Muscarinic attenuation of mnemonic rule representation in macaque dorsolateral prefrontal cortex during a pro- and anti-saccade task

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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MUSCARINIC ATTENUATION OF MNEMONIC RULE REPRESENTATION IN MACAQUE DORSOLATERAL PREFRONTAL CORTEX DURING A PRO- AND ANTI-SACCADE TASK

(Thesis format: Monograph)

by

Alex J. Major

Graduate Program in Neuroscience

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

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Abstract

Maintenance of context is necessary for execution of appropriate responses to diverse environmental stimuli. The dorsolateral prefrontal cortex (DLPFC) plays a pivotal role in executive function, including working memory and representation of abstract rules, and is modulated by the ascending cholinergic system through nicotinic and muscarinic receptors. Muscarinic receptors’ effect on local primate DLPFC neural activity in vivo during cognitive tasks remains poorly understood. Here we examined the effects of muscarinic receptor blockade on rule-related activity in the macaque prefrontal cortex by combining iontophoretic application of the general muscarinic receptor antagonist scopolamine with single-unit recordings while monkeys performed a rule-guided saccade task. We found that scopolamine reduced overall neuronal firing rate and impaired rule discriminability of task-selective cells. Saccade and visual direction selectivity measures were also reduced by muscarinic antagonism. These results demonstrate that blockade of muscarinic receptors in DLPFC creates deficits in working memory representation of rules in primates.

Keywords
Muscarinic, acetylcholine, prefrontal cortex, dorsolateral prefrontal cortex, working memory, iontophoresis, single neuron electrophysiology, antisaccade, macaque, rule
Co-Authorship

Alex J. Major, Susheel Vijayraghavan, Kevin Johnston, and Stefan Everling

As primary author of this thesis, Alex J. Major was responsible for designing the experiments, data collection, analysis, interpretation of results, and manuscript composition. Susheel Vijayraghavan offered expert guidance and also assisted in all aspects of this project mentioned above. Kevin Johnston offered support in data analysis and performed surgical procedures. Stefan Everling was the principal investigator for this project, and oversaw the experimental design, analysis, interpretation of results, manuscript composition, and surgical procedures.
Acknowledgements

I am deeply grateful to my colleague and friend, Dr. Susheel Vijayraghavan, for teaching me the art of single-unit electrophysiological recording and iontophoresic electrode construction. His leadership and seemingly endless stream of knowledge has set the foundation for my academic future.

Many thanks to Dr. Stefan Everling for allowing me to take part in his cutting-edge research into the cognitive control of eye movements. His mentorship has directed every aspect of this project and its timely execution. Dr. Kevin Johnston and Dr. Sahand Babapoor-Farrokhran were valued sources of advice for both data analysis and methodological techniques. I also appreciate the valuable insights into analysis and future directions provided by Dr. Brian Corneil and Dr. Stan Leung.

I would also like to thank Nicole Hague, Darren Pitre, Katherine Green, and Ashley Kirley for their expert management and care of the animal facility. Lastly, I extend a special thanks to all members of the Everling, Johnston, and Corneil labs for being a constant source of support and friendship.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACC</td>
<td>anterior cingulate cortex</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>acetylcholinesterase</td>
</tr>
<tr>
<td>AUROC</td>
<td>area under the receiver operating characteristic</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>ChAT</td>
<td>choline acetyltransferase</td>
</tr>
<tr>
<td>D1R</td>
<td>dopamine receptor D1</td>
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<tr>
<td>D2R</td>
<td>dopamine receptor D2</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DISC1</td>
<td>disrupted in schizophrenia 1 protein</td>
</tr>
<tr>
<td>DLPFC</td>
<td>dorsolateral prefrontal cortex</td>
</tr>
<tr>
<td>DMS</td>
<td>delayed match-to-sample</td>
</tr>
<tr>
<td>FEF</td>
<td>frontal eye fields</td>
</tr>
<tr>
<td>FR</td>
<td>firing rate</td>
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<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GIRK</td>
<td>G protein-coupled inwardly-rectifying potassium channel</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>HCN</td>
<td>hyperpolarization-activated cyclic nucleotide-gated channel</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol triphosphate</td>
</tr>
<tr>
<td>KCNQ</td>
<td>KQT-related voltage-gated delayed rectifier potassium channel</td>
</tr>
<tr>
<td>LFP</td>
<td>local field potential</td>
</tr>
<tr>
<td>M1R</td>
<td>muscarinic acetylcholine receptor M1</td>
</tr>
<tr>
<td>M2R</td>
<td>muscarinic acetylcholine receptor M2</td>
</tr>
</tbody>
</table>
NBM  nucleus basalis of Meynert
NMDA  N-methyl-D-aspartate
ODR  oculomotor delayed response
PDE4A  cAMP-specific 3',5'-cyclic phosphodiesterase 4A
PDE4B  cAMP-specific 3',5'-cyclic phosphodiesterase 4B
PFC  prefrontal cortex
PIP2  phosphatidylinositol 4,5-bisphosphate
PIP5K2A  phosphatidylinositol-5-phosphate 5-kinase type 2-alpha
PKC  protein kinase C
PLC  phospholipase C
PQCA  1-(4-cyano-4-(pyridine-2-yl)piperidin-1-yl)methyl-4-oxo-4-
      H-quinolizine-3-carboxylic acid
RGS4  regulator of G protein signaling 4
SC  superior colliculus
SRT  saccadic reaction time
V1  primary visual area
WM  working memory
Chapter 1 – Introduction

Many archaic, automatic functions of the human brain do not require conscious thought to be accomplished. For example, looking towards a loud noise or flash of light is a simple automatic response involving direct connections between our primary sensory systems and motor brain regions. Through evolution of the primate brain, human behaviour is not limited to these elementary functions and is able to integrate sensory inputs, prioritize goals, and coordinate appropriate and complex responses.

The prefrontal cortex (PFC) is often described as the locus of this ‘top-down’ influence. Knowledge from the past or related to a specific goal is used to influence ‘lower’ brain regions (e.g., sensory areas and motor outputs). For example, PFC has descending projections to extrastriate cortex (Webster et al., 1994) that can influence attention during visual discrimination tasks. Barceló et al. (2000) observed that patients with prefrontal lesions had aberrant event-related potentials in extrastriate cortex and an associated decrease in visual discrimination performance, suggesting that removal of prefrontal inputs can disrupt efficient processing of visual information. PFC is thus regarded as the head of the cognitive hierarchy, influencing other brain areas to perform in a contextually appropriate manner (Miller and Cohen, 2001; Fuster, 2008).

Neuropsychological Disambiguation of PFC Function

The human brain has proven very resilient to injury, showcasing an ability to compensate in response to lesions and even repurpose cortical tissue (Glees and Cole, 1949; Thaler et al., 2011). Studying the effects of lesions can provide foundational clues to the underlying functionality. For example, the topography of area V1 was initially mapped by observing
how V1 scotomas produced blindness to specific portions of the visual field (Henschen, 1892; Lister and Holmes, 1916).

The most well-known case of damage to PFC, and perhaps the entire brain, is that of railroad construction foreman Phineas Gage in 1848. As he was preparing an explosive charge to clear some rock, the assembly ignited prematurely, rocketing a three-foot, pointed iron bar through the left side of his face and out the top of his head. Large volumes of grey and white matter in the left frontal lobe were damaged, but to everyone’s surprise, he was able to stand up, talk, and stay conscious during the 1.2 km trip to the local doctor’s office. Phineas retained all of his primary sensory and motor functions, but his personality and intellectual faculties did suffer, leading many of his friends and close relatives to claim he was “no longer Gage” (Harlow, 1868). The fame of the accident influenced scientific thought of the time and spurred discussion over the cerebral localization of certain cognitive functions.

More recently, lesions in humans to a specific part of the frontal lobe have illuminated its role in working memory (WM) functionality. Patients with lesions to the dorsolateral PFC (DLPFC) consistently perform significantly poorer in delayed spatial WM tasks (Ptito et al., 1995; D'Esposito and Postle, 1999). Virtual lesions using repetitive transcranial magnetic stimulation elicit similar deficits on WM (Hamidi et al., 2008), possibly due to disruption of local field potential (LFP; Hamidi et al., 2009).

Observations of human lesions patients are of course limited to accidents or conditions requiring surgical ablation (e.g., epileptic patients unresponsive to pharmacological treatment). As one of the most common non-human primate models in the neurosciences, studies of the macaque brain have led to many conclusions about human cognition and neurophysiology. Recent studies have bolstered the use of this
model, as it bears considerably more homology with the human brain (Hutchison and Everling, 2012) than others, such as the rodent brain (Preuss, 1995).

The macaque brain has comparable patterns of global anatomical connectivity (Parker et al., 2002; Croxson et al., 2005; Kelly et al., 2010), functional organization (Rees et al., 2000; Koyama et al., 2004; Petrides et al., 2005; Nakahara et al., 2007), resting-state networks (Hutchison et al., 2011; Hutchison et al., 2012), and proportion of neocortex (Passingham, 2009). More focally, prefrontal cortices of macaques and humans share connectivity (Croxson et al., 2005) and cytoarchitectural patterns (Petrides and Pandya, 1999, Petrides and Pandya, 2002; Ongur et al., 2003).

Lesion studies in macaques have thus offered an exceptional substitute. Ablation of monkey DLPFC, particularly around the principal sulcus, causes spatial WM deficits similar to human lesions (Jacobsen and Nissen, 1937; Butters and Pandya, 1969). In a study by Levy and Goldman-Rakic (1999), bilateral DLPFC and dorsomedial PFC were lesioned. Removal of dorsomedial PFC did not impair spatial or nonspatial WM performance. DLPFC ablation, focused around the principal sulcus, produced deficits in spatial WM, but left nonspatial WM performance intact.

**Working Memory**

Use of the macaque model over the past few decades has allowed investigators to substantiate the role of DLPFC in higher-order processing and WM function, and to begin the untangling of responsible cellular mechanisms.

For example, Kubota and Niki (1971) observed that as monkeys performed a WM task requiring maintenance of task set over a delay period, a subset of neurons around the principal sulcus of DLPFC fired preferentially during the delay period. A great number of
publications followed, reaffirming the existence of these DLPFC ‘delay cells’, which fire in correspondence to maintenance of task information in WM (Fig. 1A; Fuster and Alexander, 1971; Fuster, 1973; Niki, 1974b; Niki, 1974c; Niki, 1974a; Niki and Watanabe, 1976; Fuster et al., 1982; Kojima and Goldman-Rakic, 1982; Kojima and Goldman-Rakic, 1984; Batuev et al., 1985). Delay cells of the DLPFC are unique due to their ability to fire in the absence of visual stimuli (Funahashi et al., 1989) and despite introduction of various distractors (Miller et al., 1996; Everling et al., 2002; Jacob and Nieder, 2014). This is in contrast to other associational areas such as the parietal cortex, which produces stronger responses to distractors (Suzuki and Gottlieb, 2013).

The oculomotor delayed response (ODR) task has been prominently utilized for detailing the function of DLPFC neurons. This paradigm has revealed that DLPFC delay cells do not simply fire during the delay period, but they only display delay firing when the trial cue was localized to a particular spatial location in the visual field, termed its ‘memory field’ (Fig. 1B; Funahashi et al., 1989). This specificity of receptive field may explain why a proportion of recorded DLPFC neurons are typically found to be unresponsive to the behavioural task (i.e., cell may have been responsive for a visual location wherein no cue was presented).

In addition to spatial WM, DLPFC neurons represent information on task set (Sakai, 2008) and display discriminable activity profiles between rules in context-dependent tasks (White and Wise, 1999; Asaad et al., 2000; Wallis et al., 2001; Mian et al., 2014). The pro- and anti-saccade task (Fig. 2A; Hallett, 1978) requires subjects to execute a saccade towards (prosaccade) or away from (antisaccade) a peripheral stimulus, depending on the colour of an initial visual cue. This task is a useful paradigm for characterizing DLPFC function, as it involves spatial and delayed response components,
Figure 1. DLPFC cell types and the oculomotor delayed response task. A, The oculomotor delayed response (ODR) task begins with a flashed cue in one of eight visual angles. The subject must maintain central fixation throughout the cue (C) and delay (D) epochs. The response epoch (R) begins when the fixation point (FP) is extinguished, instructing the subject to make a saccade towards the direction of the previously shown cue. This task reveals three task-responsive cell types: cue-selective, delay-selective, and response-selective. Note that delay-selective cells are able to fire in the absence of visual stimulation. Modified from Goldman-Rakic (1995). B, Rasters and spike histograms for a single DLPFC neuron are shown for eight visual angles during the ODR task. This neuron displays delay and response period firing for only the 270° visual angle. This neuron is considered to have a ‘memory field’ for the 270° visual angle. Modified from Funahashi et al. (1989).
Figure 2. Experimental paradigm and recording technique. A, Shown is a schematic of representative pro- and anti-saccade trials. Animals were required to perform correct responses towards (prosaccade) or away from (antisaccade) a peripheral stimulus to receive liquid reward. Dashed circles indicate gaze of the animal and arrows indicate direction of saccade. Each trial is followed by a 1700 – 2200 ms intertrial interval (ITI). B, Single-unit extracellular recordings were performed in rhesus dorsolateral prefrontal cortex using glass iontophoretic electrodes. Beige area represents recording locus. Muscarinic antagonist scopolamine (represented in blue) is shown. AS, arcuate sulcus; PS, principal sulcus.
as well as recognition and maintenance of a cued rule. DLPFC neurons can indeed display preferential delay period firing profiles for either pro- or anti-saccade trials (‘rule cells’; Everling and DeSouza, 2005). The presence of rule cells and delay period activity in DLPFC likely represents a cellular component of the cognitive circuit coordinating accurate performance in the pro- and anti-saccade task. Consistently, increased error rates are observed after DLPFC lesions (Guitton et al., 1985; Pierrot-Deseilligny et al., 2003; Ploner et al., 2005), muscimol injections into cortex around principal sulcus (Condy et al., 2007), and cryogenic deactivation (Koval et al., 2011).

Cytoarchitecture of DLPFC

Originally labeled simply as area 9 by German neuroanatomist Korbinian Brodmann (1905), this dorsolateral area of monkey cortex was then segmented by adding area 46 (Walker, 1940), largely due to the distinctly thick, ‘granular’, appearance of layer IV, whereas layer IV of area 9 was described as narrower with sparse cellular density. This nomenclature held for many years, although some heterogeneities were noted in the newly termed area 46 (Barbas and Pandya, 1989; Preuss and Goldman-Rakic, 1991). More recently, Petrides and Pandya (1999) suggested a further subdivision of Walker’s area 46, by leaving the more rostral segment of tissue as area 46 and defining the caudal region surrounding the principal sulcus as area 9/46 (Fig. 3). This delineation was motivated by the large, deeply stained pyramidal neurons found in deep layer III of area 9/46, similar to the dorsal area 9 and in contrast to the rostral area 46. Dorsal area 9/46 was this project’s locus of interest and recording location.

The structure and interconnectivity of DLPFC begin to explain its well-studied role in WM. One model of how information is maintained in WM over short periods of
Figure 3. Human and rhesus macaque prefrontal cortices. Lateral views of human (A) and rhesus macaque (B) prefrontal cortices show regions considered homologous between species. The macaque dorsal area 9/46 was the recording locus for this project. Modified from Petrides and Pandya (1999).
time is via recurrent connections (Wang, 2001). Indeed, histological examinations have revealed that DLPFC has bidirectional connections with both local PFC (Levitt et al., 1993; Kritzer and Goldman-Rakic, 1995; Pucak et al., 1996) and distant brain regions, such as parietal cortex (Leichnetz, 1980; Goldman-Rakic and Schwartz, 1982; Schwartz and Goldman-Rakic, 1984). Further, these sites of proposed recurrent activity are found in layer III and also layer V, in which large pyramidal cells exhibit delay period activity in WM tasks. These pyramidal neurons tend to make corticocortical connections in layer III and subcortical projections from layer V.

Some DLPFC neurons retain memory fields during WM tasks, wherein they display delay period activity (i.e., activity after removal visual cue) for a specific area of their visual field. As described by Patricia Goldman-Rakic (1995), DLPFC can be partitioned into discrete cortical columns (similar to that of primary visual cortex), each with distinct memory fields. Further, cortical columns make excitatory horizontal connections with other DLPFC columns of similar memory field specificity, and elicit feed-forward inhibition of cortical columns with opposing memory fields (e.g., opposite visual hemifield) via depolarization of nearby nonpyramidal cells (Williams et al., 1992; Wilson et al., 1994). For example, cortical columns with memory fields for a portion of the right visual hemifield may inhibit columns with memory fields in the opposite hemifield.

Observations of WM-related activity in DLPFC ultimately led to a model of the ‘cellular basis of WM’ by Patricia Goldman-Rakic (1995), in which recurrent horizontal connections (synapsing on dendritic spines, Melchitzky et al., 1998) between layer III pyramidal neurons in DLPFC support delay period activity (Fig. 4).
Figure 4. Goldman-Rakic’s model of the working memory circuit. Information representing the memory field for a certain direction during the oculomotor delayed response task is maintained in working memory through recurrent excitatory connections between tuned DLPFC layer III pyramidal neurons (triangles) of similar angular representation. Cortical columns of a certain angle (e.g., 90°) inhibit columns representing memory fields of differing or opposite visual angles (e.g., 270°) via inhibitory nonpyramidal neurons (circles). Modified from Goldman-Rakic (1995).
Analysis of task-selective activity at different cortical depths of DLPFC was conducted by Sawaguchi et al. (1989) and agrees that visual- and cue-related activity appears most prominently in superficial layers of DLPFC. They also found that stimulus- and saccade-related activity was more localized to infragranular layers V and VI. This is consistent with tracer injections into the superior colliculus (SC), which found retrogradely-labeled pyramidal cells in layer V of the DLPFC (Leichnetz et al., 1981). A generalization would be that layer III, through connections with other cortical areas (Kawamura and Naito, 1984; Schwartz and Goldman-Rakic, 1984; Levitt et al., 1993), receives and maintains (via local horizontal recurrent connections) cue-related information over the delay period, and upon stimulus onset directs layer V projection neurons (Song et al., 2012) to generate an appropriate output (Fig. 5).

The role of nonpyramidal cells in delay-related DLPFC activity is also being investigated. Simultaneously recorded putative pyramidal and nonpyramidal neurons have been found to have similar directional tuning in an ODR task (Rao et al., 1999), whereas more distant pairs can display opposite directional tuning (Wilson et al., 1994). This suggests that pyramidal neurons may stimulate GABAergic neurons within the same cortical column (Goldman-Rakic, 1995), which then inhibit distant pyramidal neurons of differing directional tuning, thus strengthening the circuit’s tuning for a current trial’s memory field. This is supported by iontophoresis of GABA_A receptor antagonist bicuculline methiodide, which abolished tuning of rhesus DLPFC pyramidal neurons (Rao et al., 2000). Putative nonpyramidal neurons also lost tuning, suggesting baseline levels of inhibition may effect both neuronal types. Supporting this, inhibitory inputs onto GABAergic neurons of macaque DLPFC were later described electrophysiologically
Figure 5. Model of cell type laminar distribution in DLPFC. The densities of cue-, delay- and response-selective neurons in DLPFC vary depending on cell layer. Cue neurons have been reported in greater proportion in superficial layers. Delay cells are typically found in layer III, but also in layer V. Deep layers are typically known for output, which was supported by both retrograde tracers from superior colliculus and an increased proportion of response-selective neurons. The inhibitory role of nonpyramidal neurons (red circles) is not fully understood, but one function may be to suppress other cortical columns off differing tuning (e.g., 0° column may inhibit 90° column in layer III during the oculomotor delayed response task). Modified from Arnsten et al. (2012).
Individual functions of nonpyramidal neuron types are beginning to be elucidated (DeFelipe et al., 2013).

The dendritic spine of layer III DLPFC pyramidal neurons has become an emerging focus of prefrontal circuitry. Dendritic spines have experienced a dramatic increase in prominence over evolution (Elston et al., 2001), suggesting they may be central to intelligence. Investigators have now identified an assembly of functional proteins that can quickly modify the facilitation of synaptic inputs (Fig. 6; Arnsten et al., 2012; Paspalas et al., 2013), by opening or closing of ion channels in a process termed dynamic network connectivity (Arnsten et al., 2010). Electron microscopy of these primate PFC spines has illuminated the presence of numerous drug receptors such as α2-adrenergic receptors (Aoki et al., 1998), metabotropic glutamate receptors (Muly et al., 2003), NMDA receptors (Wang et al., 2013), and D1 dopamine receptors (D1Rs; Paspalas et al., 2013); ion channels including KQT-related voltage-gated delayed rectifier potassium (KCNQ) channels (Arnsten et al., 2012) and hyperpolarization-activated cyclic nucleotide-gated (HCN) channels (Paspalas et al., 2013); and intracellular messengers RGS4 (Paspalas et al., 2009), DISC1, PDE4A, and PDE4B (Paspalas et al., 2013). This complex assembly is thought to provide increased functional plasticity depending on the cognitive state and levels of endogenous neuromodulators.

The KCNQ channel is a noteworthy player in this assembly because it is a downstream target of muscarinic receptors, the pharmacological receptors of interest in this project. It is a voltage-gated delayed rectifier potassium channel and hyperpolarizes the cell at the end of an action potential via efflux of potassium ions. Its ability to regulate cell excitability and its position at the dendritic spine make it an interesting pharmacological target in this model circuit of working memory. Iontophoretic blockade
Figure 6. Model of prefrontal pyramidal spine. Numerous functional proteins have been localized to the dendritic spine of primate prefrontal pyramidal neurons. This complex of proteins, including drug receptors, ion channels, and intracellular messangers, can dynamically change the excitability of the cell by either facilitating or interrupting excitatory postsynaptic currents into the spine from presynaptic action potentials. In this example, stimulation of M1Rs is blocking KCNQ channels, and thus membrane excitation via iontrophic glutamate channels is allowed to spread through the spine and towards the cell body. AC, adenyl cyclase; α2A-R, α2-adrenergic receptor; Ca^{++}, calcium ion; cAMP, cyclic adenosine monophosphate; DISC1, disrupted in schizophrenia 1; HCN, hyperpolarization-activated cyclic nucleotide-gated; KCNQ, KQT-related voltage-gated delayed rectifier potassium channel; M1R, M1 muscarinic acetylcholine receptor; Na^+, sodium ion; PKA, protein kinase A; RGS4, regulator of G protein signaling 4. Modified from Arnsten et al. (2012).
of KCNQ channels has indeed been shown to augment DLPFC delay period activity of monkeys performing an ODR task (Wang et al., 2011).

**DLPFC Influence on Saccadic Output**

The pro- and anti-saccade task has also been used as a tool to explore the circuitry of saccadic control. Compared to prosaccades, antisaccades are thought to require additional levels of cognitive control. During prosaccade rule trials, subjects must simply retain the rule in WM to look towards an upcoming stimulus. Antisaccade trials require maintenance of the rule in WM, but also suppression of visually-guided saccades, and calculation of a saccade trajectory with opposite angle and equal eccentricity to the stimulus. Suitably, antisaccades have longer saccadic reaction times (SRTs), suggestive of additional underlying computational elements. Erroneous prosaccades during antisaccade trials (i.e., subject looked towards the stimulus instead of away) were interpreted as failures of saccade generation centers to inhibit the accustomed tendency to look towards a flashed stimulus (Pierrot-Deseilligny et al., 1991).

The SC, gateway between cortex and the saccadic output of the brainstem (Hanes and Wurtz, 2001), receives projections from multiple neocortical areas such as DLPFC, frontal eye fields (FEF), supplementary eye fields, anterior cingulate cortex (ACC), and lateral intraparietal area (Selemon and Goldman-Rakic, 1988; Johnston and Everling, 2006). The DLPFC is thought to play a less direct role since, unlike other areas (e.g., FEF; see Johnston and Everling, 2008), low current microstimulations do not elicit saccades (Wegener et al., 2008). Partially based on the propensity for DLPFC lesion patients to execute increased antisaccade errors (Fukushima et al., 1994; Walker et al., 1998), Pierrot-Deseilligny et al. (1991) proposed that PFC has an inhibitory influence on
SC, such that during a correct antisaccade, PFC suppresses the generation of visually-evoked saccades towards the stimulus.

After closer examination of DLPFC and SC interaction (Koval et al., 2011; Johnston et al., 2014), Everling and Johnston (2013) amended models of prefrontal influence on SC, from that of inhibitory control to facilitation of goal-directed saccades. This is consistent with previous associations of the PFC with representations of task sets and rules (Bunge et al., 2005; Sakai, 2008). Thus, pyramidal rule cells in layer V DLPFC (Everling and DeSouza, 2005) may influence the SC to generate appropriate saccades. An overview of the circuitry involved in the antisaccade task is illustrated in Figure 7 (Munoz and Everling, 2004).

Muscarinic System

Acetylcholine (ACh), the first neurotransmitter to be identified (Dale, 1914; Ewins, 1914), acts upon ionotropic nicotinic and metabotropic muscarinic receptors throughout the central and peripheral nervous system. The general muscarinic receptor antagonist scopolamine, a secondary metabolite of many plants of the Solanaceae family, was noted for its clinical utility in the early 20th century for its potent amnesic properties (Gaus, 1906). Since then, scopolamine has been used for many purposes (of varying appropriateness), including obstetric analgesia (Davis et al., 1952), facilitating interrogation (Geis, 1961), pupil dilation (Maus et al., 1994), a potential antidepressant (Furey and Drevets, 2006), treating Parkinsonism tremor (Perez et al., 2011), alleviation of postoperative nausea (Apfel et al., 2010), treating motion sickness (Parrott, 1989), and as a research tool for inducing states of cognitive deficit (for review see Klinkenberg and Blokland, 2010).
Figure 7. The antisaccade circuit. As reviewed by Munoz and Everling (2004), the DLPFC receives thalamic inputs and also visual inputs via parietal cortex, and then influences saccade generation in the brainstem through the superior colliculus. CN, caudate nucleus; FEF, frontal eye fields; GPe, globus pallidus; LGN, lateral geniculate nucleus; LIP, lateral intraparietal cortex; SCi, superior colliculus intermediate layers; SCs, superior colliculus superficial layers; SEF, supplementary eye fields; SNpr, substantia nigra pars reticulata; STN, subthalamic nucleus. Modified from Munoz and Everling (2004).
Of particular interest, the revelation that manipulation of muscarinic receptors can cause cognitive deficits in humans (Drachman and Leavitt, 1974), as well as monkeys (Bartus and Johnson, 1976) and rodents (Wiener and Messer, 1973), influenced the now widely accepted ‘cholinergic hypothesis’ of Alzheimer’s disease (Bartus et al., 1982). This was ultimately reconciled with the discovery of substantial degeneration of cholinergic neurons in the brains of Alzheimer’s patients (Whitehouse et al., 1982). Blockade of muscarinic receptors with scopolamine can elicit performance deficits in multiple cognitive domains such as spatial (Rupniak et al., 1991; Green et al., 2005) and nonspatial WM (Thienel et al., 2009), attention (Spinelli et al., 2006; Fredrickson et al., 2008; Furey et al., 2008), sensory discrimination (Evans, 1975), and maintenance of rules (Saar et al., 2001; Thomas et al., 2008; Snyder et al., 2014).

ACh-producing projection neurons originate in the basal forebrain (Mesulam and Van Hoesen, 1976). In humans, it is specifically the nucleus basalis of Meynert (NBM) that projects to neocortex (Mesulam and Geula, 1988). Several investigators have explored the inputs of NBM, wondering if prefrontal afferents could elicit top-down stimulation. Although projections from medial PFC to basal forebrain have been reported in rats (Gaykema et al., 1991; Zaborszky et al., 1997), results of DLPFC projections to basal forebrain in monkeys have been weak or negative (Leichnetz and Astruc, 1977; Mesulam and Mufson, 1984; Russchen et al., 1985), and no such study has been attempted in humans (Mesulam, 2013). Instead, it appears the majority of basal forebrain inputs are associated with the limbic-paralimbic system and other neurotransmitter nuclei (e.g., locus coeruleus, raphe nuclei, ventral tegmental area; Smiley and Mesulam, 1999; Smiley et al., 1999b), intuitively suggesting that cholinergic release has an emotional basis (Mesulam, 2013).
DLPFC, along with the rest of neocortex, receives substantial input from ascending NBM cholinergic projection neurons (Robbins, 2005), which are thought to modulate cognitive processing. Using an immunotoxin selective for cholinergic neurons, Croxson et al. (2011) showed that ablation of basal forebrain cholinergic projections to rhesus PFC decreased performance in delay length-dependent cognitive tasks. Similar results have been observed in rats (Chudasama et al., 2004). Cholinergic input to PFC is evidently important for executive function, particularly WM.

The laminar distribution of cholinergic input to DLPFC is not homogenous. Using immunohistochemical staining of acetylcholinesterase (AChE) in DLPFC, Mesulam et al. (1984) reported the highest density in layers I, V, and VI. However, after further experimentation using choline acetyltransferase (ChAT) staining (DeKosky et al., 1985; Lewis, 1991; Mrzljak et al., 1995) and scrutiny of the legitimacy of AChE as a marker (Mesulam and Geula, 1992), the consensus of cholinergic input to primate DLPFC is that the highest densities are seen in superficial layers I-III and also in layer V.

In one immunohistological assay, Mrzljak et al. (1995) noted that over half of cholinergic afferents did not form a definite synaptic junction, and instead ACh may need to travel through the extracellular medium before binding a receptor or being metabolized by AChE. Indeed, the debate of whether cortical ACh release is synaptic or utilizes varicosities for volume transmission has toiled for many years, and will likely remain unresolved for some time due to technological limitations (Sarter et al., 2009).

The plurality of muscarinic receptor subtypes was not realized for decades after the discovery of ACh (Hammer et al., 1980). Through the advancement of genetic techniques, the five muscarinic receptors (M1 – M5) can now be properly acknowledged (Hulme et al., 1990). Muscarinic receptors are metabotropic and often broadly classified
into two groups (see Caulfield, 1993), the M1 class (including M1, M3, and M5 receptor subtypes) and M2 class (including M2 and M4 subtypes). Receptors of the M1 class are GPCRs coupled with G\textsubscript{q} proteins, leading to activation of PLC, cleavage of PIP\textsubscript{2} into IP\textsubscript{3} and DAG, intracellular release of Ca\textsuperscript{2+} from the endoplasmic reticulum, and activation of PKC. Activation of M1 class receptors typically leads to excitation of cells via closure of inward-rectifying K\textsuperscript{+} channels and opening of cation channels (Horowitz et al., 2005; Brown, 2010). Conversely, M2 class GPCRs are coupled to G\textsubscript{i/o} proteins, resulting in inhibition of adenyl cyclase, and ultimately depression of membrane excitability via opening of inward-rectifying K\textsuperscript{+} channels and closure of high-voltage-activated Ca\textsuperscript{2+} channels (Allen and Brown, 1993; Dascal, 1997).

Although other muscarinic subtypes are found to some extent in neocortex (Thiele, 2013), M1 and M2 muscarinic acetylcholine receptors (M1Rs and M2Rs) are the most prominent (Levey, 1993). Specifically in DLPFC, M1Rs are found in all layers. Barring one aberrant report, claiming highest M1R density in layer IV (Lidow et al., 1989), most investigations into monkey and human laminar distribution agree that the highest densities of M1R are in the superficial layers I-III (Zilles et al., 1989; Vannucchi and Goldman-Rakic, 1991; Rodriguez-Puertas et al., 1997), and perhaps another band of increased expression in layer V (Mrzljak et al., 1993). Distribution of M2Rs have been described more consistently. In lower densities compared to M1Rs, M2Rs are seen in supragranular layers, especially layer III, and also layer V, with little to zero expression in layer IV (Lidow et al., 1989; Zilles et al., 1989; Mrzljak et al., 1993; Rodriguez-Puertas et al., 1997; Mrzljak et al., 1998; Medalla and Barbas, 2012).

Mrzljak et al. (1993) first reported the cell type-specific expression of DLPFC muscarinic receptors using antibody immunohistochemistry. M1Rs were largely found
postsynaptically on pyramidal spines of layer III and V, but also on nonpyramidal dendrite shafts. M2Rs were found in a similar pattern, though also found presynaptically. A portion of these presynaptic M2Rs were recognized from rat (Yonehara et al., 1980) and later monkey studies (Smiley et al., 1999a), as autoreceptors on cholinergic afferents (Zhang et al., 2002). These M2R results were verified later in the decade with in situ hybridization (Mrzljak et al., 1998), further revealing that M2R-positive nonpyramidal neurons were more prominent in infragranular layers V and VI than supragranular layers.

Although behavioural outcomes of systemic muscarinic antagonism have been extensively studied, effects of local scopolamine application on primate DLPFC neurons engaged in a cognitive context remain relatively unknown. Here, we performed in vivo single-unit electrophysiology and concurrent iontophoresis of scopolamine in the DLPFC of monkeys performing a pro- and anti-saccade task to investigate the role of muscarinic receptors in the maintenance of task rules. This work will elucidate the role of muscarinic receptors in DLPFC during executive performance. We hypothesize that local application of scopolamine will decrease overall and task-related neuronal firing of DLPFC neurons.
Chapter 2 – Materials and Methods

Experimental procedures were performed on two adult male rhesus macaque monkeys (*Macaca mulatta*) in accordance with the Canadian Council of Animal Care policy and a protocol approved by the Animal Use Subcommittee of the University of Western Ontario Council on Animal Care.

Both animals underwent surgery for placement of a head restraining post and plastic recording chambers above their right lateral prefrontal cortices. Animals were sedated with ketamine hydrochloride (15 mg/kg, i.m.), anesthetized with propofol (2.5 mg/kg, i.v.), and endotracheally intubated. Atropine (0.05 mg/kg, s.c.) was given to monkey O to reduce bradycardia and salivary secretions. Anesthesia was maintained with propofol (0.1 – 0.2 mg/kg/min, i.v.) and isoflurane (1 – 2% in oxygen, 1 L/min). Heart rate, blood oxygen, respiratory rate, end-tidal carbon dioxide, blood pressure, and body temperature were monitored throughout the duration of the surgery. Postoperatively, animals were given cefazolin (25 mg/kg, i.m., 5 days) to prevent infection and buprenorphine hydrochloride (0.01 – 0.03 mg/kg, i.m., 3 days) to alleviate any potential discomfort. Metacam (0.2 mg/kg loading dose, 0.1 mg/kg maintenance dose, i.m., 3 – 5 days) was provided as an additional analgesic.

Plastic head posts for head restraint were fixed with dental acrylic cement, which was anchored to the skull using titanium screws. Craniotomies were performed over right DLPFC, based on stereotaxic coordinates. Plastic recording chambers (Crist Instruments, Hagerstown, MD) were placed over the trephination and firmly attached using dental acrylic cement.
**Behavioural task**

Both animals were trained on the gap variant of a pro- and anti-saccade task (Fig. 2A). Monkeys were seated in a primate chair in a shielded chamber with their heads restrained and faced a 21 inch cathode ray tube monitor 51 cm in front of them. Horizontal and vertical eye movements were recorded at 1 kHz with an EyeLink 1000 infrared eye tracker and software package (SR Research Ltd., Ottawa, Ontario, Canada). The task, behaviour monitoring, and reward delivery were controlled using CORTEX (NIMH, Bethesda, MA, USA). Trials began with presentation of a central gray filled fixation circle (0.5° diameter). After an initial 300 ms fixation period, the fixation stimulus briefly changed colour to red or green for 100 ms, indicating the task rule (prosaccade or antisaccade) of the current trial. Rule colours were counterbalanced between subjects. The subjects were required to remember the rule through a delay period (800 – 1300 ms) during which the fixation spot reverted to gray. The fixation spot was then extinguished for 150 – 300 ms (gap period) and a peripheral stimulus (17.5° from center, 0.5° diameter) was then presented. The gap was introduced to increase task difficulty (Fischer and Weber, 1992; Everling et al., 1998). The subjects were required to make the appropriate saccade towards (prosaccade) or away from (antisaccade) the stimulus, depending on the current trial’s rule. Rule and stimulus combinations were presented in pseudorandom order. In order to obtain a liquid reward, the subjects had to maintain fixation during the fixation, cue, and delay periods, make the appropriate saccade within 500 ms, and maintain fixation on the stimulus (or blank space in the case of antisaccade trials) for 120 ms. Trials were separated by a 1700 – 2200 ms intertrial interval. Trials in which animals broke central fixation before the stimulus period or made a saccade to the wrong direction were labeled erroneous. Saccade onset was defined as the moment eye
velocity surpassed 30°/s and SRT was defined as the time from the peripheral stimulus onset to saccade onset.

*In vivo extracellular recordings and iontophoresis*

Scopolamine was iontophoretically administered using custom seven-barreled glass iontophoretic electrodes (Fig. 8). The design and fabrication of the electrodes were similar to Vijayraghavan et al. (2007). A 50 μm pitch tungsten wire, 110 mm in length, (Midwest Tungsten Service, Willowbrook, IL) was electrochemically etched (Model EE-ID, Bak Electronics Inc., Sanford, FL) using a sodium nitrite and potassium hydroxide solution as described previously (Thiele et al., 2006), creating a fine wire tip. This wire was inserted into the central barrel of a multibarreled pipette (Friedrich and Dimmock Inc., Millville, NJ) and the assembly was pulled using a PMP107L-e Multipipette Puller (MicroData Instrument Inc., South Plainfield, NJ), resulting in a 10.5 cm-long electrode shaft and a thin glass tip on the order of 15 – 30 μm. Typical impedances were between 0.5 and 1 MΩ (measured at 1 kHz; IMP-1, Bak Electronics Inc., Sanford, FL). Neuronal signals were amplified, digitized, and filtered (300 Hz – 6 kHz, four-pole Bessel) with an OmniPlex Neural Data Acquisition System (Plexon Inc., Dallas, TX).

Scopolamine hydrobromide (Tocris Bioscience, Bristol, UK; 100 mM in pH 3 deionized water) was stored in 30 μL aliquots at -20°C. Before use, scopolamine was thawed and inserted into peripheral glass capillaries of the iontophoretic electrode, then pushed to the tip of the electrode using compressed air. Tungsten wires (FHC Inc., Bowdoin, ME) were inserted into each peripheral capillary and connected to a NeuroPhore BH-2 iontophoretic ejection system (Harvard Apparatus, Holliston, MA). DC
Figure 8. Idealized illustration of custom-made glass iontophoretic electrode tip. Tungsten wire for electrophysiological recording is in central glass barrel, surrounded by six drug barrels. Tip diameter is approximately 15 – 30 µm. Scopolamine hydrobromide is dissolved in water (represented in blue) and is ejected near the tip of the tungsten recording electrode.
impedances of drug barrels varied, typically between 50 and 300 MΩ. The electrode was mounted on a hydraulic micromanipulator (MO-95, Narishige Group, Tokyo, Japan) and lowered into cortex through a 25-gauge dura-penetrating stainless steel guide tube. A plastic recording grid (1 mm spacing; Crist Instrument Co. Inc., Hagerstown, MD) was used to direct guide tube placement. A schematic of the approximate recording locus around the principal sulcus in DLPFC is shown in Figure 2B. Charged drug was ejected from the drug barrels into tissue by passing constant ejection currents ranging from +10 to +100 nA, which were manually set by the experimenter during the course of scopolamine conditions. An example of iontophoretic ejection of excitatory neurotransmitter glutamate is displayed in Figure 9. A retention current of -8 nA was passed over each drug barrel during control periods. Current balancing was not required at ejection currents of this magnitude (Vijayraghavan et al., 2007). At these currents, drug ejection did not create noise in the system or affect unit physiology. This was tested by Vijayraghavan et al. (2007), who passed up to 100 nA over drug barrels filled with sodium chloride and observed that in the absence of drug, neurons did not exhibit firing rate changes. After control periods of at least 10 – 15 min, a drug condition followed with comparable duration. Multiple scopolamine doses and post-scopolamine recovery sessions were occasionally conducted. Cells were rejected if a sufficient number of correct trials were not obtained (at least 8 per rule-saccade direction combination). Multiple doses of scopolamine were occasionally applied to the same neuron to observe dose-dependent effects on neuronal activity.

Data analysis

Neuron waveforms were sorted using principal component cluster space segregation
Figure 9. Iontophoretic ejection of glutamate. In this example, we see the typical response of a prefrontal neuron to application of the excitatory neurotransmitter glutamate. Firing rate shows a noticeable and immediate increase. In this case, negative current is used because glutamate is a negatively charged molecule. Each vertical line represents an action potential from an isolated DLPFC neuron.
Data analysis was performed with custom-written programs in MATLAB (MathWorks, Natick, MA). Spike density functions were constructed by convolving spike trains with a 50 ms Gaussian activation function. Trial-wise rasters and spike density functions were aligned to peripheral stimulus onset. Delay epoch was defined as time from cue offset to stimulus onset + 70 ms (i.e., to allow time for visual information to reach DLPFC, see Johnston et al., 2009). Alpha value of 0.05 was used throughout the analysis. The rule eliciting greater firing rate (FR) during the delay epoch of the control condition was set as the preferred rule. Rule selectivities (e.g., does a neuron fire with a preference for either prosaccade or antisaccade rules) for control and drug conditions were evaluated using area under the receiver operating characteristic (AUROC). AUROC is a nonparametric measure of discriminability between two distributions, considering both true positive rate and false positive rate at various discrimination thresholds (Green and Swets, 1966). AUROCs were computed for the preferred rule versus the non-preferred rule. Completely overlapping distributions have an AUROC of 0.5, and completely distinct distributions would have an AUROC of 1. AUROCs (1000 steps) were obtained using mean FR from preferred and non-preferred rule trials during the entire delay period. The significance of the AUROC metric obtained was estimated using a bootstrapping procedure, whereby control trial FRs were randomly assigned to a preferred or non-preferred rule trial and the AUROC for shuffled trial FR distribution was computed (Everling and DeSouza, 2005). A neuron’s firing profile was deemed significantly selective if the original AUROC was greater than the 95th percentile of the shuffled AUROCs from 10,000 iterations. The same analysis was performed during the stimulus epoch (stimulus onset + 70 ms to saccade onset + 120 ms) for preferred and non-preferred rule, saccade direction, and visual stimulus direction to evaluate selectivity.
and significance. As a decrease in AUROC can be explained by either decreased mean FR difference or increased trial-to-trial FR variance, the Fano factor was calculated to inspect changes to neuronal reliability. This was calculated as trial-wise FR variance divided by the mean for control and drug epochs. Normalized population spike density functions across all rule-selective neurons were constructed in the delay epoch for the preferred and non-preferred rule using the following:

\[ FR' = \frac{FR - FR_{\text{min}}}{FR_{\text{max}} - FR_{\text{min}}} \]

where \( FR_{\text{min}} \) and \( FR_{\text{max}} \) are the minimum and maximum of the smoothed spike density functions in both control and scopolamine conditions. Normalized spike density functions of all rule cells in the delay epoch were then averaged. Identical normalizations were performed for rule, directional, and visual neurons in the stimulus epoch.
Chapter 3 – Results

Combined single-unit recordings and iontophoretic scopolamine applications were performed in 76 experimental sessions (41 from monkey O, 35 from monkey T) and 117 total neurons were recorded (65 from monkey O, 52 from monkey T). Monkeys performed the behavioural saccade task during control and scopolamine conditions, each typically lasting a minimum of 10 – 15 min. No significant differences in performance (prosaccade: control 86.2 ± 1.1% vs. scopolamine 87.8 ± 0.9%, p = 0.096; antisaccade: 76.4 ± 1.1% vs. 74.4 ± 1.3%, p = 0.094, paired t-test) or SRT (prosaccade: 147.1 ± 2.1 ms vs. 149.5 ± 2.0 ms, p = 0.090; antisaccade: 198.1 ± 2.0 ms vs. 198.9 ± 2.3 ms, p = 0.59) were observed between control and scopolamine conditions.

Scopolamine decreases overall FRs in DLPFC neurons

The most conspicuous effect of scopolamine was strong and significant attenuation of neuronal FR in all task epochs. Figure 10A shows the mean FRs of all recorded neurons during the delay, stimulus, and intertrial interval epochs. Scopolamine application attenuated population FR in all epochs (Fig. 10A; p < 0.0001, Wilcoxon signed rank test with Bonferroni correction). This inhibitory effect was dose-dependent (Fig. 10B). Figure 10B shows the normalized mean activity for 20 neurons on which successive doses of scopolamine were applied. Drug condition average FRs were normalized by dividing by their respective session’s control condition average FR. Two dose ranges were defined (<= 30 nA and > 30 nA). Low (<= 30 nA) doses exhibited a nonsignificant decrease to FR (p = 0.50) and higher doses (up to 100 nA) elicited strong inhibition during the delay epoch (p = 0.0019, Wilcoxon signed rank test with Bonferroni correction), at times
Figure 10. Effect of scopolamine on neuronal firing. 

A, Shown are mean FRs from 117 PFC neurons (control, grey bars; scopolamine, green bars) in the delay, stimulus, and intertrial interval (ITI) epochs of the task. Scopolamine significantly decreased FRs of recorded neurons in both prosaccade and antisaccade trials in the delay, stimulus, and ITI epochs. The indicated significance value applies to all comparisons. 

B, Shown are normalized mean FRs for 20 neurons where progressive doses of scopolamine were tested. Higher doses of scopolamine led to greater inhibition of FR, compared to control. Population includes any recorded neuron with scopolamine doses of both 15 – 30 nA and 31 – 100 nA and a control mean FR greater than 2 Hz. *p = 0.0022; **p = 0.0019. 

C,
Recovery of FR upon cessation of scopolamine ejection. Although recovery condition FR was significantly greater than scopolamine condition, it did not reach control levels. Includes all cells given a recovery condition and a control mean FR greater than 2 Hz. *p = 0.0038; **p = 0.0025; ***p < 0.0001. Error bars indicate SEM. Significance determined by Wilcoxon signed rank test with Bonferroni correction.
leading to complete collapse of excitability. In order to determine whether the effects of scopolamine were selectively physiological in nature, and not a consequence of loss of isolation or other unrelated effects, we examined recovery after cessation of drug application in 34 neurons. Figure 10C shows the normalized mean FRs for scopolamine application followed by recovery for 34 neurons. During recovery after scopolamine-induced suppression, population activity was significantly greater than during the scopolamine conditions (p = 0.0025, Wilcoxon signed rank test with Bonferroni correction).

**Scopolamine reduces rule selectivity in the delay epoch of DLPFC neurons**

We examined the rule selectivity (i.e., increased activity for preferred rule compared to non-preferred rule) of DLPFC neuronal firing before and after scopolamine application (Fig. 11). Figure 11A shows an example of scopolamine administration on a single rule-selective neuron. Rasters and spike density functions for prosaccade (blue traces) and antisaccade (red traces) trials are shown in control and scopolamine conditions. This neuron had greater activity during prosaccade trials than antisaccade trials in the delay epoch (prosaccade: 30.0 ± 0.84 Hz vs. antisaccade: 20.8 ± 0.90 Hz, p < 0.0001, Wilcoxon rank sum test). Rule selectivity was quantified in the delay epoch using the AUROC metric (see Materials and Methods). Iontophoretic administration of scopolamine resulted in a decrease in rule selectivity for this neuron, as determined by AUROC (control: 0.85 vs. scopolamine: 0.68). Of our sample of 117 DLPFC neurons, 22 (14 from monkey O, 8 from monkey T; 15 prosaccade-preferring, 7 antisaccade-preferring) were found to be significantly rule-selective based on the AUROC analysis. Population normalized spike density functions were constructed for these 22 neurons, which are shown in Figure 11B.
Figure 11. Effect of scopolamine on rule selectivity in the delay epoch. A, Single neuron spike rasters (top panels) and smoothed spike density functions (bottom panels) for
prosaccade (blue) and antisaccade (red) rule trials, for both control (left panels) and 70 nA scopolamine (right panels) conditions are shown. Rasters and spike density functions were aligned to stimulus onset. Each row of dots represents a trial raster and each dot represents a spike. Delay epoch begins at offset of rule cue (black diamonds) and ends 70 ms after stimulus onset. 

**B**, Normalized population spike density functions of preferred (blue) and non-preferred (red) rule activity in control (left) and scopolamine (right) conditions for 22 significantly rule-selective neurons are shown. Scopolamine decreased both FR for preferred and non-preferred rule trials and discriminability in the delay epoch.

**C**, Scatter plot of control AUROC values (abscissa; monkey O, open circles; monkey T, filled circles) compared to AUROC values in the scopolamine condition (ordinate). Dashed line indicates equality line. Greater AUROC deviation from 0.5 indicates greater selectivity for the preferred rule. AUROC values after scopolamine application were below the equality line, indicating reduction in rule selectivity. Population AUROC values were significantly reduced by scopolamine.

**D**, Scopolamine elicited a stronger decrease in population FR for the preferred rule in the delay epoch, compared to non-preferred rules. Error bars indicate SEM. Significance determined by Wilcoxon signed rank test.
Scopolamine substantially reduced the population firing and abolished selectivity in the delay epoch. Figure 11C shows a scatter plot of AUROC values (preferred vs. non-preferred rule) in the control condition vs. the scopolamine condition. 20 of 22 recorded neurons showed lower AUROC values in the scopolamine condition compared with the control condition. AUROC values were significantly reduced in the scopolamine condition (control: 0.65 ± 0.020 vs. scopolamine: 0.55 ± 0.019, p = 0.0015, Wilcoxon signed rank test). Next, we examined whether rule selectivity reduction induced by scopolamine was due to changes in preferred or non-preferred rule firing. Reduction in FR was found to be greater for the preferred rule than for the non-preferred rule (Fig. 11D; preferred: -8.2 ± 2.4 Hz vs. non-preferred: -5.9 ± 1.9 Hz, p = 0.00043, Wilcoxon signed rank test).

Scopolamine reduces rule selectivity in the stimulus epoch of DLPFC neurons

We further examined rule selectivity in the stimulus epoch. Figure 12A shows a DLPFC neuron with rule selectivity in the stimulus epoch, with greater stimulus epoch activity during antisaccade trials (prosaccade: 9.0 ± 0.71 Hz vs. antisaccade: 12.1 ± 0.72 Hz, p = 0.0018, Wilcoxon rank sum test). Scopolamine suppressed neuronal activity, leading to a reduction in rule-related activity in the stimulus epoch (control: 10.7 ± 0.52 Hz vs. scopolamine: 4.5 ± 0.30 Hz, p < 0.0001, Wilcoxon rank sum test). 30 neurons (18 from monkey O, 12 from monkey T; 11 prosaccade-preferring, 19 antisaccade-preferring) were determined to be significantly rule-selective in the stimulus epoch based on AUROC analysis. The average normalized population spike density functions for these 30 neurons (Fig. 12B) show decrement in selectivity upon scopolamine application. AUROC values decreased after scopolamine application in 25 of 30 neurons (Fig. 12C) with a significant
Figure 12. Effect of scopolamine on rule selectivity in the stimulus epoch. A, Single neuron spike rasters and smoothed spike density functions of prosaccade and antisaccade
rule trials, for both control and 15 nA scopolamine conditions are shown. Stimulus epoch begins 70 ms after stimulus onset and ends 120 ms after saccade onset (black diamonds).

**B.** Normalized population spike density functions of preferred and non-preferred rule activity in control and scopolamine conditions for 30 significantly rule-selective neurons are shown. Scopolamine decreased both FR for preferred and non-preferred rule trials and discriminability in the stimulus epoch. 

**C.** Scatter plot of control AUROC values (monkey O, open circles; monkey T, filled circles) compared to AUROC values in the scopolamine condition. AUROC values after scopolamine application were below the equality line, indicating reduction in rule selectivity. Population AUROC values were significantly reduced by scopolamine.

**D.** Scopolamine elicited a stronger decrease in population FR for the preferred rule in the stimulus epoch, compared to non-preferred rules. Error bars indicate SEM. Significance determined by Wilcoxon signed rank test.
reduction in overall stimulus rule selectivity (control: 0.65 ± 0.012 vs. scopolamine: 0.55 ± 0.013, p < 0.0001, Wilcoxon signed rank test). Again, scopolamine decreased rule selectivity by decreasing the FR for the preferred rule more than the non-preferred rule (Fig. 12D; preferred: -11.9 ± 2.7 Hz vs. non-preferred: -6.9 ± 1.9 Hz, p < 0.0001, Wilcoxon signed rank test).

Scopolamine reduces saccadic and visual selectivity of DLPFC neurons

Next, we characterized the effects of scopolamine application on DLPFC neurons displaying motor-related peri-saccadic activity and sensory visual activity related to the peripheral stimulus.

Figure 13A is an example of a saccade direction-selective DLPFC neuron. Trials are separated based on the direction of saccades (pooled pro- and anti-saccades; contralateral, blue trace; ipsilateral, red trace). This neuron showed greater peri-saccadic activity for the contralateral direction (23.0 ± 1.7 Hz) than the ipsilateral direction (18.9 ± 2.1 Hz, p = 0.027, Wilcoxon rank sum test). Of 117 total neurons, 45 (24 from monkey O, 21 from monkey T; 32 contralateral saccade-preferring, 13 ipsilateral saccade-preferring) demonstrated significant saccade direction tuning, based on AUROC analysis.

Normalized spike density functions, constructed based on preferred and non-preferred saccade direction, show a marked reduction in saccade-related firing in the stimulus epoch (Fig. 13B). Scopolamine application significantly decreased saccade-direction selectivity in these neurons as shown by the shift in AUROC values (Fig. 13C; control: 0.69 ± 0.012 vs. scopolamine: 0.58 ± 0.015, p < 0.0001, Wilcoxon signed rank test). Loss of selectivity was caused by preferentially greater inhibition to the preferred saccade
Figure 13. Effect of scopolamine on saccade direction selectivity in the stimulus epoch.

A. Single neuron spike rasters and smoothed spike density functions of contralateral
(blue) and ipsilateral (red) saccade direction trials, for both control and 70 nA scopolamine conditions are shown. Stimulus epoch begins 70 ms after stimulus onset and ends 120 ms after saccade onset (black diamonds). B, Normalized population spike density functions of preferred and non-preferred saccade direction activity in control and scopolamine conditions for 45 significantly saccade direction-selective neurons are shown. Scopolamine decreased both FR for preferred and non-preferred saccade direction and discriminability in the stimulus epoch. C, Scatter plot of control AUROC values (monkey O, open circles; monkey T, filled circles) compared to AUROC values in the scopolamine condition. AUROC values after scopolamine application were below the equality line, indicating reduction in saccade direction selectivity. Population AUROC values were significantly reduced by scopolamine. D, Scopolamine elicited a stronger decrease in population FR for the preferred saccade direction in the stimulus epoch, compared to non-preferred saccade direction. Error bars indicate SEM. Significance determined by Wilcoxon signed rank test.
direction (Fig. 6D; preferred: -12.5 ± 2.2 Hz vs. nonpreferred: -8.0 ± 1.9 Hz, p < 0.0001, Wilcoxon signed rank test).

Figure 14A shows a neuron displaying stimulus epoch selectivity for the contralateral peripheral stimulus. This neuron showed greater activity following contralateral stimulus onset than after the ipsilateral stimulus (contralateral: 29.9 ± 1.4 Hz vs. ipsilateral: 23.6 ± 1.3 Hz, p = 0.0054, Wilcoxon rank sum test). AUROC analysis of visual selectivity in the stimulus epoch revealed 35 neurons with significant hemispheric discriminability to stimulus presentation in the control condition (17 from monkey O, 18 from monkey T; 24 contralateral stimulus-prefering, 11 ipsilateral stimulus-prefering). Population normalized spike density functions of these visual neurons, shown in Figure 14B, demonstrate the pronounced suppression induced by scopolamine. AUROC values for these neurons were significantly decreased upon scopolamine administration (Fig. 14C; control: 0.65 ± 0.011 vs. scopolamine: 0.54 ± 0.013, p < 0.0001, Wilcoxon signed rank test). This deterioration of visual selectivity was again due to greater collapse in excitation for the preferred stimulus direction compared to the nonpreferred stimulus direction (Fig 14D; p < 0.0001, Wilcoxon signed rank test).

**Scopolamine does not change reliability of neuronal firing**

Since the AUROC is a nonparametric measure accounting for changes both in the mean and the variance of the distributions being compared, we wished to ascertain whether AUROC reductions upon scopolamine application were due to changes in the FR mean or changes in trial-to-trial variability. To exclude possible changes in reliability of neuronal firing, delay epoch Fano factor was calculated, yielding no significant differences between control and scopolamine conditions (prosaccade: control 2.9 ± 0.32 vs.
Figure 14. Effect of scopolamine on selectivity for peripheral stimulus direction in the stimulus epoch. A, Single neuron spike rasters and smoothed spike density functions of
contralateral and ipsilateral peripheral stimulus trials, for both control and 15 nA scopolamine conditions are shown. Stimulus epoch begins 70 ms after stimulus onset and ends 120 ms after saccade onset (black diamonds). \textbf{B}, Normalized population spike density functions of preferred and non-preferred stimulus direction activity in control and scopolamine conditions for 35 significantly visual stimulus-selective neurons are shown. Scopolamine decreased both FR for preferred and non-preferred visual direction and discriminability in the stimulus epoch. \textbf{C}, Scatter plot of control AUROC values (monkey O, open circles; monkey T, filled circles) compared to AUROC values in the scopolamine condition. AUROC values after scopolamine application were below the equality line, indicating reduction in direction selectivity. Population AUROC values were significantly reduced by scopolamine. \textbf{D}, Scopolamine elicited a stronger decrease in population FR for the preferred stimulus direction in the stimulus epoch, compared to non-preferred stimulus direction. Error bars indicate SEM. Significance determined by Wilcoxon signed rank test.
scopolamine $2.9 \pm 0.42$, $p = 0.37$; antisaccade: $3.1 \pm 0.42$ vs. $2.6 \pm 0.25$, $p = 0.63$, Wilcoxon signed rank test).
Chapter 4 – Discussion

The PFC receives substantial inputs from the ascending cholinergic system (Robbins, 2005), which influences WM, attention, arousal, and sensory discrimination (Luchicchi et al., 2014). This project attempts to address a gap in our knowledge of the physiological underpinnings of cholinergic modulation of DLPFC neurons underlying executive functions. We found that local muscarinic blockade of monkey DLPFC markedly suppressed neuronal firing and reduced selectivity for rule-mnemonic, saccade- and sensory-related activity.

Cognitive domains influenced by the cholinergic system appear to be manifold (Klinkenberg and Blokland, 2010; Bubser et al., 2012). WM function is a commonly reported target of muscarinic modulation, especially in conjunction with PFC. As discussed earlier, Croxson et al. (2011) tested monkeys with cholinergic ablation of basal forebrain on a number of cognitively demanding tasks. Consistent with rodent data (Chudasama et al., 2004), they primarily found deficits in performance involving a delay period, supporting a specific role of cholinergic input in prefrontal WM.

A recent study by Zhou et al. (2011) performed systemic scopolamine injections and concurrent electrophysiological recordings of neurons in macaque DLPFC. They found consistent WM deficits in delayed-response performance and associated decreases in DLPFC neuronal firing during the delay period. We did not observe any significant decreases in task performance or SRT, likely due to the different administration technique of scopolamine. Although previous studies have reported that iontophoresis of drugs can influence behaviour (Herrero et al., 2008; Herrero et al., 2013; Ott et al., 2014), they are a minority with effects of minuscule magnitude.
The results of Zhou et al. (2011) are consistent with our observed suppression of delay period selectivity in rule neurons, and indeed, such suppression may be the basis of WM and rule maintenance deficits observed with scopolamine. In agreement with this, a human imaging study found scopolamine-induced degradation in persistent activity, albeit in parahippocampal gyrus (Schon et al., 2005). Slice physiology also lends support to a putative cholinergic role in the physiology of recurrent activity (Hasselmo and McGaughy, 2004).

Our observation of scopolamine-induced neuronal suppression is in accord with DLPFC recordings after systemic scopolamine application (Zhou et al., 2011) and V1 recordings after iontophoretic ejection of scopolamine (Herrero et al., 2008). However, Miller and Desimone (1993) found a paradoxical increase in stimulus responsive activity of rhesus inferotemporal neurons after systemic scopolamine administration during a DMS task. Although spontaneous neuronal firing remained unchanged, this suggests scopolamine-induced suppression is not universal. However, our results, which are the first to report iontophoretic application of scopolamine to DLPFC, show that prefrontal suppression due to systemic muscarinic blockade (Zhou et al., 2011) can be a direct consequence of DLPFC muscarinic antagonism and not necessarily through indirect network-effects.

ACh has been shown to affect the physiology of macaque middle temporal neurons and their motion discriminability (Thiele et al., 2012), suggesting a role of cholinergic signaling in effective filtering of information (Thiele, 2013). This is also evident from the attenuation of attentional modulation of visual receptive fields in V1 by iontophoretic application of scopolamine (Herrero et al., 2008). Rat thalamocortical slices
have further demonstrated that cortical cholinergic receptors can differentially filter information from intracortical or distant (e.g., thalamic) afferents (Gil et al., 1997).

Here, we examined the effects of muscarinic modulation on neuronal selectivity for mnemonic rule representation. Scopolamine-induced reduction in rule selectivity found in this study is consistent with the results from Zhou et al. (2011), wherein delay activity of DLPFC neurons was degraded during a spatial WM task and a DMS task. They also found delay-dependent deficits in behavioural performance after scopolamine application, which were interestingly unaffected by distractor load. Stimulus selectivity of DLPFC neurons was found to be unaffected by systemic cholinergic blockade (ibid). In contrast, we found that peripheral stimulus selectivity was also reduced. We hypothesize that this difference is explained by the different dosing context of systemic administration in that study and the focal administration here. Furthermore, the suppression of visual-related activity found herein could potentially shed light on the lack of interaction of scopolamine modulation and distractor load found in that study. If visual activity is suppressed, then the salience of the distractors may be comparably reduced, which may contribute to the lack of interaction between distractor load and scopolamine-induced behavioural degradation found in spatial WM (ibid) and found in DMS performance by Miller and Desimone (1993), where scopolamine effects were independent of the number of intervening stimuli (analogous to distractors). This is also in accord with Parikh et al. (2007), who found transient increases in ACh release in medial PFC of rats after cue presentation in a cue-detection task, and lack thereof for undetected cues. This indicates that salient visual stimuli (e.g., cues or distractors) evoke ACh release, which may then modulate neuronal visually evoked transients. Cholinergic blockade would disrupt this modulation of visual-stimulus salience.
We report that scopolamine suppressed the selectivity of DLPFC neurons possessing peri-saccadic activity. Takeda and Funahashi (2002) observed peri-saccadically active neurons in DLPFC, which may be a manifestation of influence on saccade generation circuitry (Watanabe et al., 2006), corollary discharge feedback about eye position (Sommer and Wurtz, 2008), or saccadic remapping, which updates cortical maps prior to an impending saccade (Colby et al., 1995).

Zhou et al. (2011) also reported marginal scopolamine-induced changes in saccadic latency (~5 ms) and increased saccadic dispersion in the delayed response task, but saccades to visual stimuli with zero delay were unaffected. Since scopolamine was given systemically, it is uncertain whether scopolamine’s direct influence on saccadic neurons contributed to these small effects. Subtle changes in the WM task saccadic latencies are consistent with effects on DLPFC, which is indirectly involved in saccade generation (Everling and Johnston, 2013). Similarly, D1R agonist infusions in DLPFC had effects on ODR performance, while sparing visually guided saccades (Gamo et al., 2015). Conversely, iontophoretic activation of D2Rs selectively modulates peri-saccadic activity and not delay activity (Williams and Goldman-Rakic, 1995; Wang et al., 2004a). Thus, the contribution of neurons with peri-saccadic activity to DLPFC circuitry and behavioural performance is fraught with interest, and further elucidation of neuromodulatory influences on these cells is required.

Microiontophoretic and systemic injection studies of other modulatory systems, such as catecholaminergic (Wang et al., 2004a; Vijayraghavan et al., 2007; Gamo et al., 2010), nicotinic (Yang et al., 2013), serotonergic (Williams et al., 2002) and glutamatergic receptors (Skoblenick and Everling, 2012; Wang et al., 2013) have yielded valuable insights upon the physiological basis of neuromodulation of cognitive circuitry.
Our results suggest that in addition to other neurotransmitter systems, muscarinic receptors also modulate DLPFC. An emerging focus in the study of cognitive neuromodulation is the dendritic spine of layer III PFC pyramidal cells, where a constellation of receptors, ion channels, and intracellular signaling molecules are found in proximity, to augment or shunt spinal synaptic input and its influence on excitability, leading to dynamic network connectivity (Armsten et al., 2012; Paspalas et al., 2013). Immunohistochemical localization of muscarinic receptors on layer III spines of prefrontal pyramidal neurons supports that cholinergic input also engages dynamic network connectivity (Mrzljak et al., 1993).

Using an eloquent combination of tracer dyes and immunohistochemical labeling for electron microscopy, Medalla and Barbas (2012) recently localized macaque area 9 M2Rs in reference to cell type, laminar distribution, synaptic localization, and afferents from either ACC or area 46 DLPFC. Consistent with previous reports, they found M2Rs in superficial layers I-III and deep layers V and VI, expressed mostly on neural processes (but also on cell bodies) of both pyramidal and nonpyramidal neurons, both presynaptically and postsynaptically, at extra- and peri-synaptic sites. Overall, M2Rs were found on a minority of tracer-labeled presynaptic and postsynaptic targets, but when they were found postsynaptically, it was more so on pyramidal spines than on nonpyramidal shafts. These findings suggest that M2Rs can presynaptically inhibit glutamate release (e.g., from ACC) and postsynaptically inhibit incoming signals from both local and distant afferents.

M2Rs are known to inhibit their effector, adenyl cyclase, thereby reducing intracellular cAMP (Hildebrandt et al., 1984). Blockade of M2Rs, may result in increased cAMP and subsequent opening of HCN channels (Chen et al., 2001). Also located on
layer III pyramidal spines, excessive opening of HCN channels may lead to the reduced neuronal firing observed in our experiments.

However, in contrast to the inhibitory effect of M2R stimulation, M1Rs have mostly depolarizing effects and are expressed more prominently in primate DLPFC (Mrzljak et al., 1993), especially on pyramidal spines. Thus, although both subtypes may be present on the dynamic layer III spines, scopolamine-induced general suppression found here is more likely mediated by antagonism of postsynaptic M1Rs. Stimulation of these receptors can augment synaptic inputs and increase neuronal excitability through closure of KCNQ channels, also located on spines (Arnsten et al., 2012). Although not tested in cortical tissue, M1Rs colocalize with KCNQ channels in human HEK293 cells (Oldfield et al., 2009).

The DLPFC pyramidal circuit for WM, as proposed by Goldman-Rakic (1995), is integrated by layer III spines (Melchitzky et al., 1998). This circuitry, hypothesized to support delay activity (e.g., during an ODR task), may overlap with the prefrontal network for accurate maintenance of rules during the delay period of the pro- and antisaccade task. We further hypothesize that our observed reduction in delay period rule selectivity is due to blockade of layer III spinal M1Rs, and subsequent shunting of depolarizing current through opened KCNQ channels on dendritic spines. Consistent with this hypothesis, iontophoretic KCNQ blockade augments persistent activity in macaque DLPFC delay-neurons (Wang et al., 2011). Alternatively, blockade of M2Rs and the eventual shunting of EPSPs through opened HCN channels may cause disruption of this network. Of note, muscarinic receptors are conveniently located to influence this proposed WM circuit and its output, with high densities in layers III and V of macaque DLPFC, but not in layer IV (Mrzljak et al., 1993). This may begin to explain the cellular
mechanisms of deficits to WM and rule maintenance after scopolamine administration (Green et al., 2005; Snyder et al., 2014).

There are many unresolved questions about muscarinic receptors in this circuit. For example, antagonism of muscarinic autoreceptors typically leads to augmented release of ACh (Kilbinger, 1984), which could theoretically compete with the antimuscarinic effects of iontophoresed scopolamine. Although antagonism of M2Rs can lead to opening of HCN channels and reduced excitability, blockade of the fast membrane-delimited pathway of M2R would reduce opening of GIRK channels (Reuveny et al., 1994; Yamada et al., 1998), producing the opposite effect. As a further source of intricacy, these suppositions are focused on pyramidal cells, even though nonpyramidal cells express muscarinic receptors (Mrzljak et al., 1993; Medalla and Barbas, 2012) and are involved in WM circuits (Rao et al., 1999; Constantinidis et al., 2002; Wang et al., 2004b). Of note, nonpyramidal neurons are thought to be involved in coordinating LFP oscillations (Lytton and Sejnowski, 1991; Buzsaki and Chrobak, 1995; Cobb et al., 1995; Whittington and Traub, 2003), which may influence cognitive control such as maintenance of rules in DLPFC (Buschman et al., 2012). Future work such as iontophoresis of subtype-specific muscarinic ligands, investigation of muscarinic influence on LFP, and laminar histology of DLPFC muscarinic subtype distribution among different neuronal types will further illuminate the role of muscarinic receptors in WM circuits.

There has been burgeoning interest in pharmacological targeting of muscarinic receptors in the treatment schizophrenia and Alzheimer’s disease. It is noteworthy that allelic variants of genes encoding several intracellular messengers, which have been localized to PFC pyramidal spines, are linked to psychiatric disorders (Kirkpatrick et al.,
Alleles of PIP5K2A, a phosphoinositol pathway regulator of KCNQ function, are associated with schizophrenia (Fedorenko et al., 2008). Thus, M1R and its downstream mediators offer an attractive target for pharmaceutical intervention in these disorders of cognition. Alzheimer’s disease is characterized by degeneration of ACh-producing basal forebrain neurons, and post-mortem histology has revealed abnormal muscarinic receptor expression in PFC, including decreased M1R protein (Flynn et al., 1995). Further, schizophrenic patients have decreased M1R expression in DLPFC (Dean et al., 2002). Some of the efficacy of clozapine-like atypical antipsychotics can be attributed to muscarinic regulation of dopamine signaling (Bymaster et al., 2003; Tzavara et al., 2004). The M1R agonist xanomeline (Bodick et al., 1997; Shekhar et al., 2008) has been investigated for clinical efficacy in treatment of schizophrenia and Alzheimer’s disease and the muscarinic allosteric modulator, PQCA, ameliorates scopolamine-induced deficits in cognitive performance in macaques (Uslaner et al., 2013). The present results further support a role of muscarinic receptors in higher-order cognitive processing in primates and encourage future examination of subtype-specific contributions.
Chapter 5 – References


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Chapter 6 – Curriculum Vitae

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Education

2015 The University of Western Ontario (London, ON)
M.Sc. Neuroscience
Supervisor: Prof. Stefan Everling
Thesis: Muscarinic attenuation of mnemonic rule representation in macaque dorsolateral prefrontal cortex during a pro- and anti-saccade task.

2013 The University of Western Ontario (London, ON)
B.M.Sc. Honors Specialization in Physiology and Pharmacology
Supervisor: Prof. Melvyn Goodale
Thesis: Localization of areas responsible for wrist posture selection in ambiguous situations.

Research Experience

July 2013–Present The University of Western Ontario, London, ON (Prof. Stefan Everling). Investigating the effects of cholinergic and dopaminergic drugs on isolated neurons in rhesus macaque dorsolateral prefrontal cortex. Experimental techniques employed:
• Iontophoretic single-unit electrophysiology and pharmacology
• Systemic injection and multi-electrode array electrophysiology
• Multi-barrel glass iontophoretic electrode construction
• Handling, chairing, and cleaning rhesus macaque monkeys

September 2012–August 2013 The University of Western Ontario, London, ON (Prof. Melvyn Goodale). Investigating wrist posture selection in the visuomotor system. Experimental techniques employed:
• Behavioural: kinematics (object grasping)
• Imaging: MRI
• Stimulation: TMS

May–August 2012 The University of Western Ontario, London, ON (Prof. Michael Poulter). Investigating the role of DNMT3B protein transfection on the expression of α1 subunit of GABA_A receptor in rat B35 neuroblastoma cells. Experimental techniques employed:
• Cell culturing, transfection, and Western blot
• RNA, DNA, and protein extraction
• Reverse transcription real-time quantitative polymerase chain reaction

May–August 2011 University of Waterloo, Waterloo, ON (Prof. Guy Guillemette). Investigating interactions between N-terminal spatial calcium transforming element of voltage-dependent calcium channels and calmodulin. Experimental techniques employed:
• Restriction digestion
• Recombinant protein expression and gel electrophoresis

Manuscripts in Progress


Programming Experience

2014 JavaScript
2013 MATLAB
2012 Java
Teaching Experience

**September 2014–December 2014** Physiology 4710A Online Teaching Assistant: Physiology of the Senses, Prof. Tutis Vilis, The University of Western Ontario.

**September 2013–April 2014** Psychology 1000 Online Teaching Assistant: Introduction to Psychology, Prof. Terrence C. Biggs, The University of Western Ontario.

Leadership

2015 Schulich Graduate Council Neuroscience Representative, The University of Western Ontario.

2015 Biological Sciences Chief Steward and Finance Committee Member, PSAC610 Graduate Teaching Assistant and Postdoc Union, The University of Western Ontario.

2014 Neuroscience Departmental Steward and Finance Committee Member, PSAC610 Graduate Teaching Assistant and Postdoc Union, The University of Western Ontario.

Awards

2015/2016 Ontario Graduate Scholarship. $15,000.

2013/2014 & 2014/2015 Western Graduate Research Scholarship. $6100 per year.


2006 Tied for 26th internationally in University of Waterloo Pascal Math Contest.

Posters

