward currents in SSC ($15.3 \pm 3.3 \text{ pA, } n = 11 \text{ neurons, } t_{23} = 2.3, P = 0.03$, unpaired two-tailed $t$-test) and M1 ($14.2 \pm 3.9 \text{ pA, } n = 13 \text{ neurons, } t_{25} = 2.4, P = 0.02$, unpaired two-tailed $t$-test), compared to those observed in
mPFC (26.5 ± 3.4 pA, n = 14 neurons). Significant regional differences were also observed in acetylcholine-elicited depolarization (100 µM; F2,26 = 10.1, P = 0.0006, one-way ANOVA).

Acetylcholine elicited significantly smaller average depolarization in SSC (6.1 ± 1.5 mV, n = 9 neurons, t18 = 4.8, P = 0.0001, unpaired two-tailed t-test) and M1 (8.8 ± 2.5 mV, n = 9 neurons, t18 = 3.0, P = 0.008, unpaired two-tailed t-test), compared to mPFC (17.8 ± 1.8 mV, n = 11 neurons). Of note, this milder cholinergic stimulus was sufficient to elicit action potentials in the majority of neurons in mPFC.
Figure 3.2 Region specificity in the cholinergic excitation of layer 6 neurons in primary and associative cortex. A) The average inward current elicited by acetylcholine (ACh) differs in layer 6 of SSC, M1, and mPFC (F_{2,86} = 10.5, *** \( P < 0.0001 \), one-way ANOVA). Neurons in SSC (** \( P < 0.0001 \), unpaired two-tailed t-test) and M1 (* \( P = 0.008 \), unpaired two-tailed t-test) have smaller ACh-elicited currents than those in mPFC. B) Representative examples of ACh-elicited currents in SSC, M1, and mPFC. Of note, two examples are provided to show the greater heterogeneity observed within SSC. C) Averaged plot of all recorded ACh-elicited currents in neurons of SSC, M1, and mPFC. D) Excitation from rest elicited by ACh differs significantly in layer 6 of SSC, M1, and mPFC (F_{2,83} = 19.2, *** \( P < 0.0001 \), one-way ANOVA). Depolarization of layer 6 neurons by cholinergic stimulation is smallest in SSC (vs. M1: * \( P = 0.02 \), unpaired two-tailed t-test; vs. mPFC: ** \( P < 0.0001 \), unpaired two-tailed t-test), larger in M1 (vs. mPFC: * \( P = 0.003 \), unpaired two-tailed t-test), and largest in mPFC. E) Representative examples of ACh-elicited depolarization in SSC, M1, and mPFC. Layer 6 neurons of SSC were least likely to depolarize to threshold by cholinergic stimulation, compared to M1 (* \( P < 0.001 \), Fisher’s exact test) and mPFC (** \( P < 0.0001 \), Fisher’s exact test). s: spiking, ns: non-spiking (in response to ACh).
3.4.2 Region-specific effects of acetylcholine on layer 6 neurons appear postsynaptic in nature

Since acetylcholine can affect both pre- and post-synaptic aspects of cortical circuitry (Bloem et al., 2014; Proulx et al., 2014), we examined the effects of acetylcholine in the presence of antagonists of synaptic transmission (20 µM CNQX, 50 µM APV, 10 µM bicuculline, in bath).

Of note, the inward currents elicited by bath application of 1 mM acetylcholine persist during treatment of glutamatergic and GABAergic antagonists (examples from each region are shown in Figure 3.3A). Furthermore, significant regional differences are seen when synaptic transmission is blocked (F2,16 = 10.9, P = 0.001, one-way ANOVA; Figure 3.3B). Under these conditions, acetylcholine also elicits smaller responses in SSC (17.2 ± 6.5 pA, n = 7 neurons, t11 = 4.2, P = 0.001, unpaired two-tailed t-test) and M1 (43.6 ± 6.4 pA, n = 6 neurons, t10 = 2.2, P = 0.05, unpaired two-tailed t-test) than in mPFC (75.9 ± 13.2 pA, n = 6 neurons). Likewise, depolarization by acetylcholine in the presence of glutamatergic and GABAergic antagonists was also significantly different across primary and associative cortical regions (F2,16 = 8.2, P = 0.003, one-way ANOVA; Figure 3.3C). Neurons in SSC (5.7 ± 2.6 mV, n = 7 neurons) were significantly less depolarized by acetylcholine compared to those in M1 (16.5 ± 3.2 mV, n = 6 neurons, t11 = 2.7, P = 0.02, unpaired two-tailed t-test) and mPFC (23.6 ± 3.9 mV, n = 6 neurons, t11 = 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 we observe in layer 6 neurons of primary and associative cortex.
Figure 3.3 Region dependent effects of acetylcholine are preserved in the presence of antagonists of synaptic transmission. A) Example traces from layer 6 neurons in primary and associative regions of inward currents elicited by acetylcholine. Traces on the left show responses to acetylcholine in standard ACSF. Traces on the right show responses to acetylcholine in the same neuron in the presence of glutamatergic and GABAergic receptor antagonists (20 µM CNQX, 50 µM APV, 10 µM bicuculline) after a 10 minute wash-in period. B) Prominent regional differences in the inward currents elicited by acetylcholine are seen in the presence of synaptic blockers (F$_{2,16} = 10.9$, ***$P = 0.001$, one-way ANOVA). Currents are smallest in SSC (vs. M1: *$P = 0.01$, unpaired two-tailed t-test; vs. mPFC: **$P = 0.001$, unpaired two-tailed t-test), larger in M1 (vs. mPFC: $P = 0.05$, unpaired two-tailed t-test), and largest in mPFC. C) Depolarization by acetylcholine in the presence of synaptic blockers are significantly different across SSC, M1, and mPFC (F$_{2,16} = 8.2$, ***$P = 0.003$, one-way ANOVA). Depolarization in SSC neurons was significantly smaller compared to M1 (*$P = 0.02$, unpaired two-tailed t-test) and mPFC (**$P = 0.002$, unpaired two-tailed t-test).
### 3.4.3 The balance of nicotinic and muscarinic receptor contributions depends on cortical region

To determine the respective contributions of nicotinic and muscarinic acetylcholine receptors to the regional dependence of the acetylcholine responses in layer 6, we tested the effects of acetylcholine in the continued presence of either the non-specific muscarinic receptor antagonist atropine (200 nM) or the selective nicotinic receptor antagonist DHβE (3 µM). In a subset of experiments, a combination of both of these antagonists blocked all responses to acetylcholine in layer 6 pyramidal neurons of all three cortical regions examined (data not shown).

Nicotinic receptor-mediated inward currents elicited by acetylcholine in the presence of atropine differed significantly across the cortical regions ($F_{2,48} = 13.3$, $P < 0.0001$, one-way ANOVA; **Figure 3.4A**). Nicotinic receptor stimulation of layer 6 neurons resulted in smaller inward currents on average in SSC (21.5 ± 4.6 pA, $n = 15$ neurons) than M1 (49.1 ± 4.5 pA, $n = 13$ neurons, $t_{26} = 4.2$, $P = 0.0002$, unpaired two-tailed $t$-test) and mPFC (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 SSC compared to the other two regions (two examples are shown in **Figure 3.4B**).

Depolarization by acetylcholine in the presence of atropine was significantly different across regions ($F_{2,42} = 32.9$, $P < 0.0001$, one-way ANOVA; **Figure 3.4D**). Significantly smaller average depolarization was observed in SSC (6.2 ± 1.2 mV, $n = 15$ neurons) compared to M1 (15.7 ± 2.0 mV, $n = 12$ neurons, $t_{25} = 4.2$, $P = 0.0003$, unpaired two-tailed $t$-test) and mPFC (22.3 ± 1.3 mV, $n = 19$ neurons, $t_{32} = 8.8$, $P = 0.0001$, unpaired two-tailed $t$-test). Consistently, layer 6 neurons in mPFC were also more likely to depolarize to threshold (15/19 neurons, 78%)
than neurons in M1 (3/12 neurons, 25%, $P = 0.008$, Fisher’s exact test) and SSC (1/15 neurons, 7%, $P < 0.0001$, Fisher’s exact test).

Figure 3.4 There is region specificity in the nicotinic receptor-mediated responses to acetylcholine (ACh) in layer 6 of primary and associative cortex. A) The average inward current elicited by nicotinic receptor stimulation differs among SSC, M1, and mPFC ($F_{2,48} = 13.3$, *** $P < 0.0001$, one-way ANOVA). Nicotinic receptor-evoked inward currents are smallest in SSC (vs. M1: * $P = 0.0002$, unpaired two-tailed t-test; vs. mPFC: ** $P < 0.0001$, unpaired two-tailed t-test), and larger in M1 (vs. mPFC: # $P = 0.09$, unpaired two-tailed t-test) and mPFC. B) Representative examples of nicotinic currents in SSC, M1, and mPFC. C) Averaged plot of all recorded nicotinic responses in neurons of SSC, M1, and mPFC. D) Depolarization by nicotinic receptor stimulation differed significantly between layer 6 neurons of primary and associative cortex ($F_{2,42} = 32.9$, *** $P < 0.0001$, one-way ANOVA). This depolarization is smallest in SSC (vs. M1: * $P = 0.0003$, unpaired two-tailed t-test; vs. mPFC: ** $P = 0.0001$, unpaired two-tailed t-test), larger in M1 (vs. mPFC: * $P = 0.007$, unpaired two-tailed t-test), and largest in mPFC. E) Representative examples of nicotinic receptor-mediated depolarization in SSC, M1, and mPFC. Layer 6 neurons of SSC were least likely to depolarize to threshold by nicotinic receptor stimulation compared to M1 (* $P = 0.008$, Fisher’s exact test) and mPFC (** $P < 0.0001$, Fisher’s exact test).
Next, we investigated muscarinic receptor stimulation of layer 6 neurons by acetylcholine in the presence of the nicotinic receptor antagonist DHβE. The response to muscarinic receptor stimulation was regionally specific ($F_{2,38} = 5.6, P = 0.007$, one-way ANOVA; **Figure 3.5A**). The largest average currents were observed in layer 6 neurons of SSC ($12.5 \pm 1.3$ mV, $n = 12$ neurons), which were significantly greater than those in mPFC ($6.3 \pm 1.1$ mV, $n = 19$ neurons, $t_{29} = 3.3, P = 0.003$, unpaired two-tailed $t$-test), with M1 neurons showing intermediate responses ($9.8 \pm 1.5$ mV, $n = 10$ neurons). Unlike the acetylcholine and nicotinic responses, muscarinic responses were relatively homogenous within SSC (**Figure 3.5B**).

Despite our observation that muscarinic receptor-mediated inward currents were significantly different across the three regions, the depolarization by acetylcholine in the presence of DHβE did not differ across regions ($F_{2,39} = 1.6, P = 0.2$, one-way ANOVA; **Figure 3.5D**). As such, there are no regional differences in the ability of acetylcholine to depolarize neurons to threshold by muscarinic receptor stimulation (SSC: 1/19 neurons, 5%; M1: 0/10 neurons, 0%, mPFC: 1/13 neurons, 8%; **Figure 3.5E**). These paradoxical findings may be explained by the significantly higher average input resistance of layer 6 neurons in mPFC compared to M1 and SSC, as shown in **Table 3.1**.

Based on our pharmacological dissection of the cholinergic response in layer 6 neurons across primary and associative cortices, there appear to be significant differences in the respective contributions of nicotinic and muscarinic receptors to the overall cholinergic response within each region. These differences are illustrated in **Figure 3.6**, and calculated based on the mean magnitudes of acetylcholine-elicited inward currents in the presence of either atropine or DHβE. On average, there appears to be a shift from a combination of moderate nicotinic and muscarinic responses in layer 6 neurons of SSC (nAChR: 63%, mAChR: 37%) to larger and
more dominant nicotinic responses in M1 (nAChR: 83%, mAChR: 17%, \( P = 0.002 \), Fisher’s exact test), and the strongest and most dominant nicotinic responses in mPFC (nAChR: 91%, mAChR: 9%, \( P < 0.0001 \), Fisher’s exact test).

Figure 3.5 There is some region-specificity, albeit less pronounced, in muscarinic receptor-mediated responses to acetylcholine (ACh) in layer 6 of primary and associative cortex. A) Inward currents elicited by muscarinic receptor stimulation differ among SSC, M1, and mPFC (\( F_{2,38} = 5.6, ** P = 0.007 \), one-way ANOVA). Muscarinic receptor-mediated currents were largest in SSC and M1 and smallest in mPFC (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 SSC, M1, and mPFC. C) Averaged plot of all recorded muscarinic currents in neurons of SSC, M1, and mPFC. D) Depolarization from rest by muscarinic receptor stimulation did not differ significantly between layer 6 neurons of SSC, M1, and mPFC (\( F_{2,39} = 1.6, P = 0.2 \), one-way ANOVA). E) Examples of representative responses to muscarinic receptor stimulation. There are no differences the proportion of neurons reaching threshold by muscarinic receptor-mediated excitation.
Figure 3.6 Cortical region determines the overall cholinergic excitation of layer 6 neurons and the degree of nicotinic and muscarinic contribution to the average response. A) Superimposed average plots of all nicotinic (nAChR), muscarinic (mAChR), and overall responses to ACh in layer 6 neurons of SSC, M1, and mPFC. B) Pie charts represent the relative contributions of nicotinic and muscarinic receptor-mediated responses to ACh in neurons of SSC, M1, and mPFC. Layer 6 neurons are more dependent on nicotinic receptor stimulation in M1 (* P = 0.002, Fisher’s exact test) and mPFC (**) P < 0.0001, Fisher’s exact test) than SSC.
3.4.4 Characterization of two distinct populations of cholinergic responses among SSC neurons

We observed greater heterogeneity in SSC than M1 and mPFC in terms of the responses to cholinergic ([Figure 3.2B and E]) and nicotinic receptor stimulation ([Figure 3.4B and E]), but not to muscarinic receptor stimulation ([Figure 3.5B and E]). Based on these results, we hypothesized that layer 6 neurons in SSC could be subdivided into two subtypes based on their responses to acetylcholine. As summarized in [Figure 3.7], ‘Type 1’ neurons show prominent inward currents (57%, 22/39 total SSC neurons) and ‘Type 2’ neurons show more subtle and complex responses (43%, 17/39 total SSC neurons). The averaged acetylcholine-elicited currents of Type 1 and Type 2 neurons are shown in [Figure 3.7A] and compared to the overall average response in SSC. Interestingly, some evidence indicates that these subgroups may reflect distinct subclasses of layer 6 neurons, as characterized by their responses to depolarizing current injections. Previous work suggests that layer 6 corticothalamic neurons have regular-spiking firing patterns, whereas corticocortical neurons have initial-doublet firing patterns (Mercer et al., 2005; Kumar and Ohana, 2008; Petrof et al., 2012). Here, we find 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 (22/27 neurons) yielding a Type 1 response to acetylcholine and all initial-doublet neurons yielding a Type 2 response (12/12 neurons).

Acetylcholine-elicited inward currents are 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; [Figure 3.7C and D]). These inward currents also have different kinetics in terms of speed of onset (Type 1: 10.8 ± 0.6 sec, $n = 23$ neurons; Type 2: 31.8 ± 4.6 sec, $n = 13$ neurons; $t_{34} = 6.0, P < 0.0001$, unpaired two-tailed $t$-test; [Figure 3.7F]) and duration of response (Type 1: 42.7 ± 5.3 sec, $n = 23$ neurons; Type 2: 149.2 ± 20.4 sec, $n = 13$ neurons; $t_{34}$
= 6.4, \( P < 0.0001 \), unpaired two-tailed \( t \)-test; **Figure 3.7G**). In the presence of the muscarinic antagonist atropine, fast inward currents are found in 63% (10/16) of SSC neurons, whereas 37% (6/16) show minimal or no response. Of note, the Type 2 neurons in SSC consistently show a transient outward current at the beginning of the acetylcholine response, which is only infrequently observed in Type 1 SSC neurons (**Figure 3.7C**). Such outward currents are also prominent among the muscarinic responses in all regions observed in the presence of the nicotinic antagonist DHβE. Based on the heterogeneity in the acetylcholine and nicotinic responses and the homogeneity in the muscarinic responses in 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 acetylcholine in primary SSC contrasts with the relative homogeneity of the responses in primary M1 and the associative mPFC.
Figure 3.7 Further characterization of the two distinct cholinergic responses observed in layer 6 neurons of SSC. A) Averaged plots of acetylcholine (ACh) elicited currents in these “Type 1” and “Type 2” neurons of SSC. B) These different responses to acetylcholine may arise from two distinct subclasses of layer 6 neurons, as illustrated by their typical spiking pattern in response to injection of depolarizing current. C) Scatter plot showing uni- 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 outward currents are plotted as zeroes. D,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,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.
3.4.5 Mouse attention deficit model has disrupted associative and preserved primary responses

Associative cortical regions such as mPFC are thought to be necessary for attention tasks only under conditions of high demand (Marti et al., 2012). Here, we examined acetylcholine responses in layer 6 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 α5 nicotinic acetylcholine receptor subunit expressed in layer 6 across the cerebral cortex (Wada et al., 1990; Salas et al., 2003; Winzer-Serhan and Leslie, 2005). Recordings from α5−/− mice and their wild-type controls show a significant interaction in the effects of genotype on acetylcholine responses by cortical region (*F*₂,₆₃ = 3.9, *P* = 0.03, two-way ANOVA; Figure 3.8A and B). This interaction arises not from changes to acetylcholine responses within primary cortex but from a significant reduction in the responses within associative mPFC (*t* = 3.9, *P* < 0.001, Bonferroni post-test).

A similar interaction is found in the effects of genotype on acetylcholine-elicited depolarization across regions (*F*₂,₅₇ = 7.8, *P* = 0.01, two-way ANOVA; Figure 3.8C and D). Again, this interaction arises from a significant decrease in acetylcholine-elicited depolarization only within mPFC (*t* = 3.5, *P* < 0.01, Bonferroni post-test). Likewise, layer 6 mPFC neurons in α5−/− mice appear less likely to depolarize to threshold compared to wildtype controls (WT: 12/15 neurons, 80%; α5−/−: 3/8 neurons, *P* = 0.07, Fisher’s exact test). This decrease in cholinergic excitation is not observed in layer 6 neurons of M1 or SSC. 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 acetylcholine-elicited responses in associative cortex and not those in primary cortices.
Figure 3.8 Region-specificity of cholinergic responses is attenuated in the absence of the nicotinic receptor α5 subunit. A) Inward currents elicited by acetylcholine (ACh) in primary and associative cortex of α5−/− mice and wildtype controls. There is a significant interaction in the effects of genotype on cholinergic responses across regions (F2,63 = 3.9, P = 0.03, two-way ANOVA). ACh-elicited inward currents in layer 6 neurons of mPFC are significantly smaller in α5−/− mice compared to wildtype controls (* P < 0.001, Bonferroni post-tests), but a genotype difference is not observed in primary cortical regions. B) Representative examples of cholinergic responses across primary and associative cortex in wildtype and α5−/− mice. C) Depolarization by ACh in layer 6 neurons within SSC, M1, and mPFC of α5−/− mice and wildtype controls. A significant interaction exists in the effects of genotype on ACh-elicited depolarization across regions (F2, 57 = 7.8, P = 0.01, two-way ANOVA). Depolarization by ACh in layer 6 neurons of mPFC, but not primary cortices, are significantly smaller in α5−/− mice than wildtype controls (* P < 0.01, Bonferroni’s post-tests). D) Representative recordings of depolarization by cholinergic stimulation in SSC, M1, and mPFC. E) Proportion of neurons depolarized to threshold across primary and associative cortex in wildtype and α5−/− mice. Layer 6 neurons of mPFC in α5−/− mice trend toward being less likely to depolarize to threshold compared to wildtype controls (# P = 0.07, Fisher’s exact test).
3.5 Discussion

There are prominent regional differences in the strength and nature of cholinergic responses in layer 6 pyramidal neurons. These responses are primarily mediated by postsynaptic mechanisms, and are not a consequence of acetylcholine-elicited changes in synaptic transmission. Pharmacological dissection of the cholinergic responses reveals that the balance between nicotinic and muscarinic acetylcholine receptor-mediated responses is region specific. Layer 6 neurons in primary SSC cortex showed the weakest average response to cholinergic stimulation. Their responses were not homogenous: slightly more than half the neurons responded to both nicotinic and muscarinic receptors; however, a substantial proportion of neurons showed only muscarinic responses. By contrast, neurons in primary M1 responded more strongly and homogeneously to acetylcholine, with both nicotinic and muscarinic receptors contributing to cholinergic excitation. Finally, layer 6 neurons in the associative mPFC 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 α5 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.

3.5.1 Layer 6 neurons, attention, and the importance of cholinergic neurotransmission

We chose to target our recordings to layer 6 neurons since they are a major source of top-down feedback to the thalamus, innervating both primary and higher order thalamic nuclei (Bourassa et al., 1995; Guillery and Sherman, 2002; Gabbott et al., 2005). Coupled with thalamocortical inputs, a loop considered essential for normal attentional function is formed between cortex and thalamus (Parikh et al., 2010; Thomson, 2010). The strong cholinergic
stimulation we observe 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 acetylcholine upon cue detection during attention tasks, observed in both primary (Fournier et al., 2004; Parikh and Sarter, 2008) and associative cortices (Parikh et al., 2007; Rasmusson et al., 2007). Within SSC, acetylcholine 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 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 M1, evoked tonic release of acetylcholine through periods of attentional demand has been linked to performance on attention tasks (Parikh and Sarter, 2008), and functionally correlated to motor planning or the execution of necessary movements that contribute to normal performance on these tasks (Sirota et al., 2005; Conner et al., 2010). On the other hand, acetylcholine 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 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 may be employed only under 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 we report here show that layer 6 neurons across cortex do not respond uniformly to acetylcholine. The heightened influence of muscarinic receptor mediated responses to acetylcholine in the primary regions suggest 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 and Marrocco, 2000). In particular, we find two distinct populations of neurons within layer 6 of SSC based on their cholinergic response, which may reflect differences in this response among neurons within SSC with different projection targets (Mercer et al., 2005; Kumar and Ohana, 2008). The known heterogeneity of neurons within layer 6 (Andjelic et al., 2009; Briggs, 2010; Thomson, 2010) raises questions about their potentially distinct contributions, in terms of timing and direction of response, in the performance of somatosensory attention tasks. In addition to the postsynaptic effects of acetylcholine reported here, cholinergic stimulation can elicit changes at presynaptic sites in cerebral cortex (Bloem et al., 2014). While acetylcholine can elicit strong postsynaptic responses in the presence of antagonists to synaptic transmission, acetylcholine 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 SSC, are necessary to better understand the general circuitry within this region and how their unique responses to acetylcholine can modulate attentional behavior.

3.5.2 Acetylcholine response in associative cortex and the nicotinic receptor α5 subunit

The larger overall responses to cholinergic stimulation and the dominant nature of the nicotinic receptor-mediated component sets layer 6 neurons of the associative mPFC apart from the primary regions. Our results suggest that the nicotinic receptor α5 subunit is required for
maintaining these large cholinergic responses within layer 6 of mPFC in adulthood but does not play a role in the acetylcholine responses in the primary cortical regions examined. These results are broadly consistent with studies that have suggested that the α5 subunit shows not only laminar specificity in its expression (Birtsch et al., 1997; Salas et al., 2003; Winzer-Serhan and Leslie, 2005) but also some degree of regional specificity within cortex (Wada et al., 1990). While incorporation of the α5 subunit into α4β2* receptors can modulate nicotinic responses directly through increasing the channel Ca^{2+} permeability (Kuryatov et al., 2008), our experiments do not conclusively show a direct effect of this subunit. The constitutive deletion of α5 in our mice does not preclude the potential for developmental effects of α5 subunits. Indeed, the nicotinic response is strongest during the juvenile period in rodent mPFC, peaking during weeks two and three of postnatal life (Kassam et al., 2008; Bailey et al., 2010; Bailey et al., 2012). As such, cholinergic signaling mediated by α5-containing receptors either directly within layer 6 neurons of mPFC, or within other regions during development, may dictate long-lasting consequences on the strength of cholinergic responses in adulthood.

3.6 Conclusions

These findings provide functional data to demonstrate how layer 6 neurons respond to cholinergic stimulation across several regions important in attentional performance. There is clear regional specificity in the direct effect of acetylcholine on layer 6 neurons of primary and associative cortices. Such neurons in M1 and SSC have smaller responses to acetylcholine with a substantial muscarinic component, whereas SSC is unusual for having two populations of layer 6 neurons which show distinct responses to acetylcholine. By contrast, layer 6 neurons of the associative mPFC show dramatically greater cholinergic excitation driven predominantly by
nicotinic receptors. This region-specific pattern appears dependent on the presence of the
nicotinic α5 subunit and is lost in a mouse model of attention deficits under demanding
conditions.
4 Plasticity of prefrontal attention circuitry: Upregulated muscarinic excitability in response to decreased nicotinic signaling following deletion of α5 or β2 subunit

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4.1 Abstract

Attention depends on cholinergic stimulation of nicotinic and muscarinic acetylcholine receptors in the medial prefrontal cortex. Pyramidal neurons in layer 6 of this region express cholinergic receptors of both families and play an important role in attention through their feedback projections to the thalamus. Here, we investigate how nicotinic and muscarinic cholinergic receptors affect the excitability of these neurons using whole cell recordings in acute brain slices of prefrontal cortex. Since attention deficits have been documented in both rodents and humans having genetic abnormalities in nicotinic receptors, we focus in particular on how the cholinergic excitation of layer 6 neurons is altered by genetic deletion of either of two key nicotinic receptor subunits, the accessory α5 subunit or the ligand-binding β2 subunit. We find that the cholinergic excitation of layer 6 neurons is dominated by nicotinic receptors in wild type mice and that the reduction or loss of this nicotinic receptor stimulation is accompanied by a surprising degree of plasticity in excitatory muscarinic receptors. These findings suggest that disrupting nicotinic receptors fundamentally alters the mechanisms and timing of excitation in prefrontal attentional circuitry.
4.2 Introduction

Prefrontal acetylcholine (ACh) release increases with attentional effort (Passetti et al., 2000; Dalley et al., 2004) and correlates with detection of cues on attention tasks (Parikh et al., 2007). The loss of prefrontal ACh afferents, by contrast, substantially lowers cue detection in attention tasks (McGaughy et al., 1996). Attentional processing depends on both ionotropic nicotinic receptors (Bailey et al., 2010; Guillem et al., 2011) and metabotropic muscarinic receptors (Robbins et al., 1998), types of cholinergic receptors that may have synergistic effects (Ellis et al., 2006). The corticothalamic neurons of layer 6 are sensitive to nicotinic receptor stimulation (Kassam et al., 2008; Bailey et al., 2010). These neurons are thought to control the attentional “search light” of the brain through their various feedback projections to the thalamus (Crick, 1984; Zikopoulos and Barbas, 2006; Briggs and Usrey, 2011). Layer 6 neurons express the relatively-rare $\alpha5$ nicotinic accessory subunit (Wada et al., 1990; Salas et al., 2003), in addition to the $\alpha4$ and $\beta2$ subunits that form the high-affinity nicotinic receptors. In layer 6 neurons, the $\alpha5$ subunit is incorporated into the $\alpha4\beta2$ subtype of nicotinic receptors, greatly enhancing their conductance (Ramirez-Latorre et al., 1996) and currents (Bailey et al., 2010). Of particular interest, loss of the $\alpha5$ nicotinic receptors results in attention deficits in mice (Bailey et al., 2010). As layer 6 neurons also express muscarinic ACh receptors (Buckley et al., 1988), we investigate the combined effects of nicotinic and muscarinic ACh stimulation on excitability of these neurons. Since genetic abnormalities in nicotinic receptors are linked to attentional dysfunction (Rigbi et al., 2008; Bailey et al., 2010; Guillem et al., 2011), we examine how the cholinergic excitation of layer 6 neurons is altered by genetic deletion of two key nicotinic receptor subunits, the $\alpha5$ subunit, which increases the conductance of the high affinity nicotinic receptor, or the $\beta2$ subunit, which is required for assembly and function of these receptors.
4.3 Methods

Homozygous mice derived from heterozygous parents were used to generate α5 (Salas et al., 2003) and β2 (Picciotto et al., 1995) knockout mice for experiments. Wild type (WT) mice were bred in this manner from both α5 and β2 lines. Neurons from both WT groups were combined for analysis since no statistically significant differences were observed in our experiments.

Adult male mice from postnatal day (P) 60 to P180 were used to prepare 400 µm thick coronal slices of the prefrontal cortex (2.34 to 1.34 mm anterior to Bregma, Paxinos and Franklin, 2001) using a protocol approved by the University of Toronto Animal Care and Use Committee. In brief, the excised brain was cooled with 4°C oxygenated sucrose-based ACSF before slicing with a Dosaka Linear Slicer. Slices were transferred to 30°C oxygenated ACSF (128 mM NaCl, 10 mM D-glucose, 24 mM NaHCO3, 2 mM CaCl2, 2 mM MgSO4, 3 mM KCl, 1.25 mM NaH2PO4; pH 7.4). For recordings, slices were placed in a chamber on the stage of an Olympus BX50WI microscope. Oxygenated ACSF at room temperature flowed over the slice at 3-4 ml/minute.

4.3.1 Electrophysiology

Pipettes (3 MΩ) containing 120 mM K-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 6 pyramidal neurons in prelimbic cortex (Paxinos and Franklin, 2001). These neurons were selected based on their proximity to white matter (< 200 µm; Alves et al., 2010) together with known morphological distinguishing features of layer 6 (on average, the pyramidal neurons are smaller and closer together than in layer 5). Neurons
were recorded in current clamp using an EPC10 (HEKA) and corrected for the liquid junction potential.

Across the genotypes, there were no significant differences in resting potential ($F_{2,177} = 0.43, P = 0.6$), input resistance ($F_{2,177} = 1.2, P = 0.3$), or spike amplitude ($F_{2,177} = 1.6, P = 0.2$). Recordings were made either at resting membrane potential or with current injected to elicit a steady ~1 Hz action potential firing at baseline. ACh-induced depolarizations were measured relative to the resting potential. Changes in action potential frequency were determined using Clampfit software (Molecular Devices) by comparing the rate of firing over a 30 second period during the peak of the ACh response to that at baseline. As a control experiment, a pharmacologically-identified subgroup of layer 6 pyramidal neurons was selected based on their response to hypocretin (Bayer et al., 2004). There were no significant differences in cell properties between neurons responsive to hypocretin and the general population of layer 6 pyramidal neurons.

### 4.3.2 Pharmacology

We probed cholinergic currents by applying 1 mM ACh to the bath after a period of baseline recording. Either atropine (200 nM), or a combination of DHβE (3 µM) and MLA (10 nM) were applied in ACSF to examine nicotinic and muscarinic effects respectively. No antagonists were used in cholinergic experiments examining combined nicotinic and muscarinic effects. The subset of experiments testing responses to hypocretin 2 (100 – 300 nM), in addition to ACh, were conducted at 32°C (Bayer et al., 2004). All compounds were obtained from Sigma or Tocris.
4.3.3 Statistical analysis

Effects of ACh on membrane potential, rate of action potential firing, and the kinetics of these responses were assessed for all genotypes with one-way ANOVAs, as were comparisons of cell properties across genotypes. We used Fisher’s exact tests to evaluate genotype differences in the proportions of neurons depolarized to threshold by ACh. Unpaired $t$ tests were used to compare components of the ACh response. The effect of atropine on the muscarinic response was analyzed by paired $t$ test. The interaction between nicotinic and muscarinic effects across genotypes was analyzed by two-way ANOVA, and the difference within each genotype was assessed with Bonferroni post hoc tests.

4.4 Results

4.4.1 Nicotinic excitability is reduced in $\alpha5^{-/-}$ mice and eliminated in $\beta2^{-/-}$ mice

First, we examined the nicotinic depolarization of layer 6 pyramidal neurons in WT, $\alpha5^{-/-}$, and $\beta2^{-/-}$ mice since these subunits are known to be the principal constituents of the nicotinic receptors expressed in this layer along with the $\alpha4$ subunit. In the presence of atropine (200 nM) to block muscarinic receptors, stimulation with ACh (1 mM, 15 s) resulted in significantly different depolarization across the genotypes ($F(2,49) = 47.89$, $P = 0.0001$). As illustrated in Figure 4.1, ACh depolarized layer 6 neurons in WT mice to the greatest extent ($n = 24$), depolarized $\alpha5^{-/-}$ neurons to a lesser degree ($n = 21$), and did not alter the membrane potential in $\beta2^{-/-}$ neurons ($n = 7$). Nicotinic excitation was sufficient to elicit action potentials in the majority of WT neurons, fewer $\alpha5^{-/-}$ neurons ($P < 0.05$), and none of the $\beta2^{-/-}$ neurons. These differences are likely not due to differences in electrophysiological properties between neurons of these mice. There were no significant differences in resting potential ($F_{2,177} = 0.43$, $P = 0.6$), input resistance ($F_{2,177} = 1.2$, $P = 0.3$), or spike amplitude ($F_{2,177} = 1.6$, $P = 0.2$).
To examine nicotinic effects on the excitability of already-depolarized neurons, we injected neurons with positive current to elicit action potentials (~ 1 Hz) at baseline. The current required did not differ significantly across genotypes, consistent with the lack of significant difference in input resistance. As illustrated in Figure 4.1C, ACh increased the frequency of action potential firing in a genotype-dependent manner ($F_{(2,31)} = 11.78, P = 0.0002$). This treatment resulted in a large increase in the firing frequency of WT (n = 15) and $\alpha5^{-/-}$ neurons (n = 14) but not $\beta2^{-/-}$ neurons (n = 5).
Figure 4.1 Nicotinic excitability of layer 6 pyramidal neurons is reduced in α5−/− and eliminated in β2−/− mice. A) Stimulation of only nicotinic receptors following blockade of muscarinic receptors by atropine (200 nM) resulted in significant differences in depolarization across all genotypes (P < 0.0001), with markedly less depolarization of α5−/− and β2−/− compared to wild type (WT) neurons (***P < 0.001). B) Sample traces show nicotinic responses in neurons across all genotypes, including the percentages of neurons with suprathreshold (top) and subthreshold (bottom) responses. A smaller proportion of neurons were depolarized to threshold in α5−/− and β2−/− neurons (α5−/−: P < 0.05, β2−/−: P < 0.01) than in WT. C) In neurons already firing action potentials by current injection, nicotinic receptor stimulation affects spiking frequency differently across genotypes (P < 0.0001), with a smaller change in action potential frequency in α5−/− and β2−/− compared to WT neurons (*P < 0.05, **P < 0.001). D) Sample traces show nicotinic responses in neurons depolarized to fire action potentials by current injection. Examples (2 seconds in duration) of baseline and peak responses are shown above each trace.
4.4.2 Cholinergic excitation of layer 6 cortical neurons primarily involves nicotinic receptors unless nicotinic drive is impaired

The striking differences across genotypes in the response to nicotinic receptor stimulation raised the question of how layer 6 neurons normally respond to ACh in the absence of atropine, when it can stimulate both nicotinic and muscarinic receptors together. This experiment is shown in Figure 4.2 (we use ‘cholinergic’ to identify conditions where both nicotinic and muscarinic receptors are stimulated). In layer 6 neurons from WT mice, we find that cholinergic depolarization does not differ greatly from nicotinic depolarization alone (cholinergic: 20.3 ± 1.3 mV, n = 23; nicotinic: 19.2 ± 1.0 mV, n = 24; t = 0.7, P = 0.5). By contrast, layer 6 neurons from α5\(^{-/-}\) mice show greater depolarization when both nicotinic and muscarinic receptors are stimulated (cholinergic: 15.3 ± 1.1 mV, n = 26; nicotinic: 11.9 ± 1.1 mV, n = 21; t = 2.2, P = 0.03). More strikingly in β2\(^{-/-}\) mice, the muscarinic component appears dominant since the response to nicotinic receptor stimulation is negligible in comparison with the response to cholinergic stimulation (cholinergic: 4.3 ± 0.6 mV, n = 23; nicotinic: 0.1 ± 0.2 mV, n = 7; t = 3.8, P = 0.0005). Yet, there are significant differences in the magnitude of depolarization elicited by cholinergic stimulation across the genotypes (F(2,69) = 63.12, P = 0.0001). Compared to nicotinic receptor stimulation alone, cholinergic stimulation had a significantly greater effect on the ability to elicit action potentials from rest in neurons from α5\(^{-/-}\) mice, and was able to elicit spiking in some neurons from β2\(^{-/-}\) mice.

In contrast to the differences in nicotinic effects in layer 6 neurons across genotypes, cholinergic stimulation increased the frequency of action potential firing similarly across all three groups. As illustrated in Figure 4.2C, we observed no significant differences in the percent increase in action potential frequency at the peak of the cholinergic response between layer 6 neurons from WT (n = 22), α5\(^{-/-}\) (n = 17), and β2\(^{-/-}\) (n = 24) mice (F(2,60) = 0.67, P = 0.5). The
similar effects of ACh across genotypes on spike frequency cannot be attributed to a ceiling effect, since depolarizing current was consistently able to elicit faster spiking across the genotypes (paired t-test, \( n = 59, t = 20.7, P < 0.0001 \)).

Notably, the time course of cholinergic effects was genotype dependent, with slower onset and substantially longer currents in layer 6 neurons from \( \beta_2^{-/-} \) mice. The 10-90% rise times of cholinergic responses in \( \beta_2^{-/-} \) mice were slower for depolarization (WT: \( 13.3 \pm 1.1 \) s, \( \alpha_5^{-/-} \): \( 14.7 \pm 0.6 \) s, \( \beta_2^{-/-} \): \( 25.8 \pm 2.9 \) s; \( F_{(2,45)} = 10.1, P = 0.0002; P < 0.01 \) for WT versus \( \beta_2^{-/-} \)) and action potential frequency (WT: \( 14.6 \pm 0.8 \) s, \( \alpha_5^{-/-} \): \( 13.4 \pm 1.3 \) s, \( \beta_2^{-/-} \): \( 20.7 \pm 2.6 \) s; \( F_{(2,42)} = 4.1, P = 0.02 \)). Yet, the peak cholinergic effects lasted significantly longer in \( \beta_2^{-/-} \) neurons, as shown by the \( \tau \) (63% decay time) of depolarization (WT: \( 53.0 \pm 8.3 \) s, \( \alpha_5^{-/-} \): \( 53.4 \pm 9.2 \) s, \( \beta_2^{-/-} \): \( 257.3 \pm 22.8 \) s; \( F_{(2,45)} = 48.3, P < 0.0001; P < 0.001 \) for WT versus \( \beta_2^{-/-} \)) and action potential frequency (WT: \( 42.7 \pm 4.4 \) s, \( \alpha_5^{-/-} \): \( 56.1 \pm 10.8 \) s, \( \beta_2^{-/-} \): \( 111.6 \pm 25.5 \) s; \( F_{(2,38)} = 5.5, P < 0.01; P < 0.01 \) for WT versus \( \beta_2^{-/-} \)).
Figure 4.2 Cholinergic (nicotinic and muscarinic receptor) stimulation depolarizes wild type (WT) neurons most but increases spike frequency similarly across genotypes. A) Cholinergic stimulation depolarizes neurons to a different degree across genotypes ($P < 0.0001$), with less depolarization in $\alpha^5^-/-$ and $\beta^2^-/-$ compared to WT neurons ($* P < 0.01$, $** P < 0.001$). B) Sample traces showing cholinergic responses in neurons of all genotypes. C) Cholinergic stimulation increases action potential firing frequency to a similar degree across all genotypes ($P = 0.5$). D) Sample traces showing cholinergic responses in neurons of all genotypes depolarized to fire action potentials by current injection. B, D) Note the slower onset and prolonged peak response in $\beta^2^-/-$ neurons.
4.4.3 Muscarinic responses are enhanced in layer 6 of α5⁻/⁻ and β2⁻/⁻

The difference between responses to nicotinic-only and total cholinergic stimulation in layer 6 neurons of α5⁻/⁻ and β2⁻/⁻ mice suggests potential plasticity in muscarinic-only cholinergic effects. To address this question, we tested the effects of muscarinic-only stimulation using ACh in the presence of nicotinic blockers (DHβE 3 µM, MLA 10 nM), as well as antagonists for the AMPA and NMDA glutamate receptors (CNQX 10 µM, APV 50 µM) to assess whether functional upregulation of muscarinic currents occurred in layer 6 neurons lacking specific nicotinic receptor subtypes. Changes in membrane potential following muscarinic receptor stimulation were significantly different across the genotypes ($F(2,47) = 4.20$, $P = 0.02$), as illustrated in Figure 4.3. The depolarization elicited in layer 6 neurons from WT mice were small ($n = 17$) compared to the larger depolarization in α5⁻/⁻ ($n = 16$; $t = 2.6$, $P = 0.01$) and β2⁻/⁻ ($n = 17$) mice. The muscarinic antagonist atropine (200 nM, 10 min) suppressed these responses in all genotypes ($n = 11$, $t = 3.4$, $P = 0.006$). Furthermore, selective antagonists for the excitatory M1 and M3 muscarinic receptors (pirenzepine, 500 nM; J-104129 fumerate, 50 nM) suppressed the depolarization ($22 ± 17\%$ of that seen previously; $n = 5$).

There were also significant genotype differences in the increase in firing frequency elicited by muscarinic receptor stimulation ($F_{(2,46)} = 3.86$, $P = 0.03$). Muscarinic receptor stimulation increased peak action potential firing in layer 6 neurons from WT mice ($n = 14$), but this increase was significantly larger in layer 6 neurons from both α5⁻/⁻ ($n = 16$; $t = 2.1$, $P = 0.04$) and β2⁻/⁻ ($n = 19$; $t = 3.3$, $P = 0.002$) mice. Again, these muscarinic responses were suppressed by atropine across the genotypes ($n = 7$, $t = 6.5$, $P = 0.0006$). Similarly, M1/M3 antagonists eliminated the increase in action potential firing ($-5 ± 6\%$ of that seen previously, $n = 7$).

With muscarinic-only stimulation, all three genotypes showed the longer 10-90% rise times and 63% decays characteristic of responses mediated only by G-protein-coupled receptors. These
results contrast with the kinetics of the cholinergic responses where the fast, nicotinic responses dominated in the wildtype and α5⁻/⁻ mice, and the slow, muscarinic response dominated in the β2⁻/⁻ mice.

4.4.4 Hypocretin-responsive neurons show same pattern of muscarinic responses

The differences in the magnitude of the muscarinic responses across the genotypes raises the question of whether the layer 6 neurons in the mice deleted for nicotinic subunits are indeed the same type of neurons as in WT mice. To address this question, we examined a pharmacologically-identified subgroup of neurons within layer 6 of cortex (Bayer et al., 2004). In these neurons excited by hypocretin (100-300 nM, 1 min), which were recorded at 32°C (based on Bayer et al., 2004), muscarinic responses showed the same pattern across genotypes as in the previous room temperature recordings of the general population of layer 6 neurons (change in membrane potential: WT, 2.5 ± 0.6 mV, n = 9; α5⁻/⁻, 8.7 ± 2.3 mV, n = 9; β2⁻/⁻, 7.9 ± 1.4 mV, n = 13; F(2,28) = 4.2, P = 0.02); change in spiking frequency: WT, 341 ± 94 %, n = 9; α5⁻/⁻, 763 ± 160 %, n = 9; β2⁻/⁻, 651 ± 113 %, n = 10; F(2,25) = 2.9, P = 0.07). These findings are consistent with the interpretation that layer 6 neurons, which normally display large nicotinic responses to ACh, upregulate their muscarinic responses after loss or reduction of nicotinic excitation. To ascertain the specificity of this M1/M3 muscarinic upregulation, we compared the response to hypocretin across the genotypes since it is also mediated by a Gαq-coupled receptor. However, these responses did not differ by genotype (F(2,82) = 0.85, P = 0.4).
Figure 4.3 Muscarinic responses are enhanced in α5⁻/⁻ and β2⁻/⁻ compared to wild type (WT) neurons. A) Stimulation of only muscarinic receptors on layer 6 neurons following blockade of nicotinic receptors (3 μM DHBE, 10 nM MLA) and glutamate receptors (10 μM CNQX, 50 μM APV) resulted in significant differences in depolarization across genotypes ($P < 0.05$), with greater muscarinic depolarization seen in α5⁻/⁻ than WT neurons (*$P < 0.05$). B) Sample traces show the muscarinic response in neurons from all genotypes. No WT neurons, but some α5⁻/⁻ and β2⁻/⁻ neurons are depolarized to threshold. C) Muscarinic receptor stimulation increases action potential firing differently across the genotypes ($P < 0.001$), with action potential frequency increasing to a greater degree in β2⁻/⁻ compared to WT neurons (*$P < 0.05$). D) Sample traces showing muscarinic responses in neurons depolarized to fire action potentials by current injection.
4.4.5 The balance of muscarinic to nicotinic excitation differs across genotypes

Differences in the responses to nicotinic and muscarinic receptor stimulation across genotypes are illustrated in Figure 4.4. Analysis of changes in membrane potential shows an interaction between the responses to nicotinic and muscarinic receptor stimulation ($F_{(2,96)} = 41.75, P = 0.0001$) (Figure 4.4A), with the genotypes varying greatly in the ratio of the muscarinic to the nicotinic responses (WT: 0.2, $\alpha_5^{-/-}$: 0.6, $\beta_2^{-/-}$: 61). As illustrated in Figure 4.4B, we observed a similar interaction in the increase in action potential frequency due to nicotinic and muscarinic receptor stimulation ($F_{(2,77)} = 15.4, P = 0.0001$), with the genotypes varying substantially in the ratio of the muscarinic to the nicotinic response (WT: 0.5, $\alpha_5^{-/-}$: 1.3, $\beta_2^{-/-}$: 4.1). There appears to be a minor contribution of muscarinic receptors in WT mice, a balance between nicotinic and muscarinic contributions in $\alpha_5^{-/-}$, and a predominant contribution of muscarinic receptors in $\beta_2^{-/-}$ mice.
Figure 4.4 The balance of nicotinic to muscarinic excitation is shifted in α5⁻/⁻ and β2⁻/⁻ compared to wild type (WT) neurons. A) There is a significant interaction in the degree of nicotinic and muscarinic depolarization across genotypes ($P < 0.0001$). Nicotinic receptor stimulation contributes more to membrane depolarization in WT and α5⁻/⁻ neurons (*$P < 0.01$). B) A significant interaction is found in the increase in action potential firing by nicotinic versus muscarinic receptor stimulation across genotypes ($P < 0.0001$). Nicotinic receptor stimulation increases spiking frequency to a greater degree in WT neurons, while muscarinic receptor stimulation makes a larger contribution in β2⁻/⁻ neurons (*$P < 0.01$, **$P < 0.001$).


4.5 Discussion

We have found that ACh predominantly excites layer 6 pyramidal neurons in WT mice through nicotinic receptors. Impairment in nicotinic receptor stimulation through genetic deletion of either the conductance-enhancing α5 subunit or the ligand-binding β2 subunit is accompanied by an increase in the cholinergic excitation of these neurons through the metabotropic muscarinic family of ACh receptors. This muscarinic excitation is suppressed by antagonists of M1 and M3 receptors and significantly alters the timing of the peak cholinergic response in mice deleted for the ligand-binding β2 subunit. Our results suggest that disrupting nicotinic receptor function can fundamentally alter the mechanisms and timing of excitation in prefrontal attentional circuitry. Humans and rodents with aberrant expression or function of nicotinic receptors are at higher risk for attention deficits (Rigbi et al., 2008; Bailey et al., 2010; Guillem et al., 2011). Such changes in nicotinic receptors can result from genetic polymorphisms which reduce function (Bierut et al., 2008; Kuryatov et al., 2011) or through developmental exposure to the drug nicotine (Poorthuis et al., 2009). The observed upregulation of the typically-smaller muscarinic component of cholinergic activation in neurons of α5−/− and β2−/− mice allows a substantial response to ACh despite the reduction or loss of nicotinic receptor function, and highlights the functional significance of nicotinic signaling within attention pathways. Our results suggest that humans with reduced prefrontal nicotinic receptor function may have a larger muscarinic contribution towards the overall cholinergic response in layer 6 neurons. This plasticity of cholinergic signaling may, in fact, reduce the apparent severity of the attention deficits resulting from genetic or developmental alterations in nicotinic receptors. Indeed, in mice performing an attention task, α5−/− mice show only significantly lower accuracy compared to wildtype controls under challenging conditions (Bailey et al., 2010), while β2−/− mice demonstrate only a higher
level of omissions but no decreases in accuracy (Guillem et al., 2011). Yet, having an atypical muscarinic component, which is slower and longer than the normal nicotinic excitation, may result in more complex changes to prefrontal attention circuitry and function than previously anticipated.

Our results indicate that synergistic effects of prefrontal nicotinic and muscarinic receptors on attentional performance (Ellis et al., 2006) do not normally reflect shared activation of layer 6 neurons. Instead, these receptors may act predominantly on different cortical output layers. In mice with genetically-induced nicotinic dysfunction, we observed increased excitatory muscarinic responses in layer 6 neurons that act in a homeostatic manner to preserve their cholinergic response. However, our results suggest that this atypical muscarinic excitability of layer 6 neurons is not uniformly upregulated. The ACh depolarization from rest is only partially rescued, yet the increase in action potential frequency of already-depolarized neurons appears normal. Since cortical muscarinic receptor binding is not altered in β2⁻/⁻ mice (Zoli et al., 1999), the differential rescue of two aspects of cortical excitability suggests a locus of plasticity downstream of the muscarinic receptors.

The medial prefrontal cortex and its cholinergic afferents are essential for efficient attentional processing. Here, we show that the cholinergic excitation of layer 6 neurons is of sufficient functional importance that compensatory processes provide this excitation even in the absence of the nicotinic receptors normally utilized in wild-type mice. While the upregulation of muscarinic excitability may ameliorate the severity of attention deficits resulting from alterations in nicotinic receptors, it would likely alter the speed of attentional processing and render layer 6 attention circuitry vulnerable to the effects of anti-muscarinic medications.
5 Serotonergic suppression of mouse prefrontal circuits implicated in task attention

Acknowledgements and Contributions

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5.1 Abstract

Serotonin (5-HT) regulates attention by neurobiological mechanisms that are not well understood. Layer 6 (L6) pyramidal neurons of prefrontal cortex play an important role in attention and express 5-HT receptors, but the serotonergic modulation of this layer and its excitatory output is not well understood. Here, we performed whole-cell recordings and pharmacological manipulations in acute brain slices from wildtype and transgenic mice expressing either eGFP or eGFP-channelrhodopsin in prefrontal L6 pyramidal neurons. Excitatory circuits between L6 pyramidal neurons and L5 GABAergic interneurons, including a population of interneurons essential for task attention, were investigated using optogenetic techniques. Our experiments show that prefrontal L6 pyramidal neurons are subject to strong serotonergic inhibition and demonstrate direct 5-HT-sensitive connections between prefrontal L6 pyramidal neurons and two classes of L5 interneurons. This work helps to build a neurobiological framework to appreciate serotonergic disruption of task attention and yields insight into the disruptions of attention observed in psychiatric disorders with altered 5-HT receptors and signaling.
5.2 Introduction

The medial prefrontal cortex (mPFC) is critical for “top-down” executive control of attention (Miller and Cohen, 2001; Knudsen, 2007), and disruption of its signaling impairs normal performance on attention tasks (Muir et al., 1996; Miner et al., 1997; Granon et al., 1998). Layer 6 (L6) of mPFC, in particular, plays an important role in attention (Alitto and Usrey, 2003; West et al., 2006; Zikopoulos and Barbas, 2006; Kassam et al., 2008; Bailey et al., 2010; Guillem et al., 2011), and is a major source of cortico-thalamic output (Guillery and Sherman, 2002; Thomson et al., 2002; Mercer et al., 2005; Watts and Thomson, 2005; West et al., 2006; Zikopoulos and Barbas, 2006; Parikh et al., 2007; Sherman, 2007; Kassam et al., 2008; Bailey et al., 2010; Thomson, 2010). Much less, however, is known about the cortico-cortical collaterals of L6 pyramidal neurons in mPFC. This question is increasingly urgent in view of recent work from primary sensory cortex showing L6 pyramidal neurons send excitatory projections to fast-spiking cortical interneurons which achieve gain control over the cortical column (Olsen et al., 2012), together with recent work from mPFC showing that fast-spiking prefrontal interneurons are essential for attention (Kim et al., 2016). In mPFC, there remains much to be understood about the local targets of prefrontal L6 pyramidal neurons and the susceptibility of these attention circuits to neuromodulators, such as serotonin.

Serotonin is known to shape and bias attention in human and nonhuman primates, with reduction of brain 5-HT enhancing attention (Schmitt et al., 2000; Gallagher et al., 2003; Booij et al., 2005; Wingen et al., 2007) and elevation of brain 5-HT impairing attention task performance (Riedel et al., 2005; Oranje et al., 2008; Watson et al., 2015). Yet, the mechanisms underlying this relationship between 5-HT and attention are not well understood. Like primates, the deep layers of mPFC in rodents are well innervated by serotonin afferents (Wilson and Molliver,
1991; Linley et al., 2013; Goodfellow et al., 2014; Muzerelle et al., 2016). Therefore we can ask in a mouse model whether serotonin modulates prefrontal L6 pyramidal neurons known to play a role in task attention (Bailey et al., 2010; Guillem et al., 2011). In rodents, a substantial proportion of prefrontal L6 pyramidal neurons express 5-HT$_{1A}$ and/or 5-HT$_{2A}$ receptors (Chalmers and Watson, 1991; Pompeiano et al., 1992, 1994; Cornea-Hébert et al., 1999; Amargós-Bosch et al., 2004), with broad similarities in the expression of these 5-HT receptors in prefrontal L6 of human and nonhuman primates (de Almeida and Mengod, 2007; de Almeida and Mengod, 2008; Mengod et al., 2015). Co-expression of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors is common in mPFC pyramidal neurons (Amargós-Bosch et al., 2004) and is observed in 48% of pyramidal neurons in L6 of mouse mPFC (Table 3 in (Amargós-Bosch et al., 2004)). Roles for 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors in attentional performance have been suggested (Carli and Samanin, 2000; Koskinen et al., 2000; Winstanley et al., 2003; Wingen et al., 2007). Furthermore, the serotonergic system is dysregulated in several brain disorders that are accompanied by disruptions of attention, including autism (Chugani, 2002; Kane et al., 2012), schizophrenia (Luck and Gold, 2008), and mood disorders (Marvel and Paradiso, 2004; Jans et al., 2007; Murrough et al., 2011).

Here, we investigated how mPFC L6 pyramidal neurons are modulated by 5-HT, how L6 excitation affects the two major classes of L5 interneurons, and how 5-HT modulates these internal mPFC circuits. Our results reveal a robust 5-HT-elicited inhibition of L6 pyramidal neurons mediated by 5-HT$_{1A}$ receptors and a lesser, state-dependent, and somewhat unexpected, contribution by 5-HT$_{2A}$ receptors. We find that L6 pyramidal neurons robustly activate fast-spiking (FS) as well as non-fast-spiking (nFS) interneurons in L5. Lastly, we show that these intra-cortical circuits in mPFC are strongly suppressed by 5-HT.
5.3 Methods

5.3.1 Experimental Animals

We employed BAC transgenic Swiss Webster mice with expression of eGFP driven by the synaptotagmin 6 promoter (Syt6-EGFP EL71, MMRRC; RRID:MMRRC 010557-UCD) made by the GENSAT Project (Gong et al., 2003). L6 pyramidal neurons in mPFC express eGFP, and facilitates visual targeting of these neurons for recording (Tian et al., 2014). Syt6 mice were kept heterozygous and there were no significant differences in their 5-HT responses compared to their wildtype littermate controls or wildtype C57BL/6 mice ($F_{(2,64)} = 0.58, P = 0.56$, one-way ANOVA). To investigate the downstream synaptic connection of prefrontal L6 pyramidal neurons, we crossed GENSAT epiphycan BAC transgenic mice expressing cre-recombinase (Epyc-Cre KR363, a gift from Dr. Nathaniel Heintz at Rockefeller University; RRID:MMRRC_036145-UCD) with Ai:32 mice (Jackson Laboratories; RRID:IMSR_JAX:024109) to achieve eGFP-channelrhodopsin-2 expression in prefrontal L6 neurons (Epyc-ChR2). Wildtype littermates of the Epyc-ChR2 were used as controls to ensure that the UV light did not have effects in brain slices from mice lacking channelrhodopsin-2. Translating Ribosome Affinity Purification and quantitative RT-PCR were used to confirm that Syt6 and Epyc-cre neurons indeed represent an overlapping population of L6 glutamatergic neurons. All experimental animal procedures were performed in accordance with the University of Toronto and The Rockefeller University Institutional Animal Care and Use Committee’s regulations.

5.3.2 Electrophysiology

Coronal brain slices (400 µm) for electrophysiological recordings were obtained from adult male mice (postnatal 60 to 170 days; mean ± SEM; 101 ± 4 days; n = 41 mice). 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 (400 μm thick, 2.34 – 0.74 mm from Bregma) were cut on a Dosaka Linear Slicer (SciMedia, Costa Mesa CA) and recovered 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 hours.

Recovered slices were transferred to a perfusion chamber on the stage of a BX50W1 microscope (Olympus). ACSF was bubbled (95% O₂, 5% CO₂ at room temperature) and perfused the chamber at a rate of 3-4 ml/min. In addition to recording from L6 pyramidal neurons based on neuronal morphology and anatomical landmarks in wildtype mice, L6 in Syt6 mice was landmarked with fluorescently-identified eGFP-positive neurons (X-cite Series 120; Lumen Dynamics; Tian et al., 2014). Recording electrodes (2-4 MΩ) 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 L6 pyramidal neurons. Interneurons in L5 were identified visually based on their unique morphology in IR-DIC (small, circular somata) in contrast to L5 pyramidal neurons (oriented, triangular shaped somata, relatively thick apical dendrites towards pia). A subset of patched interneurons was filled with Alexa-594 (20 μM) or Texas red dextran (0.15%) in the patch solution for morphological confirmation of these criteria. Interneurons were further sub-classified as FS or nFS based on their electrophysiological spike pattern and maximal spike frequency. Multiphoton images were acquired with a Ti:sapphire laser (Mai Tai, Spectra Physics) using an Olympus Fluoview FV1000 microscope and an Olympus XLPlan N 25x water-immersion objective. Neuronal membrane potential and holding current were recorded
with an EPC10 (HEKA Electroniks), and corrected for the liquid junction potential (14 mV). All data were acquired at 20 kHz and low-pass filtered at 3 kHz with pClamp software (Molecular Devices). Threshold potential for action potentials were detected using a derivative threshold of at least 20 mV/ms, and action potential amplitude was calculated as the change in membrane potential from threshold to the peak of the action potential. Intrinsic properties of L6 pyramidal neurons, as well as L5 FS and nFS interneurons are summarized in Table 5.1.

To examine the effects of 5-HT on L6 pyramidal neurons near rest and during spiking, we performed whole cell patch clamp recording in voltage clamp at -75 mV and in current clamp with current injections to elicit either constant spiking (2-3 Hz) at baseline or an initial membrane potential of -75 mV before depolarizing current injections (1 sec, 25 pA steps, 15 s intervals) were used to assess input-output relationships. For the latter experiment, the frequency of action potential firing was measured for each depolarizing current step, and plotted against the magnitude of the injected current step.

### 5.3.3 Pharmacology

Acute responses to 5-HT were probed by bath application of 5-HT (serotonin creatinine sulphate, Sigma; 10 µM; 30 s) in ACSF. To examine the effect of 5-HT on the excitability of L6 pyramidal neurons, 5-HT (10 µM) was bath applied until a steady state response is reached, and remained in bath throughout the duration of the input-output test protocols (~2 min total application). Selective antagonists and agonist were from Tocris, except where mentioned. Antagonists for 5-HT<sub>1A</sub> receptors (30 nM WAY100635, 10 µM NAN-190) and 5-HT<sub>2A/C</sub> receptors (30 nM MDL100907; 2 µM ketanserin; 300 nM – 3 µM ritanserin) were applied in bath for 10 minutes before further experiments with 5-HT. There were no significant differences between effects of 300 nM and 3 µM ritanserin, and results were grouped for analysis. TCB-2
was used as a specific agonist of 5-HT$_{2A}$ receptors (300 nM – 1 µM). Other agonists and antagonists used for characterization of the 5-HT response in L6 neurons were as follows: 2 µM TTX (Alomone), 20 µM CNQX, 50 µM D-APV, 100 µM picrotoxin, CGP52432 1 µM, and 8-OH-DPAT 10 µM.

### 5.3.4 Optogenetic stimulation

Channelrhodopsin-expressing neurons in *Epyc*-ChR2 mice were stimulated by blue LED light (473 nm) delivered by optic fiber (Thorlabs) mounted on a mechanical micromanipulator (Narishige). Light stimulation was directed directly to L6 of mPFC by targeted positioning of the optic fiber. Twenty light pulses (2-5 ms each) were delivered at 20 Hz to stimulate L6 neurons. This stimulation profile was sufficient to elicit robust activation of L6 pyramidal neurons expressing channelrhodopsin. In control experiments with brain slices from littermate mice lacking channelrhodopsin, light stimulation did not elicit a response in either L6 pyramidal neurons or L5 interneurons. Responses to light stimulation in L6 pyramidal neurons and L5 interneurons were measured in current-clamp from a baseline membrane potential of -75 mV held by continuous injection of depolarizing current. Response latency in L6 pyramidal neurons expressing channelrhodopsin was calculated from the time of light-on to the onset of the corresponding membrane potential change. Time-to-spike for L6 neurons from light-on was also calculated using the peak of the first resulting action potential. In L5 interneurons, the latency to response from L6 activation was calculated in voltage clamp as the time taken from light-on to the onset of the post-synaptic current, then corrected by the time-to-spike in L6 pyramidal neurons. Pairwise analysis of the effects of 5-HT on the excitation of L5 interneurons by optogenetic activation of L6 were performed using light stimulus that was able to elicit at least 4 action potentials in patched L5 interneurons. Light stimulus intensity to elicit a baseline of at
least 4 action potentials did not differ between FS and nFS interneurons ($t_{14} = 1.4, P = 0.18$, unpaired $t$-test).

5.3.5 Statistical analysis

All recordings were analyzed using Clampfit software (Molecular Devices). Statistical analyses were performed with GraphPad Prism (GraphPad Software). Analyses performed were: one-sample $t$-test, unpaired Student’s $t$-test, paired Student’s $t$-test, one-way ANOVA, two-way ANOVA, two-way repeated measures ANOVA. All tests were two-sided. Dunnett’s multiple comparison tests were performed post hoc to compare changes in action potential firing in L6 neurons elicited by 5-HT. Sidak’s multiple comparison tests were used to compare differences in spike frequency at individual injected current steps in the presence of 5-HT. All data are presented as mean ± SEM.

5.3.6 Translating Ribosome Affinity Purification and Quantitative RT-PCR

Translating ribosome affinity purification and quantitative RT-PCR experiments were performed by Dr. Eric Schmidt at Rockefeller University. Detailed methods are outlined in Chapter 7.1 Supplementary Methods.
5.4 Results

5.4.1 Serotonin robustly inhibits L6 pyramidal neurons of mPFC

Here, we investigated the electrophysiological consequences of 5-HT on pyramidal neurons in L6 of mPFC. Experiments in voltage clamp showed robust and replicable outward currents (58.3 ± 6.4 pA, n = 28, Figure 5.1A and B) in response to bath application of 5-HT (10 µM, 30 s). These 5-HT-elicited currents were dose-dependent, with an EC50 of 5.7 ± 0.1 µM (n = 7, r² = 0.9). The lack of significant change in these responses to blocking voltage-gated sodium channels with TTX (2 µM, 10 minutes in bath; t₃ = 1.2, P = 0.3, n = 4, paired t-test), to blocking AMPA, NMDA, and GABA-A receptors (CNQX [20 µM], APV [50 µM], picrotoxin [100 µM], t₆ = 0.5, P = 0.6, n = 7, paired t-test, Figure 5.1A and D), as well as to blocking these synaptic receptors together with GABA-B blockade with CGP52432 (10 µM) (t₃ = 0.27, P = 0.8, n = 4, paired t-test) suggest direct mediation by 5-HT receptors on L6 pyramidal neurons themselves.

We found that the specific 5-HT₁A antagonist WAY100635 (30 nM, 10 minutes in bath) significantly reduced the 5-HT mediated current in L6 pyramidal neurons (~70% reduction to 18.3 ± 2.6 pA, t₁₈ = 4.9, P < 0.0001, n = 19, unpaired t-test, Figure 5.1B, C, and D). The 5-HT₁A agonist 8-OH-DPAT (10 µM) elicited outward currents of similar magnitude to the 5-HT current in L6 pyramidal neurons (5-HT: 61.2 ± 12.8 pA; 8-OH-DPAT: 48.5 ± 11.1 pA, t₅ = 1.6, P = 0.2, n = 6, paired t-test).
Figure 5.1 Serotonin (5-HT) inhibits L6 pyramidal neurons of medial prefrontal cortex. Responses to 5-HT were probed in voltage-clamp and current-clamp by bath application of 5-HT. Representative voltage-clamp traces of the 5-HT response in L6 pyramidal neurons show that responses to 5-HT are: A) stable and persist in the presence of synaptic blockers, B) significantly suppressed by WAY100635, and C) similarly suppressed by a combination of WAY100635 and synaptic blockers. D) 5-HT elicits strong outward currents on L6 pyramidal neurons of mPFC (n = 28) that can be pharmacologically modulated (F(2, 53) = 11.8, P < 0.0001, one-way ANOVA). Post hoc analyses show that these currents persist in the presence of synaptic blockers (q = 0.2, P > 0.05, n = 7, Dunnett’s Multiple Comparison Test), but are significantly suppressed by WAY100635 (q = 4.6, P < 0.0001, n = 19). Responses to 5-HT were probed in current-clamp in L6 pyramidal neurons of medial prefrontal cortex with current injection to elicit steady firing (~2-3 Hz) at baseline. Representative current-clamp traces show that responses to 5-HT are: E) inhibitory, repeatable, and unaffected by synaptic blockers, F) not
fully blocked by WAY100635, nor with G) WAY100635 and synaptic blockers. H) L6 pyramidal neurons of mPFC are strongly hyperpolarized by 5-HT (n = 26). Post hoc analyses of pharmacological effects on this hyperpolarization (F(2, 50) = 17, P < 0.0001, one-way ANOVA) show that this inhibition is unaffected by synaptic blockers (q = 0.8, P > 0.5, n = 6, Dunnett’s Multiple Comparison Test), but greatly reduced by WAY100635 (q = 5.4, P < 0.0001, n = 21). I) Action potential firing was significantly affected by 5-HT (F(4, 103) = 37, P < 0.0001, one-way ANOVA). Post hoc analyses reveal that baseline firing was suppressed by 5-HT (q = 9, P < 0.0001, n = 17, Dunnett’s Multiple Comparison Test), remain suppressed by 5-HT in synaptic blockers (q = 6.1, P < 0.0001, n = 6). The suppression is not blocked by WAY100635 (q = 8.8, P < 0.0001, n = 16), and returns to baseline levels following washout of 5-HT (q = 2.3, P > 0.05, n = 36).

To investigate the functional effects of 5-HT on L6 pyramidal neurons during excitation, we used current-clamp and bath applied 5-HT in the presence of injected positive depolarizing current sufficient to elicit action potential firing (2-3 Hz). Under these conditions, 5-HT hyperpolarized L6 neurons (-16.3 ± 1.6 mV, n = 17) and fully and significantly inhibited action potential firing in every recorded neuron (t7 = 13.5, P < 0.0001, n = 8, paired t-test) (Figure 5.1E, H, and I). This suppression was repeatable in the same neuron after washout, and was not affected by the presence of synaptic blockers (Figure 5.1E, H and I). Antagonism of 5-HT1A receptors by WAY100635 significantly reduced the 5-HT mediated hyperpolarization (-7.8 ± 0.7 mV, t18 = 5.0, P < 0.0001, n = 19, unpaired t-test; Figure 5.1F - I). Unexpectedly, however, 5-HT still robustly and significantly inhibited action potential firing in every neuron (t13 = 12, P < 0.0001, n = 14, paired t-test) (Figure 1F-I). This significant suppression of L6 spiking by 5-HT was also observed in the presence of synaptic blockers (t3 = 4.8, P = 0.02, n = 4, paired t-test). These data show a robust and repeatable 5-HT inhibition of L6 neurons by 5-HT with a component mediated by 5-HT1A receptors. However, the continued suppression of action potential firing by 5-HT after blockade of 5-HT1A receptors suggests the involvement of an additional 5-HT-mediated mechanism for inhibition of mPFC L6 pyramidal neurons.
5.4.2 Serotonergic 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors co-operate in inhibiting L6 pyramidal neurons

To interrogate this unidentified component of 5-HT inhibition of L6 pyramidal neurons, action potentials were elicited by a series of incremental square depolarizing pulses before and during 5-HT application. Despite the stronger activation, 5-HT still significantly reduced spike frequency at each injected current step, as shown by the significant right-shift in the input-output curve (inhibitory effect of 5-HT: $F_{(1, 168)} = 31, P < 0.0001$, n = 22, repeated measures two-way ANOVA, Figure 5.2A). Post hoc tests show that significantly fewer action potentials were elicited by input current in 5-HT compared to baseline at every step ($P < 0.05$, Sidak’s multiple comparisons test). Of note, the 25 pA step was supra-threshold for 17/22 neurons at baseline and only for 3/22 neurons in 5-HT ($P < 0.0001$, Fisher’s exact test). Spiking was significantly restored following a 5 minute washout of 5-HT ($F_{(1, 168)} = 28, P < 0.0001$, repeated measures two-way ANOVA). Activation of 5-HT$_{1A}$ receptors by 8-OH-DPAT also significantly suppressed firing of L6 neurons (50 pA current injection, baseline: 4.0 ± 1.1 Hz; 8-OH-DPAT: 0.8 ± 0.6 Hz; $t_5 = 5.3, P = 0.003$, n = 6, paired t-test), as did 8-OH-DPAT across a range of depolarizing steps, ($F_{(5,12)} = 35.7, P < 0.0001$, two-way repeated measures ANOVA, data not shown).

Yet, further experiments suggest that 5-HT recruits an additional receptor beyond 5-HT$_{1A}$ to inhibit the excitability of L6 pyramidal neurons. Significant 5-HT suppression of L6 neuronal excitability continued after antagonism of 5-HT$_{1A}$ receptors by WAY100635, with a significantly right-shifted input-output (inhibitory effect of 5-HT in WAY100635: $F_{(1, 72)} = 72, P < 0.0001$, n = 10, repeated measures two-way ANOVA, Figure 5.2B). Consistent with our above data, this result suggests the participation of at least one additional subtype of 5-HT receptor in inhibiting L6 pyramidal neurons. The 5-HT$_{2A}$ receptors that are co-expressed with 5-HT$_{1A}$ receptors in
48% of L6 pyramidal neurons in mouse mPFC (Table 3 in (Amargós-Bosch et al., 2004) are an unusual candidate to underlie the 5-HT-mediated supra-threshold suppression of spiking. These receptors typically recruit excitatory effectors (Lambe and Aghajanian, 2001; Zhang and Arsenault, 2005; Weisstaub et al., 2006; Benekareddy et al., 2010; Weber and Andrade, 2010; Avesar and Gulledge, 2012); although previous work has demonstrated the capacity of serotonin and 5-HT2A agonists to exert direct inhibitory effects through 5-HT2A receptors or heteromers (Carr et al., 2002; Kurrasch-Orbaugh et al., 2003; González-Maeso et al., 2007; Moreno et al., 2011). We found that adding the selective 5-HT2A receptor antagonist MDL100907 abolished the remaining inhibitory effects elicited by 5-HT on the input-output of L6 neurons (no significant effects of 5-HT in WAY100635 and MDL100907: F(1,32) = 0.8, P = 0.4, n = 5, repeated measures two-way ANOVA, Figure 5.2C). A similar blockade of the inhibitory effects of 5-HT on L6 neurons was also seen when other 5-HT2A antagonists were applied together with WAY100635, such as ketanserin (2 µM) or ritanserin (300 nM - 1 µM) (F(1,48) = 0.3, P = 0.6, n = 7, repeated measures two-way ANOVA).

To probe further the power of 5-HT2A receptors to inhibit L6 pyramidal neurons in mPFC, we applied a potent 5-HT2A agonist, TCB-2 (300 nM - 1µM). Here, we observed a robust inhibition of L6 neuronal excitability, with a significant right-shift of the input-output relationship (inhibitory effect of TCB-2: F(1,80) = 24, P < 0.0001, n = 11, repeated measures two-way ANOVA, Figure 5.2D). Pre-treatment with MDL100907 abolished the inhibitory effect of TCB-2 (F(1,16) = 1.2, P = 0.3, n = 3, repeated measure two-way ANOVA). Taken together, our results suggest that 5-HT inhibition of mPFC L6 pyramidal neurons is mediated by a combination of 5-HT1A and 5-HT2A receptors acting in concert. However, substantial future work
will be needed to elucidate the mechanisms by which these receptors can work individually and cooperatively to suppress the excitability of L6 pyramidal neurons.

Figure 5.2 Combined activation of serotonergic 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors mediate inhibition of L6 neuronal excitability at suprathreshold potentials. Incremental current steps were injected into patched L6 pyramidal neurons and their output in firing frequency was measured. Shown are representative recordings of the response to a 150 pA current step in single L6 pyramidal neurons (left), and the response to the same 150 pA current step in the presence of 5-HT (middle). The input-output relationship for each group is plotted (right). A) The input-output relationship of L6 pyramidal neurons is significantly right-shifted by 5-HT (F$_{1,}$
Post hoc analysis showed significantly fewer elicited action potentials at every input step ($P < 0.05$, Sidak’s multiple comparisons test). B) L6 excitability is significantly suppressed by 5-HT in the presence of WAY100635 ($P < 0.0001$, $F_{(1, 72)} = 72$, repeated measures two-way ANOVA), an effect especially prominent at higher input steps ($125 – 200$ pA steps, $P < 0.05$, Sidak’s multiple comparisons test). C) L6 suppression by 5-HT is fully blocked by simultaneous blockade of both 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors by specific antagonists WAY100635 and MDL100907 ($F_{(1,32)} = 0.8$, $P = 0.4$, repeated measures two-way ANOVA). D) TCB-2, a selective 5-HT$_{2A}$ receptor agonist, inhibits L6 neuronal firing ($F_{(1,80)} = 24$, $P < 0.0001$, repeated measures two-way ANOVA), also more prominently at higher input steps ($125 – 200$ pA steps, $P < 0.05$, Sidak’s multiple comparisons test).

5.4.3 Transgenic mouse for examining the effect of L6 activation on L5 interneurons

It has been shown that L6 pyramidal neurons in primary sensory cortex exert robust gain modulation over superficial layers of the cortical column (Olsen et al., 2012) through connections to FS interneurons (Vélez-Fort and Margrie, 2012). In mPFC, recent work has demonstrated the importance of fast-spiking interneurons in L5 for performance on attention tasks (Kim et al., 2016). To investigate the effects of prefrontal L6 activation on its targets in the cortical column, we utilized the Epyc-Cre BAC transgenic mice that target Cre recombinase to L6 cells in mPFC, then generated Epyc-cre;Ai:32 mice (Epyc-ChR2) to obtain expression of channelrhodopsin in those cells. Figures 5.3A and B show a similar distribution of L6 cells labeled by Syt6-eGFP fluorescence, used in the initial electrophysiology experiments, and by Epyc-cre, used for the optogenetic experiments. Due to the lack of reliable histological markers for L6 pyramidal neurons in mPFC, the translating ribosome affinity purification (TRAP) technique (Doyle et al., 2008; Heiman et al., 2008) was used to interrogate the identity of the Epyc-cre cells. An adeno-associated virus (AAV) vector (AAV-FLEX-EGFPL10a) to express EGFP-tagged ribosomal protein L10a (EGFPL10a) in a Cre-dependent manner was injected into the mPFC of Epyc-Cre mice and anti-EGFP immunoprecipitations (IPs) were performed to
isolate tagged polysomes. Bound mRNAs were then purified and analyzed by quantitative RT-PCR (qPCR). These data are plotted in Figure 5.3C. There was a significant enrichment for the excitatory neuron marker, Slc17a7 (VGluT1), in the Epyc TRAP IP when compared to whole PFC input. Two genes known to be expressed in L6 corticothalamic cells, Ntsr1 (Gong et al., 2007; Olsen et al., 2012; Mease et al., 2014) and Foxp2 (Ferland et al., 2003), were also significantly enriched in IP samples. In contrast, genes that label inhibitory interneurons (Gad1), astrocytes (Aldh1l1), or oligodendrocytes (Cnp) were significantly depleted from the IPs. Taken together, these data suggest that Epyc-Cre labels a population of L6 corticothalamic pyramidal cells. Importantly, the qPCR also revealed that Syt6 was highly enriched in the Epyc cells, demonstrating that the Syt6-eGFP and Epyc-Cre mice label an overlapping population of neurons. By contrast, levels of the housekeeping gene Gapdh were found to be similar between the TRAP IPs and whole PFC input. Similar results were obtained for the 5-HT receptors, Htr1a and Htr2a, suggesting these genes are expressed but not enriched in the Epyc cells, which was not surprising given the expression of 5-HT1A and 5-HT2A in other populations of neurons in mouse mPFC beyond L6 (Chalmers and Watson, 1991; Pompeiano et al., 1992, 1994; Cornea-Hébert et al., 1999; Amargós-Bosch et al., 2004).

5.4.4 Optogenetic activation of L6 pyramidal neurons is sensitive to serotonin

In electrophysiological experiments from Epyc-ChR2, we found that L6 pyramidal neurons, but not non-pyramidal neurons, were strongly depolarized upon light stimulation (473 nm, train of 2-ms duration pulses at 20 Hz for 1 s), which was targeted to L6 mPFC with optic fiber (Figure 5.4A, B). In contrast, prefrontal L6 neurons of littermate controls lacking channelrhodopsin did not respond to light stimulation. To verify that L6 pyramidal neurons were directly activated by light stimulation, we measured the kinetics of their light-evoked excitation.
L6 pyramidal neurons rapidly responded to light (< 1 ms latency to onset of excitation), consistent with direct activation through expressed channelrhodopsin (Ernst et al., 2008). This response rises to threshold, giving an action potential peak at 4.7 ± 0.7 ms (time-to-L6-spike; n = 5).

The channelrhodopsin-expressing L6 neurons from *Epyc*-ChR2 mice showed similar sensitivity to 5-HT as the *Syt6*-eGFP cells in the above experiments. Light-mediated excitation of *Epyc*-ChR2 L6 neurons was significantly suppressed in the presence of 5-HT (F(1,24) = 10.3, \( P < 0.004 \), repeated measures two-way ANOVA).

### 5.4.5 Optogenetic activation of L6 drives excitation of L5 interneurons

Since fast-spiking GABAergic neurons in mPFC are important to normal performance in attention tasks (Kim et al., 2016), we patched mPFC L5 interneurons in *Epyc*-ChR2 mice as potential downstream projection targets of L6 pyramidal neurons. We anticipated that light-mediated activation of L6 pyramidal neurons by targeted optic fiber would elicit postsynaptic responses in patched L5 interneurons. GABAergic interneurons were visually identified by their morphology and intrinsic properties, and their spiking patterns in response to depolarizing current steps were documented. This experimental protocol yielded two distinct populations of interneurons: FS cells with characteristic action potential firing >40 Hz and nFS cells which displayed low-threshold firing characteristics. The intrinsic properties of these neurons are illustrated in Table 5.1. A subset of patched interneurons (n = 6 FS interneurons, n = 5 nFS interneurons) was filled with Alexa-594 (20 µM) or Texas red dextran (0.15%) in the patch solution to verify their morphology. Filled FS (6/6) and nFS (5/5) interneurons were morphologically characteristic of the respective subtypes of interneurons in cortex (Markram et al., 2004; Ascoli et al., 2008).
Figure 5.3 Characterization of L6 neurons in medial prefrontal cortex (mPFC) expressing synaptotagmin 6 and epiphycan. A) Neurons expressing eGFP driven by the synaptotagmin-6 (Syt6) BAC promoter are localized to L6 pyramidal neurons in the prelimbic region of mPFC. B) EGFP is also seen in L6 pyramidal neurons in prelimbic mPFC by anti-EGFP immunohistochemistry in Epyc-Cre mice crossed to a Cre-dependent eGFP reporter. Image is adapted from www.gensat.org. C) Quantification (mean+SEM) by qRT-PCR of the expression of selected genes in mPFC Epyc-vTRAP IP samples compared to whole PFC input. Positive values indicate enrichment in the IP and negative values indicate depletion. Dotted lines indicate a 2-fold difference in either direction. Green bars represent genes that are >2-fold enriched in the Epyc cells, red bars represent genes that are >2-fold depleted, and gray bars are genes expressed at similar levels to the rest of PFC. Of note, mRNA for serotonin receptors Htr1a and Htr2a were expressed, but not enriched in Epyc cells, a not unexpected finding given the expression of 5-HT\textsubscript{1A} and 5-HT\textsubscript{2A} in other populations of neurons in mouse mPFC beyond L6. *P < 0.05; **P < 0.01 by Student’s t-test.

Note: Figure 5.3B was adapted from GENSAT website. Dr. Eric Schmidt performed the experiments in Figure 5.3C.
Figure 5.4 Optogenetic activation of L6 pyramidal neurons of medial prefrontal cortex excites L5 interneurons. A) Schematic representation of light activation of L6 pyramidal neurons of medial prefrontal cortex in Epyc-ChR2 mice with axons projecting to L5 interneurons. B) Channelrhodopsin-expressing pyramidal L6 neurons were robustly excited by targeted light stimulation over L6. The effects of increasing L6 light power are shown for one example L6 pyramidal neuron. C) Light activation of L6 robustly excited L5 interneurons. The effects of increasing L6 light power are shown for three different L5 interneurons. D) Top, close-up of the initial light-evoked action potential in L6 to show the timing from onset of light (blue dotted line) to peak of the spike (black dotted line). Scale bar: 20 mV, 1 ms. Note: in L6 the onset of depolarization from light is < 1 ms. Bottom, voltage-clamp recording showing the light-evoked postsynaptic response in a L5 interneuron to demonstrate response latency. Scale bar: 40 pA, 1 ms. Post-synaptic responses in L5 interneurons were initiated 1.1 ± 0.3 ms following initial spike of L6 pyramidal neurons, as indicated by an arrow.
Table 5.1 Intrinsic electrophysiological properties of three groups of neurons recorded: pyramidal neurons in L6, fast-spiking (FS) interneurons in L5, and non-fast-spiking (nFS) interneurons in L5. Neuronal properties shown are: resting membrane potential (RMP), input resistance, spike amplitude, and peak firing frequency upon injection of a maximal suprathreshold current. Data are shown as mean ± SEM. Comparisons between L5 FS and nFS interneurons: *P < 0.05, **P < 0.001 by unpaired t-tests.
All of the L5 FS (n = 19) and nFS (n = 22) interneurons recorded responded to light stimulation positioned over L6 (20 Hz, 2 – 5 ms pulse duration, 20 pulse train). Light stimulation over other cortical layers did not produce a response. Latency between time-to-L6-spike and response onset in L5 interneurons was 1.1 ± 0.3 ms, consistent with a monosynaptic connection (Markram et al., 1997; Feldmeyer et al., 2005; Frick et al., 2008) from L6 (Figure 5.4C).

Activation of both FS and nFS interneurons by optogenetic stimulation of L6 were substantially and significantly reduced by TTX (F(1,80) = 19, P < 0.0001, two-way ANOVA). Together with the need for light activation over L6, it appears that channelrhodopsin is predominantly localized in the L6 pyramidal cell bodies and not in axon terminals impinging on the L5 interneurons.

Light stimulation over L6 elicited action potential firing in 100% of FS cells (Figure 5.4D) and 70% of nFS cells. The firing pattern elicited in these two types of interneurons was different, with a greater number of spikes seen at the start of L6 stimulation in FS neurons and a more evenly-distributed firing pattern observed in the nFS neurons (Figure 5.5). The minimal L6 light to elicit a suprathreshold excitatory response did not differ significantly between FS and nFS L5 interneurons (t14 = 0.2, P = 0.8, unpaired t-test), despite a significant difference in input resistance (t14 = 4.3, P = 0.0006, unpaired t-test; Table 1). In response to maximal L6 light stimulation of L6, FS interneurons fired more action potentials than nFS interneurons (t14 = 4.4, P = 0.0007, unpaired t-test).
Figure 5.5 Two distinct groups of interneurons are found in L5 and are activated by light stimulation of L6 pyramidal neurons. A) Fast-spiking (FS) interneurons of L5 characterized by injection of current steps. B) Representative trace of a L5 FS interneuron activated by L6. Note the rapidly depressing response to L6 activation. C) Activation of FS interneurons by L6 elicited action potential firing primarily during the initial phase of activation that rapidly depressed over the duration of the stimulation (# of elicited action potentials in first half of stimulation vs. second half: $t_9 = 7.2, P < 0.0001$, unpaired $t$-test). D) Non-fast-spiking (nFS) interneurons of L5 characterized by injection of current steps. E) Representative trace of a L5 nFS interneuron activated by L6, demonstrating a more regular firing pattern. F) L5 nFS interneurons were activated by L6, and fired in a regular pattern over the course of the stimulation (# of elicited action potentials in first half of stimulation vs. second half: $t_7 = 1.9, P = 0.1$, unpaired $t$-test).
5.4.6 Serotonin suppresses L6 activation of L5 interneurons

Since optogenetic stimulation in L6 resulted in robust and highly stable excitation of interneurons in L5 that did not decrease over time at baseline conditions ($t_{11} = 0.8$, $P = 0.4$, paired $t$-test; Figure 5.6A), it was straightforward to test the effect of 5-HT on this local circuit. We found that 5-HT significantly suppressed the number of action potentials elicited in L5 interneurons by optogenetic activation of L6 (FS cells: $t_8 = 3.8$, $P = 0.005$, $n = 9$, paired $t$-test; nFS cells: $t_6 = 5.7$, $P = 0.001$, $n = 7$, paired $t$-test; Figure 5.6B). Of note, this suppression appeared to arise from 5-HT effects in L6 since interneurons in L5 showed minimal direct responses to 5-HT at -75 mV ($2.7 \pm 6.0 \text{ pA}$, $P = 0.9$, $n = 29$, one sample $t$-test). Furthermore, these interneurons showed no change to spiking elicited by depolarizing steps of current amplitudes similar to those elicited by optogenetic stimulation of L6 (FS interneurons: $F_{(1,9)} = 3.1$, $P = 0.1$, repeated measures two-way ANOVA; nFS interneurons: $F_{(1,18)} = 3.4$, $P = 0.1$, repeated measures two-way ANOVA, data not shown). The excitation of L5 interneurons by L6 optogenetic activation was no longer sensitive to 5-HT upon blockade of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors ($t_{15} = 0.9$, $P = 0.4$, paired $t$-test, $n = 10$ FS interneurons, $n = 6$ nFS interneurons; Figure 5.6C). Overall, these results demonstrate the ability of L6 pyramidal neurons to excite a diverse group of inhibitory interneurons in L5 and the sensitivity of this effect to suppression by serotonergic 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors.
Figure 5.6 L6 activation of L5 interneurons in medial prefrontal cortex is stable over time, but suppressed by 5-HT. A) Excitatory effects on L5 interneurons by optogenetic activation of L6 were stable and repeatable over time. Shown here are the postsynaptic responses in a L5 interneuron to L6 light-stimulation repeated over 15 minutes. The number of spikes elicited initially and upon repetition in L5 interneurons are plotted on the bar graph at the right (mean ± SEM). There was no significant difference ($t_{11} = 0.8$, $P = 0.4$, paired $t$-test), showing that the postsynaptic effect in L5 interneurons does not decrease over time under baseline conditions. B) L6 activation of L5 FS interneurons were significantly suppressed by 5-HT ($t_8 = 3.8$, $P = 0.005$, paired $t$-test, $n = 9$). Shown here are repeated recordings (baseline, 5-HT, washout) from 3 different L5 interneurons. The number of spikes elicited at baseline and in the presence of 5-HT in L5 interneurons are illustrated in the bar graph on the right (mean ± SEM). C) Antagonists of serotonergic 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors blocked the inhibitory effects of 5-HT on L6 activation of L5 interneurons ($t_{15} = 0.9$, $P = 0.4$, paired $t$-test, $n = 16$). Shown here is one representative L5 interneuron excited by L6 stimulation, which is suppressed by 5-HT applied alone, and no longer suppressed by 5-HT in the presence of WAY100635 and MDL100907. The results are plotted on the bar graph at the right (mean ± SEM).
5.5 Discussion

In this study, we show robust serotonergic inhibition of L6 pyramidal neurons and their output to L5 interneurons. This suppression of L6 activity by 5-HT is driven by the combined effects of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors. Using transgenic mice and optogenetic techniques, we illustrate a functional link between L6 pyramidal neurons and L5 interneurons potentially important to performance on attention tasks. Light stimulation in L6 strongly excited L5 interneurons. This excitatory connection was inhibited by 5-HT and was restored in the presence of 5-HT$_{1A}$ and 5-HT$_{2A}$ antagonists. Taken together, these results suggest that 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors mediate an inhibitory drive in L6 that can suppress its local activation of cortical targets in L5, which others have shown to be critical to attention (Kim et al., 2016).

5.5.1 Prefrontal L6 pyramidal neurons excite a diverse group of interneurons in L5

We found that L6 pyramidal neurons excited both FS and nFS interneurons in L5 of mPFC. These groups of interneurons likely represent the parvalbumin- (PV) and/or somatostatin-expressing (SOM) groups of interneurons, which together form the majority of interneurons in cortical L5 (Kawaguchi and Kubota, 1997; Rudy et al., 2011). Both perisomatic PV and dendrite-targeting SOM interneurons are mediators of activity on downstream cortical pyramidal output (Kawaguchi and Kubota, 1997; Glickfeld et al., 2009; Kvitsiani et al., 2013; Hangya et al., 2014), with mPFC PV interneuron activity particularly important to normal performance on attention tasks (Kim et al., 2016). Our finding of a functional connection between L6 and L5 interneurons suggests a means by which L6 could influence mPFC cortical gain modulation, as has been observed in primary sensory cortices (Olsen et al., 2012; Bortone et al., 2014). These findings are in agreement with previous work in primary sensory cortex that examined
anatomical and functional connections within the cortical column (Zhang and Deschênes, 1998; Thomson et al., 2002; Mercer et al., 2005; Watts and Thomson, 2005; West et al., 2006; Kim et al., 2014). This research suggests that activation of L6 is a driver of intracortical inhibition leading to a widespread suppression of cortical targets, observed in vivo in visual cortex (Beierlein et al., 2003; Olsen et al., 2012; Bortone et al., 2014). The L6 excitation of nFS in addition to FS interneurons in mPFC suggests additional complexity in association cortex. Our data show for the first time that excitatory output from L6 can drive interneuron activity in L5 in mPFC, a region critical for attention and other executive functions.

5.5.2 Serotonergic inhibition of this L6 to L5 intracortical circuit

We found that 5-HT, via stimulation of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors, inhibited L6 pyramidal neurons and their activation of L5 interneurons. These two receptors show substantial co-localization in L6 pyramidal neurons in mPFC of mouse (Table 3 in (Amargós-Bosch et al., 2004), yet typically appear to exert opposing electrophysiological effects in other cortical layers (Benekareddy et al., 2010; Avesar and Gulledge, 2012; Stephens et al., 2014). While 5-HT$_{1A}$ receptors are known inhibitory receptors acting via Kir3 channels (Goodfellow et al., 2014; Johnston et al., 2014), 5-HT$_{2A}$ receptors act through a less well-characterized set of channels to excite certain populations of neurons, including a subset of L5 pyramidal neurons in mPFC (Willins et al., 1999; Benekareddy et al., 2010; Weber and Andrade, 2010; Avesar and Gulledge, 2012). Direct inhibition of L6 pyramidal neurons by 5-HT$_{2A}$ receptors could arise from a number of possible mechanisms: including through the suppression of sodium channels (Carr et al., 2002) or via 5-HT$_{2A}$ heteromers with inhibitory signaling (González-Maeso et al., 2007; Moreno et al., 2011; Viñals et al., 2015). Pyramidal neurons in L6 also have prominent afterhyperpolarizations (Proulx et al., 2015), known to affect excitability. Accordingly, the $\text{G}_\alpha_q$-
coupled 5-HT$_{2A}$ receptors may affect the excitability of these neurons by modulating channels contributing to different phases of the afterhyperpolarization (Gulledge and Stuart, 2005; Villalobos et al., 2005; Gulledge et al., 2009; Villalobos et al., 2011). Complex and carefully controlled future work will be necessary to identify the mechanisms underlying the 5-HT$_{2A}$ receptor mediated inhibition of L6 pyramidal neuron excitability.

In investigating FS and nFS interneurons in L5, we found that the majority of these cells did not respond strongly to 5-HT. A minority showed electrophysiological responses (FS: 4/13; nFS: 2/16), predominantly inward currents (< -20 pA) that were insufficient to elicit spiking. These proportions are consistent with the literature on the expression of 5-HT receptors only in a small proportion of L5 interneurons (Abi-Saab et al., 1999; Santana et al., 2004; Rudy et al., 2011; Celada et al., 2013). Our findings were not significantly altered by the inclusion or exclusion of these neurons. Control experiments with GABA-A and GABA-B blockers suggest that 5-HT receptors on interneurons are not significantly involved in the 5-HT suppression of L6 pyramidal neurons. Taken together, our data support the hypothesis that the combined activation of both 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors can inhibit neuronal excitability in L6 neurons of prefrontal cortex. However, further pharmacological work is required to examine the specific downstream mechanisms underlying this inhibition of L6 pyramidal neurons. Furthermore, additional investigations into the consequences of serotonergic inhibition of L6 on local network dynamics will provide more insight into the nature of these important associative circuits and how they control attention.
5.5.3 Serotonin, prefrontal attention circuitry, and attention in psychiatric illness

Prefrontal attention circuitry is complex and attentional performance can be perturbed by extremes of mPFC activity in either direction (Pezze et al., 2014). Serotonin shapes and biases attention in humans and rodents: with low levels of 5-HT enhancing attention (Schmitt et al., 2000; Gallagher et al., 2003; Booij et al., 2005) and higher levels of 5-HT disrupting attention (Riedel et al., 2005; Wingen et al., 2007; Watson et al., 2015) through 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors (Wingen et al., 2007). Stress is well known to raise prefrontal serotonin levels (Adell et al., 1997; Fujino et al., 2002; Bland et al., 2004) and similar behavioral manipulations disrupt attention (Minor et al., 1984; Sänger et al., 2014). Intriguingly, elevation of intra-cortical 5-HT has been associated with deficits in attention (Puumala and Sirviö, 1998) and increases in impulsivity (Dalley et al., 2002). Specific activation of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors result in similar attention deficits (Carli and Samanin, 2000; Koskinen et al., 2000). Conversely, infusion of antagonists to 5-HT$_{2A}$ receptors into mPFC improved performance on attention tasks in rodents and reduced impulsivity (Passetti et al., 2003; Winstanley et al., 2003).

While manipulation of 5-HT receptors specifically in mPFC can manipulate attention, certain key experiments (i.e. attention under stress or in models of dysregulated 5-HT signaling) remain to be performed. Based on our findings, rising levels of cortical 5-HT may increase cortical noise due to suppression of L6 mediated cortical inhibition. Increasing the signal-to-noise ratio is correlated with attentional focus (Briggs et al., 2013; Pratte et al., 2013), whereas deficits can arise from increasing cortical noise in prefrontal cortex (Pezze et al., 2014), Similarly, disruptions to normal excitation of L6 neurons of mPFC can lead to attention deficits in rodents, although previous studies have focused on cholinergic stimulation of L6 (Bailey et al., 2010; Guillem et al., 2011). Our study is the first to demonstrate the robust inhibitory
modulation exerted on L6 by 5-HT, and the resultant decrease in its ability to stimulate interneuron activity in L5. Taken together, we provide evidence that L6 of mPFC is a candidate locus of action for the modulatory effects of 5-HT on attention. Based on recent work in nonhuman primates (Watson et al., 2015), it is tempting to speculate that 5-HT levels in deep mPFC may modulate the balance between social vigilance and attentional task performance, a phenomenon that is impaired in several neuropsychiatric illnesses.
6 General Discussion

6.1 Summary of findings

The prefrontal cortex is essential to attention. The position of prefrontal layer 6 within the cortex allows for it to control the activity of downstream thalamic and cortical targets. However, the cellular functions of layer 6 and its response to neuromodulation are not well understood. Furthermore, the functional connections made between layer 6 and downstream cortical targets have not been demonstrated in medial prefrontal cortex. In this thesis, I investigated the question of how layer 6 neurons of medial prefrontal cortex are modulated by acetylcholine and serotonin, two neurotransmitters known to influence normal attention. In addition, I identify a functional local connection between layer 6 pyramidal neurons of medial prefrontal cortex and layer 5 interneurons. Furthermore, I show that modulation of layer 6 activity by serotonin can influence layer 5 interneuron activation.

In Chapter 3, I examined the distribution of cholinergic responses in layer 6 neurons across associative and primary regions of prefrontal cortex. I found a pattern of cholinergic responsiveness across these regions, not only in the amplitude of the responses, but in the composition of receptors involved in the response. In particular, I show that the associative medial prefrontal cortex is unique amongst these regions in that the acetylcholine response is dominated by nicotinic receptors, with only a minor muscarinic influence. This is in contrast to primary motor and somatosensory cortices in which nicotinic and muscarinic receptors play a more equivalent role in mediating the neuronal response to acetylcholine. Additionally, the nicotinic-driven responses in medial prefrontal cortex are dependent on the expression of the nicotinic receptor α5 subunit.
In Chapter 4, I demonstrated the presence of plasticity within the neuronal composition of nicotinic and muscarinic acetylcholine receptor responses contributing to the overall cholinergic signal within layer 6 pyramidal neurons of medial prefrontal cortex (Figure 6.1). Using knockout mice lacking either the nicotinic receptor α5 or β2 subunits, resulting in a disruption or the complete abolition of nicotinic responses to acetylcholine respectively in these neurons, I uncovered a compensatory increase in muscarinic receptor-mediated responses to acetylcholine in layer 6 pyramidal neurons. Whilst the compensation does not fully recover the depolarizing currents in these neurons, the functional excitability at threshold potentials was not compromised in the knockout mice despite significantly reduced nicotinic currents. The degree of compensation matches the severity of nicotinic receptor disruption, and is mediated by muscarinic M1 and M3 receptors.

In Chapter 5, I demonstrated an inhibitory effect of serotonin on layer 6 neurons of medial prefrontal cortex, opposing the excitatory drive of acetylcholine that has previously been identified in these neurons. I found a co-operative contribution of the inhibitory 5-HT₁A receptors and, surprisingly, the typically excitatory 5-HT₂A receptors to the overall inhibition of layer 6 pyramidal neurons. Furthermore, I examined the consequences of silencing layer 6 neurons with serotonin on layer 5 interneurons, downstream targets of layer 6 neurons that are important to attention (Figure 6.2). I found that activation of layer 5 interneurons by layer 6 pyramidal neurons is suppressed by serotonin, also mediated through both 5-HT₁A and 5-HT₂A receptors.

Collectively, the findings presented in this thesis serves as a foundation which places layer 6 pyramidal neurons of medial prefrontal cortex as a potential modulator of local cortical activity. In addition, I investigated functional means by which these neurons can be modulated by neurotransmitters known to influence attention. In the following sections, I will discuss in
more detail the findings presented in this thesis with respect to current literature, and future works.

Figure 6.1 Plasticity of muscarinic acetylcholine receptor function in layer 6 of medial prefrontal cortex. Disruption or elimination of nicotinic acetylcholine receptor function in transgenic mice triggers a compensatory increase in muscarinic acetylcholine receptor function in layer 6 neurons of medial prefrontal cortex.
Figure 6.2 Connectivity and modulation of layer 6 neurons in medial prefrontal cortex, and their suggested relevance to attention. Top: layer 6 pyramidal neurons in medial prefrontal cortex send excitatory axonal projections to interneurons of layer 5, and possible other upper layers. Modulation of layer 6 neuronal firing either positively by cholinergic stimulation or negatively by serotonergic stimulation affects activity of downstream interneurons of other layers. Bottom: hypothesized role of acetylcholine and serotonin in the mediation of certain forms of attention by acting through layer 6 of medial prefrontal cortex. Release of either neurotransmitter based on environmental and attentional demands can shift the signal-to-noise ratio in both directions, thus directly influencing top-down control of attentiveness within the medial prefrontal cortex.
6.2 Layer 6 as a modulator of cortical activity and implications for attention

Cortical layer 6 is less studied than other layers, in particular within associative areas (Thomson, 2010). Layer 6 notably receives both thalamo-cortical input and sends cortico-cortical and reciprocal cortico-thalamic outputs. While much of the research into layer 6 has revolved around primary sensory regions, less attention has been paid to these neurons within associative areas important for executive function. In medial prefrontal cortex, a large proportion of layer 6 neurons send and receive reciprocal connections to the medial dorsal thalamus (Gabbott et al., 2005; Kassam et al., 2008). In primary sensory areas, a similar connection is found between the layers, although evidence shows that connections are made between layer 6 pyramidal neurons and layer 5 pyramidal neurons, rather than layer 5 interneurons (Mercer et al., 2005). Furthermore, connections are made between layer 6 and layer 4 (Anderson et al., 1994; Staiger et al., 1996), and also locally with layer 6 interneurons (West et al., 2006). However, the nature of the cortico-cortical connections made by layer 6 of medial prefrontal cortex had yet to be studied in detail.

6.2.1 Connectivity between layer 6 and other cortical layers

Using transgenic mice expressing channelrhodopsin-2 in layer 6 pyramidal neurons, I found a monosynaptic connection between these layer 6 pyramidal neurons of medial prefrontal cortex and various subtypes of interneurons in layer 5 of the same cortical column (Chapter 5). Expression of channelrhodopsin-2 in layer 6 was driven by the Epyc (epiphycan) promoter, which is localized within pyramidal neurons of layer 6. These neurons can be visually identified for targeting during electrophysiological experiments, although expression of the reporter is weak. However, a high percentage of blindly patched layer 6 pyramidal neurons in mPFC...
expressed channelrhodopsin-2, on the basis of their activation by light, and the < 1 ms timing of
this activation following light-on. *Epyc* positive neurons were further confirmed to be
representative of cortico-thalamic pyramidal neurons from layer 6 by Translating Ribosome
Affinity Purification and qRT-PCR experiments performed by our collaborator Dr. Eric Schmidt.
These experiments examined the relative expression of marker genes in the *Epyc* positive
neurons relative to rest of cortex. As such, finding a relative enhancement in genes such as
*Slc17a7, Ntsr1*, and *Foxp2* confirm that *Epyc*-expressing neurons are layer 6 corticothalamic
pyramidal neurons (Ferland et al., 2003; Gong et al., 2007; Olsen et al., 2012; Mease et al.,
2014), while an enhancement of *Syt6* expression in these neurons demonstrate that an
overlapping population of neurons express both *Epyc* and *Syt6*. These findings demonstrate that an
overlapping population of neurons expressing both *Epyc* and *Syt6*. These findings demonstrate that in
distinct subclasses of cortico-thalamic and cortico-cortical projecting neurons in
layer 6, cortico-thalamic projecting neurons can send additional axon collaterals towards other
layers within the cortical column.

The connection with layer 5 interneurons was probed by light-mediated activation of
layer 6 neurons, in which the delay from light-on to an action potential in layer 6 neurons was
under 1 ms. The observation that the connection between layer 6 and layer 5 interneurons was
monosynaptic was then based on the latency between light-on, the peak of the action potential in
layer 6 neurons, and the initialization of the excitatory post-synaptic potential in layer 5
interneurons. The delay on average was 1 ms, indicative of a typical monosynaptic connection
(Markram et al., 1997; Feldmeyer et al., 2005; Frick et al., 2008), whereas a polysynaptic
response would have resulted in longer latency between the light and postsynaptic response.
Further experiments will be necessary to morphologically and functionally map the connections
of layer 6 to its downstream targets to verify its monosynaptic nature, and to confirm other
possible connections originating from layer 6. Using a combination of fluorescence microscopy and electrophysiology, future work can make use of either transgenic or viral expression of markers for layer 6 (promoters such as *Epyc, Syt6, Ntsr*) and downstream targets (possibly interneurons using *PV, SOM, VIP*, etc.) to elucidate these connections. Using these transgenic mice, the question of direct connectivity between these two neuronal types across layers can be probed through FRET experiments or staining and higher resolution imaging. More precise targeting for electrophysiology and optogenetics can be achieved through the reporter-tagged neurons. Resolving this direct connection as well as other cortico-cortical connectivity arising from layer 6 will allow for a better understanding of the impact of neuromodulation within this layer.

### 6.2.2 Impact of layer 6 activity on attention circuitry within prefrontal cortex

The described monosynaptic nature and the direct target of the layer 6 to layer 5 interneuron connection described in this thesis fits in accordance to previous literature describing the role that layer 6 plays in the circuit in sensory cortex (Olsen et al., 2012; Bortone et al., 2014). Here, a fast signal to mediate the activation of local interneurons is necessary to either inhibit or disinhibit activity in other cortical layers, and thus alter the signal-to-noise ratio of areas or layers important for attention processing (Briggs et al., 2013; Pratte et al., 2013). In this thesis, my characterization of the connection between layer 6 pyramidal neurons and layer 5 interneurons in the medial prefrontal cortex places layer 6 neurons in a position to regulate the activity of layer 5 pyramidal neurons, an output layer important to executive function. Likewise, an increase in layer 6 activity would lead to an increase in layer 5 interneuron activation, subsequent release of GABA, and consequently suppression of the downstream neuronal targets of these interneurons within the cortical column. The net effect of layer 6 activation would be an
overall decrease in cortical activity, especially if future work can demonstrate similar connections between layer 6 and interneurons of other layers. On the other hand, decreases in layer 6 activity will disinhibit layer 5 pyramidal neurons, since target interneurons will be less active resulting in less GABA release. This phenomenon is key to attention as increasing the signal-to-noise ratio of cortical activity is correlated to improved attentional focus (Briggs et al., 2013; Pratte et al., 2013), whereas a decrease in signal-to-noise ratio can lead to attention deficits (Pezze et al., 2014). Recent work by Kim et al. (2016) has shown that the activity of fast-spiking interneurons in prefrontal cortex is critical to normal attention performance. Similarly, the impact of layer 6 activity on cortical gain has been previously shown in visual cortex (Olsen et al., 2012; Bortone et al., 2014). In the medial prefrontal cortex, where pharmacologically induced changes in activity have been shown to influence performance on attention tasks in humans and rodents (Schmitt et al., 2000; Robbins, 2002; Hahn et al., 2003b; Chudasama et al., 2004; Wingen et al., 2007), it is increasingly important to understand the role of layer 6 in controlling these changes in cortical activity.

6.2.3 Future work: layer 6 activity and mapping out direct effects on attention performance

As mentioned above, layer 6 acts as a gain modulator of cortical activity across numerous sensory regions (Olsen et al., 2012; Bortone et al., 2014). The findings from this thesis also indicate that layer 6 neurons of mPFC are well positioned to control the cortical activity of other layers. This modulatory activity arises from its connections to inhibitory interneurons in other layers. Within the mPFC, fast-spiking interneurons in layer 5 are particularly important to normal attention though their link to the generation of gamma oscillations (Kim et al., 2016). The findings of this thesis placed in context with the existing literature suggest that layer 6 neurons of
mPFC, through its connectivity to interneurons of layer 5 and possibly other layers, is in a unique position to influence network activity and oscillatory dynamics which are critical to normal attention. Several potential research questions can be investigated based on these preliminary findings: first, the functional consequences of layer 6 activation on oscillatory activity within the mPFC, second, the live behavioural consequences of layer 6 activity on attentional performance in vivo, and third, whether any therapeutic value can be derived from controlling layer 6 activity.

The question of what role layer 6 plays in the dynamics of mPFC attention circuits arise from the importance of oscillatory activity in normal attention (Voloh et al., 2015; Kim et al., 2016). Existing studies have only begun to identify the importance of maintaining these oscillations in normal attention, but not how they are generated intrinsically. Much work has shown that synchronous activation of fast-spiking PV neurons can be sufficient for inducing gamma oscillations (Cardin et al., 2009; Sohal et al., 2009; Buzsáki and Wang, 2012). Activity through PV neurons in sensory cortex are thought to locally modulate sensory responses in such ways representative of attention (Atallah et al., 2012; Lee et al., 2012; Wilson et al., 2012; Siegle et al., 2014). Disruption of gamma oscillations in mPFC, as well as in sensory regions, can significantly impact attentional performance (Gregoriou et al., 2014; Kim et al., 2016). As such, the finding of this thesis that layer 6 can directly influence fast-spiking interneurons in layer 5 raises the question of how gamma oscillations can be affected as a result. These questions can be approached by using similar optogenetic techniques, but with different modes of recording either in vivo or in slice. Similarly, the functional effects of layer 6 activation on this oscillatory activity can also translate into effects on attention performance. Using standard attention tasks such as the 5-CSRTT in conjunction with live recording, much more can be learned about the active role
that layer 6 can play in modulating cortical activity, oscillatory rhythms, and attention performance.

The third question of whether this network can then be modulated experimentally and therapeutically will be addressed in the following sections.

6.3 Modulation of layer 6 pyramidal neurons in prefrontal cortex

The importance of the PFC in cognition has led to much research on ways in which cognitive functions such as attention can be modulated. The findings of this thesis positions layer 6 neurons in mPFC as a modulator of the prefrontal attention circuit. While some work in the mPFC have already established the presence of cholinergic neuromodulation in deep layers of the mPFC through nicotinic acetylcholine receptors (Kassam et al., 2008; Bailey et al., 2010; Guillem et al., 2011; Poorthuis et al., 2013), there was still a substantial gap in knowledge with regards to the mechanisms underlying not just the cholinergic responsiveness, but also other neurotransmitters that may act on these neurons based on receptor expression (Buckley et al., 1988; Amargós-Bosch et al., 2004). The following sections will discuss the findings in this thesis with respect to their importance in the overall modulation of mPFC layer 6.

6.3.1 Distribution of nicotinic and muscarinic acetylcholine responses

Layer 6 neurons across primary and associative cortical regions are innervated by cholinergic fibers arising from the basal forebrain (Mechawar and Descarries, 2001). Release of acetylcholine from these terminals coincide with numerous forms of attention (Passetti et al., 2000; Fournier et al., 2004; Kozak et al., 2006; Parikh et al., 2007; Parikh and Sarter, 2008). In Chapter 3 of this thesis, I examined the distribution of these responses to acetylcholine across three cortical regions comprising of primary somatosensory cortex, primary motor cortex, and
the associative mPFC. While the presence of functional responses to acetylcholine was not unexpected based on widespread cholinergic receptor expression across cortex, the finding of region-specific signatures of cholinergic responses driven by a combination of nicotinic and muscarinic receptors demonstrates a layer of complexity in the cortical cholinergic system. Existing electrophysiological studies focusing on cholinergic responses in layer 6 primarily had focused on nicotinic responses (Kassam et al., 2008; Bailey et al., 2010; Poorthuis et al., 2013), whereas muscarinic receptor-mediated responses in layer 6 were less studied. Furthermore, few have looked at both nicotinic and muscarinic receptor-mediated responses in conjunction at the level of individual neurons (Lamour et al., 1982). My work in Chapters 3 and 4 examining both nicotinic and muscarinic receptors in conjunction aimed to investigate the overall cholinergic excitation of layer 6 neurons, and interrogate the question of whether differences in cholinergic response and receptor composition in different cortical regions can be linked to different attentional processes.

In associative mPFC, the nicotinic response was found to be the primary driver of cholinergic excitation, whereas muscarinic receptors played a relatively small role. As previously discussed, layer 6 mPFC is in positioned as a gain modulator for the local attention circuit. Likewise, the region itself appears to be important to sustained, focused, and challenging attention (Marti et al., 2012). Furthermore, disruptions to nicotinic receptors within this region have detrimental effects on performance in challenging attention tasks (Bailey et al., 2010; Guillem et al., 2011). The faster ionotropic nicotinic responses may excel at mediating this sort of attention, where the temporal profile of modulating neuronal activity must align with fast changes in attentional demands. On the other hand, there is evidence that the associative mPFC is not essential to attention during periods of lower demand (Smucny et al., 2013). In some
cases, primary cortices can carry out forms of attentional processing locally (Meyer, 2011), and their activity can correspond to attention performance (Pfurtscheller et al., 1996; Jones et al., 2010) and modulated by cholinergic stimulation (Bauer et al., 2012). The finding in this thesis of a proportionally stronger muscarinic receptor-mediated component making up the overall cholinergic excitability suggests an additional level of complexity to the function of layer 6 neurons within primary cortex relative to nicotinic responses, but across a longer temporal profile. The larger contribution of these slower, but longer-acting receptors on layer 6 neurons of primary cortex corresponds with the notion that these regions are not critical for challenging, demanding attention, but rather involved in other forms of attention. Indeed, neuroimaging studies have shown that sensory cortical regions show higher levels of activity upon perception of stimuli (Chen et al., 2003; Yoo et al., 2003; Blankenburg et al., 2006; Schaefer et al., 2006). However, in the case of attentiveness to a perceived stimuli, there is a correlation between attention and synchrony in neuronal activity, rather than increases overall firing (Meyer, 2011). Given the position of layer 6 as a gain modulator within primary sensory regions (Olsen et al., 2012), these results lend more credence to the hypothesis that a slow but longer-duration activation of layer 6 by a more muscarinic receptor-dominated response to acetylcholine release during attention drives synchronous oscillatory activity via activation of fast-spiking interneurons of other cortical layers. This is not to say that in the mPFC, the relatively smaller but still significant muscarinic receptor-mediated excitation of layer 6 neurons is not acting in a similar manner, rather an additional, powerful nicotinic receptor-mediated drive is also present for higher attentional demands.
6.3.2 Plasticity of cholinergic responses in layer 6 pyramidal neurons

The finding that both nicotinic and muscarinic receptors are present and actively contribute to the cholinergic response in layer 6 neurons of mPFC led to the question of how the two receptors typically interact. As mentioned above, existing literature of layer 6 mPFC has focused mostly on nicotinic receptor-mediated responses to acetylcholine. By pharmacologically blocking $\alpha_{4}\beta_{2}^{*}$ receptors, I presented in Chapter 4 the finding that muscarinic receptors mediate a supra-threshold excitation of layer 6 neurons. Using transgenic mice as tools to either disrupt or knock out nicotinic receptor function, the muscarinic receptor-mediated response showed a degree of plasticity in upregulating their function which compensates for decreased nicotinic receptor function. This finding demonstrates an increased level of complexity in the cholinergic machinery within layer 6 mPFC wherein an element of compensatory plasticity exists, but also that this compensation can be asymmetrical, as only supra-threshold excitation mediated by acetylcholine is rescued by this muscarinic upregulation. Bailey and colleagues (2010) found in transgenic mice constitutively lacking the nicotinic receptor $\alpha_{5}$ subunit that nicotinic responses to acetylcholine are reduced significantly in layer 6 neurons of mPFC. Likewise, the same mice performed significantly worse in terms of accuracy at the 5-CSRTT under the more challenging condition of very brief stimulus durations, but not under less difficult test conditions (Bailey et al., 2010). Guillem and colleagues (2011) used knockout mice lacking nicotinic receptor $\beta_{2}$ subunits (Picciotto et al., 1995), thereby preventing normal assembly of $\alpha_{4}\beta_{2}^{*}$ receptors. Neurons lacking the $\beta_{2}$ subunit showed little to no response to acetylcholine, and performed significantly worse in the 5-CSRTT in the form of increased omissions, but without a decrease in accuracy (Guillem et al., 2011). Moreover, a rescue of the $\beta_{2}$ subunit expression specifically in the prelimbic region of mPFC restored normal attention performance on the task. In both these cases, behavioural data presented milder-than-expected deficits in attention performance despite
either disruptions or knockout of nicotinic receptor function in the mPFC. The findings of this thesis suggest a potential compensatory mechanism that underlies these behavioural results, wherein performance under high attentional demand is disrupted, but a compensatory increase in muscarinic receptor function rescues a more drastic attention deficit during less demanding tasks. Altogether, these data place layer 6 of mPFC as potentially important site for attention modulation, and brings about more questions about other ways by which its activity can be controlled.

6.3.3 Serotonin as an inhibitory modulator of layer 6 pyramidal neurons

The ability of serotonin to negatively influence attention has been documented, but not studied in detail mechanistically in mPFC. The presence of 5-HT receptors in layer 6 led to the question of whether it can act in an opposing manner on layer 6 neurons in comparison to acetylcholine. The finding in Chapter 5 of this thesis that serotonin can inhibit the excitability of layer 6 neurons in mPFC fits with the expression of 5-HT$_{1A}$ receptors in this region. However, the unexpected supra-threshold inhibitory response mediated by 5-HT$_{2A}$ receptors differs from previous work showing that 5-HT$_{2A}$ receptor activation results in excitation, rather than inhibition in other cortical layers (Lambe and Aghajanian, 2001; Zhang and Arsenault, 2005; Weisstaub et al., 2006; Benekareddy et al., 2010; Weber and Andrade, 2010; Avesar and Gulledge, 2012). Possible mechanisms have been described by other groups: Carr and colleagues (2002) suggested persistent sodium channels can be suppressed by 5-HT$_{2A}$ activation, and González-Maeso and colleagues have proposed a potential inhibitory heteromer between 5-HT$_{2A}$ and mGluR2 receptors (González-Maeso et al., 2007; Moreno et al., 2011; Viñals et al., 2015). All of these possible mechanisms require further experiments to test in layer 6 neurons of mPFC.
to discern whether layer 6 neurons are unique relative to other layers in exhibiting this inhibitory response to 5-HT$_{2A}$ activation.

Given the role of layer 6 within the mPFC attention circuit, the question is raised about how serotonergic inhibition of layer 6 can affect its downstream targets. My finding in Chapter 5 that layer 6 neurons of mPFC are connected to interneurons of layer 5, and evidence supporting connections to other layers as well suggest that any modulation layer 6 will have a proportional downstream effect across the cortical column. While much of this work remains to be done in mPFC, activation of layer 6 in visual cortex has been shown to reduce cortical activity in other layers, and increase signal-to-noise ratio which is correlated with attentional focus (Briggs et al., 2013; Pratte et al., 2013). As such, we can expect that serotonin’s inhibition of layer 6 activity will result in the opposite – a disinhibition of cortical activity due to a reduction in interneuron activation, and thus, a decreased signal-to-noise ratio that will impair attention (Pezze et al., 2014).

Endogenous release of serotonin needs to be further verified in prefrontal cortex to provide context into the relevance to attention of the observed serotonergic response in layer 6. Previous reports suggest heightened brain serotonin levels during stress (Adell et al., 1997; Fujino et al., 2002; Bland et al., 2004), and stressful conditions can significantly disrupt attention (Minor et al., 1984; Sänger et al., 2014). A similar effect on attention also exists with other monoaminergic systems, where either too little catecholamine release in fatigued or low stress conditions (Deutch and Roth, 1990; Finlay et al., 1995), or too much catecholamine release in stressful conditions (Birnbaum et al., 2004) results in attention deficits (Arnsten and Pliszka, 2011). This inverted-U relationship requires further investigation in vivo to probe for the active effects of catecholamine levels on cortical firing and how it relates to attention.
6.3.4 Future work: investigating endogenous control of layer 6 activity

Our current understanding of layer 6 as a modulator of cortical gain is still rudimentary and requires more extensive and mechanistic research. As outlined in Chapter 6.2, the mapping of direct connections between layer 6 and its downstream targets is first required to identify prime targets for electrophysiological and translational behavioural research. Despite this, immediate attempts can be made to better understand the many ways in which layer 6 neurons can be modulated. Two existing neuromodulatory systems have been explored in this thesis, and represent ways to either activate or suppress layer 6 activity. However, numerous other receptors have been identified on layer 6 neurons of mPFC such as the dopamine D1 and D2 receptors (Gaspar et al., 1995) and the α1-adrenergic receptor (Mitrano et al., 2014), representing the dopaminergic and adrenergic systems that have both been implicated in the prefrontal control of attention performance (Granon et al., 2000; Arnsten and Pliszka, 2011; Barnes et al., 2012). Additionally, more nuanced pharmacological examinations of all of these neurotransmitters and their effects on layer 6 neurons are required. Interesting avenues of research can include the use of allosteric modulators to either enhance or reduce, but not fully remove, the effects of certain receptor subtypes to maintain physiological levels of control over layer 6 activity. These experiments can all yield tremendous therapeutic value in the preservation or enhancement of cognition in humans.

A pitfall in the methodology employed in this thesis is the way by which the responses to cholinergic and serotonergic stimulation were probed. Bath application of said neurotransmitters elicits a relatively non-physiological profile of binding activity, despite our attempts at maintaining near-physiological concentrations of neurotransmitters on the most part. However, by using established cholinergic and serotonergic neuron-specific promoters such as ChAT
(choline acetyltransferase) and Pet1 (a transcription factor specific to serotonergic neurons), the optogenetic toolkit can be expressed in said neurons to allow for experiments that involve light-evoked endogenous release of acetylcholine and serotonin from their native terminals at a high spatial and temporal resolution. These experiments will first serve to validate whether the findings in this thesis are preserved from release of endogenous levels of acetylcholine and serotonin. Next, these tools can be used to further track the progression of these signalling pathways from neurotransmitter release to downstream effects based on their high resolution.

6.4 Conclusion

Layer 6 of the medial prefrontal cortex is an important node of top-down control of executive function and attention within the brain. As such, it has become increasingly important to better understand the cellular functions, neuromodulatory mechanism, and functional connections of layer 6. This thesis has focused on a detailed examination of the connectivity and mechanisms of neuromodulation of neurons within layer 6 of medial prefrontal cortex by acetylcholine and serotonin, two neurotransmitters implicated in attentional control. The distribution, composition, plasticity, and mechanisms underlying the cholinergic and serotonergic stimulation of layer 6 neurons was examined in the experimental chapters, with additional work detailing the consequences of layer 6 activation on downstream targets. Future work will explore in more detail the endogenous effects of various neurotransmitters on layer 6 activity and aim to map out a more definitive connection between layer 6 and the rest of the cortical column. Better understanding of these aspects of prefrontal neurophysiology will provide us with clear insight into the regulation of attention and offer therapeutic value in the treatment of cognitive deficits.
References


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7 Appendices

7.1 Supplementary Methods

7.1.1 Translating Ribosome Affinity Purification and Quantitative RT-PCR

Adult (8-12 weeks old) Epyc-Cre mice under ketamine/xylazine (100/10 mg/kg) anesthesia received single bilateral stereotaxic injections of 0.25 µl AAV-FLEX-EGFPL10a virus (3.75x10^{12} gc/ml) into the mPFC (+1.54 AP from bregma, +0.4 ML, -1.80 DV from dura). Animals were sacrificed in a controlled CO₂ chamber three weeks after surgery, brains were rapidly dissected in ice-cold HBSS containing 2.5 mM HEPES-KOH (pH 7.4), 35 mM glucose, 4 mM NaHCO₃, and 100 µg/ml cycloheximide. The cortex was isolated from the rest of the brain and each hemisphere was split along the coronal plane at the level of the genu of the corpus collosum (~1.6 mm AP from bregma). The rostral portion was saved as the “PFC” and used for TRAP. Tissue from three mice (male and female) was pooled for each sample and three biological replicates were collected. Polysome immunoprecipitations were carried out as previously described (Schmidt et al., 2012; Heiman et al., 2014). Briefly, the tissue was homogenized in extraction buffer containing 10 mM HEPES-KOH (pH 7.4), 150 mM KCl, 5 mM MgCl₂, 0.5 mM DTT, 100 µg/ml cycloheximide, RNasin (Promega, Madison, WI) and SUPERase-InTM (Life Technologies) RNase inhibitors, and Complete-EDTA-free protease inhibitors (Roche), and then cleared by centrifugation at 2000 x g. IGEPAL CA-630 (NP-40, Sigma) and DHPC (Avanti Polar Lipids, Alabaster, AL) were both added to the S2 supernatant for a final concentration of 1% for each, followed by centrifugation at 20,000 x g. Polysomes were immunoprecipitated from the S20 supernatant using 100 µg monoclonal anti-EGFP antibodies (50 µg each of clones 19C8 and 19F7; see ref. 2) bound to biotinylated-Protein L (Pierce, Thermo Fisher, Waltham, MA) coated streptavidin-conjugated magnetic beads (Life
Technologies), and washed in high salt buffer containing 10 mM HEPES-KOH (pH 7.4), 350 mM KCl, 5 mM MgCl2, 1% IGEPAL CA-630, 0.5 mM DTT, 100 µg/ml cycloheximide, and RNasin RNase inhibitors (Promega). IPs were carried out overnight at 4°C. Bound RNA was purified using the Absolutely RNA Nanoprep kit (Agilent, Santa Clara, CA). RNA was also purified from the pre-IP supernatant to serve as whole-PFC “input” samples. RNA quantity was measured with a Nanodrop 1000 spectrophotometer and quality was assayed on an Agilent 2100 Bioanalyzer. Only samples with RNA integrity values >7.0 were used for qRT-PCR analysis. cDNA was synthesized from 15 ng of IP or input total RNA using the Ovation qPCR System (NuGEN Technologies) following manufacturer’s protocol. Quantitative RT-PCR was performed on an Applied Biosystems StepOnePlus Fast Real-Time PCR System using commercially available Taqman assays (Table 2) and following standard cycling conditions (50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min). Ten nanograms of cDNA were used for each qRT-PCR reaction and technical triplicates were run for each of the biological triplicates from TRAP IP and input samples. The mean C_T for technical replicates was used for quantification. Data were normalized to Gapdh by the comparative C_T (2^-\Delta\Delta CT) method (Livak and Schmittgen, 2001). Data are presented as Mean ± SEM of biological triplicates. Statistical significance was calculated between the normalized expression values (2^-\Delta CT) from the IP and input biological replicates for each gene by Student’s t-test in Microsoft Excel.