Mechanisms of Cholinergic Neuromodulation of Prefrontal Cortex in Health and Disease:

A Combined Whole-Cell Electrophysiology and Multiphoton Calcium Imaging Approach

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Physiology
University of Toronto

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Abstract

The neuromodulation of the prefrontal cortex (PFC) by acetylcholine is essential for optimal executive function, where it exerts robust excitatory actions together with complex calcium ion signals via the nicotinic and muscarinic acetylcholine receptors. This thesis examines how cholinergic calcium fluxes regulate the ability of acetylcholine to excite prefrontal circuits and how these interactions are disrupted in rodent models of psychiatric and neurological illness. Using whole-cell electrophysiology together with multiphoton calcium imaging in slices of rodent PFC, I have found that cholinergic calcium mobilization can exert important regulatory control over cholinergic excitability: by enhancing sub-threshold cholinergic excitation and by constraining supra-threshold effects via the modulation of spike output. Importantly, these distinct interactions were disrupted in two separate models of neuropsychiatric disease with documented cognitive dysfunction.

First, in pyramidal cells of prefrontal output layer 5, acetylcholine-induced calcium release was found to potentiate excitatory cholinergic currents via the electrogenic process of sodium/calcium exchange. This phenomenon was found to emerge in young
adulthood, when executive function typically reaches maturity. What is more, this developmental consolidation of cholinergic signaling was abolished subsequent to the early stress of repeated maternal separation—a model of psychiatric vulnerability—where cholinergic responses retained an adolescent phenotype accompanied by a disruption in the expression of multiple developmentally-regulated genes associated with Gαq and calcium signaling.

Second, in a well-characterized model of Alzheimer’s disease, I have found that layer 6 PFC pyramidal cells, which are known to be essential for peak attentional performance, are unable to sustain cholinergic excitation to the same extent as those of non-transgenic littermate controls as a result of the excessive activation of calcium-activated hyperpolarizing conductances. Further, I demonstrate that cholinergic excitation can be improved in the cortex of these mice by pharmacological blockade of SK channels, suggesting a novel target for the treatment of executive dysfunction.

In sum, this thesis characterizes two distinct and potentially complementary mechanisms whereby calcium can shape cholinergic excitability in prefrontal circuits essential to executive function and how these interactions are disrupted in models of brain disease with documented cognitive dysfunction.
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Table of Contents

Acknowledgments .................................................................................................................... iv

Table of Contents .................................................................................................................. v

List of Tables ........................................................................................................................... viii

List of Figures .......................................................................................................................... ix

List of Supplemental Materials .............................................................................................. xi

List of Abbreviations ............................................................................................................... xiii

Chapter 1: General Introduction ............................................................................................ 1

1.1 The Prefrontal Cortex: An Overview of Anatomy and Function ........................................ 2

1.1.1 The anatomy of the prefrontal cortex ........................................................................... 2

1.1.2 The function of the prefrontal cortex ........................................................................... 4

1.2 The Cholinergic Modulation of the Prefrontal Cortex is Essential to Working Memory and
Attention ..................................................................................................................................... 7

1.3 Acetylcholine Modulates the Deep Layers of the PFC via Nicotinic and Muscarinic
Acetylcholine Receptors ........................................................................................................... 10

1.3.1 Acetylcholine innervates the deep layers of prefrontal cortex ..................................... 10

1.3.2 The nicotinic acetylcholine receptors .......................................................................... 13

1.3.3 The muscarinic acetylcholine receptors ....................................................................... 17

1.4 Disruption of Prefrontal Executive Function in Neurological and Psychiatric Illness ....... 26

1.4.1 Early life stress and susceptibility to psychiatric illness ............................................... 27

1.4.2 Alzheimer's Disease (AD) .......................................................................................... 28

1.5 Questions & Scope of Thesis ............................................................................................ 30

Chapter 2 ................................................................................................................................ 32

2 Materials & Methods ........................................................................................................... 32

2.1 Animals ............................................................................................................................... 32

2.1.1 Early stress paradigm .................................................................................................... 34

2.2 Brain Slice Preparation ...................................................................................................... 35

2.3 Electrophysiological Recordings ....................................................................................... 35

2.4 Calcium Imaging ................................................................................................................ 36

2.5 Ion Substitution Experiments ............................................................................................ 37

2.6 Pharmacology .................................................................................................................... 38
2.7 Statistical Analyses........................................................................................................... 38

Chapter 3................................................................................................................................. 40

3 Early Stress Prevents the Potentiation of Muscarinic Excitation by Calcium Release in Adult Prefrontal Cortex........................................................................................................... 41

3.1 Abstract................................................................................................................................... 41

3.2 Introduction............................................................................................................................ 42

3.3 Materials and Methods........................................................................................................... 44

3.3.1 Animals ................................................................................................................................. 44

3.3.2 Early stress paradigm ........................................................................................................... 45

3.3.3 Brain slice preparation and recording conditions ................................................................. 45

3.3.4 Electrophysiological recordings and multiphoton Ca\textsuperscript{2+} imaging........................................ 45

3.3.5 Pharmacology ..................................................................................................................... 47

3.3.6 Statistics ............................................................................................................................. 47

3.4 Results.................................................................................................................................... 47

3.4.1 ACh-elicited somatic Ca\textsuperscript{2+}-release potentiates excitatory muscarinic currents ........... 47

3.4.2 Ca\textsuperscript{2+}-potentiation of I_{ACh} develops in young adulthood and is disrupted by early stress 53

3.4.3 Early stress produces developmentally-specific changes in PFC gene expression* ............ 54

3.4.4 Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange potentiates muscarinic excitation in healthy adult controls .... 55

3.5 Discussion.............................................................................................................................. 57

3.5.1 Mechanisms of muscarinic modulation of the PFC circuits of executive function .......... 58

3.5.2 G_{o}\textsubscript{q}-coupled receptor signaling pathway complexity and vulnerability ............... 61

3.5.3 Interactions of genes, development and early stress .......................................................... 61

Chapter 4.................................................................................................................................... 64

4 Impaired Cholinergic Excitation of Prefrontal Attention Circuitry in the TgCRND8 Model of Alzheimer’s Disease ........................................................................................................... 65

4.1 Abstract................................................................................................................................... 65

4.2 Introduction............................................................................................................................ 65

4.3 Materials and Methods........................................................................................................... 67

4.3.1 Animals ................................................................................................................................. 67

4.3.2 Brain slice preparation and recording conditions ................................................................. 68

4.3.3 Electrophysiological recordings ............................................................................................ 68

4.3.4 Calcium imaging .................................................................................................................. 69
4.3.5 Statistical analyses

4.4 Results

4.4.1 TgCRND8 mice cannot sustain peak excitation by acetylcholine in prefrontal layer 6 70
4.4.2 Probing mechanisms of altered excitability in TgCRND8 prefrontal layer 6 neurons 74
4.4.3 Enhanced supra-threshold calcium flux underlies aberrations in the cholinergic
excitation of layer 6 pyramidal neurons of TgCRND8 animals 81
4.4.4 Inhibition of SK channels improves cholinergic excitability of TgCRND8 neurons 85
4.5 Discussion

Chapter 5

5 General Discussion

5.1 Summary of Findings

5.2 Cholinergic Calcium Signaling Modulates Neurons of Prefrontal Cortical Output Layers

5.3 Calcium Shapes Cholinergic Excitability

5.3.1 Regulation of acetylcholine-elicited calcium release by cellular activity, developmental
stage and environmental factors

5.3.2 The potentiation of muscarinic excitation by agonist-induced calcium release in
prefrontal layer 5 pyramidal cells

5.3.3 The modulation of calcium-dependent afterhyperpolarization potential in prefrontal
layer 6 pyramidal cells

5.4 Towards an Understanding of Key Biochemical Pathways Underlying Executive Function and
their Susceptibility to Disruption in Neurological and Psychiatric Illness

5.4.1 Go coupled receptor signaling pathway complexity and vulnerability in prefrontal
cortex

5.4.2 Dyshomeostasis of calcium signaling in neurological and psychiatric disorders

5.5 Conclusion

References

Appendices

6 Supplementary Materials

6.1 Supplementary Methods

6.1.1 Morphological Reconstruction

6.1.2 Quantitative PCR (qPCR)*

6.2 Supplementary Figures

6.3 Supplementary Tables
List of Tables

Table 1. Ionic conductances mediating M$_1$-like muscarinic effects in neocortex and their effects on neuronal excitability. 22

Table 2. Electrophysiological properties of layer 6 pyramidal neurons of the PFC of TgCRND8 mice and WT controls. ................................................................. 69
List of Figures

**Figure 1.** Acetylcholine densely projects to deep cortical output layers 5 and 6..................12

**Figure 2.** Subunit composition and layout of nicotinic acetylcholine receptor subunits in layer 6 of medial prefrontal cortex.................................................................16

**Figure 3.** Cellular mechanisms of muscarinic modulation..............................................19

**Figure 4.** The TgCRND8 model phenotypic timeline......................................................33

**Figure 5.** Early stress (ES) paradigm of maternal separation........................................34

**Figure 6.** ACh-elicited somatic Ca\(^{2+}\) increases in layer V pyramidal cells of adult PFC. ....48

**Figure 7.** ACh-elicited somatic Ca\(^{2+}\) increases potentiate muscarinic responses in layer V pyramidal cells of adult PFC.................................................................49

**Figure 8.** Muscarinic excitation (I\(_{\text{ACH}}\)) is potentiated by ACh-elicited Ca\(^{2+}\) release from intracellular stores (dF/F\(_{\text{ACH}}\))..................................................................................................52

**Figure 9.** The Ca\(^{2+}\) potentiation of I\(_{\text{ACH}}\) emerges in young adulthood but is absent following early stress (ES).........................................................................................................54

**Figure 10.** Schematic to illustrate the hypothesis that PFC excitatory muscarinic currents are potentiated via NCX- and NCKX-mediated electrogenesis........................................60

**Figure 11.** Impaired excitation in response to acetylcholine in layer 6 prefrontal cortex of TgCRND8 mice........................................................................................................74

**Figure 12.** Reduced intrinsic excitability in L6 prefrontal pyramidal neurons of TgCRND8 mice................................................................................................................................75

**Figure 13.** The afterhyperpolarization potential (AHP) is enhanced in L6 pyramidal neurons of TgCRND8 mice........................................................................................................76
Figure 14. Spiking elicits greater Ca$^{2+}$ responses in TgCRND8 neurons. ..............................................78

Figure 15. The post-burst AHP is apamin-sensitive in WT. .................................................................80

Figure 16. Enhanced AHPs in TgCRND8 neurons result from a greater SK contribution..............81

Figure 17. Greater Ca$^{2+}$ responses accompany acetylcholine-elicited firing in the TgCRND8. .................................................................................................................................85

Figure 18. Inhibition of SK improves cholinergic excitability in TgCRND8 mice. ....................87

Figure 19. Acetylcholine elicits intracellular calcium mobilization together with the modulation of cellular excitability via the nicotinic and muscarinic receptors. .........................94

Figure 20. Early stress prevents the potentiation of muscarinic excitation by calcium release in adult prefrontal cortex. Summary of Chapter 3 findings........................................95

Figure 21. Dysregulated intrinsic and cholinergic excitability of layer 6 PFC in 3-4 month old TgCRND8 mice as a result of enhanced contributions from calcium-activated SK channels. Summary of Chapter 4 findings.................................................................96
Supplemental Figure 1. $A\text{Ch}_{Ca^{2+}}$ and $A\text{Ch}_{No\ Ca^{2+}}$ neurons are similar morphologically...... 156

Supplemental Figure 2. Developmental emergence of the potentiation of $I_{A\text{Ch}}$ by $Ca^{2+}$ release is absent following early stress....................................................................................... 157

Supplemental Figure 3. The developmental consolidation of the $Ca^{2+}$ potentiation of $I_{A\text{Ch}}$ occurs as $dF/F_{A\text{Ch}}$ responses become more precisely timed in young adulthood and fails to occur following early stress (ES).................................................................................................................. 159

Supplemental Figure 4. Early stress (ES) produces changes in the expression of genes involved in Ga<sub>q</sub>-coupled receptor signaling pathways in both adolescent and adult brain. ........................................................................................................................................ 160

Supplemental Figure 5. $dF/F_{A\text{Ch}}$ yields a supplementary $I_{A\text{Ch}}$ coinciding with $dF/F_{A\text{Ch}}$ clearance...................................................................................................................................................... 161

Supplemental Figure 6. Ion substitution experiments reveal that $dF/F_{A\text{Ch}}$ clearance is $Na^{+}$- and $K^{+}$-dependent and that $I_{A\text{Ch}}$ is $Na^{+}$-dependent........................................................................................................................................ 162

Supplemental Figure 7. Pharmacological inhibition of $Na^{+}/Ca^{2+}$ exchange reduces $I_{A\text{Ch}}$. 163

Supplemental Figure 8. Differences in post-burst AHP are most pronounced between 100-500 ms post-burst........................................................................................................................................ 164

Supplemental Figure 9. Effect of apamin (200 nM) in WT controls................................................. 165
Supplemental Table 1. Morphological properties of AChCa2+ and AChNoCa2+ pyramidal cells.

Supplemental Table 2. Interaction of development and the experience of early stress in validated gene expression.
List of Abbreviations

\( \alpha 4 \beta 2^* \)  \( \alpha 4 \beta 2 \)-containing nicotinic receptors

ACh  Acetylcholine

ACh\(_{\text{Ca}^{2+}} \)  Calcium responding cell (to acetylcholine)

ACh\(_{\text{No Ca}^{2+}} \)  Non-calcium responding cell (to acetylcholine)

ACSF  Artificial cerebrospinal fluid

AD  Alzheimer’s disease

AHP  Afterhyperpolarization potential

AOI  Area of interest

APP  Amyloid precursor protein

BA  Barrier filter

BAPTA  1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid

BDNF  Brain Derived Neurotrophic Factor

Ca\(^{2+} \)  Calcium

ChAT  Choline acetyltransferase

DAG  Diacylglycerol

dF/F\(_{\text{ACh}} \)  Acetylcholine-elicited calcium release

DSM  Diagnostic and Statistical Manual of Mental Disorders

ES  Early stress

I\(_{\text{ACh}} \)  Acetylcholine-elicited inward current

IP\(_{3} \)  Inositol 1,4,5-trisphosphate

K\(^+ \)  Potassium
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>L5</td>
<td>Layer 5</td>
</tr>
<tr>
<td>L6</td>
<td>Layer 6</td>
</tr>
<tr>
<td>LUT</td>
<td>Look up table</td>
</tr>
<tr>
<td>M₁R</td>
<td>M₁ muscarinic acetylcholine receptor</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium</td>
</tr>
<tr>
<td>NCKX</td>
<td>Potassium-dependent sodium-calcium exchanger</td>
</tr>
<tr>
<td>NCX</td>
<td>Sodium-calcium exchanger</td>
</tr>
<tr>
<td>NMDG</td>
<td>N-methyl-D-glucamine</td>
</tr>
<tr>
<td>NSCC</td>
<td>Non-selective cation channel</td>
</tr>
<tr>
<td>OGB</td>
<td>Oregon-Green BAPTA</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal cortex</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phospholipid phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potential channel</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
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<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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INTRODUCTION

Acknowledgements

Chapter 1: General Introduction

1.1 The Prefrontal Cortex: An Overview of Anatomy and Function

The prefrontal cortex (PFC) lies at the forefront of the brain and is its central executive. The PFC is evolutionarily recent and, like other association areas, it has disproportionately expanded in the mammalian cortex. This is especially true in the primate brain (Buckner and Krienen, 2013) and most distinctively so in humans, where the PFC constitutes approximately one third of the neocortex (Fuster, 2001). Ultimately, this feature has allowed us to evolve complex thoughts and actions that distinguish us from all other animals. As will be discussed, however, key anatomical and functional features of the prefrontal cortex are evolutionarily conserved.

1.1.1 The anatomy of the prefrontal cortex

Historically, the PFC was delineated based on cytoarchitectonic features after the seminal work of Brodmann (1909). The frontal cortex as a whole lacks a clear layer 4 and as such is said to be agranular or dysgranular (Wise, 2008). This feature is common to all mammals (Wise, 2008) but, in contrast with the distinctly agranular frontal motor areas, parts of the primate frontal cortex exhibit some granularity; it is this characteristic which first served to define the prefrontal cortex. This became the source of much debate with respect to the nature of the PFC as these cytoarchitectonic criteria proved untenable in establishing homologies across species, being unique to primes (Preuss, 1995; Uylings et al., 2003; Petrides, 2005). With the work of Rose & Woolsey (1948), however, greater importance was placed on neural connectivity as anatomical
criterion and the PFC became defined as that region of the frontal lobe which 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). Ultimately, the pattern and density of neural connections, along with ontogenetic and functional features indicate that all mammals share some form of prefrontal cortex (Uylings and van Eden, 1990; Uylings et al., 2003; Wise, 2008).

It is clear that none of the cognitive functions of the PFC can be understood “if taken out of a broad connectionist context” (Fuster, 2001). One of the key features of the PFC is its dense reciprocal cortico-cortical connectivity, both within the PFC itself and across several other cortical areas, including premotor, sensory and limbic cortices (Goldman-Rakic, 1988; Uylings and van Eden, 1990; Pandya and Yeterian, 1996; Barbas, 2000; Öngür and Price, 2000; Uylings et al., 2003). Major subcortical afferents to the prefrontal cortex arise from the thalamus, amygdala, basal forebrain and brainstem (Öngür and Price, 2000; Hoover and Vertes, 2007) whereas its most significant efferent pathways project to the striatum, thalamus, hypothalamus, amygdala, and brainstem (Uylings and van Eden, 1990; Öngür and Price, 2000; Vertes, 2003; Gabbott et al., 2005). Importantly, the PFC is the only cortical brain region with reciprocal connections to the neuromodulatory centres of the basal forebrain and brainstem (Uylings and van Eden, 1990; Uylings et al., 2003): to the cholinergic cells of the basal forebrain (Gaykema et al., 1991; Zaborszky et al., 1997; Ghashghaei and Barbas, 2001), the noradrenergic neurons of the locus coeruleus (Cedarbaum and Aghajanian, 1978; Luppi et al., 1995), the serotonergic cells of the dorsal raphe (Aghajanian and Wang, 1977) and the dopaminergic cells of the ventral tegmental area
(Geisler and Zahm, 2005). As such, the neuromodulation of the PFC can exert profound influences on the networks of executive function and widespread influences on the brain as a whole.

1.1.2 The function of the prefrontal cortex

The PFC constitutes the highest level of the cortical hierarchy (Fuster, 2001) and mediates executive function, defined as “the coordinated operation of various processes to accomplish a particular goal in a flexible manner” (Funahashi, 2001).

Patients with prefrontal damage—as a result of traumatic injury, stroke and/or vascular deterioration, for example—exhibit various cognitive deficits, including impairments in working memory, attention, planning, emotional regulation and cognitive control (Szczepanski and Knight, 2014). Likewise, animal studies have shown that PFC lesions lead to an array of cognitive impairments (Uylings et al., 2003, Kolb 1984, Dalley et al. 2004, Kesner and Churchwell 2011). While it does appear that the PFC exhibits subregion specialization to a certain extent, there is limited evidence of double dissociations in the PFC (Duncan and Owen, 2000; Duncan, 2001; Wilson et al., 2010; Szczepanski and Knight, 2014). Instead, neuroimaging studies have revealed that distributed prefrontal activation is associated with a broad range of cognitive demands (Duncan and Owen, 2000; Ikkai and Curtis, 2011; Cole et al., 2013). The emerging view is that the networks of the prefrontal cortex subserve executive function via parallel distributed processing (Goldman-Rakic, 1988; Mesulam, 1990; Cole et al., 2012; 2013) by virtue of its dense interconnectivity with several cortical areas and subcortical structures that essentially position it as an important hub (Buckner et al., 2009; Sporns, 2014). As such, the details of subregion connectivity underlie some region-specific
functionalization (Goldman-Rakic, 1988; Barbas, 2000) but it may be that the function of the PFC as a whole is more than the sum of its parts (Wilson et al., 2010).

Executive function is not a unitary concept (Elliott, 2003) and the details of PFC function is an area of active research where much remains to be resolved. Nonetheless, integrative theories of PFC function have been put forth and hinge on neurophysiological findings pertaining to the cellular mechanisms of working memory and attention, which I will describe next. Briefly, it has been proposed that “the active maintenance of patterns of activity in the prefrontal cortex that represent goals and the means to achieve them (Miller and Cohen, 2001)” constitutes a core feature of PFC function. The role of the PFC in complex temporal integration has also been emphasized (Fuster, 2001; Wilson et al., 2010) as well as the capability for adaptive coding, where “neurons adapt their properties to carry specifically information that is relevant to current concerns (Duncan, 2001)”. Much insight can be provided by investigations of the PFC at the cellular level, to which this thesis aims to contribute.

1.1.2.1 Cellular mechanisms of prefrontal function

The first neurophysiological studies pertaining to the cellular bases of prefrontal function were performed in monkeys trained on a well-characterized working memory task, the delayed response paradigm (for review of this task, see Rodriguez and Paule, 2009). A delayed response trial begins with the brief presentation of a cue, followed by a delay period, and is terminated by a behavioural response that is contingent on the correct retention and recall of cue information. It was previously known that animals with PFC lesions were significantly impaired on this task (Jacobsen, 1935; Butters and Pandya, 1969; Fuster and Alexander, 1970) and, building on this, Fuster and colleagues (1971)
hypothesized that changes in neuronal activity within the intact PFC should be associated with the events taking place during delayed response trials. Indeed, extracellular microelectrode unit recordings in awake behaving monkeys revealed that neurons in the PFC exhibit sustained increases in firing rate during the delay period (Fuster and Alexander, 1971). Independently, Kubota and Niki (1971) demonstrated the same on a slight variation of this task and many subsequent studies have since replicated these finding (Kubota and Niki, 1971; Funahashi et al., 1989; Carlson et al., 1997; Rao et al., 1997; Rainer et al., 1998; Funahashi, 2001). Most notably, Goldman-Rakic and her team employed an oculomotor variation of the delayed response task, using a visual cue and tracking eye saccades to the remembered cued location as behavioural response (Funahashi et al., 1989). In this way, they were able to further demonstrate that neurons exhibiting delay-period activity also have mnemonic receptive fields, which in the case of this spatial memory task entailed that visual cues in a neuron’s preferred location elicited maximal delay-period activity. This provided compelling evidence in support of the idea that that delay-period activity reflects the maintenance of relevant information in “temporary active storage” (Funahashi, 2001) and that it “bridges across time” the sensory cue with the contingent response (Fuster, 2001; Ikkai and Curtis, 2011). Since, delay activity has been observed across both spatial and non-spatial working memory tasks, reflecting retention of non-spatial features such as object shape, patterns or colors (Quintana et al., 1988; Miller et al., 1996; Rao et al., 1997; Rainer et al., 1998), for example, as well as displaying responsiveness across sensory modalities, including in response to auditory (Watanabe, 1992; Bodner et al., 1996) and tactile stimuli (Romo et al., 1999).
It became the widely accepted view that delay activity constitutes the cellular basis of working memory (Goldman-Rakic, 1995a; Funahashi, 2001). Since, delay activity has been found to be widespread throughout the PFC and to exhibit considerable overlap across a variety of task demands (Duncan, 2001; Funahashi, 2001; Postle, 2006; Wise, 2008; Ikkai and Curtis, 2011). Interestingly, Wise and colleagues have demonstrated that in most PFC neurons, delay activity in fact preferentially reflects attended locations as opposed to remembered ones (Lebedev et al., 2004). With the advent of functional neuroimaging, it became possible to measure increases in PFC activity in humans engaged in various tasks and, while not as temporally precise as their more invasive neurophysiological counterparts carried out in monkeys, these studies have revealed that delay activity is indeed a more general feature of prefrontal function. Thus, similar increases in activity are observed not only during working memory, but also as a reflection of attentional processes and motor intention, with any one executive task eliciting delay period activity in distributed regions of PFC (Ikkai and Curtis, 2011). From this work, Ikkai and Curtis (2011) have argued that “there is a single neural mechanism dependent upon persistent activity in the PFC…that is common to maintaining [working memory] representations, attention, and intentions, and perhaps a host of additional spatial cognitions”. Thus, while the meaning of delay activity may be subject to interpretation, it is clear that it is a cellular property fundamental to prefrontal executive function.

1.2 The Cholinergic Modulation of the Prefrontal Cortex is Essential to Working Memory and Attention

Working memory and attention intersect as do the cellular mechanisms that are thought to underlie them (Awh and Jonides, 2001; Lebedev et al., 2004; Postle, 2006; Ikkai and
As will be discussed, the cholinergic modulation of the prefrontal cortex is essential to these executive functions and its effects at the cellular level are consistent with delay activity.

Early pharmacological studies have demonstrated that administration of anticholinergic drugs impairs cognitive function (Deutsch and Rocklin, 1967; Deutsch, 1971; Warburton and Brown, 1971) and cholinergic cell loss has long been a known contributor to senile dementia and Alzheimer’s disease (Bartus et al., 1982; Whitehouse et al., 1982; Coyle et al., 1983). Lesion studies have further implicated the cholinergic modulation of PFC as essential to executive function. The prefrontal cortex receives dense cholinergic innervation arising from the basal forebrain, principally from the basal nucleus and parts of the diagonal band, but also from magnocellular preoptic nucleus and substantia innominata (Bigl et al., 1982; Woolf et al., 1983; Mesulam et al., 1983a; 1983b; Rye et al., 1984; Lewis, 1991; Woolf, 1991). In the 1990s, excitotoxic lesions of the cholinergic neurons of the basal forebrain—which led to significant reduction of cholinergic fibers in the cortex as revealed by decreased staining for choline acetyltransferase (ChAT) (Robbins et al., 1989b; Markowska et al., 1990; Muir et al., 1992; Voytko et al., 1994)—were shown to produce marked impairments both in attention (Robbins et al., 1989a; Dunnett et al., 1991; Muir et al., 1992; 1993; Pang et al., 1993; Voytko et al., 1994) and working memory (Markowska et al., 1990; Winkler et al., 1995). Since it was also shown that the deficits incurred by these lesions could be reversed by cortical grafts of cholinergic cells (Muir et al., 1992; Winkler et al., 1995), it was established that cholinergic innervation of the neocortex was especially important to executive function. However, it remained a possibility that non-cholinergic cell loss in the basal forebrain also contributed to the observed effects. With the development of
the cholinergic-specific immunotoxin, IgG-saporin, it was therefore possible to pinpoint
the role of the cholinergic modulation of PFC with more certainty. Intrabasalis infusions
of the cholinergic immunotoxin 192 IgG-saporin led to the loss of cortical cholinergic
afferents, reduced acetylcholine efflux in the prefrontal cortex, and significant
impairments on attention tasks (McGaughy et al., 1996; 2002). Furthermore, the
selective cholinergic deafferentation of PFC was shown to be sufficient to produce these
deficits (Dalley, 2004; Parikh et al., 2007; Newman and McGaughy, 2008) and to
correlate with the attenuation of PFC unit firing during attentional tasks (Gill et al.,
2000). Recently, the cholinergic deafferentation of PFC has also been shown to lead to
impairments on the delay-response task (Croxson et al., 2011), although a more
specific role for attention could not be ruled out. It is in fact very difficult to disentangle
working memory from attention.

Several pharmacological studies have also shown that cholinergic antagonism
impairs performance on delay-response (Penetar and McDonough, 1983; Granon et al.,
1995; Plakke et al., 2008; Zhou et al., 2011) as well as attentional tasks (Muir et al.,
1992; Mirza and Bright, 2001). Zhou and colleagues (2011) have further demonstrated
that prefrontal muscarinic receptor antagonism impairs not only behavioural
performance on delay response tasks, but also neuronal delay activity in the PFC.
Moreover, studies utilizing null mutations of ACh receptor subtypes have shown
selective deficits on prefrontal-dependent delay non-matching to sample working
memory tasks (Anagnostaras et al., 2003) and cholinergic receptors in the deep layers
of the PFC, in particular, have recently been proven to be necessary for optimal
attentional performance (Bailey et al., 2010; Guillem et al., 2011; Proulx et al., 2013).
Finally, the importance of prefrontal cholinergic modulation was further suggested by microdialysis studies showing robust acetylcholine efflux within the prefrontal cortex during the performance of attention tasks (Himmelheber et al., 2000; Passetti et al., 2000; Dalley et al., 2001) and the development of choline-sensitive microelectrodes, which offer greater temporal resolution than microdialysis probes, have further revealed that acetylcholine release in the prefrontal cortex increases rapidly and transiently—on the timescale of seconds to minutes—during the performance of attention tasks (Parikh et al., 2007).

1.3 Acetylcholine Modulates the Deep Layers of the PFC via Nicotinic and Muscarinic Acetylcholine Receptors

1.3.1 Acetylcholine innervates the deep layers of prefrontal cortex

The cholinergic innervation of the PFC is present in all cortical layers (Lewis, 1991) but is most pronounced in layer 5 (L5) and layer 6 (L6) (Ghashghaei and Barbas, 2001; Henny and Jones, 2008; Bloem et al., 2014). There, it exerts robust excitatory actions via two classes of acetylcholine receptors: the ionotropic nicotinic receptors and the metabotropic muscarinic acetylcholine receptors, which are G-protein coupled. Acetylcholine likely exerts a particularly important influence on the circuits of executive function given that L5 and L6 constitute the major cortical output layers. Interestingly, there appears to be laminar specificity to cholinergic effects in PFC. Whereas L5 neurons are primarily subject to postsynaptic mscarinic influences (Gulledge et al., 2009; Proulx et al., 2014), L6 neurons are preferentially subject to robust nicotinic excitation (Kassam et al., 2008; Tian et al., 2011; Poorthuis et al., 2012).
Layer 5 mainly receives feedforward information from the upper cortical layers 2/3 (Douglas and Martin, 2004; Thomson and Lamy, 2007; Weiler et al., 2008). Approximately 30% of L5 PFC neurons project to the lateral hypothalamus, ~20% to the striatum and ~10% to the basolateral amygdala (Gabbott et al., 2005). By contrast, a large proportion of L6 pyramidal cells are corticothalamic (Gabbott et al., 2005; Thomson, 2010) and constitute the major source of excitatory afferents to the thalamus (Sherman, 2007), where they affect the inhibitory reticular thalamic neurons (Zhang, 2003) and the excitatory thalamocortical projection neurons (Krosigk et al., 1999), including those of the midline and intralaminar thalamic nuclei that have long been implicated in awareness and attention (Berendse and Groenewegen, 1991; Van der Werf et al., 2002; Vertes, 2006; Hoover and Vertes, 2007). These neurons integrate highly processed information from layer 5 pyramidal cells, from layer 6 cortico-cortical neurons, and from direct thalamic inputs (Thomson, 2010). Importantly, there are 10 times more corticothalamic feedback projections than there are thalamocortical afferents (Cudeiro and Sillito, 2006). While not all neurons in layer 6 are corticothalamic, it has recently been shown that layer 6 neurons as a class exert powerful gain control over all the other cortical layers (Olsen et al., 2012) and that they can strongly activate cortical output layer 5 (Kim et al., 2014).

Pyramidal cells constitute ≈ 80% of neurons in L5 and L6 PFC (Gabbott et al., 2005), consistent with the rest of the cerebral cortex (Larkum, 2013). A schematic of the cholinergic innervation of PFC is shown in Figure 1.
Figure 1. Acetylcholine densely projects to deep cortical output layers 5 and 6. Dense cholinergic innervation of the deep prefrontal layers is highlighted in red. Arrows indicate weighted efferent projections. Inset, acetylcholinesterase-positive terminals and fibers within the PFC, adapted from Ghashghaei & Barbas (2001). L5 pyr, layer 5 pyramidal; L6 pyr, layer 6 pyramidal. Cresyl violet stained cortical section adapted from McNeil, Mori and Cheng (1999).
1.3.2 The nicotinic acetylcholine receptors

Nicotinic acetylcholine receptors are pentameric ligand-gated cation channels (Albuquerque et al., 2009; Gotti et al., 2009), permeable to Na\(^+\), K\(^+\) and Ca\(^{2+}\) ions (Fucile, 2004; Gotti et al., 2009). Two families of subunits can contribute to the pentameric structure necessary for functional nicotinic receptors: the α subunits (α2-10) and the β subunits (β 2-4) (Albuquerque et al., 2009; Gotti et al., 2009). They are arranged in a pinwheel around a central pore, assembled either as α-containing homomers or α / β heteromers. Nicotinic receptors are widely expressed in the central nervous system and subunit composition differs from one region to the next (Albuquerque et al., 2009; Gotti et al., 2009). The subunit composition and stoichiometry of nicotinic receptors influence their functional properties, with important implications for nicotinic signaling (Moroni, 2006; Tapia et al., 2007; Bailey et al., 2010; Marks et al., 2010; Grady et al., 2012).

The most widely expressed nicotinic acetylcholine receptors in the brain are the α4β2-containing receptors (α4β2*) (Léna and Changeux, 1999; Ferreira et al., 2001; Perry et al., 2002; Gotti et al., 2009), which are prominently expressed throughout cortex (Wada et al., 1989; Hill et al., 1993; Nakayama et al., 1995; Gotti and Clementi, 2004). The α4β2* receptors have high affinity for nicotinic agonists (including acetylcholine and nicotine) and desensitize slowly, on the timescale of seconds (Quick and Lester, 2002; Gotti et al., 2006; 2009). The α4β2* nicotinic receptors can assume different stoichiometries, including \((α4)_2(β2)_3\) and \((α4)_3(β2)_2\), but they can also incorporate the accessory α5 subunit to form \((α4)_2(β2)_2(α5)\) receptors (α4α5β2) (Wada et al., 1990; Conroy et al., 1992; Ramirez-Latorre et al., 1996; Kuryatov et al., 2008; Albuquerque et al., 2009; Gotti et al., 2009). The accessory α5 subunits cannot form
functional channels by themselves, since they do not contribute to the acetylcholine binding site, and thus require co-assembly with other α and β subunits (Gotti and Clementi, 2004; Gotti et al., 2009). However, inclusion of α5 can alter α4β2* nicotinic receptor properties substantially (Ramirez-Latorre et al., 1996; Tapia et al., 2007; Kuryatov et al., 2008): it can enhance receptor assembly and expression (Ramirez-Latorre et al., 1996), modulate receptor sensitivity to acetylcholine (Moroni, 2006; Kuryatov et al., 2008; Gotti et al., 2009; McClure-Begley et al., 2009; Bailey et al., 2010), increase Ca^{2+} permeability (Kuryatov et al., 2008), and confer sensitivity to allosteric modulation by galanthamine (Kassam et al., 2008; Kuryatov et al., 2008).

Immunohistochemistry for the YFP-tagged α4 subunit suggests that high affinity nicotinic receptors will be densely expressed in layer 6 prefrontal cortex (Alves et al., 2010), where the accessory α5 subunit is also prominently expressed (Wada et al., 1990; Marks et al., 1992; Salas et al., 2003). Interestingly, while only one fifth of all α4β2* nicotinic receptors in the brain are estimated to contain the α5 accessory subunit (Brown et al., 2007b; Gotti et al., 2009), prefrontal layer 6 nicotinic receptors appear to incorporate α5 to a disproportionately large extent (Bailey et al., 2010). Indeed, functional concentration-response analyses of prefrontal corticothalamic neurons from WT and α5 knockout mice (α5^{-/-}) suggest that the vast majority of α4β2* nicotinic receptors of its layer 6 neurons express this subunit (Bailey et al., 2010). This unique expression pattern has ramifications for attentional signaling and behavior (Bailey et al., 2010; Guillem et al., 2011; Proulx et al., 2013).

At the cellular level, acetylcholine elicits robust excitatory responses in the layer 6 corticothalamic neurons of the medial prefrontal cortex that appear to be directly
mediated by stimulation of somatodendritic postsynaptic α4α5β2 nicotinic receptors (Kassam et al., 2008; Bailey et al., 2010; Tian et al., 2011). Acetylcholine binding to the nicotinic receptor leads to rapid conformational changes that result in channel opening and the flow of Na⁺, K⁺ and Ca²⁺ cations through the pore (Albuquerque et al., 2009; Gotti et al., 2009). When sufficiently large, this membrane depolarization can lead to the generation of action potentials. Acetylcholine depolarizes the vast majority of layer 6 pyramidal cells in this way (Kassam et al., 2008), but these excitatory nicotinic responses are completely eliminated in β2⁻/⁻ mice (Guillem et al., 2011; Tian et al., 2011), which lack functional α4β2* nicotinic receptors, and are significantly reduced in α5⁻/⁻ mice (Tian et al., 2011).

A rapid α7-mediated nicotinic response has been documented in the prefrontal cortex of juvenile mice (Poorthuis et al., 2012). Importantly in this layer, α4β2*-containing nicotinic receptors on thalamocortical terminals strongly facilitate thalamic excitation of layer 5 pyramidal neurons (Gioanni et al., 1999; Lambe et al., 2003, 2005), an indirect effect that translates into a large increase in the frequency of rapid, glutamatergic excitatory post-synaptic currents. Of note, a positive feedback relationship has been demonstrated between nicotinic-elicited prefrontal glutamatergic release and the release of acetylcholine itself from cholinergic terminals in prefrontal cortex (Parikh et al., 2008, 2010; Howe et al., 2010).
Figure 2. Subunit composition and layout of nicotinic acetylcholine receptor subunits in layer 6 of medial prefrontal cortex.  

**Fig. 2A** Schematics showing three possible compositions of α4β2* nicotinic receptors within layer 6 neurons of medial prefrontal cortex. Figure adapted from McKay et al. 2007 **Fig. 2B** Photomicrograph of mouse medial prefrontal cortex immunostained for YFP-tagged nicotinic acetylcholine receptor α4 subunits, putatively expressed in α4β2*-containing cells as shown at lower resolution by Marks and colleagues (1992). White matter on the right and the medial pial surface is on the left; adapted from Alves et al. (2010). Scale bar 200 µm. **Fig. 2C** In situ hybridization showing a dense band of α5 nicotinic subunit mRNA expression in layer 6 of the medial prefrontal cortex; adapted from Wada et al. (1995). Figure from Proulx et al. (2013).
1.3.3 The muscarinic acetylcholine receptors

The muscarinic acetylcholine receptors (mACHR) are metabotropic 7-transmembrane-spanning G-protein coupled receptors (Felder, 1995), of which there are 5 subtypes: the excitatory M₁-like muscarinic receptors (M₁, M₃, M₅), which couple to phospholipase C and stimulate Ca²⁺ release from intracellular stores, and the inhibitory M₂-like muscarinic receptors (M₂, M₄), which attenuate adenylyl cyclase activity (Felder, 1995; Caulfield and Birdsall, 1998). Cloning of the muscarinic receptors was achieved in the late 1980s (Kubo et al., 1986; Bonner et al., 1987) and their crystal structure has recently been revealed (Haga et al., 2012; Kruse et al., 2012). The muscarinic receptor subtypes display profound sequence homology with a strictly conserved orthosteric binding pocket located in a large extracellular vestibule (Kruse et al., 2012). Small differences in the distance between transmembrane domains 5 and 6, particularly on the intracellular side, likely confer G-protein specificity (Kruse et al., 2012).

The M₁ receptor is the predominant muscarinic receptor subtype expressed in the PFC, much like the rest of the neocortex (Buckley et al., 1988; Levey et al., 1991). Both pyramidal cells and interneurons are subject to cholinergic innervation (Mrzljak et al., 1995) but cortical M₁ receptors are mainly expressed in the perisomatic and dendritic compartments of pyramidal cells (Mrzljak et al., 1993; Yamasaki et al., 2010). Functionally, Allan Gulledge and colleagues (2009) have confirmed by use of M₁, M₃, M₅ and dual M₃/M₅ knockout mice, that the M₁ receptors mediate the muscarinic modulation of PFC pyramidal cells (Gulledge et al., 2009). Activation of the M₁ muscarinic receptors leads to the dissociation of the bound G<sub>q/11</sub>-protein complex into α and βγ′ subunits, which in turn couple to downstream effectors that include ion channels and second messengers (Felder, 1995). Phospholipase C (PLC) is the primary effector
of muscarinic \(M_1\) receptors (Berstein et al., 1992) and its activation leads to the breakdown of phosphatidylinositol 4,5-biphosphate (PIP\(_2\)) into diacylglycerol (DAG) and inositol triphosphate (IP\(_3\)) (Downes, 1982; Berridge and Irvine, 1989; Felder, 1995), leading to release of Ca\(^{2+}\) from IP\(_3\)-sensitive stores (Berridge and Irvine, 1984; Power and Sah, 2002; Gulledge, 2005). In addition, activation of muscarinic \(G_{\alpha q}\)-coupled receptors exerts robust and prolonged excitatory actions in cortical neurons (Krnjević et al., 1971; McCormick and Prince, 1986; Haj-Dahmane and Andrade, 1996; Gulledge et al., 2009) and has been shown to stimulate persistent activity (Egorov et al., 2002; Zhang and Seguela, 2010; Rahman and Berger, 2011; Zhou et al., 2011). Indeed, muscarinic stimulation of cortical neurons elicits a slow and prolonged depolarization of the membrane potential that can lead to spiking (Krnjević et al., 1971; McCormick and Prince, 1986; Haj-Dahmane and Andrade, 1996; Klink and Alonso, 1997; Shalinsky et al., 2002; Egorov et al., 2003; Carr et al., 2007) and promote spike acceleration (Krnjević et al., 1971; McCormick and Prince, 1986; Schwindt et al., 1988; Haj-Dahmane and Andrade, 1998; Gulledge et al., 2009; Santini et al., 2012). A slow inward current underlies these excitatory actions as revealed in voltage-clamp (McCormick and Williamson, 1989; Nishikawa et al., 1994; Haj-Dahmane and Andrade, 1996; Shalinsky et al., 2002; Carr et al., 2007). These excitatory effects are occasionally preceded by a rapid and transient inhibition that results from the activation of the Ca\(^{2+}\)-dependent K\(^{+}\) channel, SK, in response to release of Ca\(^{2+}\) from intracellular stores (Gulledge, 2005). Furthermore, in response to a burst of action potentials, the muscarinic stimulation of cortical pyramidal neurons can lead to the appearance of a pronounced slow after depolarization potential (ADP) that has the ability to sustain persistent firing outlasting stimulus duration, in a manner reminiscent of delay activity (Schwindt et al., 1988;
Andrade, 1991; Haj-Dahmane and Andrade, 1998; Gulledge et al., 2009; Yan et al., 2009; Dasari et al., 2013).

Figure 3. Cellular mechanisms of muscarinic modulation. Activation of M\textsubscript{1} muscarinic acetylcholine (ACh) receptors leads to release of calcium (Ca\textsuperscript{2+}) from intracellular stores and the modulation of ion channels. Both the closure of K+ channels and/or the opening of non-selective cation channels (NSCC) are known to contribute to muscarinic excitation. The opening of Ca\textsuperscript{2+}-activated K+ channels of the SK subtype can also elicit a transient inhibition following release of Ca\textsuperscript{2+} from intracellular stores. DAG, diacylglycerol; IP\textsubscript{3}, inositol 1,4,5-trisphosphate, M\textsubscript{1}R, M\textsubscript{1} muscarinic acetylcholine receptor; PIP\textsubscript{2}, phospholipid phosphatidyl inositol; PLC, phospholipase C. Figure is adapted from Proulx et al. (2014)
1.3.3.1 Mechanisms of muscarinic excitation

The mechanisms whereby muscarinic excitation is achieved have proven complex and the jury is still out on the mechanisms of cortical muscarinic modulation. Early studies probing the mechanisms of muscarinic excitation revealed that it was associated with the decrease of a resting K$^+$ conductance (Weight and Votava, 1970; Krnjević et al., 1971). That cellular excitation could be achieved by the closing of ion channels was an important novel concept at the time, as excitatory effects had been shown only to result from increased membrane conductance associated with channel opening up until that point (Weight and Votava, 1970). In a seminal study by Brown & Adams (1980), the underlying M-current ($I_M$) was identified and characterized by means of dual electrode voltage-clamp recordings in frog sympathetic neurons. The M-current was shown to be a voltage-gated K$^+$ current that was active at rest from ~ -60 mV and with half-maximal activation at membrane potentials of approximately -30 mV. Most importantly, it was demonstrated to be subject to inhibition by muscarinic stimulation, thereby producing membrane depolarization (Brown and Adams, 1980). It is now known that the channels mediating the M-current are composed of subunits of the Kv7 family of K$^+$ channels (Wang et al., 1998) and that in addition to being voltage-gated, these channels are PIP$_2$-dependent (Suh and Hille, 2005; Gamper and Shapiro, 2007). As such, M-channels close as PIP$_2$ reserves become rapidly depleted upon muscarinic stimulation (Brown et al., 2007a; Brown, 2010).

Interestingly, multiple conductances have since been shown to contribute to the muscarinic excitation of cortical neurons and its underlying mechanisms remain
somewhat controversial. Table 1 outlines the ionic conductances thought to mediate M₁-like muscarinic effects in neocortex and their effects on neuronal excitability. The closing of leak (Krnjevic et al., 1971), M-type (Santini et al., 2012), and constitutively active inwardly rectifying K⁺ channels (Carr and Surmeier, 2007) have all been shown to play a role. Contrasting evidence has also implicated transient receptor potential (TRP)-like, non-selective cation channels as the mediators of cortical muscarinic excitation (Haj-Dahmane and Andrade, 1996; Klink and Alonso, 1997; Shalinsky et al., 2002; Yan et al., 2009) but a recent report has cast significant doubt on TRPC involvement (Dasari et al., 2013).

In summary, activation of M₁ muscarinic receptors leads to release of Ca²⁺ from intracellular stores together with cellular excitation that results from either the closing of K⁺ channels, the opening of non-selective cation channels or both (Figure 3). How the prolonged excitatory component of the muscarinic response relates to internal Ca²⁺ mobilization is not understood.
Table 1. Ionic conductances mediating M₁-like muscarinic effects in neocortex and their effects on neuronal excitability.

<table>
<thead>
<tr>
<th>Channel activities</th>
<th>Preparation</th>
<th>Neuronal response</th>
<th>References</th>
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<tbody>
<tr>
<td>Closing of $K^+$ channels</td>
<td><em>In vivo</em> intracellular recordings combined with extracellular iontophoresis in post-cruciate cortex of anesthetized cat</td>
<td>Iontophoretically applied ACh elicits a slow depolarization, delays the repolarization of the action potential &amp; promotes repetitive firing</td>
<td>(Krnjević et al., 1971)</td>
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<tr>
<td>Closing of M₁-channels and of the Ca²⁺-activated K⁺ channels that underlie the AHP</td>
<td>Intracellular recordings from layer 5 pyramidal cells in slices of anterior cingulate cortex of adult guinea pig</td>
<td>ACh application by pressure injection elicits a barrage of postsynaptic potentials, produces a short latency hyperpolarization (presumed synaptic) followed by a slow excitatory response, and results in the inhibition of the slow afterhyperpolarization potential (AHP) that follows a train of action potentials</td>
<td>(McCormick and Prince, 1986)</td>
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<tr>
<td>Closing of Ca²⁺-activated K⁺ channels</td>
<td>Intracellular and voltage-clamp recordings of layer 5 pyramidal neurons from slices of cat sensorimotor cortex</td>
<td>Muscarine inhibits both a Ca²⁺-dependent and a Ca²⁺-independent AHP elicited by high frequency spike trains, and produces an afterdepolarization potential (ADP) that can trigger repetitive firing</td>
<td>(Schwindt et al., 1988)</td>
</tr>
<tr>
<td>Closing of M₁-channels and of the K⁺ channel that underlies the AHP</td>
<td>Intracellular recordings of neurons from surgically removed human cortical tissue and from slices of anterior cingulate, sensorimotor and temporal cortices of guinea pig</td>
<td>Application of ACh or of a muscarinic agonist results in a slow depolarization of the membrane potential and a substantial reduction of the AHP together with reduced spike frequency adaptation. In voltage-clamp, muscarinic agonists elicit a long-lasting slow inward current that results from the suppression the M-current.</td>
<td>(McCormick and Williamson, 1989)</td>
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<td>Event</td>
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<tr>
<td>Closing of a voltage-independent K⁺ channel and of a voltage- and Ca²⁺-sensitive K⁺ channel</td>
<td>Intracellular and extracellular recordings in corticotelal and corticopontine burst-generating neurons of layer 5 sensorimotor and primary visual cortices of guinea pig in the slice preparation.</td>
<td>Application of ACh elicits a depolarization and subsequent shift in the firing pattern of neurons from spontaneously bursting to single-spike activity. In voltage-clamp, the underlying excitatory current of this response elicits an inward current resulting from a decrease in potassium conductances.</td>
<td>(Wang and McCormick, 1993)</td>
</tr>
<tr>
<td>Closing of M-channels</td>
<td>Nyastatin perforated patch clamp recordings in pyramidal neurons acutely dissociated from young rat cerebral cortex (P7-14)</td>
<td>Application of ACh or of muscarinic agonists elicits a long lasting inward current that is pirenzepine-sensitive</td>
<td>(Nishikawa et al., 1994)</td>
</tr>
<tr>
<td>Closing of PIP₂⁻-dependent Kir2 channels</td>
<td>Whole-cell current-clamp and voltage-clamp recordings in L5 pyramidal cells of prefrontal cortical slices of young rats (P16-22)</td>
<td>Bath application of carbachol elicits a strong depolarization leading to tonic firing in current clamp; inward current in voltage-clamp</td>
<td>(Carr and Surmeier, 2007)</td>
</tr>
<tr>
<td>Closing of M-channels</td>
<td>Whole-cell patch clamp electrophysiology in prefrontal brain slices of young rats (P25-28)</td>
<td>Muscarine enhances intrinsic excitability</td>
<td>(Santini et al., 2012)</td>
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**Opening of K⁺ channels**

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<th>Event</th>
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<tr>
<td>Activation of Ca²⁺-activated K⁺ channel (SK channels)</td>
<td>Whole-cell and cell-attached recordings from layer 5 neurons in slices of rat somatosensory cortex</td>
<td>Activation of M1-type muscarinic receptors with pressure injection of ACh elicits a strong and direct transient inhibition</td>
<td>(Gulledge, 2005)</td>
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**Opening of non-selective cation channels**

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<th>Event</th>
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<tr>
<td>Activation of a voltage-dependent, non-selective cation current</td>
<td>Whole-cell recordings in layer 5 pyramidal cells of slice from adult rat prefrontal cortex</td>
<td>Pressure application of ACh, muscarine/oxotremorine or bath application of carbachol elicited strong depolarization of the membrane potential in current clamp and robust inward currents in voltage-clamp.</td>
<td>(Haj-Dahmane and Andrade, 1996)</td>
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<td>Activation of a Ca²⁺-dependent cationic conductance largely permeable to Na⁺</td>
<td>Intracellular recordings in layer 2 stellate and non-stellate cells in slices of medial entorhinal cortex</td>
<td>Pressure application of carbachol elicits membrane depolarization</td>
<td>(Klink and Alonso, 1997)</td>
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<tr>
<td>Activation of a $\text{Ca}^{2+}$- and voltage-sensitive non-selective cation conductance</td>
<td>Sharp micro electrode and whole-cell recordings in layer 5 pyramidal cells of PFC slices</td>
<td>Application of muscarinic agonists results in a slow depolarization and the appearance of a slow afterdepolarization potential in response to spike bursts that has the ability to generate sustained firing</td>
<td>(Haj-Dahmane and Andrade, 1998; 1999)</td>
</tr>
<tr>
<td>Activation of a non-selective cation channel and block of a $K^+$ conductance</td>
<td>Whole-cell patch clamp recordings of layer 2 principal neurons in slices of medial entorhinal cortex</td>
<td>Bath application of carbachol elicits slowly developing and prolonged depolarization in current clamp, and a slow inward current in voltage-clamp</td>
<td>(Shalinsky et al., 2002)</td>
</tr>
<tr>
<td>Activation of a non-selective cation channel</td>
<td>Intracellular recordings in layer 5 neurons in slices of entorhinal cortex</td>
<td>Pressure-pulse application of carbachol elicits depolarizations of the membrane potential and synaptically driven epileptiform activity</td>
<td>(Egorov et al., 2003)</td>
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<td>Activation of TRPC channels</td>
<td>Whole-cell recordings in acute slice preparation, organotypic culture or HEK-293 cells in combination with dominant negative, over expression, gene deletion, and reconstitution strategies. In cortical neurons, the effect was only observed in cells from animals aged P19-P24 but not younger.</td>
<td>Bath application of carbachol induces a slow afterdepolarization capable of sustaining repetitive spiking activity in response to a burst of action potentials.</td>
<td>(Yan et al., 2009)</td>
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1.4 Disruption of Prefrontal Executive Function in Neurological and Psychiatric Illness

The prefrontal cortex is a critical node in widespread and dynamic brain networks that sustain higher cognitive function in health and that perpetuate executive dysfunction in psychiatric illness (Tekin and Cummings, 2002; Meyer-Lindenberg and Weinberger, 2006; Stam, 2014). Executive deficits have been reported in normal human aging (Gazzaley et al., 2005) as well as a multitude of neurological and psychiatric disorders (Tekin and Cummings, 2002), including depression (Austin, 2001; Price and Drevets, 2009), schizophrenia (Tan et al., 2009; Meyer-Lindenberg, 2010), bipolar disorder (Arts et al. 2007) and attention deficit hyperactivity disorder (Castellanos et al., 2006), all of which have been hypothesized to developmental origins. Aspects of executive function have also been proposed as intermediate phenotypes for these complex disorders in the quest to identify more quantitative biological indices and their more penetrant neural substrates (Meyer-Lindenberg and Weinberger, 2006). Yet, however ubiquitous in neuropsychiatric illness, the underlying neurobiology of executive dysfunction remains poorly understood.

As we have seen, the cholinergic modulation of the PFC is integral to executive function. It is especially powerful in its ability to subsequently influence downstream cortical and subcortical networks (Funahashi, 2001; Nelson et al., 2005; Arnsten et al., 2012), as well as being uniquely positioned to exert feedback control on neuromodulatory centers (Uylings et al., 2003), including the cholinergic nuclei (Gaykema et al., 1990; Zaborszky et al., 1997). Dysregulation of the cholinergic system is thought to contribute to the executive deficits that are prevalent in neurological and
psychiatric disorders (Terry and Buccafusco, 2003; Sarter and Paolone, 2011). This thesis considers cellular mechanisms of prefrontal cholinergic function and dysfunction in models of (1) psychiatric vulnerability and of (2) Alzheimer’s disease (AD). In addition to commonalities in executive dysfunction, these disorders share abnormalities both in prefrontal involvement (In AD: Johannsen et al., 1999; Grady, 2001; Salat et al., 2001; Tekin and Cummings, 2002; Grady et al., 2003; In psychiatric disease: Weinberger, 1986; Baxter, 1989; Drevets et al., 1997; Tekin and Cummings, 2002; Sheline, 2003; Price and Drevets, 2009; Mayberg et al., 1999) as well as in cholinergic mechanisms (In AD: Davies and Maloney, 1976; Perry et al., 1978; Bartus et al., 1982; Henke and Lang, 1983; Zubenko et al., 1989; Davis et al., 1999; Bartus, 2000; Selkoe, 2001; Auld et al., 2002; In psychiatric disease: Dursun and Kutcher, 1999; Furey and Drevets, 2006; Mineur et al., 2007; Bacher et al., 2010; Drevets and Furey, 2010; Dagytė et al., 2011; Mineur et al., 2013; van Enkhuizen et al., 2014). What is more, it is interesting to note that a history of depression can significantly increase the risk for Alzheimer’s disease and other dementias (Ownby et al., 2006; Dotson et al., 2010; Saczynski et al., 2010).

1.4.1 Early life stress and susceptibility to psychiatric illness

The prefrontal cortex is one of the last brain regions to mature (Huttenlocher and Dabholkar, 1997; Sowell et al., 2001; Gogtay et al., 2004) and, accordingly, peak performance of its executive functions is only achieved in mid- to late adolescence (De Luca et al., 2003; Luciana et al., 2005; Conklin et al., 2007). Brain regions requiring protracted development may be especially vulnerable to the effects of early stress (Pechtel and Pizzagalli 2010), which in itself is a risk factor for PFC dysfunction, deficits in cognitive performance, and psychiatric illness (Heim and Nemeroff, 2001; Hedges and Woon, 2010; Kessler et al., 2010; Pechtel and Pizzagalli, 2010; Green et al.,
Early stress takes the form of parental loss, parental maladjustment due to mental illness, substance disorder, or criminal behaviour, and maltreatment (neglect or abuse) (Kessler et al., 2010). It is a devastating environmental disruption with lasting repercussions as it correlates strongly with DSM-IV psychiatric disorders such as anxiety and depression (Ronald C Kessler, 2009; Kessler et al., 2010; Pechtel and Pizzagalli, 2010; Green et al., 2010b). The lifetime prevalence of DSM-IV disorders is estimated at 18-36% worldwide (Ronald C Kessler, 2009) and adverse childhood experiences account for 30-45% of these (Green et al., 2010b). Furthermore, early stress contributes to 67% of suicide attempts (Dube et al., 2001). While the role of the PFC in psychiatric illness is well-appreciated (Ressler and Mayberg, 2007; Drevets et al., 2008; Engel et al., 2008; Price and Drevets, 2009; Shin and Liberzon, 2009; Shin et al., 2009), much is still unknown about the normal maturation of the PFC and the cellular mechanisms underlying its vulnerability to disruption.

1.4.2 Alzheimer’s Disease (AD)

Alzheimer’s disease (AD) is a neurodegenerative disorder that constitutes the main cause of dementia and now affects more than 25 million people worldwide, a number expected to quadruple by 2050 (Brookmeyer et al., 2007). It is associated with profound cognitive impairments that include severe memory loss and early attention deficits (Grady et al., 1988; Perry and Hodges, 1999; Baddeley et al., 2001; Romberg et al., 2013a). Impaired attention negatively impacts the day-to-day life of AD patients, contributing to distraction, confusion and poor executive control (Perry and Hodges, 1999). It is also likely that disruptions in attention further exacerbate the memory deficits that constitute a telltale sign of the disease: if you can’t attend, you can’t encode (Romberg et al., 2013a; 2013b). Yet, little is understood of the cellular mechanisms that
underlie the non-mnemonic deficits of executive function in AD and their contribution to
the disease.

Histologically, AD is associated with the formation and accumulation of plaques
and tangles that constitute the hallmark of the disease and that result from disruptions in
amyloid precursor protein (APP) processing and the hyperphosphorylation of
microtubule associated tau protein, respectively (Selkoe, 2001; LaFerla, 2002). β-
amyloid peptides can impair both muscarinic (Kelly et al., 1996) and nicotinic (Liu et al.,
2001; Pettit et al., 2001) receptor function and indeed interfere with the cholinergic
system as a whole (Auld et al., 2002).

Manifestations of AD in people and animal models have long been linked to
aberrations in cholinergic enzymatic activity (Davies and Maloney, 1976; Perry et al.,
1978; Henke and Lang, 1983; Zubenko et al., 1989; Davis et al., 1999; Selkoe, 2001;
Auld et al., 2002; DeKosky et al., 2002), receptor binding (Nordberg et al., 1988;
Whitehouse et al., 1988; Marutle et al., 1999; Nordberg, 2001; O'Brien et al., 2006;
Kendziorra et al., 2010), and associated signalling pathways (Jope et al., 1997, Jope
findings of cholinergic cell loss in Alzheimer’s disease (Davies and Maloney, 1976;
Whitehouse et al., 1982) prompted the cholinergic hypothesis of AD (Bartus et al., 1982)
and even pointed to the important involvement of acetylcholine in mediating cognitive
functions (Bartus et al., 1982; Bartus, 2000). To date, acetylcholinesterase inhibitors
constitute the standard of care for AD (Citron, 2010), although loss of cholinergic cells is
an end-stage manifestation of the disease that is unlikely to account for cognitive
deficits that may arise much earlier (Davis et al., 1999; Terry and Buccafusco, 2003).
Significant reductions in nicotinic receptor binding have been documented in the AD brain and this holds particularly true for the $\alpha_4\beta_2^*$ nicotinic receptor subtype (Nordberg et al., 1988; Whitehouse et al., 1988; Marutle et al., 1999; Nordberg, 2001; O'Brien et al., 2006; Kendziorra et al., 2010) that is highly expressed in L6 PFC (Wada et al., 1989; 1990; Kassam et al., 2008; Alves et al., 2010). On the other hand, the bulk of the evidence points to the fact that the expression and binding of $M_1$ muscarinic receptors, which mediate postsynaptic cholinergic action in cortex (Gulledge et al., 2009), are unchanged (Mash et al., 1985; Araujo et al., 1988; Levey, 1996; Rossner et al., 1998; Jope, 1999). Reductions in the functional coupling of the $M_1$ receptor to downstream cellular effectors, however, have been reported (Jope et al., 1997; Jope, 1999).

Interestingly, early disruptions in intracellular calcium have been documented in several models of AD, prompting the interesting proposal that AD may represent a “calciumopathy”. Under this paradigm, calcium anomalies would arise very early in disease progression, surreptitiously disrupting cellular function until normal physiological function can no longer be sustained (LaFerla, 2002; Stutzmann, 2005; 2007; Bezprozvanny and Mattson, 2008). How such disruptions might contribute to cholinergic signalling, however, has not previously been investigated.

1.5 Questions & Scope of Thesis
The broad questions of how acetylcholine mediates its actions on pyramidal cells of the PFC and how these neuromodulatory influences are affected in neurological and psychiatric illness are addressed in this thesis.
First, I will examine how calcium release from intracellular stores interacts with cellular excitation following muscarinic stimulation of L5 PFC pyramidal neurons. I will characterize the developmental maturation of this interaction and its susceptibility to environmental disruption by early life stress, an established model of psychiatric vulnerability.

Second, I will examine mechanisms of sustained cholinergic excitation in pyramidal neurons of L6 PFC in healthy control and in a mouse model of Alzheimer's Disease with documented attentional impairments.
Chapter 2

2 Materials & Methods

2.1 Animals

Sprague Dawley rats were used for the characterization of the interaction between the ACh-elicited Ca\(^{2+}\)-release (dF/F\(_{ACh}\)) and the ACh-elicited inward current (I\(_{ACh}\)) in healthy adults, in development and following exposure to early life stress (Chapter 3). TgCRND8 mice and their WT littermates, which are on a hybrid C3H-C57/Bl6 background, were used for studying abnormalities in prefrontal cellular excitability in a mouse model of Alzheimer’s disease (Chapter 4). All experiments were performed at the University of Toronto and approved by the University of Toronto Animal Care Committee.

In the first part of the Results (Chapter 3), the characterization of the normal interaction between the ACh-elicited calcium increases (dF/F\(_{ACh}\)) and the cholinergic currents (I\(_{ACh}\)) was conducted in 31 young adult male rats (age: 60 ± 13 days, range 40–95 days). The second set of experiments examined how the above interaction matures and whether it is susceptible to disruption by early stress. For these experiments, we used repeated maternal separation to elicit early stress (see below), and control animals were born and raised within the same animal room during the same time period. Recordings were performed in adolescence (postnatal day [P]30–P45; n = 5 control rats, n = 4 early stress [ES]), young adulthood (P60–P100; n = 9 control rats, n = 6 ES), or adulthood (P130–P175; n = 6 control rats, n = 8 ES).
For the characterization of the cholinergic excitation of layer 6 prefrontal cortex in a model of Alzheimer’s disease (Chapter 4), we used adult male and female TgCRND8 mice on a mixed C57/Bl6-C3H background, as previously reported (Chishti et al., 2001), along with their littermate WT siblings as controls (Age, WT: 109 ± 2 days, N=17 animals; Tg: 107 ± 2 days, N=23 animals). The TgCRND8 mouse model is widely used in Alzheimer’s research and encodes both the Swedish and Indiana mutations of amyloid precursor protein (APP) found in inherited familial AD (Chishti et al., 2001). Importantly, it permits the use of littermate controls and recapitulates several of the neuropathological and cognitive features of AD, including amyloid plaque deposition and memory impairments by 3 months of age (Chishti et al., 2001), followed by deficits in sustained attention by 4-5 months of age (Romberg et al., 2013b). Of note, the attention deficits appear to precede the cholinergic cell loss, which is observed much later, at 7 months of age (Bellucci et al., 2006). The phenotypic timeline is depicted in Figure 4.

Figure 4. The TgCRND8 model phenotypic timeline.
2.1.1 Early stress paradigm

The early stress (ES) paradigm of maternal separation is a well-validated model of psychiatric vulnerability (Plotsky and Meaney, 1993; Liu et al., 2000; Meaney, 2001; Sánchez et al., 2001; Gross and Hen, 2004). Pregnant primiparous dams delivered pups within the animal housing facility and litters were randomly assigned to ES or control groups on P1. Pups in the ES litters were separated from their dams for a period of 3 hours at the same time each day from P2 to P14. Control litters were left undisturbed during this time. All litters were handled briefly at 3- to 4-day intervals to allow for cage cleaning and weighing. Once weaned, all pups were housed in same-sex sibling groups of two to three rats. A schematic of this paradigm is shown in Figure 5.

![Maternal Separation Diagram](image)

**Figure 5. Early stress (ES) paradigm of maternal separation.** Pups in the ES litters are separated from their mothers for a period of 3 h at the same time in the morning each day from P2 to P14. Control litters are left undisturbed during this time. Animals exposed to maternal separation develop a phenotype of psychiatric vulnerability and are more prone to develop anxiety and depression phenotypes in adulthood.
2.2 Brain Slice Preparation

Animals were deeply anesthetized with chloral hydrate (400 mg/kg) delivered intraperitonally and subsequently decapitated. Each brain was rapidly cooled with 4°C oxygenated sucrose artificial cerebrospinal fluid (254 mM sucrose substituted for sodium chloride). Coronal slices (400 µm) of the PFC were cut on a Dosaka Linear Slicer (SciMedia, Costa Mesa, California) and were transferred to 30°C oxygenated artificial cerebrospinal fluid (ACSF, containing, in mM: 128 sodium chloride, 10 D-glucose, 26 sodium bicarbonate, 2 calcium chloride, 2 magnesium sulfate, 3 potassium chloride, 1.25 monosodium phosphate, pH 7.4) in a prechamber (Automatic Scientific, Berkeley, California) and allowed to recover for at least 1.5 hours before the beginning of an experiment. Slices were placed in a chamber on the stage of an upright microscope for whole-cell recordings. Artificial cerebrospinal fluid was bubbled with 95% oxygen and 5% carbon dioxide and flowed over the slice at 30°C with a rate of 3 mL to 4 mL per minute.

2.3 Electrophysiological Recordings

Deep layer 5 (Chapter 3) and layer 6 (Chapter 4) pyramidal neurons were patched under visual control using infrared differential interference contrast microscopy in the cingulate and prelimbic regions of the prefrontal cortex. Whole-cell patch electrodes (2–3 MΩ) contained the following (in mM): 120 K-gluconate, 5 potassium chloride, 2 magnesium chloride, 4 dipotassium adenosine triphosphate, 0.4 disodium-guanosine triphosphate, 10 disodium-phosphocreatine, and 10 4-(2-hydroxyethyl)piperazin-1-yl ethanesulfonic acid (HEPES) buffer (adjusted to pH 7.33 with potassium hydroxide). The liquid junction potential was 15 mV. Data was acquired and low-pass filtered at 2
kHz with an Axopatch 200b amplifier (Molecular Devices, Sunnyvale, California) and Digidata1440 digitizer/pClamp10.2 software (Molecular Devices). Membrane potential, membrane capacitance, input resistance, spike amplitude and spike threshold were measured immediately after gaining access to a cell. Cholinergic responses were subsequently assessed either in current clamp or in voltage-clamp mode. In voltage-clamp, neurons were held at −75 mV, near the calculated equilibrium potential for chloride under these conditions.

2.4 Calcium Imaging

For calcium imaging experiments, the calcium dye Oregon Green BAPTA-1 (OGB-1, 100 µM) was included in the pipette, along with Alexa-594 hydrazide (20 µM) to aid in the visualization of the neuron. OGB-1 is a high affinity dye that has a $K_d$ of approximately 180 nM. Pilot experiments were also conducted with the calcium indicators fluo-4F ($K_d \sim 345$ nM) and OGB-6F ($K_d \sim 3$ µM). This work indicated that ACh-elicited calcium increases span a broad range, likely from a few hundred nM to low µM concentrations, making OGB-1 an adequate choice of indicator. It should be noted, however, that it remains a possibility that I failed to detect calcium increases larger than ~ 2 µM, where this dye is known to saturate (Hendel et al., 2008). Multiphoton imaging was performed using a Ti:sapphire laser (Newport) tuned to wavelength 800 nm and an Olympus Fluoview FV1000 microscope with a 60X water-immersion 0.90 NA objective. The emitted fluorescence was separated into green (OGB-1 signal) and red (A-594 signal) channels with a dichroic mirror at 570 nM and filtered (green: BA 495-540; red: BA 570-620) before detection. Images were acquired at a rate of ~10 frames/second and analyzed with Fluoview software. A pan-somatic area of interest was selected for
analysis and green fluorescence increases were calculated relative to baseline fluorescence (dF/F₀). Calcium responders (AChₐCa²⁺) were identified as cells where the ACh-elicited Ca²⁺ increase was at least five times the standard deviation of the baseline fluorescence signal; whereas, cells that lacked such a response to ACh were considered non-responders (AChₐNoCa²⁺). Pseudocoloring was achieved post hoc for illustrative purposes with look-up tables adjusted to the maximal signal bandwidth in Fiji (ImageJA v.1.45b; http://fiji.sc/Fiji). The green to red fluorescence ratio (G/R) was calculated by dividing the green signal by that of the red channel for the purpose of evaluating potential differences in basal calcium levels between groups.

2.5 Ion Substitution Experiments

The composition of the ACSF was modified in a subset of experiments to help examine the ionic dependence of the Ca²⁺ clearance as well as the ionic mechanisms of the supplementary muscarinic currents. In particular, the roles of Na⁺ and K⁺ were probed. Eighty percent sodium (Na⁺) substitution was achieved by substituting the extracellular NaCl with choline-chloride or NMDG and contained the following, in mM: 0 NaCl, 128 choline-chloride (or NMDG), 10 D-glucose, 26 NaHCO₃, 2 CaCl₂, 2 MgSO₄, 3 KCl, 1.25 NaH₂PO₄, pH 7.4. Dual Na⁺ and K⁺ substitution was achieved by using Cs-gluconate containing intracellular solution and substituting extracellular Na⁺ and K⁺ with choline chloride, in mM: 0 NaCl, 131 choline-chloride, 10 D-glucose, 26 NaHCO₃, 2 CaCl₂, 2 MgSO₄, 0 KCl, 1.25 NaH₂PO₄, pH 7.4. Cesium intracellular solution contained the following (in mM): 120 gluconic acid, 115 CsOH, 5 CsCl, 2 MgCl, 4 K-ATP, 0.4 Na₂GTP, 10 Na₂₃-phosphocreatine and 10 HEPES (adjusted to pH 7.33 with CsCl). The
liquid junction potential was 18.3 mV and 18.2 mV in Na\(^+\) and dual Na\(^+\) and K\(^+\) substitution conditions, respectively, and was corrected post-hoc.

2.6 Pharmacology

All drugs were bath applied. Acetylcholine chloride and caffeine were obtained from Sigma. Pirenzepine, thapsigargin, and KB- R7943 were obtained from Tocris. Apamin was obtained from Alomone Labs, and cadmium chloride was obtained from Emerald BioSystems.

2.7 Statistical Analyses

Results are expressed as mean ± SEM, and all statistical comparisons were made at a significance level of 0.05 (Prism versions 5.0d/6.0, GraphPad). Average current and fluorescent increases were generated with Axograph X (Axograph Scientific, Sydney, Australia). For the purpose of quantifying ACh-elicited firing, input-output curves and post-burst afterhyperpolarization potentials (AHPs), action potentials were automatically detected with a derivative threshold of 20 mV/ms. To assess the ability of cells to sustain near-maximal firing in response to ACh, the cumulative probability of the instantaneous firing frequency of individual action potentials was normalized to the maximum instantaneous firing within each acetylcholine-elicited train of action potentials. Genotype and treatment differences of cumulative probabilities were assessed with K-S tests. The peak and area under the curve of post-burst potentials were measured from the peak of the last action potential in each burst up to 1 s post-burst.
RESULTS
Early stress prevents the potentiation of muscarinic excitation by calcium release in adult prefrontal cortex

Acknowledgements & Contributions

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3 Early Stress Prevents the Potentiation of Muscarinic Excitation by Calcium Release in Adult Prefrontal Cortex

3.1 Abstract

**Background:** The experience of early stress contributes to the etiology of several psychiatric disorders and can lead to lasting cognitive deficits. Late-maturing executive functions, such as working memory and attention, are particularly vulnerable. These executive functions require activation of the prefrontal cortex (PFC) by muscarinic M₁ acetylcholine (ACh) receptors. Such Gαq-protein coupled receptors trigger the release of calcium (Ca²⁺) ions from internal stores and elicit prolonged neuronal excitation.

**Methods and Results:** In brain slices of PFC, we employ multiphoton Ca²⁺ imaging simultaneously with whole-cell electrophysiological recordings to demonstrate that ACh-induced Ca²⁺ release potentiates ACh-elicited excitatory currents. In the healthy PFC, this potentiation of muscarinic excitation emerges in young adulthood, when executive function typically reaches maturity. However, such developmental consolidation of muscarinic ACh signaling is abolished subsequent to the early stress of repeated maternal separation, where ACh responses retain an adolescent phenotype accompanied by a disruption in the expression of multiple developmentally-regulated genes associated with Gαq and Ca²⁺ signaling. Pharmacological and ionic manipulations reveal that the enhancement of muscarinic excitation in the healthy adult PFC results from the extrusion of ACh-mediated Ca²⁺ release via the electrogenic process of Na⁺/Ca²⁺ exchange. **Conclusions:** This work illustrates a long-lasting disruption in ACh-
mediated cortical excitation following early stress, and raises the possibility that such cellular mechanisms may disrupt the maturation of executive function.

3.2 Introduction

The prefrontal cortex (PFC) is one of the last brain regions to mature (Huttenlocher and Dabholkar, 1997; Sowell et al., 2001; Gogtay et al., 2004) and, accordingly, peak performance of executive function is only achieved in mid- to late adolescence (De Luca et al., 2003; Luciana et al., 2005; Conklin et al., 2007). Executive function is essential for performing complex tasks such as solving novel problems, planning, decision making, and the modification of behavior in light of new information (Baddeley, 1992; Funahashi, 2001; Elliott, 2003). Disruptions in PFC activity and impairments in executive function are observed in several psychiatric disorders (Tekin and Cummings, 2002; Elliott, 2003), including depression (Austin, 2001; Price and Drevets, 2009), schizophrenia (Forbes et al., 2008; Tan et al., 2009; Meyer-Lindenberg, 2010), bipolar disorder (Arts et al., 2007) and attention deficit hyperactivity disorder (Castellanos et al., 2006), all of which have been hypothesized to have developmental origins (Weinberger, 1995; Gross and Hen, 2004; Ansorge et al., 2007; Shaw et al., 2007; Sanches et al., 2008). Brain regions requiring protracted development may be especially vulnerable to the effects of early stress (Pechtel and Pizzagalli, 2010), which in itself is a risk factor for PFC dysfunction, deficits in cognitive performance, and psychiatric illness (Heim and Nemeroff, 2001; Sanchez, 2006; Pechtel and Pizzagalli, 2010). Yet, much is still unknown about the normal maturation of the PFC and the cellular mechanisms underlying its vulnerability to disruption.
Acetylcholine (ACh) modulation of the PFC is essential for executive functions such as working memory and attention (Winkler et al., 1995; Parikh et al., 2007; Croxson et al., 2011; Hasselmo and Sarter, 2011) and dysregulation of the cholinergic system is thought to contribute to the executive deficits that are prevalent in psychiatric disorders (Hasselmo and Sarter, 2011; Sarter and Paolone, 2011). At the cellular level, the ability of PFC neurons to sustain persistent activity forms the basis of working memory (Goldman-Rakic, 1995a) and involves muscarinic acetylcholine receptors (Egorov et al., 2002; Zhang and Seguela, 2010; Zhou et al., 2011). Layer V pyramidal cells of the PFC receive a dense cholinergic innervation arising from the basal forebrain (Houser et al., 1985; Henny and Jones, 2008) and respond to ACh principally via M₁ muscarinic receptors (Gulledge et al., 2009). Activation of these Gα₉-coupled receptors exerts robust excitatory actions (Krnjević et al., 1971; McCormick and Prince, 1986; Haj-Dahmane and Andrade, 1996; Gulledge et al., 2009) in addition to the release of Ca²⁺ from IP₃-sensitive stores (Berridge and Irvine, 1984). Although the ionic mechanisms underlying these responses have been extensively studied, they remain incompletely understood. In particular, the role of agonist-induced Ca²⁺ release in shaping muscarinic excitation is not known. Although intracellular Ca²⁺ has been posited to play an important role in shaping persistent activity (Teramae and Fukai, 2005; Fransén et al., 2006; Zhang and Seguela, 2010), no study to date has simultaneously examined the release of intracellular Ca²⁺ together with the magnitude and timing of muscarinic excitation. Furthermore, it is not known how these aspects of PFC cholinergic signaling change during postnatal development or their vulnerability to disruption by environmental factors such as early stress.
Using multiphoton Ca\(^{2+}\) imaging with concurrent whole-cell electrophysiological recordings in brain slices of PFC, we demonstrate for the first time that agonist-induced Ca\(^{2+}\) release from intracellular stores significantly potentiates the excitatory effects of muscarinic ACh receptors. We further provide mechanistic insight into the source of these excitatory effects. Importantly, we show that such potentiation of muscarinic receptor activity is associated with key developmental stages for executive function and is subject to disruption by early stress.

### 3.3 Materials and Methods

#### 3.3.1 Animals

Sprague Dawley rats were used for all experiments. Experiments were approved by the University of Toronto Animal Care Committee or the Tata Institute of Fundamental Research Animal Ethics Committee. The characterization of the normal interaction between the ACh-elicited Ca\(^{2+}\) release and the ACh-elicited inward current (I\(_{ACh}\)) was conducted in 31 young adult male rats (age: 60 ± 13, range 40-95 days). The second set of experiments examined how the above interaction matures and whether it was susceptible to disruption by early stress. For these experiments we used repeated maternal separation to elicit early stress (Berton and Nestler, 2006; Benekareddy et al., 2010), and control animals were born and raised within the same animal room during the same time period. Recordings were performed in adolescence (P30-45; \(n = 5\) controls, \(n = 4\) ES), young adulthood (P60-100; \(n = 9\) controls, \(n = 6\) ES) or adulthood (P130-175; \(n = 6\) controls, \(n = 8\) ES). The third set of experiments employed the same maternal separation paradigm and examined gene expression in the PFC at either P21 (\(n = 7-8\) controls, \(n = 8-9\) ES) or P60 (\(n = 7\) controls, \(n = 7-8\) ES).
3.3.2 Early stress paradigm

Pregnant primiparous dams delivered pups within the animal housing facility and litters were randomly assigned to ES or control groups on postnatal (P) day P1. Pups in the ES litters were separated from their dams for a period of 3 h at the same time each day from P2 to P14. Control litters were left undisturbed during this time. All litters were handled briefly at 3–4 d intervals to allow for cage cleaning and weighing. Once weaned, all pups were housed in same-sex sibling groups of 2–3 rats.

3.3.3 Brain slice preparation and recording conditions

Each brain was cooled as rapidly as possible with 4 °C oxygenated sucrose artificial cerebrospinal fluid (ACSF) (254 mM sucrose substituted for NaCl). Coronal slices (400 μm thick) of the PFC were cut on a Dosaka Linear Slicer (SciMedia), and were transferred to 30 °C oxygenated ACSF (containing the following, in mM: 128 NaCl, 10 D-glucose, 26 NaHCO₃, 2 CaCl₂, 2 MgSO₄, 3 KCl, 1.25 NaH₂PO₄, pH 7.4) in a pre-chamber (Automate Scientific) and allowed to recover for at least 1.5 h before the beginning of an experiment. Slices were placed in a chamber on the stage of an upright microscope for whole cell recordings. ACSF was bubbled with 95% oxygen and 5% carbon dioxide and flowed over the slice at 30 °C with a rate of 3–4 ml/min.

3.3.4 Electrophysiological recordings and multiphoton Ca²⁺ imaging

Layer V pyramidal neurons were patched under visual control using infrared differential interference contrast microscopy in the cingulate and prelimbic regions. Intracellular patch solution contained the following (in mM): 120 K-gluconate, 5 KCl, 2 MgCl₂, 4 K-ATP, 0.4 Na₂-GTP, 10 Na₂-phosphocreatine, and 10 HEPES buffer (adjusted to pH 7.33 with KOH). The high affinity Ca²⁺ dye Oregon Green BAPTA-1 (OGB-1, 100 μM)
was included in the pipette along with the Ca\(^{2+}\) insensitive dye Alexa-594 hydrazide (20 µM), which was used for the visualization of the neuron and subsequent morphological reconstruction (Supplementary Methods). Currents were recorded with an Axopatch 200b (Molecular Devices), acquired and low-pass filtered at 2 kHz with pClamp10.2/Digidata1440.

Multiphoton imaging was performed using a Ti:sapphire laser (Newport) tuned to wavelength 800 nm and an Olympus Fluoview FV1000 microscope with a 60X water-immersion 0.90 NA objective. The emitted fluorescence was separated into green (OGB-1 signal) and red (A-594 signal) channels with a dichroic mirror at 570 nm and filtered (green: BA 495-540; red: BA 570-620) before detection. Images were acquired at a rate of ~10 frames/second and analyzed with Fluoview software. A pan-somatic area of interest (AOI) was selected for analysis and green fluorescence increases were calculated relative to baseline fluorescence (dF/F\(_0\)). Calcium responders (ACh\(_{Ca^{2+}}\)) were identified as cells where the ACh-elicited Ca\(^{2+}\) increase was at least 5 times the standard deviation of the baseline fluorescence signal; whereas cells that lacked such a response to ACh were considered nonresponders (ACh\(_{No \ Ca^{2+}}\)). Pseudo-coloring was achieved post-hoc for illustrative purposes with look up tables (LUTs) adjusted to the maximal signal bandwidth in Fiji (ImageJA v.1.45b).

Intrinsic cell properties were assessed in current clamp mode. ACh\(_{Ca^{2+}}\) cells were not significantly different from ACh\(_{No \ Ca^{2+}}\) cells as assessed by membrane potential (ACh\(_{Ca^{2+}}\): \(-80 \pm 2\) mV, \(n = 16\); ACh\(_{No \ Ca^{2+}}\): \(-81\pm 1\) mV, \(n = 14\); \(p = 0.9\)), input resistance (ACh\(_{Ca^{2+}}\): \(118 \pm 12\) MΩ, \(n = 16\); ACh\(_{No \ Ca^{2+}}\): \(144 \pm 14\) MΩ, \(n = 14\); \(p = 0.2\)), and
membrane capacitance ($\text{ACh}_{\text{Ca}^{2+}}$: $180 \pm 10$ pF, $n = 16$; $\text{ACh}_{\text{No Ca}^{2+}}$: $167 \pm 9$ mV, $n = 14$; $p = 0.4$). Examination of cholinergic currents was performed in voltage-clamp mode at a LJP-corrected holding potential of $-75$ mV.

3.3.5 Pharmacology

Acetylcholine chloride and caffeine were obtained from Sigma. Pirenzepine, thapsigargin and KB-R7943 were obtained from Tocris. All drugs were bath applied.

3.3.6 Statistics

Results are expressed as mean ± SEM, and all statistical comparisons were made at a significance level of 0.05 (Prism versions 5.0d/6.0, GraphPad). Average current and fluorescent increases were generated with Axograph X. Gene expression analysis experiments with two groups were analyzed using the unpaired Student’s $t$ test. To analyze the influence of ES on the developmental expression profile of genes involved in $\text{Ca}^{2+}$ signaling, data was subjected to two-way ANOVA, followed by a Bonferroni post hoc test for group comparisons.

3.4 Results

3.4.1 ACh-elicited somatic $\text{Ca}^{2+}$-release potentiates excitatory muscarinic currents

The stimulation of muscarinic receptors can elicit IP$_3$-induced somatic $\text{Ca}^{2+}$ increases (Power and Sah, 2002) that initiate gene transcription (Ghosh and Greenberg, 1995; Dolmetsch et al., 1998; Li et al., 1998). Initial experiments in layer V pyramidal neurons revealed that bath application of ACh (1 mM, 15 s) elicited $\text{Ca}^{2+}$ release as measured by increased OGB-1 fluorescence ($\text{dF/F}_{\text{ACh}}$; Figure 6). When observed, this somatic increase was seen in multiple regions of interest (ROI), as illustrated on the $\text{dF/F}_{\text{ACh}}$
overlay in Figure 6. Accordingly, we selected a pan-somatic ROI for our investigation of the relative magnitude and timing of the two distinct responses to acetylcholine (ACh): the increase in Ca$^{2+}$ within the soma ($dF/F_{ACh}$) and the excitatory inward current ($I_{ACh}$).

**Figure 6.** ACh-elicited somatic Ca$^{2+}$ increases in layer V pyramidal cells of adult PFC. Left, a schematic of the recording area is shown in gray. Right, Bath application of 1 mM ACh (15 s, red) elicits pan-somatic Ca$^{2+}$ increases ($dF/F_{ACh}$). Various AOIs are depicted on the red channel image and their respective Ca$^{2+}$ signals are overlapped below. Changes in fluorescence measured in four smaller AOIs do not significantly differ from that measured in a single, pan-somatic AOI. Sample OGB-1 fluorescence increases from baseline (1), peak Ca$^{2+}$ release (2) and washout (3) are pseudocolored for illustrative purposes. Scale bar, 10 µm.

Electrophysiologically, an $I_{ACh}$ was observed in all cells (-73 ± 7 pA, $n = 30$); however, the $dF/F_{ACh}$ varied among neurons: monophasic $dF/F_{ACh}$ were observed in 13/30 (43%) cells (Figure 7A), multi-peaked $dF/F_{ACh}$ in 3/30 (10%) cells (Figure 7B) and no $dF/F_{ACh}$ in 14/30 (47%) cells (ACh$_{No Ca^{2+}}$; Figure 7C). In addition to the prolonged excitatory inward $I_{ACh}$, a subpopulation of ACh$_{Ca^{2+}}$ neurons showed a transient outward current (Figure 7A), a previously-described M$_1$ muscarinic receptor-mediated phenomenon (Gulledge,
2005; Gulledge et al., 2009). Yet, examining the relationship between the magnitudes of \( \frac{dF}{F_{ACh}} \) and \( I_{ACh} \), we found that increased Ca\(^{2+} \) was associated with greater muscarinic excitation. Peak \( I_{ACh} \) amplitudes were significantly larger in \( ACh_{Ca2+} \) cells (Figure 7D, unpaired \( t \) test, \( p = 0.02 \)) and were significantly correlated with \( \frac{dF}{F_{ACh}} \) (Figure 7E, \( R^2 = 0.41, p = 0.01 \)). While neuromodulatory responses of PFC layer V pyramidal neurons have been shown to exhibit cell type specificity (Dembrow et al., 2010), \( ACh_{Ca2+} \) and \( ACh_{No Ca2+} \) cells were neither electrophysiologically (see Methods) nor morphologically distinct (Supplemental Figure 1, Supplemental Table 1).

**Figure 7.** \( ACh \)-elicited somatic Ca\(^{2+} \) increases potentiate muscarinic responses in layer V pyramidal cells of adult PFC. \( ACh \) elicits 3 types of responses: **A**, mono-peaked \( \frac{dF}{F_{ACh}} \) (47%), **B**, multi-peaked \( \frac{dF}{F_{ACh}} \) (10%) and **C**, non-response (47%, \( ACh_{No Ca2+} \)). **D** Peak excitatory inward currents (\( I_{ACh} \)) are greater in cells where Ca\(^{2+} \) release is also stimulated. **E** Correlation of peak \( I_{ACh} \) and \( \frac{dF}{F_{ACh}} \) is significant (\( p = 0.01 \)), with the exclusion of one outlier. * \( p < 0.05 \)
Both $dF/F_{ACh}$ and $I_{ACh}$ were abolished by the $M_1$ muscarinic receptor antagonist pirenzepine (Figure 8A,B; 500 nM, 10 min), consistent with a genetic deletion study that has demonstrated a predominant role for this receptor subtype in mediating cholinergic responses in PFC layer V pyramidal cells (Gulledge, 2005; Gulledge et al., 2009). Furthermore, $Ca^{2+}$ store depletion achieved by bath application of the SERCA pump inhibitor thapsigargin (10 µM) almost completely eliminated $dF/F_{ACh}$ (Figure 8C; $dF/F_{ACh}$ reduction: 91 ± 5%, $n = 7$), confirming the involvement of intracellular release from the endoplasmic reticulum following $M_1$ receptor activation. Consistent with the hypothesis that $Ca^{2+}$ release potentiates $I_{ACh}$, thapsigargin treatment led to a significant $I_{ACh}$ reduction (Figure 3D; matched-pair t-test, $p = 0.02$; percent reduction: 27 ± 10%, $n = 7$), with the remainder of $I_{ACh}$ likely reflecting the direct effects of muscarinic $M_1$ receptors on ion channels (Krnjević et al., 1971; McCormick and Prince, 1986; Haj-Dahmane and Andrade, 1996; Carr and Surmeier, 2007; Yan et al., 2009; Dasari et al., 2013).

Of note, $ACh_{NoCa^{2+}}$ neurons (that did not initially show a $dF/F_{ACh}$) could be “primed” prior to $ACh$ application by a depolarizing pulse (to 0 mV), which would replenish intracellular $Ca^{2+}$ stores by means of voltage-gated $Ca^{2+}$ entry (Stutzmann et al., 2003). Importantly, successful priming resulted in a potentiated $I_{ACh}$ in 9/14 cells (Figure 8E,F; matched-pair t-test, $p = 0.002$). The potentiated $I_{ACh}$ observed following priming was not likely to be attributed to the priming event per se, given that $I_{ACh}$ was unchanged in the 5/14 cells where priming was ineffective at eliciting a subsequent $dF/F_{ACh}$ (data not shown).
We next tested whether recruiting an additional mechanism of Ca$^{2+}$ release would alter $I_{ACh}$. For this experiment, we used caffeine, a ryanodine receptor agonist that can elicit Ca$^{2+}$ release from intracellular stores (McPherson et al., 1991) (data not shown). The presence of caffeine significantly prolonged the $dF/F_{ACh}$ (Decay time$_{ACh}$: 2.3 ± 0.8 s, Decay time$_{ACh+caff}$: 10.8 ± 2.9 s; matched-pair t-test, $p = 0.03$, $n = 9$) and significantly enhanced the $I_{ACh}$ amplitude (Peak $I_{ACh}$: −75 ± 6 pA, Peak $I_{ACh+caff}$: −111 ± 15 pA; matched-pair t-test, $p = 0.02$, $n = 9$) and $I_{ACh}$ area under the curve (area-$I_{ACh}$: 4545 ± 551 pA*s, area-$I_{ACh+caff}$: 6556 ± 874 pA*s; matched-pair t-test, $p = 0.01$; $n = 9$). These data emphasize that intracellular Ca$^{2+}$ is an important modulator of excitatory muscarinic responses.
Figure 8. Muscarinic excitation ($I_{ACh}$) is potentiated by ACh-elicited Ca$^{2+}$ release from intracellular stores ($dF/F_{ACh}$). A, B Muscarinic M$_1$ receptor antagonist pirenzepine (500 nM, 10 min) abolished $I_{ACh}$ and $dF/F_{ACh}$ in layer V pyramidal cells of adult PFC (lower and upper traces, respectively). The middle trace schematically depicts changes in holding potential. Note the brief depolarizing step at the end of the trace, which serves as a control. C, D Depletion of intracellular Ca$^{2+}$ stores with thapsigargin (10 µM, 10 min) attenuates $dF/F_{ACh}$ and significantly reduces $I_{ACh}$ in ACh$_{Ca^{2+}}$ cells. E, F Conversely, in ACh$_{No_{Ca^{2+}}}$ cells, a depolarizing pulse (to 0 mV) delivered prior to ACh application could trigger Ca$^{2+}$ entry and subsequent $dF/F_{ACh}$, presumably due to the replenishment of intracellular stores.
3.4.2 \textit{Ca}^{2+}-potentiation of $I_{ACh}$ develops in young adulthood and is disrupted by early stress

Peak performance of ACh-dependent executive functions is only achieved as the PFC matures (Sowell et al., 2001; De Luca et al., 2003; Gogtay et al., 2004; Luciana et al., 2005; Conklin et al., 2007) and this late maturation is thought to enhance vulnerability to early stress (Hedges and Woon, 2010; Pechtel and Pizzagalli, 2010). Therefore, we next examined the relationship of the $I_{ACh}$ and $dF/F_{ACh}$ across a broader developmental period and tested whether it is altered by the early stress of repeated maternal separation (Figure 9, Supplemental Figure 2) (Berton and Nestler, 2006; Benekareddy et al., 2010). In this independent replication, we found that peak $I_{ACh}$ were significantly greater in cells where somatic Ca$^{2+}$ release could be detected, both in young and mature adult controls. However, the potentiation of $I_{ACh}$ by $dF/F_{ACh}$ was absent in adolescents (Figure 9B, Supplemental Figure 2), although the proportion of AChCa$^{2+}$ cells did not differ significantly with developmental stage ($\chi^2_{df=2} = 4.4, p = 0.1$). Thus, it appears that the potentiation of muscarinic excitation by ACh-induced Ca$^{2+}$ release is a phenomenon that emerges in early adulthood.

Following the experience of early stress, this expected developmental emergence of the Ca$^{2+}$ potentiation of $I_{ACh}$ was absent (Figure 9B, Supplemental Figure 2). Yet, the proportion of AChCa$^{2+}$ cells between control animals and those that had undergone the experience of early stress remained unchanged in the adolescent (Fisher’s exact test, $p = 1.00$), young adult (Fisher’s exact test, $p = 0.4$) and mature adult (Fisher’s exact test, $p = 1.00$). Interestingly, these developmental changes susceptible to early stress were accompanied by marked differences in the timing but not the amplitude of the individual $dF/F_{ACh}$ responses (Supplemental Figure 3).
3.4.3 Early stress produces developmentally-specific changes in PFC gene expression*

We hypothesized that differences in the timing of dF/F_Ach in development and early stress reflect differences in G_{aq} and Ca^{2+}-mediated signaling. To test this hypothesis, we performed qPCR for a number of genes that had previously been shown to be

![Figure 9](image-url)
dysregulated in the PFC in adult animals with a history of early stress (Benekareddy et al., 2010). As shown in Supplemental Figure 4 and Supplemental Table 2, we found that early stress significantly altered the pattern of developmental expression of genes associated with Ca\(^{2+}\)-mediated signaling, including a voltage-gated Ca\(^{2+}\) channel subunit, Ca\(^{2+}\)-dependent enzymes, Ca\(^{2+}\)-sensitive adhesion molecules and, notably, K\(^+\)-dependent Na\(^{+}/Ca^{2+}\) exchangers (NCKX). Many of these genes have been shown to structurally or functionally interact with the IP\(_3\) receptor (Cameron et al., 1995; Pattni et al., 2003; Delmas et al., 2004; Patterson et al., 2004; Singleton and Bourguignon, 2004; Power and Sah, 2005; Berridge, 2006; Bourguignon and Jin 1995) and together may contribute to the complex developmental regulation of ACh-induced Ca\(^{2+}\) release and its dysregulation following early stress.

* These data were collected and analyzed by Dr. Deepika Suri under the supervision of Dr. Vidita Vaidya at the Tata Institute of Fundamental Research in Mumbai, India. Methods are outlined in Appendices.

3.4.4 Na\(^{+}/Ca^{2+}\) exchange potentiates muscarinic excitation in healthy adult controls

We next sought to further elucidate the mechanism whereby the dF/F\(_{\text{ACh}}\) may potentiate I\(_{\text{ACh}}\) in healthy adult controls. While mindful that optimal muscarinic function likely depends on a multitude of G\(_{\alpha_q}\) and Ca\(^{2+}\) signaling-related genes, the differences in NCKX expression patterns raised the interesting possibility that Na\(^+\)/Ca\(^{2+}\) exchange might contribute to muscarinic function in the PFC. Specifically, we hypothesized that electrogeneric Na\(^+\)/Ca\(^{2+}\) exchange might be responsible for the potentiation of I\(_{\text{ACh}}\) by dF/F\(_{\text{ACh}}\) in the PFC of adult control animals. While this phenomenon has not been investigated in the cerebral cortex, it has been suggested to contribute to muscarinic signaling in the medial septum (Xu et al., 2006) and the tuberomammillary nucleus.
Molecular mediators of \( \text{Na}^+/\text{Ca}^{2+} \)-exchange include two families of electrogenic exchangers that increase cellular excitation in the process of removing \( \text{Ca}^{2+} \): the \( \text{Na}^+ \)-dependent \( \text{Ca}^{2+} \) exchanger (NCX), which couples the extrusion of 1 \( \text{Ca}^{2+} \) in exchange for 3 \( \text{Na}^+ \) ions, and the \( \text{Na}^+ \)- and \( \text{K}^+ \)-dependent \( \text{Ca}^{2+} \) exchanger (NCKX), which couples the extrusion of 1 \( \text{Ca}^{2+} \) and 1 \( \text{K}^+ \) in exchange for 4 \( \text{Na}^+ \) ions (Blaustein and Lederer, 1999; Lytton, 2007). These proteins are highly expressed in the brain (Lytton et al., 2002; Lytton, 2007) and the cerebral cortex (Gibney et al., 2002).

To evaluate the possibility of \( \text{Na}^+/\text{Ca}^{2+} \) exchanger involvement in muscarinic potentiation, we examined the relative timing of \( I_{\text{ACh}} \) and the decay of \( dF/F_{\text{ACh}} \), a measurement of \( \text{Ca}^{2+} \) clearance and potential indicator of the extrusion of \( \text{Ca}^{2+} \). For this analysis, we averaged the traces obtained in \( \text{ACh}_{\text{Ca}^{2+}} \) (\( n = 16 \)) and \( \text{ACh}_{\text{No Ca}^{2+}} \) neurons (\( n = 14 \)) of adult control animals from our original dataset (Figure 7). This analysis highlights that the presence of a \( dF/F_{\text{ACh}} \) yielded a \textit{supplemental} \( I_{\text{ACh}} \), in addition to the basal \( I_{\text{ACh}} \) observed in \( \text{ACh}_{\text{No Ca}^{2+}} \) neurons (Supplemental Figure 5). Interestingly, this supplemental \( I_{\text{ACh}} \) coincided with the decay, or clearance, of the \( dF/F_{\text{ACh}} \) (Supplemental Figure 5), which is consistent with a contribution of electrogenic exchangers to PFC cholinergic modulation.

To probe further the potential involvement of \( \text{Na}^+/\text{Ca}^{2+} \) exchange, we reduced the concentration gradient of \( \text{Na}^+ \) on which electrogenic \( \text{Ca}^{2+} \) extrusion depends. Eighty percent \( \text{Na}^+ \) substitution reduced \( I_{\text{ACh}} \) to a degree that suggests the \( \text{Na}^+ \)-dependence of both the basal and supplemental \( I_{\text{ACh}} \) (Supplemental Figure 6). Importantly, \( \text{Na}^+ \) substitution prolonged the \( dF/F_{\text{ACh}} \) response, an effect enhanced with dual \( \text{Na}^+ \) and \( \text{K}^+ \) substitution (Supplemental Figure 6). These observations further suggested that
clearance of Ca\(^{2+}\) by electrogenic Na\(^{+}\)/Ca\(^{2+}\) exchange contributes to \(I_{ACh}\) following agonist-induced Ca\(^{2+}\) release in PFC, and that members of the NCX and/or NCKX family may be involved. Accordingly, we found that a NCX and NCKX inhibitor (Altimimi et al., 2012), KB-R7943 (50 µM, 5 min), reduced the \(I_{ACh}\) by an average of 34 ± 10 % (Supplemental Figure 7) and that non-specific inhibition of Na\(^{+}\)/Ca\(^{2+}\) exchange with NiCl\(_{2}\) (3 mM) (Xu et al., 2006) reduced the \(I_{ACh}\) by an average of 63 ± 7% (Supplemental Figure 7). The schematic in Figure 6 shows the mechanism hypothesized to underlie muscarinic excitation of layer V PFC neurons in healthy adults.

### 3.5 Discussion

Here, we provide novel insights into the mechanisms and developmental changes of cortical muscarinic modulation and its vulnerability to disruption by early stress. First, we demonstrated that muscarinic excitation is potentiated by ACh-induced Ca\(^{2+}\) release. Second, we found that this phenomenon emerges in young adulthood as agonist-induced Ca\(^{2+}\) release becomes more precisely timed. Third, we discovered that the experience of early stress disrupts this developmental consolidation of muscarinic signaling, leading to the retention of the adolescent phenotype with potential implications for executive function. We further report that these changes in cellular function are compounded by developmental changes in the PFC expression of genes associated with Ca\(^{2+}\) signaling. We conclude by implicating Na\(^{+}\)/Ca\(^{2+}\) exchange-mediated electrogensis in the potentiation of muscarinic excitation in healthy adult PFC.
3.5.1 Mechanisms of muscarinic modulation of the PFC circuits of executive function

ACh modulation of the PFC is essential for executive functions such as working memory and attention (Winkler et al., 1995; Parikh et al., 2007; Croxson et al., 2011; Hasselmo and Sarter, 2011) and disruption of such modulation is thought to contribute to the executive deficits prevalent in psychiatric disorders (Hasselmo and Sarter, 2011; Sarter and Paolone, 2011). Muscarinic ACh receptors in particular are necessary to sustain persistent activity, which is thought to underlie working memory (Goldman-Rakic, 1995b; Egorov et al., 2002; Zhang and Seguela, 2010; Zhou et al., 2011) and to stabilize attention circuitry (Hasselmo and Sarter, 2011). Here, we demonstrate for the first time that agonist-induced Ca\(^{2+}\) release from intracellular stores can significantly potentiate the excitatory effects of PFC muscarinic ACh receptors.

There has been considerable debate over the specific mechanisms underlying cortical muscarinic excitation. The inhibition of several subtypes of K\(^+\) channels (Krnjević et al., 1971; McCormick and Prince, 1986; Carr and Surmeier, 2007) and/or the activation of transient receptor potential (TRP)-like, non-selective cation channels (Haj-Dahmane and Andrade, 1996; Klink and Alonso, 1997; Shalinsky et al., 2002; Yan et al., 2009) have been implicated as the mediators of muscarinic excitation. The evidence provided in this study suggests that electrogenic clearance of dF/F\(_{ACh}\) contributes a supplemental excitatory I\(_{ACh}\) that lasts well beyond any transient inhibitory Ca\(^{2+}\) effects and clearly enhances the excitatory component of the muscarinic response. We suggest that in healthy adult PFC, members of the NCX and/or NCKX families potentiate muscarinic excitation by coupling extrusion of ACh-induced Ca\(^{2+}\) release to net Na\(^+\) influx, as summarized in Figure 10. NCX and NCKX proteins have been shown
to play a major role in Ca\textsuperscript{2+} clearance (Lee et al., 2002; Kim, 2005; Altimimi and Schnetkamp, 2007; Lytton, 2007) and their low affinity and high transport properties position them especially well for the clearance of larger cytosolic Ca\textsuperscript{2+} increases resulting from signaling events (Visser and Lytton, 2007). Many different isoforms are expressed in adult rat cerebral cortex, including NCX1.4, NCX1.5, NCX2, and NCX3 (Canitano et al., 2002; Gibney et al., 2002; Minelli et al., 2007), as well as NCKX2, NCKX3, and NCKX4 (Yu and Colvin, 1997; Gibney et al., 2002; Lytton et al., 2002). NCKX6 has also been shown to be expressed in brain (Cai, 2003). This apparent complexity may be increased further by the differential sensitivity of these exchanger isoforms to post-translational modulation (Lytton, 2007). Of note, a growing body of evidence suggests that the individual exchanger isoforms make unique contributions to normal cognition (Jeon et al., 2003; Li et al., 2006; Molinaro et al., 2011). Further studies will be necessary to elucidate the molecular mechanisms of ACh-induced Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange in the PFC and to investigate its role in shaping cellular and network activity in health and disease.

Overall, the further characterization of dF/F\textsubscript{ACh}, its regulation and its activity-dependence is a timely subject for further investigation. The observation of multi-peaked Ca\textsuperscript{2+} responses may reflect regenerative Ca\textsuperscript{2+} release, either due to the increased sensitivity of IP\textsubscript{3} receptors by Ca\textsuperscript{2+} or to the recruitment of local ryanodine receptors (Berridge, 1998). Different dF/F\textsubscript{ACh} responses may also reflect differences in muscarinic receptor phosphorylation state (Kawabata et al., 1996). Furthermore, the ability to elicit dF/F\textsubscript{ACh} in ACh\textsubscript{NoCa2+} by “priming” the cells with a depolarizing pulse, which would replenish intracellular Ca\textsuperscript{2+} stores by means of voltage-gated Ca\textsuperscript{2+} entry (Stutzmann et
al., 2003), suggests that the propensity for ACh to elicit a somatic Ca\(^{2+}\) response does not reflect cell type but instead may reflect Ca\(^{2+}\) store readiness.

Figure 10. Schematic to illustrate the hypothesis that PFC excitatory muscarinic currents are potentiated via NCX- and NCKX-mediated electrogenesis. Activation of M\(_1\) muscarinic acetylcholine receptors leads to release of Ca\(^{2+}\) from intracellular stores and modulation of a Na\(^{+}\)-dependent ion channel conductance to generate the primary \(I_{ACh}\). Na\(^{+}\)-Ca\(^{2+}\) exchanger (NCX) and/or Na\(^{+}\)-K\(^{+}\)-Ca\(^{2+}\) exchanger (NCKX)-mediated electrogenesis underlies the supplementary \(I_{ACh}\) observed in the presence of dF/F\(_{ACh}\). PIP2, phospholipid phosphatidylinositol 4,5-bisphosphate; DAG, diacyl glycerol; IP3, inositol 1,4,5-trisphosphate; NSCC, non-selective cation channel.
3.5.2  $\text{G}_\alpha_q$-coupled receptor signaling pathway complexity and vulnerability

G-protein signaling involves a cascade of several second messengers, including Ca$^{2+}$ (Berridge et al., 2003), that can interact to yield responses on a variety of timescales (Hamm, 1998; Delmas et al., 2004; Ritter and Hall, 2009). The complexity of muscarinic signaling, and of $\text{G}_\alpha_q$-protein coupled receptors more generally, may help confer the flexibility that has been deemed essential to executive function at the neuronal level (Funahashi, 2001). Disruptions in the phospholipase C pathway, which is activated by $\text{G}_\alpha_q$-protein coupled receptors, have been implicated in the pathophysiology of mental illness (Arnsten, 2009). Here, we present evidence that the experience of early stress dysregulates $\text{G}_\alpha_q$-coupled muscarinic signaling and alters the expression of a network of proteins that structurally and/or functionally interact with the IP$_3$ receptor (Delmas et al., 2004; Patterson et al., 2004; Berridge, 2006), including calcineurin (Cameron et al., 1995; Patterson et al., 2004), the L-type Ca$^{2+}$ channel β2 subunit (Power and Sah, 2005), CD44 (Singleton and Bourguignon, 2004; Bourguignon and Jin 1995), calpain 8 (Pattini et al., 2003; Patterson et al., 2004), ANK FY1 (Bourguignon and Jin 1995) and AKAP1 (Berridge et al., 2003; Delmas et al., 2004).

3.5.3  Interactions of genes, development and early stress

Peak performance on tasks of executive function is only achieved as the PFC matures in mid- to late adolescence (Huttenlocher and Dabholkar, 1997; Sowell et al., 2001; De Luca et al., 2003; Gogtay et al., 2004; Luciana et al., 2005; Conklin et al., 2007) and brain regions of such protracted development are especially vulnerable to the effects of early stress (Pechtel and Pizzagalli, 2010). Here, we investigated aspects of the normal maturation of muscarinic acetylcholine responses in the PFC. We found that
intracellular Ca\(^{2+}\) release normally potentiates muscarinic excitation in adulthood but not in the adolescent period. This developmental profile was not observed in the brains of animals that have undergone the experience of early stress. Gene expression differences suggest that the timing of muscarinic-elicited Ca\(^{2+}\) dynamics may depend on, and be vulnerable to, developmental disruptions of the molecular machinery associated with Ca\(^{2+}\) release-related microdomains (Berridge et al., 2003; Delmas et al., 2004; Patterson et al., 2004; Berridge, 2006).

The maintenance of a juvenile phenotype into adulthood following early stress is an interesting possibility; however, this notion appears overly simplistic. Our data show not only that early stress can lead to expression changes in genes relating to G\(\alpha_q\) Ca\(^{2+}\) signaling, but also that early life experience interacts with development (Supplemental Table 2, Supplemental Figure 4). In particular, the potentiation of muscarinic excitation by Ca\(^{2+}\) release appears to require the maturation of a network of G\(\alpha_q\)- and Ca\(^{2+}\) signaling-related genes whose susceptibility to early stress suggest experience-dependent modulation. Therefore, rather than the retention of a juvenile phenotype, it is the developmental trajectory of gene expression and cellular function that is altered following early stress.

In sum, this work illustrates cellular mechanisms and molecular pathways that may allow early stress to disrupt cognitive performance on tasks requiring mature executive function. Additionally, these findings elucidate a mechanism contributing to PFC muscarinic excitation which shows delayed developmental consolidation. It is hoped that a deeper understanding of the cholinergic modulation of PFC will ultimately
help improve treatment strategies for cognitive deficits in neurological and psychiatric disorders.
Chapter 4

Impaired cholinergic excitation of prefrontal attention circuitry in the TgCRND8 model of Alzheimer’s disease

Acknowledgements & Contributions

Éliane Proulx and Evelyn Lambe designed the experiments and wrote the manuscript. Éliane Proulx performed all experiments and analyzed the data under the supervision of Dr. Evelyn Lambe at the University of Toronto, Canada. Paul Fraser and JoAnne McLaurin provided materials and expertise. We thank Ms. Mary Hill and Ms. Rosemary Ahrens for expert technical assistance. This work was funded by grants from the Canadian Institutes of Health Research: MOP-89825 (EKL), TAD-117950 (PF), and MOP-102467 (JM); the Canada Research Chairs Program (EKL); an Ontario Early Researcher Award (EKL); the Canadian Foundation for Innovation (EKL), the Alzheimer’s Society of Ontario (PF), and the Cryptic Rite Charitable Foundation (JM).
4 Impaired Cholinergic Excitation of Prefrontal Attention Circuitry in the TgCRND8 Model of Alzheimer’s Disease

4.1 Abstract

Attention deficits in Alzheimer’s Disease (AD) can exacerbate other executive symptoms, yet are not well understood. As these deficits precede changes in cholinergic neurons, it has been assumed that they arise from multiple alterations in prefrontal cholinergic stimulation, as a result of aberrant expression of acetylcholinesterase and nicotinic acetylcholine receptors. Here, in the TgCRND8 mouse model of AD, we demonstrate and characterize a disruption of cholinergic excitation in the major corticothalamic layer of the prefrontal cortex, where modulation by acetylcholine is essential for optimal attentional function. Using electrophysiology with concurrent multiphoton imaging, we show that layer 6 pyramidal cells are unable to sustain cholinergic excitation to the same extent as their non-transgenic littermate controls, as a result of the excessive activation of calcium-activated hyperpolarizing conductances. We report that cholinergic excitation can be improved in TgCRND8 cortex by pharmacological blockade of SK channels, suggesting a novel target for the treatment of executive dysfunction in AD.

4.2 Introduction

Alzheimer’s disease (AD) is a neurodegenerative disorder that constitutes the main cause of dementia. It is associated with profound cognitive impairments that include severe memory loss and early attention deficits (Grady et al., 1988; Perry and Hodges,
Impaired attention negatively impacts the day-to-day life of AD patients, contributing to distraction, confusion and poor executive control (Perry and Hodges, 1999). It is also likely that disruptions in attention further exacerbate the memory deficits that constitute a telltale sign of the disease: if you can’t attend, you can’t encode (Romberg et al., 2013a; 2013b). Yet, little is understood of the cellular mechanisms that underlie the non-mnemonic deficits of executive function in AD and their contribution to the disease.

To date, acetylcholinesterase inhibitors constitute the standard of care for AD (Citron, 2010), although loss of cholinergic cells is an end-stage manifestation of the disease that is unlikely to account for cognitive deficits that may arise much earlier (Davis et al., 1999; Terry and Buccafusco, 2003). We now know that the cholinergic modulation of the prefrontal cortex (PFC) is essential for optimal attentional performance (Dalley, 2004; Parikh et al., 2007; Bailey et al., 2010; Guillem et al., 2011; Proulx et al., 2013). Acetylcholine is released in the PFC during attention tasks (Parikh et al., 2007), where it exerts robust direct excitation of the deep cortical layers (Kassam et al., 2008; Poorthuis et al., 2012; Proulx et al., 2014). The powerful and prolonged activation of layer 6 pyramidal neurons by acetylcholine is consistent with the sustained firing necessary for attention and other executive functions (Funahashi, 2001; Hasselmo and Sarter, 2011; Ikkai and Curtis, 2011; Zhou et al., 2011) and depends on a complex interplay of nicotinic- and muscarinic-mediated signalling (Bailey et al., 2010; Tian et al., 2011). Manifestations of AD in people and animal models have long been linked to aberrations in cholinergic enzymatic activity (Davies and Maloney, 1976; Perry et al., 1978; Henke and Lang, 1983; Zubenko et al., 1989; Davis et al., 1999; Selkoe, 2001; Auld et al., 2002; DeKosky et al., 2002), nicotinic receptor binding (Nordberg et al.,
1988; Whitehouse et al., 1988; Marutle et al., 1999; Nordberg, 2001; O'Brien et al., 2006; Kendziorra et al., 2010), and cholinergic signalling pathways (Jope et al., 1997, 1999; Kelly et al., 1996; Pettit et al., 2001; Liu et al., 2001; Auld et al., 2002). How do these broad changes affect the activation and sustained firing of layer 6 prefrontal attention circuitry?

To address this question, we examined cholinergic signalling in prefrontal attention circuitry of a well-characterized animal model of AD. The TgCRND8 mouse model is widely used in Alzheimer’s research and encodes both the Swedish and Indiana mutations of amyloid precursor protein (APP) found in inherited familial AD (Chishti et al., 2001). Importantly, it permits the use of littermate controls and is ideal for this investigation because it recapitulates several of the neuropathological and cognitive features of AD, including amyloid plaque deposition and memory impairments by 3 months of age (Chishti et al., 2001), followed by deficits in sustained attention by 4-5 months of age (Romberg et al., 2013b). Of note, the attention deficits appear to precede the cholinergic cell loss, which is observed much later, at 7 months of age or older (Bellucci et al., 2006; Choi et al., 2013). Here, we report significant impairments in the cholinergic excitation of layer 6 prefrontal neurons of TgCRND8 mice by 3-4 months of age and show that these deficits can be pharmacologically rescued.

4.3 Materials and Methods

4.3.1 Animals

All experiments were approved by the University of Toronto Animal Care Committee. We used adult male and female TgCRND8 mice on a mixed C57/Bl6-C3H background, as previously reported (Chishti et al., 2001), along with their non-transgenic littermate
siblings (WT) as controls (Age, mean ± SEM, WT: 109 ± 2 days, N=17 animals; Tg: 107 ± 2 days, N=23 animals).

4.3.2 Brain slice preparation and recording conditions

Each brain was cooled as rapidly as possible with 4 °C oxygenated sucrose artificial cerebrospinal fluid (ACSF) (254 mM sucrose substituted for NaCl). Coronal slices (400 µm thick) of the PFC were cut on a Dosaka Pro-7 Linear Slicer (SciMedia), and were transferred to 30 °C oxygenated ACSF (containing the following, in mM: 128 NaCl, 10 D-glucose, 26 NaHCO₃, 2 CaCl₂, 2 MgSO₄, 3 KCl, 1.25 NaH₂PO₄, pH 7.4) in a pre-chamber (Automate Scientific) and allowed to recover for at least 1.5 h before the beginning of an experiment. Slices were placed in a chamber on the stage of an upright microscope for whole-cell recordings. ACSF was bubbled with 95% oxygen and 5% carbon dioxide and flowed over the slice at 30 °C with a rate of 3-4 ml/min.

4.3.3 Electrophysiological recordings

Layer 6 pyramidal neurons were patched under visual control using infrared differential interference contrast microscopy in the cingulate and prelimbic regions of the PFC. Intracellular patch solution contained the following (in mM): 120 K-gluconate, 5 KCl, 2 MgCl₂, 4 K-ATP, 0.4 Na₂-GTP, 10 Na₂-phosphocreatine, and 10 HEPES buffer (adjusted to pH 7.33 with KOH). Data was acquired and low-pass filtered at 20 kHz with an Axopatch 200b amplifier (Molecular Devices) and Digidata1440 digitizer/pClamp10.2 software (Molecular Devices). Intrinsic cellular properties were assessed in current clamp mode and did not differ significantly between genotypes (Table 2; mean ± SEM; sample size for this analysis: n = 49 WT neurons; n =58 TgCRND8 neurons).
All drugs were bath applied. Acetylcholine chloride and atropine were obtained from Sigma, apamin from Alomone Labs, dihydro-ß-erythroidine hydrobromide from R&D Systems and cadmium chloride was obtained from Emerald BioSystems.

**Table 2. Electrophysiological properties of layer 6 pyramidal neurons of the PFC of TgCRND8 mice and WT controls.**

<table>
<thead>
<tr>
<th></th>
<th>WT (N = 49 cells)</th>
<th>Tg (N = 58 cells)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capacitance (pF)</td>
<td>72 ± 2</td>
<td>71 ± 2</td>
<td>0.5</td>
</tr>
<tr>
<td>Input resistance (MΩ)</td>
<td>313 ± 18</td>
<td>285 ± 16</td>
<td>0.2</td>
</tr>
<tr>
<td>Membrane potential (mV)</td>
<td>-83 ± 1</td>
<td>-85 ± 1</td>
<td>0.3</td>
</tr>
<tr>
<td>Spike threshold (mV)</td>
<td>-52 ± 0.4</td>
<td>-51 ± 0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Spike amplitude (mV)</td>
<td>79 ± 1</td>
<td>80 ± 1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

4.3.4 Calcium imaging

The calcium dye Oregon Green BAPTA-1 (OGB-1, 100 μM) was included in the pipette in a subset of cells, along with Alexa-594 hydrazide (20 μM) to aid in the visualization of the neuron. Multiphoton imaging was performed using a Ti:sapphire laser (Newport) tuned to wavelength 800 nm and an Olympus Fluoview FV1000 microscope with a 60X water-immersion 0.90 NA objective. The emitted fluorescence was separated into green (OGB-1 signal) and red (A-594 signal) channels with a dichroic mirror at 570 nM and filtered (green: BA 495-540; red: BA 570-620) before detection. Images were acquired at a rate of ~10 frames/second and analyzed with Fluoview software. A pan-somatic
area of interest was selected for analysis and green fluorescence increases were calculated relative to baseline fluorescence (dF/F₀). The green to red fluorescence ratio (G/R) was calculated by dividing the green signal by that of the red channel for the purpose of evaluating potential differences in basal calcium levels between genotypes.

4.3.5 Statistical analyses

Results are expressed as mean ± SEM, and all statistical comparisons were made at a significance level of 0.05 (Prism versions 5.0d/6.0, GraphPad). Average traces of post-burst potentials and fluorescent increases were generated with Axograph X. For the purpose of quantifying acetylcholine-elicited firing, input-output curves and post-burst afterhyperpolarization potentials (AHPs), action potentials were automatically detected with a derivative threshold of 20 mV/ms. To assess the ability of cells to sustain near-maximal firing in response to acetylcholine, the cumulative probability of the instantaneous firing frequency of individual action potentials was normalized to the maximum instantaneous firing within each acetylcholine-elicited train of action potentials. Genotype and treatment differences of cumulative probabilities were assessed with K-S tests. The peak and area under the curve of post-burst potentials were measured from the peak of the last action potential in each burst up to 1 s post-burst.

4.4 Results

4.4.1 TgCRND8 mice cannot sustain peak excitation by acetylcholine in prefrontal layer 6

Since there is a panoply of cholinergic-related deficits in AD, we investigated the effects of known concentrations of bath-applied acetylcholine on brain slices from littermate WT
and TgCRND8 mice at an age when attention deficits may be developing. In layer 6 pyramidal neurons of the prefrontal cortex (PFC), acetylcholine elicited cellular depolarization in a concentration-dependent manner in both WT and TgCRND8 mice (Figure 11). Unexpectedly, spiking was elicited in fewer cells in TgCRND8 than WT mice (Figure 11A, three-way contingency test, $G^2_{df=7} = 70.72$, $P < 0.0001$). What is more, in cells where acetylcholine (1 mM) elicited supra-threshold depolarizations, acetylcholine-elicited firing patterns were revealed to be distinct. The distribution of instantaneous frequency measurements for individual action potentials were significantly different between groups (Figure 11B, $P < 0.004$, Mann-Whitney test) and individual TgCRND8 neurons failed to sustain near-maximal firing frequencies to the same extent as WT cells (Figure 11C): the cumulative probability of action potential frequency—normalized to maximal instantaneous frequency within each cell—was dramatically reduced in TgCRND8 neurons ($K$-S test, $P < 0.00001$). The maximal instantaneous firing frequency (WT: $9 \pm 1$ Hz, $N=17$; Tg: $11 \pm 3$ Hz, $N=15$; $t$-test, $P = 0.4$) and duration of spiking (WT: $47 \pm 11$ s, $N=17$; Tg: $42 \pm 9$ s, $N=15$; $t$-test, $P = 0.7$) achieved, however, were similar between genotypes. Further probes of the electrophysiological effects of acetylcholine in layer 6 pyramidal cells in voltage-clamp found that near resting membrane potential ($V_{holding} = -75$ mV), acetylcholine currents were modestly but significantly smaller in TgCRND8 mice (WT: $-121 \pm 8$ pA, $N=23$; TgCRND8: $-92 \pm 9$ pA, $N = 28$; $P = 0.02$). However, pharmacological dissection of these current revealed similar nicotinic (WT$_{atropine}$: $-83 \pm 12$ pA, $N = 7$; TgCRND8$_{atropine}$: $-86 \pm 12$ pA, $N = 9$; $P = 0.9$) and muscarinic contributions (WT$_{DHBE}$: $-33 \pm 7$ pA, $N =8$; TgCRND8$_{DHBE}$: $-22 \pm 6$ pA, $N = 7$; $P = 0.3$), suggesting that the acetylcholine current may be decreased in the TgCRND8 mice through an interaction of nicotinic and
muscarinic signaling. Therefore, to compensate for any potential differences in the cholinergic drive toward threshold between the genotypes, we applied current to the cells to elicit baseline firing at 1-3 Hz. Under these conditions, TgCRND8 neurons also failed to achieve equivalent levels of peak firing (Maximal instantaneous frequency, WT: 21 ± 2 Hz, N = 10 cells; TgCRND8: 15 ± 2 Hz, N = 13 cells; P < 0.05; cumulative probability of action potential instantaneous frequencies, K-S test, P < 0.00001; data not shown). Altogether, these data suggest that the deficit in layer 6 neurons at this early stage in TgCRND8 mice appears to be selectively limited to a marked impairment in the ability to maintain peak excitation to acetylcholine.
Figure 11. Impaired excitation in response to acetylcholine in layer 6 prefrontal cortex of TgCRND8 mice. Bath application of acetylcholine (ACh) elicits cellular depolarization from resting membrane potential in a concentration-dependent manner in both WT and TgCRND8 neurons. ACh was applied at 10 µM, 100 µM and 1 mM in WT (N=19 matched-pairs) and TgCRND8 (N=23 matched-pairs).

**Fig. 11A.** Representative example of responses in a WT cell, top (blue traces), and a TgCRND8 neuron, bottom (red traces). Pie charts depict the proportion of cells where ACh elicited depolarizations that led to spiking. ACh elicited spiking in 0/19 cells in WT and 0/23 cells in TgCRND8 when applied at 10 µM, in 10/19 (53%) cells in WT and 8/23 (35%) cells in TgCRND8 at 100 µM, and in 17/19 (89%) cells in WT and 15/23 (65%) cells in TgCRND8 mice when applied at 1 mM (Three-way contingency test, $G^2_{df=7} = 70.72$, $P < 0.0001$). **Fig. 11B,** frequency histograms of instantaneous firing frequencies elicited by acetylcholine (1 mM) in all cells ($P < 0.004$, Mann-Whitney test). **Fig. 11C,** The cumulative probability of action potential firing frequency elicited by 1 mM ACh (normalized to maximal instantaneous frequency ($F_{inst}$) within each cell) reveals that TgCRND8 neurons fail to sustain near maximal firing frequencies to the same extent as WT cells ($K$-S test, $P < 0.00001$). Note that the distribution is left-skewed in TgCRND8 neurons, with a greater number of observations falling below half-maximal values. **Fig. 11D,** mean depolarization was 7 ± 1 mV in WT and 5 ± 1 mV in TgCRND8 at 10 µM, 17 ± 2 mV in WT and 15 ± 2 mV in TgCRND8 at 100 µM and, at 1 mM ACh, 21 ± 2 mV in WT and 22 ± 1 mV in TgCRND8. The depolarization achieved was not significantly different between genotypes as revealed by two-way repeated measures ANOVA (effect of genotype, $F_{1,40} = 0.29$, $P = 0.6$).

4.4.2 Probing mechanisms of altered excitability in TgCRND8 prefrontal layer 6 neurons

Given the observed differences in sustaining acetylcholine-elicited peak firing, we investigated whether there were differences in intrinsic excitability in prefrontal layer 6 pyramidal neurons. We generated input-output curves by applying depolarizing current steps of 500 ms duration in 50 pA increments. A clear difference between genotypes was evident in the layer 6 neurons at stronger depolarizations, with fewer action potentials elicited in TgCRND8 mice (Figure 12, effect of genotype, two-way ANOVA, $F_{1,253} = 8.12$, $P = 0.005$). However, at rheobase there were no significant differences in action potential amplitude (WT: 79 ± 3 mV, N=13; TgCRND8: 77 ± 2, N=12; $P = 0.5$), rise time (WT: 282 ± 12 µs, N=13; TgCRND8: 289 ± 11 µs, N=12; $P = 0.7$), half-width (WT: 1 ± 0.03 ms, N=13; TgCRND8: 1 ± 0.03 ms, N=12; $P = 0.4$) nor current applied (WT: 66 ± 10 pA, N =13; TgCRND8: 84 ± 11 pA, N =12; $P = 0.2$) in the same subset of cells.
Figure 12. Reduced intrinsic excitability in L6 prefrontal pyramidal neurons of TgCRND8 mice. Input-output curves were generated by applying depolarizing current steps of 500 ms duration in 50 pA increments from resting membrane potential. **Fig. 12A**, representative example of a train of action potentials elicited by a 250 pA current step in WT (top, blue trace) and TgCRND8 neurons (bottom, red trace). Below, a schematic representation of the current injection. **Fig. 12B**, input-output curves in WT cells, (blue, N=13) and TgCRND8 cells (red, N=12 cells). Data points represent mean ± SEM. Effect of genotype is significant as revealed by two-way ANOVA ($F_{1,253} = 8.12$, $P = 0.005$).

The neuronal ability to sustain repetitive spike firing is dependent upon the electrophysiological phenomenon known as the afterhyperpolarization potential (AHP) (Kandel and Spencer, 1961; Sah, 1996; Disterhoft and Oh, 2006; Bean, 2007), which constitutes the undershoot of the action potential and results from hyperpolarizing conductances activated during spiking. Accordingly, we systematically examined whether there were genotype differences in the AHP that occurs in response to the identical numbers of action potentials in TgCRND8 cells and WT controls. Action potentials were elicited by applying brief depolarizing current pulses (2 nA, 2 ms) at 50 Hz from a membrane potential of -70 mV (Figure 13). Both the peak AHP and post-burst area were significantly greater in TgCRND8 mice as revealed by two-way ANOVA (Figure 13B, C; effect of genotype, peak AHP, $F_{1,259} = 8.35$, $P = 0.004$; post-burst area, $F_{1,222} = 18.53$, $P < 0.0001$). A more detailed analysis further revealed that the greatest
differences were observed between 100-500 ms post-burst (Supplemental Figure 8), suggesting differences in what is considered the medium AHP (mAHP) (Sah, 1996; Faber and Sah, 2003).

**Figure 13.** The afterhyperpolarization potential (AHP) is enhanced in L6 pyramidal neurons of TgCRND8 mice. Action potentials were elicited by applying brief depolarizing current pulses (1 nA, 2 ms) at 50 Hz from a membrane potential of -70 mV. **Fig. 13A** depicts the overlay of the average responses in WT (blue, N = 16 cells) and TgCRND8 (red, N = 23 cells). A schematic of the spike burst protocol is shown below. Spikes have been truncated. **Fig. 13B,** Peak AHP in WT and TgCRND8 (two-way ANOVA, effect of genotype, \( F_{1,259} = 8.35 , P = 0.004 \)). **Fig. 13C,** mean post-burst area under the curve (AUC) in WT and TgCRND8 (two-way ANOVA, effect of genotype, \( F_{1,222} = 18.53 , P < 0.0001 \)). Data represented in Fig 13B,C are mean ± SEM.
Since several components of the AHP exhibit calcium-dependence (Sah, 1996; Faber and Sah, 2003), we sought to determine whether enhanced post-burst AHPs were correlated with increased calcium responses in layer 6 TgCRND8 neurons. Accordingly, this subset of our AHP experiments were performed with concurrent multiphoton imaging of the intracellular calcium indicator Oregon-Green BAPTA-1 (OGB-1, 100 µM) in the recorded layer 6 pyramidal neurons. The spike burst protocols elicited changes in intracellular calcium levels, as measured by changes in fluorescence with respect to baseline levels (dF/F). These changes depended on the number of spikes in both genotypes (Effect of no. of spikes, peak dF/F, $F_{5,222} = 93.06$, $P < 0.0001$; dF/F Area, $F_{5,122} = 80.48$, $P < 0.0001$), but were significantly greater in layer 6 TgCRND8 neurons (Figure 14, Peak dF/F, effect of genotype, $F_{1,222} = 11.82$, $P = 0.0007$; dF/F Area, effect of genotype, $F_{1,122} = 12.61$, $P = 0.0005$). Baseline fluorescence was not different between genotypes, as assessed by the G/R ratio (WT: $233 \pm 13$ units, N=16; TgCRND8: $230 \pm 15$ units, N=23; $P = 0.9$). Moreover, the linear regression of mean peak AHP against mean peak dF/F in WT and TgCRND8 neurons were similar between genotypes, suggesting that AHPs are enhanced in TgCRND8 mice as a result of enhanced calcium increases (slopes of the linear regressions, $P = 0.2$).
Figure 14. Spiking elicits greater Ca^{2+} responses in TgCRND8 neurons. **Fig. 14A**, In the same subset of cells as Fig. 13, changes in calcium with respect to baseline levels (dF/F) elicited by the spike burst protocol detected by the calcium indicator Oregon-Green BAPTA-1 (OGB-1, 100 µM) with 2-photon microscopy. An overlay of the average responses in WT (blue, N=16) and TgCRND8 (red, N=23) is shown and a schematic of the spike burst protocol is depicted below. **Fig. 14B**, Quantification of the peak dF/F (mean ± SEM) in WT and TgCRND8 neurons (Two-way ANOVA, effect of genotype, F_{1,222} = 11.82, P = 0.0007). **Fig. 14C**, Area under the curve of dF/F in WT and TgCRND8 (Two-way ANOVA, effect of genotype, F_{1,122} = 12.61, P = 0.0005).

One candidate channel for a calcium-dependent medium AHP is the small conductance calcium-activated potassium channel known as SK (Sah, 1996; Faber and Sah, 2003). We found that in WT layer 6 PFC pyramidal neurons, the post-burst AHP was reduced by the SK-specific toxin, apamin, particularly between 50-100 ms post-
burst (Figure 15, Supplemental Figure 9). In TgCRND8 layer 6 neurons, the post-burst AHP was also reduced by apamin (TgCRND8control v. TgCRND8apamin, Peak AHP, $F_{1,258} = 39.14$, $P < 0.0001$; AUC, $F_{1,258} = 33.59$, $P < 0.0001$). Moreover, between 100-500 ms—where the genotype difference was most pronounced (Supplemental Figure 8)—differences were abolished in the presence of apamin (Figure 16; WTapamin v. TgCRND8apamin, Peak AHP100-500 ms, $F_{1,234} = 0.04$, $P = 0.84$; AUC100-500 ms, $F_{1,234} = 0.40$, $P = 0.53$), suggesting that the enhanced AHPs in TgCRND8 mice result from a greater SK contribution. Interestingly, the apamin experiments did reveal a residual, apamin-resistant early AHP in TgCRND8 neurons (1-4 spikes). Overall, however, the SK inhibitor apamin restored AHP area to WT control levels in TgCRND8 neurons (WTcontrol v. TgCRND8apamin, two-way ANOVA, $F_{1,216} = 1.03$, $P = 0.3$) and also abolished genotype differences in intrinsic excitability as assessed by input-output curves (WTcontrol v. TgCRND8apamin two-way ANOVA, $F_{1,168} = 0.01$, $P = 0.9$; data not shown).
Figure 15. The post-burst AHP is apamin-sensitive in WT. **Fig. 15A**, an example of post-burst AHPs elicited under control condition in WT and, below, an example of post-burst AHPs elicited in the presence of the SK antagonist apamin (200 nM) in another layer 6 PFC neuron from WT. **Fig. 15C,D**. In control v. apamin conditions, measurements of the peak AHP (Two-way ANOVA, treatment effect, $F_{1,198} = 39.62$, $P < 0.0001$) and post-burst area (Two-way ANOVA, treatment effect, $F_{1,198} = 10.70$, $P = 0.001$).
Figure 16. Enhanced AHPs in TgCRND8 neurons result from a greater SK contribution. Fig. 16A, Overlay of the post-burst AHPs elicited by the spike burst protocol in the presence of apamin (200 nM) in WT (blue, N=19 cells) and TgCRND8 mice (red, N = 22 cells). Fig. 16 B, C, measurements of the peak AHP (Two-way ANOVA, effect of genotype, $F_{1,234} = 4.90$, $P = 0.03$) and post-burst area (two-way ANOVA, effect of genotype, $F_{1,234}=5.94$, $P = 0.02$). Apamin was most effective at normalizing the post-burst AHP in TgCRND8 where the genotype difference was most pronounced (WT apamin v. TgCRND8 apamin, Peak AHP$_{100-500\text{ ms}}$, $F_{1,234} = 0.04$, $P = 0.84$; AUC$_{100-500\text{ ms}}$, $F_{1,234} = 0.40$, $P = 0.53$) but reveals a residual, apamin-resistant early AHP in TgCRND8 (1-4 spikes, arrowheads).

4.4.3 Enhanced supra-threshold calcium flux underlies aberrations in the cholinergic excitation of layer 6 pyramidal neurons of TgCRND8 animals

Cholinergic stimulation of layer 6 pyramidal neurons has the potential to raise intracellular calcium levels substantially. Not only does acetylcholine bring the majority of these neurons to spiking—thereby permitting the activation of voltage-sensitive
channels that flux calcium (i.e. NMDA receptors, voltage-gated calcium channels)—but the acetylcholine receptors themselves are poised to contribute to intracellular calcium increases. Indeed, in layer 6 prefrontal cortex, acetylcholine stimulates an unusual subtype of nicotinic receptor (Bailey et al., 2010) known to have high calcium flux (Tapia et al., 2007) and stimulates excitatory muscarinic receptors (Tian et al., 2011), which can also release calcium from intracellular stores in prefrontal neurons (Gulledge et al., 2005, 2009; Proulx et al., 2014). Thus, given the observation that cholinergic firing is impaired in TgCRND8 neurons and that calcium-dependent AHPs are enhanced in these same cells, we next investigated whether acetylcholine-elicited spiking is accompanied by greater calcium responses in the TgCRND8 neurons. Acetylcholine (1 mM, 15 s) elicited spiking in 8/9 cells in WT and 6/7 cells in TgCRND8 mice with calcium increases that were significantly greater in the TgCRND8, as detected by the calcium-sensitive dye Oregon Green BAPTA-1 (100 µM) (Figure 17; Peak dF/F, WT: 50 ± 9%, N = 8; TgCRND8: 78 ± 7%, N = 6, P = 0.04; Area of dF/F, WT: 1090 ± 240 % * s, N = 8, TgCRND8: 1950 ± 500 % * s, N = 6, P = 0.1). What is more, in this independent replication, we also observed that TgCRND8 cells failed to sustain firing frequencies to the same extent as WT neurons (Cumulative probabilities of normalized instantaneous frequency, K-S test, P < 0.00001). Thus, impairments in cholinergic firing in the TgCRND8 neurons were accompanied by increased calcium responses.

Since multiple sources of calcium may contribute to these cholinergic calcium responses in layer 6 neurons of prefrontal cortex, we examined genotype differences in both sub- and supra-threshold sources of calcium ion flux. In voltage-clamp, to prevent spiking and the opening of voltage-gated channels, calcium responses elicited by acetylcholine were not different between genotypes when the membrane potential was
clamped at -75 mV, (Peak dF/F, WT: 46 ± 6%, N = 16, TgCRND8: 48 ± 7%, N = 18; P = 0.8, Area of dF/F, WT: 638 ± 110 % * s, N =16, TgCRND8: 606 ± 65 % * s, N = 18, P = 0.8). These data suggest that cholinergic stimulation greatly enhances cellular calcium increases in the TgCRND8 mice only when coincident with trains of action potentials. To probe these genotype differences in supra-threshold calcium influx, we next examined ACh calcium responses in current clamp in the presence of the NMDA receptor channel antagonist, AP-5 (50 µM), or in the presence of the voltage-gated calcium channel blocker, CdCl₂ (100 µM). We found that ACh-elicited calcium responses were significantly reduced with respect to ACSF control in the TgCRND8 neurons but not in WT cells, either in AP-5 (Peak dF/F, WT<sub>AP-5</sub>: 68 ± 9, N = 7, P = 0.2; TgCRND8<sub>AP-5</sub>: 38 ± 7%, N = 7, P = 0.002) or in CdCl₂ (Peak dF/F, WT<sub>CdCl₂</sub>: 32 ± 9%, N = 5, P = 0.2; TgCdCl₂: 15 ± 4%, N = 7, P < 0.0001). In addition, each manipulation could improve the ability of TgCRND8 neurons to sustain cholinergic firing (K-S tests, TgCRND8<sub>control</sub> v. TgCRND8<sub>AP5</sub>, P < 0.0005; TgCRND8<sub>control</sub> v. TgCRND8<sub>CdCl₂</sub>, P < 0.00001), consistent with the interpretation that the dysregulation of a calcium-sensitive conductance underlies impairments in the cholinergic excitation of TgCRND8 neurons.
A

WT

TgCRND8

Mean ACh-elicited firing frequency over time

P < 0.005

Mean ACh-elicited calcium increases

P < 0.05
Figure 17. Greater Ca\(^{2+}\) responses accompany acetylcholine-elicited firing in the TgCRND8.
Acetylcholine (ACh, 1 mM, 15 s) elicited spiking in 8/9 cells in WT and 6/7 in TgCRND8, with calcium increases that were significantly greater in the TgCRND8 cells as detected by the calcium-sensitive dye Oregon Green BAPTA-1 (OGB-1, 100 uM). Fig. 7A, Example of whole-cell membrane potential, top, and calcium increases, bottom, simultaneously recorded in response to bath application of ACh in WT, upper panel, and TgCRND8, lower panel. Note the large AHPs in the TgCRND8 trace (arrowhead). Fig. 7B, average number of spikes per seconds, top, and calcium responses, bottom, elicited by acetylcholine in the WT (blue, N=8) and TgCRND8 cells (red, N = 6).

4.4.4 Inhibition of SK channels improves cholinergic excitability of TgCRND8 neurons
Since the layer 6 TgCRND8 neurons show reduced ability to maintain peak firing, elevated calcium levels in response to acetylcholine, and enhanced apamin-sensitive AHPs, we sought to determine whether the inhibition of SK channels could help improve cholinergic excitability in layer 6 PFC of TgCRND8 mice. In the presence of apamin, an SK-antagonist, 1 mM acetylcholine elicited spiking in 8/12 (67%) cells and, under these conditions, higher frequency spiking could now be sustained to a significantly greater extent, leading to a rightward shift of the cumulative probability distribution of acetylcholine-elicited firing frequency in TgCRND8 neurons (Figure 18B, K-S test, P < 0.00001). These data strongly support the hypothesis that impairments in cholinergic excitability arise from enhanced activity of calcium-activated SK channels in layer 6 of the TgCRND8 mice.

Taken together, our findings demonstrate early disruption of cholinergic excitation of layer 6 PFC neurons in an animal model of AD, identify the mechanisms underlying this disruption, and use this information to restore the maintenance of peak cholinergic excitability in a critical component of attention circuitry.
4.5 Discussion

We have found that layer 6 pyramidal neurons of prefrontal attention circuitry are susceptible to disruptions of cellular excitability in the TgCRND8 brain. In particular, we show that these cells are unable to sustain cholinergic excitation to the same extent as non-transgenic littermate mice as a result of the excessive activation of calcium-activated hyperpolarizing conductances. Cholinergic excitation can be improved in layer 6 pyramidal cells of TgCRND8 mice by pharmacological blockade of SK channels, suggesting a novel target for the treatment of executive dysfunction in AD.

The TgCRND8 mouse model is a widely used model of disease that recapitulates several key neuropathological and cognitive features of AD (Chishti et al., 2001; Webster et al., 2014), including attention deficits (Romberg et al., 2013b). Since TgCRND8 mice harbour amyloid precursor protein (APP) mutations without disruptions to other genetic loci of AD vulnerability, namely the tau and presenilin genes (Selkoe, 2001; Goedert and Spillantini, 2006), attentional impairments appear related to the overexpression of APP, aberrant APP processing and/or the production of Aβ pathology. The disruption of the cholinergic excitation of PFC layer 6 neurons described here is a plausible proximate cause for the attention deficits observed in TgCRND8 mice. These animals display impairments on the 5-choice serial reaction time task (5-CSRTT, for review see Bari et al., 2008) (Romberg et al., 2013b), an attentional task.
that requires cholinergic modulation of the deep layer PFC neurons for optimal performance (Bailey et al., 2010; Guillem et al., 2011). The PFC constitutes an important hub for higher cognitive function (Duncan and Owen, 2000; Buckner et al., 2009; Cole et al., 2013; Sporns, 2014), and its neuromodulation exerts powerful influences on the brain as a whole. The acetylcholine released in the PFC during attention tasks (Parikh et al., 2007) robustly and directly excites the deep cortical layers (Kassam et al., 2008; Poorthuis et al., 2012; Proulx et al., 2014) in a manner consistent with the sustained firing necessary for attention and other executive functions (Funahashi, 2001; Hasselmo and Sarter, 2011; Ikkai and Curtis, 2011; Zhou et al., 2011). The cholinergic modulation of PFC layer 6 would be especially important in this regard given its dense corticothalamic feedback projections and local influence over the activity of the cortical column (Thomson, 2010; Olsen et al., 2012; Proulx et al., 2013; Kim et al., 2014).

The impairments in cholinergic excitability we document would be exacerbated by previously-described cholinergic changes that accompany the progression of AD. Such deficits include the neurodegeneration of the cholinergic cells of the basal forebrain (Davies and Maloney, 1976; Whitehouse et al., 1982), as well as a decrease in the enzymatic activity related to the synthesis and breakdown of acetylcholine (Davies and Maloney, 1976; Perry et al., 1978; Henke and Lang, 1983; Zubenko et al., 1989; Davis et al., 1999; Selkoe, 2001; Auld et al., 2002; DeKosky et al., 2002). In the current study, however, bath application of acetylcholine would circumvent potential differences in endogenous acetylcholine levels between the genotypes, indicating that significant differences in cholinergic responses are observed in the TgCRND8 neurons independently of the disruptions in acetylcholine levels that accompany disease.
progression. Furthermore, significant reductions in nicotinic receptor binding have been documented in the AD brain and this holds particularly true for the $\alpha 4\beta 2^*$ nicotinic receptor subtype (Nordberg et al., 1988; Whitehouse et al., 1988; Marutle et al., 1999; Nordberg, 2001; O'Brien et al., 2006; Kendziorra et al., 2010) that is highly expressed in L6 PFC (Wada et al., 1989; 1990; Kassam et al., 2008; Alves et al., 2010). There is also some evidence of disrupted functional coupling of excitatory muscarinic receptors to downstream cellular effectors (Jope et al., 1997; Jope, 1999). $\beta$-amyloid peptides can impair both muscarinic (Kelly et al., 1996) and nicotinic (Liu et al., 2001; Pettit et al., 2001) receptor function and indeed interfere with the cholinergic system as a whole (see Auld 2002 for review). It is striking that we found only modest reductions in TgCRND8 acetylcholine currents near resting membrane potential, and neither differences in muscarinic or nicotinic currents between the genotypes nor in the level of depolarization achieved. Instead, the greatest difference rested in the degree to which layer 6 cells could sustain firing in response to acetylcholine. The extent to which nicotinic and muscarinic receptors contribute to this threshold effect will be critical to elucidate in future work, particularly in view of the accompanying supra-threshold calcium differences that are reported here.

Cholinergic signaling can alter intracellular calcium levels in a multitude of ways. The subtype of nicotinic receptors that play a prominent role in the excitation of PFC layer 6 cells (Kassam et al., 2008; Tian et al., 2011; Poorthuis et al., 2012) are characterized by unusual calcium permeability (Fucile, 2004; Tapia et al., 2007). While the M1 muscarinic receptors contribute only modestly to the depolarization of PFC layer 6 cells (Tian et al., 2014), they can trigger the release of calcium from intracellular stores (Berridge and Irvine, 1984; Neher et al., 1988; Gulledge, 2005; Proulx et al., 2014). Disruptions in
intracellular calcium have been documented in several models of AD (Stutzmann, 2004; 2006; Wykes et al., 2012; Chakroborty, 2014). Potential contributors suggested to be perturbed in models of AD include calcium-permeable nicotinic receptors (Liu et al., 2001; Pettit et al., 2001), excitatory muscarinic receptors (Kelly et al., 1996), voltage-gated calcium channels (Wang and Mattson, 2014), calcium-induced calcium release (Stutzmann, 2004; 2006), and extra-sensitive NMDA receptors (Shah and Haylett, 2002; Goussakov et al., 2010; Romberg et al., 2012; Wang and Mattson, 2014). Here we find that layer 6 neurons in the TgCRND8 mice do not have aberrant calcium levels at baseline and show only moderate elevation in response to strong depolarizing stimuli and, indeed, to cholinergic stimulation below threshold. Instead, the combination of cholinergic stimuli and depolarization is required to bring about strongly elevated intracellular calcium levels. Our data indicate that aberrations in voltage-gated calcium channel and NMDA receptor function may contribute to this supra-threshold effect. It is interesting to note that calcium flux through both voltage-gated calcium channels and NMDA receptors can couple to SK channels (Marrion and Tavalin 1998; Wolfart and Roeper, 2002; Ngo-Anh et al., 2005; Giessel and Sabatini, 2010), which could account for the impairments in cholinergic firing of layer 6 TgCRND8 neurons that are documented in the current study. Yet, much additional work will be necessary to determine the cellular mechanisms underlying supra-threshold calcium responses in the TgCRND8 mice and its subsequent dysregulation of cholinergic firing and whether such an interaction is specific to the cholinergic system remains an open question. Nonetheless, it is clear that the cholinergic stimulation of layer 6 pyramidal cells of prefrontal cortex can be regarded as a highly relevant physiological stimulus essential to optimal attentional performance.
In the major corticothalamic layer, where modulation by acetylcholine is essential for optimal attentional function, we have found that there is impaired cholinergic excitability in a mouse model of AD. Further, we have found that blockade of the SK class of calcium-activated potassium ion channels significantly restores persistent cholinergic excitability in PFC of TgCRND8. Consistent with this finding, local PFC administration of an SK blocker has been shown to improve executive function (Brennan et al., 2008). Of note, the SK-mediated AHP is modifiable by experience (Disterhoft and Oh, 2006), a modulatory control that appears to be essential for learning. Yet, the dynamic control of its magnitude, and even its calcium sensitivity, is complex (Bildl et al., 2004; Kramár et al., 2004; Ren et al., 2006; Allen et al., 2007; Giessel and Sabatini, 2010; El-Hassar et al., 2014). Regulation of the SK-mediated AHP by BDNF points to a potential locus of disruption in AD and the TgCRND8 model which would be correlated with early cognitive decline (Peng et al., 2005; Francis et al., 2012). Understanding the regulation of persistent cholinergic excitation in PFC attention circuits is a key step in understanding normal control of executive function and its vulnerability to disruption in AD.
Chapter 5

5 General Discussion

5.1 Summary of Findings

The cholinergic modulation of the prefrontal cortex is essential to executive function. In this thesis, I have addressed the broad question of how acetylcholine mediates its actions on the pyramidal cells of the deep layers of PFC, both in the healthy and diseased brain. Particular emphasis was placed on investigating the role of calcium in shaping cholinergic excitability. It was found that cholinergic calcium mobilization exerts important regulatory control over cholinergic excitability by enhancing sub-threshold cholinergic excitation but constraining supra-threshold effects via the modulation of spike output. These distinct interactions were disrupted in two separate models of neuropsychiatric disease with documented cognitive dysfunction. Figure 19 summarizes the complex interactions of cholinergic excitation and calcium mobilization.

In Chapter 3, I have demonstrated that the muscarinic excitation of layer 5 pyramidal neurons of PFC is potentiated by ACh-induced Ca\(^{2+}\) release via the process of Na\(^+\)/Ca\(^{2+}\) exchange-mediated electrogenesis. Interestingly, I have found that this phenomenon emerges in young adulthood as agonist-induced Ca\(^{2+}\) release becomes more precisely timed. Furthermore, I have shown that the experience of early stress — which is a key contributor to psychiatric vulnerability — disrupts this developmental consolidation of muscarinic signaling, leading to the retention of the adolescent phenotype with potential implications for executive function. These findings are summarized in Figure 20.
In Chapter 4, I have shown that in a well-characterized animal model of Alzheimer’s disease, layer 6 pyramidal cells of PFC are unable to sustain cholinergic excitation to the same extent as their non-transgenic littermate controls, as a result of the excessive activation of calcium-activated hyperpolarizing conductances (summarized in Figure 21). I further report that cholinergic excitation can be improved in the cortex of TgCRND8 mice by pharmacological blockade of SK channels, suggesting a novel target for the treatment of executive dysfunction in AD.

Taken together, these constitute novel neuromodulatory mechanisms of prefrontal executive circuits. As deep layer pyramidal output neurons exert important influences on the cortical column (Olsen et al., 2012; Kim et al., 2014) and, by virtue of widespread output connectivity (Barbas, 2000; Fuster, 2001; Vertes, 2003; Gabbott et al., 2005; Buckner and Krienen, 2013), on the brain as a whole, it is likely that these mechanisms will have important ramifications for cognition and behavior.
Figure 19. Acetylcholine elicits intracellular calcium mobilization together with the modulation of cellular excitability via the nicotinic and muscarinic receptors. These two responses interact via multiple mechanisms, as summarized here. This thesis has demonstrated two distinct mechanisms whereby calcium can modulate cholinergic excitability in prefrontal circuits: via Na\(^+\)/Ca\(^{2+}\)-exchange-mediated electrogensis and via the activation of calcium-activated SK channels.
Figure 20. Early stress prevents the potentiation of muscarinic excitation by calcium release in adult prefrontal cortex. Summary of Chapter 3 findings.
Figure 21. Dysregulated intrinsic and cholinergic excitability of layer 6 PFC in 3-4 month old TgCRND8 mice as a result of enhanced contributions from calcium-activated SK channels. Summary of Chapter 4 findings. Cholinergic excitation is achieved via stimulation of both nicotinic and muscarinic receptors in these cells.
5.2 Cholinergic Calcium Signaling Modulates Neurons of Prefrontal Cortical Output Layers

A detailed understanding of cholinergic calcium signaling is currently lacking, although it is no doubt of utmost importance for understanding the cellular mechanisms that underlie executive function and dysfunction. This thesis has therefore contributed to filling this knowledge gap. It is clear that calcium is critical to cell function. Indeed, calcium is a ubiquitous and versatile second messenger (Berridge et al., 2000) that lies at the crux of multiple signalling pathways that together orchestrate neuronal circuit function underlying behaviour (Ghosh and Greenberg, 1995; Berridge, 1998; Berridge et al., 2000; Bootman et al., 2002; Berridge, 2014). Calcium has a well-established role in synaptic plasticity, learning and memory (Bliss and Collingridge, 1993; Berridge, 1998; Martin et al., 2000) and has the ability to exert long term changes by initiating gene transcription (Ghosh and Greenberg, 1995; Dolmetsch et al., 1998; Li et al., 1998; Flavell and Greenberg, 2008). It is becoming increasingly appreciated that intracellular calcium increases following Gqα-connected receptor activation also plays an important role in working memory (Dash et al., 2007). Not only does evidence suggest that persistent activity is calcium-dependent (Teramae and Fukai, 2005; Fransén et al., 2006; Gao and Goldman-Rakic, 2006; Zhang and Seguela, 2010), but the balanced activation of downstream, Gqα-activated calcium-dependent kinases and phosphatases in the prefrontal cortex—such as CaMKII, PKC and calcineurin—is required for optimal working memory function; more specifically, calcium-activated protein phosphatase activation might improve, and excessive calcium-activated protein kinase activity might impair, working memory (Zeng et al., 2001; Birnbaum, 2004; Runyan, 2005; Dash et al., 2007; Arnsten, 2009). How the activity of these Gqα-activated calcium binding proteins
modulate cholinergic signaling in the PFC and, ultimately, executive function and
dysfunction, remains a fascinating question for future study. Evaluating the involvement
of calcineurin, in particular, would be especially interesting, given that its prefrontal
expression is developmentally-regulated and subject to environmental disruption by
early stress (Proulx et al., 2014).

As one of the most important cellular second messengers to physiological
function, intracellular calcium is tightly regulated to allow for the precise and exquisite
spatiotemporal control of cellular signaling (Ghosh and Greenberg, 1995). Calcium can
enter the cell by means of membrane-bound ion channels, either voltage-gated or
ligand-gated, or following release from intracellular stores via activation of ryanodine
receptors or IP3 receptors located on the endoplasmic reticulum (Ghosh and
Greenberg, 1995; Berridge et al., 2000), an organelle that extends throughout the cell
and forms a continuous network from the soma to dendritic spines and presynaptic
terminals (Takei et al., 1992; Spacek and Harris, 1997; Verkhratsky, 2002; Baker et al.,
2013). The release of calcium from intracellular stores can be elicited by Gaq-protein
coupled receptor activation (Downes, 1982; Berridge and Irvine, 1989), such as in the
case of the muscarinic M1 receptor activation (Neher et al., 1988; Felder, 1995; Power
and Sah, 2002; Gulledge, 2005; Proulx et al., 2014), but also as a result of regenerative
calcium-induced calcium release, which further amplifies calcium signals by positive
feedback; indeed, both the IP3 receptors and the ryanodine receptors can be further
stimulated by calcium (Berridge, 2002; Bootman et al., 2002). Finally, store-operated
calcium entry is an additional mechanism whereby calcium store depletion triggers
calcium entry via a mechanism known as store-operated calcium entry (Smyth et al.,
2010). Thus, it is clear that calcium signals are dynamic and complex.
In the prefrontal cortex and hippocampus, cholinergic stimulation elicits pan-somatic and nuclear calcium signals (Power and Sah, 2002; Proulx et al., 2014) which result from the release of Ca\(^{2+}\) from IP\(_3\) sensitive stores following the activation the metabotropic M\(_1\) muscarinic acetylcholine receptors. These postsynaptic calcium responses have been characterized in layer 5 prefrontal output neurons (Gulledge, 2005; Proulx et al., 2014) but their role in modulating neurons of layer 6 prefrontal cortex, which constitutes the major corticothalamic output, is likely to prove more complex. While also subject to muscarinic modulation (Tian et al., 2011), these neurons are robustly excited by nicotinic receptors (Kassam et al., 2008; Tian et al., 2011; Poorthuis et al., 2012), which also flux calcium (Tapia et al., 2007; Kuryatov et al., 2008). The fractional calcium current fluxing through nicotinic receptors is estimated to range from 2-12\% (Rogers and Dani, 1995; Fucile et al., 2003; Fucile, 2004), the upper range of which is comparable to that of the NMDA receptors (Burnashev et al., 1995). Calcium signals induced by nicotinic receptor stimulation can be amplified by a functional interaction with voltage-gated calcium channels together with calcium-induced calcium release through ryanodine receptors (Rathouz and Berg, 1994; Sharma and Vijayaraghavan, 2001; Tsuneki:2000tg; Dajas-Bailador et al., 2002; Shen and Yakel, 2009). Nicotinic calcium signals are involved in the regulation of neurotransmitter release (Gray et al., 1996; Soliakov and Wonnacott, 1996; Kulak et al., 2001), synaptic plasticity (Mansvelder and McGehee, 2000; Ji et al., 2001) and gene transcription (Greenberg et al., 1986).

The direct postsynaptic effects of acetylcholine are also accompanied by the indirect stimulation of ionotropic glutamate receptors following the cholinergic excitation of glutamatergic presynaptic terminals (Lambe et al., 2003; Poorthuis et al., 2012). In
turn, both the AMPA and NMDA ionotropic glutamatergic receptor subtypes that putatively mediate these postsynaptic actions (Tian:2014uf; Giessel and Sabatini, 2010; Poorthuis et al., 2012) can flux calcium to varying degrees (Giessel and Sabatini, 2010), which may or may not be able to subsequently elicit calcium-induced calcium release (Emptage et al., 1999; Kovalchuk et al., 2000; Rose and Konnerth, 2001).

Thus, cholinergic signaling has the ability to generate complex calcium signals that can substantially modulate cellular function. Yet, many details pertaining to the underlying calcium dynamics and of their complex effector pathways await further study. As will be discussed next, this thesis has contributed to this aim to show mechanisms whereby calcium shapes cholinergic excitability in prefrontal circuits essential to executive function.

5.3 Calcium Shapes Cholinergic Excitability

I have shown in this thesis that intracellular calcium increases can both stimulate (Chapter 3) and restrain (Chapter 4) cholinergic excitability. In the first case, calcium release was shown to boost excitatory muscarinic currents near resting membrane potential. By contrast, in Chapter 4, it was shown that excessive calcium responses in the AD brain correlate with the excessive recruitment of the Ca$^{2+}$-activated potassium conductance, SK, leading to enhanced afterhyperpolarization potentials that prevent the sustained increase in firing frequency of layer 6 pyramidal neurons in response to acetylcholine. While these experiments were carried out in layer 5 and layer 6 PFC, respectively, these findings indicate that calcium may serve as a important regulator of cholinergic excitability: by boosting excitatory drive to threshold level and, subsequently, by restraining supra-threshold excitation.
5.3.1 Regulation of acetylcholine-elicited calcium release by cellular activity, developmental stage and environmental factors

This is the first study to report percentage of ACh-induced calcium release with respect to developmental stage or early stress experience (Proulx et al., 2014). At concentrations comparable to those seen at the site of release (Dani and Bertrand, 2007), acetylcholine (1 mM) elicits somatic calcium increases in ~50% of layer 5 pyramidal cells of prefrontal cortex (Proulx et al., 2014), a percentage which I found to be stable across developmental stages independently of the experience of early stress (Supplemental Figure 2). Interestingly, the ability to elicit dF/F_{ACh} in a majority of ACh_{NoCa2+} neurons by “priming” the cells with a depolarizing pulse—which would replenish intracellular Ca^{2+} stores by means of voltage-gated Ca^{2+} entry (Stutzmann et al., 2003)—together with the absence of morphological differences between ACh_{Ca2+} and ACh_{NoCa2+} neurons (Supplemental Figure 1, Supplemental Table 1) suggest that the propensity for ACh to elicit a somatic Ca^{2+} response does not reflect cell type but instead may reflect a state of Ca^{2+} store readiness that may be related to recent activity. Differences in the type of dF/F_{ACh} response elicited further raise the possibility that dF/F_{ACh} are dependent on local, activity-dependent changes in the cytosolic environment. Multi-peaked calcium responses, for instance, may reflect regenerative Ca^{2+} release, either due to the increased sensitivity of IP_{3} receptors by Ca^{2+} or to the recruitment of local ryanodine receptors (Berridge, 1998), or reflect differences in muscarinic receptor phosphorylation state (Kawabata et al., 1996). Overall, the further characterization of dF/F_{ACh}, its regulation, and its activity-dependence remain interesting questions for further investigation.
An additional intriguing finding was that the **timing** of calcium release can become more tightly regulated as the brain matures to correlate with the potentiation of muscarinic excitation by calcium. In the healthy control brain, the time to peak $dF/F_{ACh}$ measured in the adolescent neurons had significantly greater variance compared to that of adult neurons (Supplemental Figure 3). After early stress, however, the greater variance of the adolescent group was maintained into adulthood (Supplemental Figure 3). Interestingly, while the time to peak $dF/F_{ACh}$ varied across development and after early stress, the time to peak $I_{ACh}$ did not. Importantly, therefore, the timing and onset of the (basal) $I_{ACh}$ is not changed while the *supplemental* $I_{ACh}$, which appears to depend on the clearance of the $dF/F_{ACh}$, is absent (Figure 9, Supplemental Figure 2). These data therefore highlight that the basal and supplemental $I_{ACh}$ likely couple to divergent effectors, as do the $I_{ACh}$ and $dF/F_{ACh}$. Furthermore, these data suggest that the regulation of intracellular events leading to calcium release are important for optimal cholinergic function and ultimately, perhaps also for executive function. As such, the details of **effector coupling efficiency**, which may be affected by precise cellular localization, trafficking and interaction of receptors, second messengers and target effectors remain an important avenue for further study.

5.3.2  The potentiation of muscarinic excitation by agonist-induced calcium release in prefrontal layer 5 pyramidal cells

In layer 5 pyramidal cells of the PFC, cholinergic responses are mediated by $M_1$ muscarinic receptors. I have shown that muscarinic excitation can be potentiated by calcium release and that this phenomenon is subject to both developmental regulation and environmental disruption. I conclude that the ACh-induced calcium release
(dF/F_{ACh}) potentiates the excitatory cholinergic current (I_{ACh}) based on 3 lines of evidence:

1. ACh_{Ca} cells have significantly greater I_{ACh} than ACh_{No Ca} cells (cross-sectional observations);

2. I_{ACh} is significantly greater when a dF/F_{ACh} is elicited in ACh_{NoCa} cells by priming (matched observations);

3. I_{ACh} is significantly smaller when a dF/F_{ACh} is abolished in ACh_{Ca} cells by depletion of intracellular calcium stores (matched observations).

These findings were further strengthened by their replication in an independent cohort (control animals, both in young adults and mature adults).

The molecular identity of the ion channel mediating ACh-induced excitation is an area of active study (see Section 1.3.3.1.). For this reason, I have distinguished between the basal and supplemental I_{ACh} to specifically draw attention to the fact that there are different components to muscarinic excitation with potentially different mechanistic underpinnings. The study of the supplementary current does not necessitate knowledge of the ionic identity of the basal I_{ACh} and this further characterization of muscarinic excitation constitutes a novel contribution to the field. I report that both basal and supplementary currents are Na^{+} dependent near resting membrane potential. The combined observation that the presence of a somatic Ca^{2+} response can enhance I_{ACh}, that its clearance correlates with the supplemental excitation, and that the I_{ACh} is sensitive to pharmacological manipulation of Na^{+}/Ca^{2+} exchange has lead me to propose a role for Na^{+}/Ca^{2+} exchange-mediated electrogenesis in ACh-mediated excitation.
5.3.3 The modulation of calcium-dependent afterhyperpolarization potential in prefrontal layer 6 pyramidal cells

I have found that in the TgCRND8 brain, enhanced activity of the SK class of calcium-activated potassium ion channels can significantly impair cholinergic excitability in a cell population known to be essential to optimal attentional function. Acetylcholine can exert profound—but usually transient—inhibitory effects on cellular excitability as a result of perisomatic SK channel activation following M₁ receptor-mediated calcium release from intracellular stores (Gulledge, 2005; Power and Sah, 2008; Gulledge et al., 2009). More broadly, however, these calcium-dependent potassium channels have been shown in other neuronal populations to regulate neuronal excitability by contributing to the afterhyperpolarization potential (AHP) (Sah, 1996; Faber and Sah, 2003). In turn, an important body of work has demonstrated that the AHP is a key regulator of excitability that is modifiable by experience (Faber and Sah, 2003; Disterhoft and Oh, 2006). The AHP has been dissected into three components based on kinetics and pharmacological profile: the fast AHP (fAHP), which occurs within 10 ms post-spike and lasts less than 5 ms, the medium AHP (mAHP), which occurs within ~100 ms of the action potential or bursts of action potentials and lasts hundreds of milliseconds, and the slow AHP (sAHP), which occurs > 1 s post-burst and lasts for seconds (for review, see Faber and Sah 2003, Disterhoft and Oh 2006). It is thought that the fAHP is primarily mediated by BK channels, the mAHP by SK channels and the sAHP by the Na+/K+ ATPase (Sah, 1996; Faber and Sah, 2003; Bond et al., 2004; Villalobos et al., 2004; Gulledge et al., 2013). In Chapter 4, I systematically examined the AHP in order to investigate potential genotype differences that could underlie the impairments in intrinsic and cholinergic excitability that were observed. I found genotype differences to be most pronounced
between 100-500 ms post-burst (Figure 13, Supplemental Figure 8) and more importantly so when more action potentials were elicited, coinciding with enhanced calcium increases (Figure 14). Together, these findings pointed to greater SK contribution, which was confirmed by the near-normalization of AHPs in TgCRND8 with apamin (Figure 16). Given that the correlation of peak AHP and peak dF/F was not different between genotypes, I posit that the greater calcium increases observed in the TgCRND8 are likely the more proximal cause of enhanced mAHPs, as opposed to increased expression of SK channels or their aberrant modulation by amyloid-β peptides, although this may also be the case. Interestingly, we observed a supplemental, calcium-independent “early” AHP in the TgCRND8 upon pharmacological manipulation (Figure 16), possibly as a result of greater fast delayed-rectifier potassium currents, which have been reported in these mice (Wykes et al., 2012). I did not, however, observe changes in action potential amplitude or half-width at rheobase, which is at odds with this possibility.

The selective SK antagonist apamin was able to reverse deficits in cholinergic excitability in the slice (Figure 18) but, whether it will prove effective as a cognitive enhancer to improve attentional performance when delivered to the deep layers of PFC, *in vivo*, will prove an important question for future study. In particular, it will be interesting to probe the effect of apamin on performance on the 5-choice serial reaction time task following delivery to the deep layers of prefrontal cortex of both WT control and TgCRND8 mice.
5.4 Towards an Understanding of Key Biochemical Pathways Underlying Executive Function and their Susceptibility to Disruption in Neurological and Psychiatric Illness

5.4.1 Gαq-coupled receptor signaling pathway complexity and vulnerability in prefrontal cortex

The complexity of muscarinic signaling, and of Gαq-protein coupled receptors more generally, may help confer the flexibility that has been deemed essential to executive function at the neuronal level (Funahashi, 2001). On the other hand, it very well may be that it is the inter-dependence of these complex signaling pathways that renders them susceptible to disruption in neurological and psychiatric illness. The Gαq-coupled signaling pathway may be especially vulnerable in neuropsychiatric dysfunction for a few reasons. First, Gαq-coupled second messengers and associated calcium signals are essential to multiple cellular functions, as outlined above; second, these signaling pathways have the ability to affect excitatory/inhibitory balance, with important repercussions for network function (Lisman, 2012; Berridge, 2013; 2014) and third, they are developmentally regulated and subject to environmental disruption (Birnbaum, 2004; Arnsten, 2009; Proulx et al., 2014). Indeed, disruptions in the phospholipase C pathway, which is activated by Gαq-protein coupled receptors, have been implicated in the pathophysiology of mental illness (Arnsten, 2009; Berridge, 2014). One critical second messenger in this pathway is IP₃, being the key effector that triggers calcium release from internal stores. IP₃ receptors are widely distributed throughout the brain (Sharp et al., 1993; Furuichi and Mikoshiba, 1995) and their regulation by calcium is complex (Bootman and Lipp, 1999). I have presented evidence that the experience of early stress dysregulates Gαq-coupled muscarinic signaling and alters the expression of
a network of proteins that structurally and/or functionally interact with the IP$_3$ receptor (Delmas et al., 2004; Patterson et al., 2004; Berridge, 2006), including calcineurin (Cameron et al., 1995; Patterson et al., 2004), CD44 (Singleton and Bourguignon, 2004; Bourguignon and Jin 1995), calpain 8 (Pattini et al., 2003; Patterson et al., 2004), ANKFY1 (Bourguignon and Jin 1995), AKAP1 (Berridge et al., 2003; Delmas et al., 2004) and the L-type Ca$^{2+}$ channel β2 subunit (Power and Sah, 2005), which itself has been implicated in several psychiatric disorders including bipolar disorder, schizophrenia and major depression (Ferreira et al., 2008; Sklar et al., 2008; Green et al., 2010a; Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013). Other effectors in this pathway that have also been implicated in mental illness include protein kinase C (PKC) (Manji and Lenox, 1999; Birnbaum, 2004; Zarate and Manji, 2009), CaMKII (Robison, 2014), neuronal calcium sensor-1 (NCS-1) (Koh et al., 2003; Schlecker et al., 2006), neurogranin (NRGN) (Ruano et al., 2008; Lisman, 2012) and diacylglycerol kinase (Baum et al., 2008). How these proteins contribute to the cholinergic modulation of the prefrontal cortex will prove an interesting question for future study. Indeed, whether and how genetic and/or pharmacological manipulation of such G$_{q}$- and calcium-signaling related proteins affects cholinergic excitation, firing and intracellular calcium mobilization may provide valuable insight into the mechanisms of cholinergic neuromodulation of prefrontal cortex, with important implications for the understanding of the cellular mechanisms of executive function and dysfunction.
5.4.2 Dyshomeostasis of calcium signaling in neurological and psychiatric disorders

Disruptions in intracellular calcium preceding amyloid-β plaque deposition have been documented in several models of Alzheimer’s disease, prompting the interesting proposal that AD may represent a “calciumopathy” (Stutzmann, 2007). Under this paradigm, calcium anomalies would arise very early in disease progression, surreptitiously disrupting cellular function until normal physiological function can no longer be sustained (Mattson et al., 2000; LaFerla, 2002; Stutzmann, 2005; 2007; Stutzmann and Mattson, 2011; Chakroborty and Stutzmann, 2014). Aberrations in calcium signaling have also been documented in aging (Kumar et al., 2009), schizophrenia (Lidow, 2003; Giegling et al., 2010) and bipolar disorder (Warsh et al., 2004; Machado-Vieira et al., 2011). Indeed, since calcium signaling lies at the crux of innumerable physiological processes (Ghosh and Greenberg, 1995; Berridge, 1998; Berridge et al., 2000; Bootman et al., 2002; Berridge, 2014) and its regulation can be vulnerable to environmental disruption (Birnbaum, 2004; Arnsten, 2009; Proulx et al., 2014), it is understandable that the cumulative effect of the consequent disruption of multiple effector systems could ultimately lead to disease. The extent to which the specific disruption of these intricate signaling cascades in the cholinergic system underlies the executive dysfunctions that are prevalent in several psychiatric and neurological illness remains an open question.

5.5 Conclusion

The prefrontal cortex is a critical node in widespread and dynamic brain networks that sustain higher cognitive function in health and that perpetuate executive dysfunction in neurological and psychiatric illness. The cholinergic neuromodulation of the prefrontal
cortex is essential to executive function, where acetylcholine exerts its actions via the nicotinic and muscarinic receptors. This thesis has detailed cellular mechanisms whereby calcium shapes cholinergic excitability in prefrontal circuits essential to executive function and elucidated how this interaction is disrupted in models of psychiatric vulnerability and of Alzheimer’s disease. Future work will further explore the nature of the interaction of nicotinic and muscarinic receptors as well as the role of key \( \text{G}_{\alpha q} \)- and calcium signaling-related genes in mediating the cholinergic modulation of prefrontal cortex, both in health and disease.
References


Baum AE et al. (2008) A genome-wide association study implicates diacylglycerol kinase eta (DGKH) and several other genes in the etiology of bipolar disorder. Molecular Psychiatry 13:197–207.


Brown DA, Hughes SA, Marsh SJ, Tinker A (2007a) Regulation of M(Kv7.2/7.3) channels in neurons by PIP(2) and products of PIP(2) hydrolysis: significance for receptor-mediated inhibition. The Journal of Physiology 582:917–925.


Green EK et al. (2010a) The bipolar disorder risk allele at CACNA1C also confers risk of recurrent major depression and of schizophrenia. Molecular Psychiatry 15:1016–1022.


Koh PO, Undie AS, Kabbani N, Levenson R, Goldman-Rakic PS, Lidow MS (2003) Up-regulation of neuronal calcium sensor-1 (NCS-1) in the prefrontal cortex of


Mirza NR, Bright JL (2001) Nicotine-induced enhancements in the five-choice serial reaction time task in rats are strain-dependent. Psychopharmacology 154:8–12.


O'Brien JT, Colloby SJ, Pakrasi S, Perry EK, Pimlott SL, Wyper DJ, McKeith IG, Williams ED (2006) α4β2 nicotinic receptor status in Alzheimer's disease using 123I-


Wang Y, Mattson MP (2014) L-type Ca2+ currents at CA1 synapses, but not CA3 or dentate granule neuron synapses, are increased in 3xTgAD mice in an age-dependent manner. Neurobiology of Aging 35:88–95.


Appendices

6 Supplementary Materials

6.1 Supplementary Methods

6.1.1 Morphological Reconstruction

Morphological reconstruction of a subset of neurons was achieved by scanning a series of 512 X 512 frames in the x-y plane along 1 µm thick z-stack sections and collecting red channel emission. Captured image stacks were subsequently stitched together using Neurolucida software (MicroBrightField Inc.) and neurons were traced and analyzed using Neurolucida AutoNeuron in the interactive mode. Sholl analyses were obtained by measuring the number of dendritic crossings at concentric spheres of increasing diameter (25 µm increments).

6.1.2 Quantitative PCR (qPCR)*

*These experiments were carried out by Dr. Deepika Suri under the supervision of Dr. Vidita Vaidya at the Tata Institute of Fundamental Research in Mumbai, India.

We assessed the developmental expression of genes involved in Ca\textsuperscript{2+} signaling using quantitative PCR (qPCR). Control and ES animals were killed by decapitation and the PFC dissected in ice cold PBS. RNA was extracted from tissue samples using Trizol reagent (Sigma, USA) and 2 µg from each sample was reverse-transcribed (High capacity cDNA reverse transcription kit, Applied Biosystems). The synthesized cDNA was subjected to qPCR with primers specific to the genes of interest, and data analyzed using the ∆∆Ct method described previously (Bookout, 2003) with normalization to the endogenous housekeeping gene hypoxanthine-guanine phosphoribosyltransferase.
(Hprt). Results were compared to age-matched controls to examine the influence of early stress on gene expression within a particular age (Figure 5), or to the P21 control group (Supplemental Table 2) to assess the influence of early stress on the developmental profile of gene expression. Results were expressed as fold change ± SEM.
6.2 Supplementary Figures

**Supplemental Figure 1.** ACh$_{Ca2+}$ and ACh$_{No Ca2+}$ neurons are similar morphologically. A Z-projection of a serially imaged neuron labelled with Alexa-594, above, and reconstructed with Neuro lucida, bottom. B Apical sholl analysis reveals no differences in dendritic length and complexity between the two groups of neurons. Additional measurements and comparisons are presented in Supplemental Table 1 and methods are outlined in Supplemental Methods.
Supplemental Figure 2. Developmental emergence of the potentiation of I$_{ACh}$ by Ca$^{2+}$ release is absent following early stress. Average dF/F$_{ACh}$ and I$_{ACh}$ traces in ACh$_{Ca2+}$ and ACh$_{NoCa2+}$ neurons in control and ES animals across development. In this independent replication in young adults (P60-100; top center panel), we observed a dF/F$_{ACh}$ in 11/28 (39%) of neurons and found that peak I$_{ACh}$ were significantly greater in cells where somatic Ca$^{2+}$ release could be detected (I$_{ACh-Ca2+}$: $-76 \pm 8$ pA, $n = 11$; I$_{ACh-No Ca2+}$: $-50 \pm 5$ pA, $n = 17$; unpaired t-test, $p = 0.005$). In the mature adult controls (P130-175, top right panel), a potentiation was also observed (I$_{ACh-Ca2+}$: $-65 \pm 10$ pA, $n = 10$; I$_{ACh-No Ca2+}$: $-38 \pm 6$ pA, $n = 6$; unpaired t-test, $p = 0.08$). By contrast, in the adolescent controls (P30-45, top left panel) we observed no potentiation of muscarinic excitation (I$_{ACh-Ca2+}$: $-55 \pm 5$ pA, $n = 16$; I$_{ACh-No Ca2+}$: $-55 \pm 6$ pA, $n = 8$; unpaired t-test, $p = 0.9$). In the early stress brains (lower panel), there were no effects of dF/F$_{ACh}$ on I$_{ACh}$ in the adolescents (I$_{ACh-Ca2+}$: $-67 \pm 9$ pA, $n = 10$; I$_{ACh-No Ca2+}$: $-53 \pm 10$ pA, $n = 5$; unpaired t-test, $p = 0.4$), young adults (I$_{ACh-Ca2+}$: $-61 \pm 9$ pA, $n = 10$; I$_{ACh-No Ca2+}$: $-62 \pm 10$ pA, $n = 8$; unpaired t-test, $p = 0.9$), nor adults (I$_{ACh-Ca2+}$: $-45 \pm 8$ pA, $n = 17$; I$_{ACh-No Ca2+}$: $-55 \pm 8$ pA, $n = 11$; unpaired t-test, $p = 0.5$). There is a suggestion of developmental variability in the percentage of control neurons exhibiting ACh-induced calcium release: adolescent (16/24 cells, or 67%), young adult (11/28 cells, or 39%) and mature adult (10/16 cells, or 63%); however, these proportions appear to fall within the same distribution by chi-square analysis ($X^2_{(df:2)} = 4.5$, $P = 0.1$). Furthermore, at each developmental stage, there were also no significant differences in the proportion of ACh-elicited Ca$^{2+}$ responders between control animals and those that had undergone the experience of early stress: adolescent (Fisher’s exact test, $P = 1.0$), young adult (Fisher’s exact test, =0.4), and mature adult (Fisher’s exact test, $P=1.0$).
A

**Average dF/F**

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<th>Adolescent</th>
<th>Young Adult</th>
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B

Control

![Graph](image)

ES

![Graph](image)

10 s

25% dF/F

C

**P < 0.05**

Adolescent

Control

![Box plot](image)

ES

![Box plot](image)

Time of peak dF/F (s)

**P < 0.1**

Adult

Control

![Box plot](image)

ES

![Box plot](image)

Time of peak dF/F (s)
Supplemental Figure 3. The developmental consolidation of the Ca\(^{2+}\) potentiation of \(I_{ACh}\) occurs as dF/F\(_{ACh}\) responses become more precisely timed in young adulthood and fails to occur following early stress (ES). A, Average dF/F\(_{ACh}\) responses in adolescent, young adult and adult brains of control and early stress (ES) animals. Note that a broader and noisier average is observed in control adolescent and across all ages of early stress animals, in contrast with the more coherent single peak averages observed in adult controls. This reflects differences in the timing of dF/F\(_{ACh}\), as shown in B with the overlap of individual dF/F\(_{ACh}\). Box plots of time of peak dF/F\(_{ACh}\) are shown in C, where the boundaries of the box depict the interquartile difference and whiskers represent the distribution spread. There was considerably more variability in the timing of dF/F\(_{ACh}\) of the adolescent brain compared to that of adult controls (F-test, \(F_{15,19} = 2.69, p = 0.04\)) but the variances were not different in adolescent and adult ES animals (\(F_{9,26} = 1.61, p = 0.3\)). The time of peak dF/F\(_{ACh}\) in adolescence and adulthood was significantly different in early stress brains compared to controls (Two-way ANOVA, significant ES effect, \(F_{1,69} = 8.45, p = 0.005\)). There were no other differences in the peak (two-way ANOVA, \(F_{1,70} = 0.05, p = 0.8\)), rise time (two-way ANOVA, \(F_{1,70} = 0.29, p = 0.6\)), half-width (two-way ANOVA, \(F_{1,70} = 1.63, p = 0.2\)), nor decay time (two-way ANOVA, \(F_{1,70} = 0.00, p = 0.9\)) of dF/F\(_{ACh}\) between groups in adolescent and adult brains. The time to peak \(I_{ACh}\) did not vary between groups neither in adolescence nor adulthood (Adolescent\(_{Control}\): 31 ± 2 s, N = 25; Adolescent\(_{ES}\): 37 ± 2 s, N = 15; Adult\(_{Control}\): 34 ± 1 s, N = 44, Adult\(_{ES}\): 34 ± 1 s, N = 46; \(F_{3,126} = 1.93, P = 0.13\); Tukey’s multiple comparison post-hoc test reveals no further differences).
Supplemental Figure 4. Early stress (ES) produces changes in the expression of genes involved in Ga\(_q\)-coupled receptor signaling pathways in both adolescent and adult brain. Functional analysis by RT-PCR reveal gene expression differences plotted as fold change with respect to age-matched controls in adolescent and adult brain. *P < 0.1, **P < 0.05, ***P < 0.001.
Supplemental Figure 5. dF/F_{ACh} yields a supplementary I_{ACh} coinciding with dF/F_{ACh} clearance. 

A The average dF/F_{ACh} and I_{ACh} traces (green and blue traces, respectively) from ACh_{Ca^{2+}} neurons is contrasted with the dF/F_{ACh} and I_{ACh} averages from cells in ACh_{NoCa^{2+}} cells (black traces). A supplemental I_{ACh} is observed in ACh_{Ca^{2+}} neurons that coincides with the decay of the dF/F_{ACh}, which is indicative of Ca^{2+} clearance. Response onset of the dF/F_{ACh} and I_{ACh} traces were similar (B, 10% onset, unpaired t-test, p = 0.3), indicating that the I_{ACh} and dF/F_{ACh} muscarinic responses were initiated simultaneously, but the 10-90 rise time of the I_{ACh} occurs with a significant delay (C, unpaired t-test, p < 0.0001) such that peak I_{ACh} in ACh_{Ca^{2+}} neurons coincides with Ca^{2+} clearance. D Sample dF/F_{ACh} and I_{ACh} traces in 3 different cells highlight that the peak I_{ACh} coincides with Ca^{2+} clearance (shaded in gray). This observed combination of increased dF/F_{ACh} together with increased I_{ACh} would be consistent with a contribution of electrogenic exchangers to PFC cholinergic modulation.
Supplemental Figure 6. Ion substitution experiments reveal that dF/FACCh clearance is Na+- and K+-dependent and that IACh is Na+-dependent. A Eighty percent Na+ substitution (see Supplemental Methods) slowed dF/FACCh responses and abolished IACh (IACh: $-82 \pm 12$ pA; IACh (low Na+): $-7 \pm 2$ pA; matched-pair t test, $p < 0.0001$, $n = 12$). B Average dF/FACCh responses under standard condition (black trace, $n = 12$), Na+ substitution (blue, $n = 12$) and dual Na+ and K+ substitution (green, $n = 9$) reveal that dF/FACCh clearance was most sensitive to dual Na+ and K+ substitution. Na+ substitution prolonged the dF/FACCh response, lengthening the half-width of the dF/FACCh (standard conditions: $5.3 \pm 1$ s; Na+ substitution: $8.2 \pm 1$ s, matched-pair t-test, $p = 0.05$, $n = 12$) and slowing decay times (standard conditions: $6.4 \pm 1$ s; Na+ substitution: $9.1 \pm 2$ s, matched-pair t-test, $p = 0.09$, $n = 12$). This effect was greater with dual Na+ and K+ substitution: the half-width of the dF/FACCh was significantly broader (Na+/K+ substitution: 12 ± 2 s, $n = 9$, unpaired t-test, $p = 0.001$) and decay time significantly slower (Na+/K+ substitution: 10 ± 1 s, $n = 9$, unpaired t-test, $p = 0.02$). Baseline Ca2+ level were not appreciably altered, as approximated by the G/R fluorescence ratio, in Na+ substitution (Before: 0.3 ± 0.06, After: 0.3 ± 0.07, $n = 12$, matched pair t-test, $p = 0.2$) or dual Na+ and K+ substitution (Before: 0.14 ± 0.01, After: 0.15 ± 0.01, $n = 9$, matched pair t-test, $p = 0.16$). These observations further suggested that clearance of Ca2+ by electrogenic Na+/Ca2+ exchange contributes to IACh following agonist-induced Ca2+ release in PFC, and that members of the NCK and/or NCKX family may be involved.
Supplemental Figure 7. Pharmacological inhibition of Na⁺/Ca²⁺ exchange reduces $I_{\text{ACh}}$. A, B
The NCX and NCKX inhibitor KB-R7943 (50 µM, 5 min) (Altimimi 2012), reduced the $I_{\text{ACh}}$ by an average of 34 ± 10 % (Before: $-98 ± 21$ pA, KBR: $-71 ± 28$ pA; matched-pair t-test, $p = 0.05$, $n = 5$).

Supplemental Figure 8. Differences in post-burst AHP are most pronounced between 100-500 ms post-burst. Post-burst amplitude at 25 ms, 50 ms, 100 ms, 250 ms, 500 ms and 1000 ms post-burst. P values of the genotype difference revealed by two-way ANOVA are shown. Asterisk denote significant difference as revealed by Bonferroni post-hoc test.
Supplemental Figure 9. Effect of apamin (200 nM) in WT controls. Post-burst amplitude at 25 ms, 50 ms, 100 ms, 250 ms, 500 ms and 1000 ms post-burst in control conditions (blue) vs. in the presence of apamin (black). Following bursts of 1-32 action potentials at 50 Hz, an apamin-sensitive component is observed from ~50 ms to 100 ms post-burst. Beyond 100 ms, however, the AHP is apamin-insensitive in WT. P values of the treatment effect revealed by two-way ANOVA are shown. Asterisk denote significant difference as revealed by Bonferroni post-hoc test.
6.3 Supplementary Tables

**Supplemental Table 1.** Morphological properties of ACh\textsubscript{Ca2+} and ACh\textsubscript{NoCa2+} pyramidal cells.

<table>
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<tr>
<th></th>
<th>Somatic Ca\textsuperscript{2+}\textsubscript{ACh} (N=12)</th>
<th>No somatic Ca\textsuperscript{2+}\textsubscript{ACh} (N =8)</th>
<th>(P) Value</th>
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<tbody>
<tr>
<td>Soma surface area ((\mu m))</td>
<td>1010 ± 170</td>
<td>1040 ± 180</td>
<td>NS</td>
</tr>
<tr>
<td>Soma volume ((\mu m^3))</td>
<td>2600 ± 680</td>
<td>2120 ± 310</td>
<td>NS</td>
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<tr>
<td>No. apical ends (N)</td>
<td>18 ± 2</td>
<td>18 ± 2</td>
<td>NS</td>
</tr>
<tr>
<td>Total apical dendritic length ((\mu m))</td>
<td>2320 ± 320</td>
<td>1950 ± 260</td>
<td>NS</td>
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<tr>
<td>Total apical dendritic surface area ((\mu m^2))</td>
<td>9420 ± 1360</td>
<td>7940 ± 1070</td>
<td>NS</td>
</tr>
<tr>
<td>Total apical dendritic volume ((\mu m^3))</td>
<td>3260 ± 540</td>
<td>2740 ± 390</td>
<td>NS</td>
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</table>

**Supplemental Table 2.** Interaction of development and the experience of early stress in validated gene expression. Data reflects fold change with respect to gene expression in young control animals (P21).

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<tr>
<th>Gene</th>
<th>Adolescence C</th>
<th>Adolescence ES</th>
<th>Adulthood C</th>
<th>Adulthood ES</th>
<th>Age P Value</th>
<th>Early Life P Value</th>
<th>Interaction P Value</th>
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<td>Akap1</td>
<td>1±0.17</td>
<td>1.42±0.06</td>
<td>0.39±0.03</td>
<td>0.29±0.06</td>
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<td>Ankfy1</td>
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<td>0.45±0.06</td>
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<td>Cancb2</td>
<td>1±0.14</td>
<td>0.87±0.05</td>
<td>0.32±0.01</td>
<td>0.41±0.02</td>
<td>0.0001</td>
<td>0.5</td>
<td>0.03</td>
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<tr>
<td>Capn8</td>
<td>1±0.18</td>
<td>0.61±0.07</td>
<td>3.51±1.74</td>
<td>2.90±1.92</td>
<td>0.002</td>
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<td>Cd44</td>
<td>1±0.19</td>
<td>2.68±0.79</td>
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<td>Cleca6</td>
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<tr>
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<td>0.17±0.02</td>
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<td>0.65</td>
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<td>0.22±0.06</td>
<td>0.0001</td>
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</table>