Sex Differences in Nicotinic Currents of Layer VI Neurons of Prefrontal Cortex During Development

by

NYRESA CYNTHIA ALVES

A thesis submitted in conformity with the requirements of the degree of Master of Science, Graduate Department of Physiology Faculty of Medicine University of Toronto

© copyright by Nyresa Cynthia Alves, 2009
There is a large sex difference in the prevalence of attention deficit disorder; yet, little is known about sex differences in prefrontal attention circuitry. We investigated sex differences in the developmental nicotinic excitation of corticothalamic layer VI neurons, which play an important role in attention. Using whole cell recording in prefrontal brain slices, we examined the inward currents elicited by nicotinic stimulation in rodents. We found a prominent sex difference in the currents during the first postnatal month when males had significantly greater $\alpha_4\beta_2^*$ nicotinic currents. Immunohistochemical analysis of $\alpha_4YFP$ mice revealed no sex difference in the pattern or proportion of YFP-positive neurons in layer VI. Further electrophysiological experiments revealed that progesterone is able to rapidly and significantly suppress nicotinic currents in layer VI neurons. This is the first illustration at a cellular level that prefrontal attention circuitry is differently excited by nicotinic stimulation in males and females during development.
Acknowledgements

I thank God for enlightening me and for blessing me with my graduate school experience.

Special thanks to my supervisor, Dr. Evelyn Lambe, for her guidance, support and friendship through the course of this project. In addition to providing me with every opportunity to learn and grow as a scientist, she has also kept my professional development a priority. I cannot express enough my gratitude towards her unrelenting dedication to her students, passion for high-quality research and strong work ethic. She has been and continues to be a positive influence in my life.

Many thanks to my friends in Dr. Lambe’s lab - Sameera Kassam, Nathalie Goodfellow, Eliane Proulx, and Dr. Craig Bailey - for their generosity, kindness, suggestions and constant support and encouragement. Their hard work and achievements have been a source of inspiration to me. Thank you to my supervisory committee members, Dr. Sheena Josselyn and Dr. Richard Horner, for their guidance and comments throughout this project. Their inputs have made my graduate school experience challenging and rewarding.

Finally, I thank my family for their love and support. My experience here leaves me with many new skills, rich experiences and a deep appreciation for research in neuroscience and physiology.
# Table of Contents

Section 1  Introduction..................................................................................1

1.1 Prefrontal Cortex.................................................................1

1.1.1 Prefrontal Cortex development......................................1

1.1.2 Prefrontal Cortex Layer VI Connections.......................2

1.1.3 Prefrontal Cortex role in Attention.................................4

1.1.4 Rodent Prefrontal Cortex...............................................4

1.2 Nicotinic Receptors ...............................................................5

1.2.1 Background on Nicotinic Receptors...............................6

1.2.2 Nicotinic Receptors in Layer VI during Development......8

1.2.3 Nicotine and Attention....................................................8

1.2.4 Developmental role of Nicotinic Receptors in Prefrontal Cortex...9

1.2.5 Potential Function of Nicotinic Excitation during Development..10

1.3 Sex Differences.................................................................11

1.3.1 Sex Differences in Nicotinic Receptors..........................11

1.3.2 Sex Differences in the Brain...........................................11

1.3.3 Sex Differences in Behaviour........................................12

1.3.4 Sex Differences in Nicotine and Acetylcholine Metabolism.....13

1.4 Sex Steroids in the Brain during Development.....................14

1.4.1 Sex Steroid-related Sex differences during Development....14

1.4.2 Sex Steroid Hormones and Nicotinic Receptors................15

Section 2  Sex Differences in Nicotinic Excitation .......................17

2.1 Introduction..............................................................................18

2.2 Materials and Methods..........................................................19

2.3 Results ..................................................................................23

2.4 Discussion ...............................................................................41

Section 3  Conclusion and Future Work............................................46

3.1 Summary of Contributions......................................................46

3.2 Ongoing work on α5 knockout mice....................................47

3.3 Future Directions.................................................................49

Section 4  Bibliography.................................................................54
List of Figures

Figure 1.1 Connections between the thalamus and cortex ........................................3

Figure 1 Sex difference in nicotinic excitation during development.................................25

Figure 2 $\alpha_4\beta_2$ nicotinic receptors mediate nicotinic currents........................................27

Figure 3 Sex differences are observed across strains and species.........................................29

Figure 4 Acetylcholinesterase does not account for sex differences........................................31

Figure 5 Effects of nicotine on layer VI neurons..........................................................33

Figure 6 Nicotinic receptors in $\alpha_4$ YFP mouse..........................................................36

Supplementary Figure 1 Multiphoton YFP image..............................................................37

Figure 7 Progesterone inhibits nicotinic currents in layer VI neurons.................................40

Figure 3.1 Ongoing experiments in $\alpha_5$ knockout mice..................................................48

Figure 3.2 Neurosteroid biosynthetic pathway.................................................................50
### List of Abbreviations

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3βHSD</td>
<td>3-β-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>α4β2*</td>
<td>alpha4 beta2 [Nicotinic Receptors] containing an accessory subunit (denoted by *)</td>
</tr>
<tr>
<td>A</td>
<td>Amperes</td>
</tr>
<tr>
<td>ACSF</td>
<td>Artificial Cerebrospinal Fluid</td>
</tr>
<tr>
<td>ADHD</td>
<td>Attention Deficit Disorder</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-l-5-methyl-4-isoxazole-propionate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>CaCl2</td>
<td>Calcium Chloride</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DHβE</td>
<td>dihydro-β-erythroidine</td>
</tr>
<tr>
<td>IQ</td>
<td>Intelligence Quotient</td>
</tr>
<tr>
<td>FO</td>
<td>First Order</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-Aminobutyric acid</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HO</td>
<td>Higher Order</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>KOH</td>
<td>Potassium Hydroxide</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>K$_2$-ATP</td>
<td>Adenosine 5’Triphosphate Dipotassium salt</td>
</tr>
<tr>
<td>$\mu$</td>
<td>micro ($10^{-6}$)</td>
</tr>
<tr>
<td>m</td>
<td>milli ($10^{-3}$)</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>MgCl</td>
<td>Magnesium Chloride</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>Magnesium Sulphate</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>Magnesium ion</td>
</tr>
<tr>
<td>MLA</td>
<td>Methyllycaconitine</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>Sodium Bicarbonate</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$</td>
<td>Sodium Dihydrophosphate</td>
</tr>
<tr>
<td>Na$_2$-GTP</td>
<td>Adenosine 5’Triphosphate Disodium salt</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>$\Omega$</td>
<td>Ohms</td>
</tr>
<tr>
<td>%</td>
<td>percent</td>
</tr>
<tr>
<td>p</td>
<td>pico ($10^{-12}$)</td>
</tr>
<tr>
<td>±</td>
<td>plus or minus</td>
</tr>
<tr>
<td>RU486</td>
<td>Mifepristone</td>
</tr>
<tr>
<td>s</td>
<td>seconds</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error Mean</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris(hydroxymethyl)aminomethane buffer saline</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>vol/vol</td>
<td>volume per volume</td>
</tr>
<tr>
<td>wt/vol</td>
<td>weight per volume</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow Fluorescent Protein</td>
</tr>
</tbody>
</table>
Section 1: Introduction

Prefrontal Cortex

The prefrontal cortex is thought to play a key role in cognitive processing, and is particularly involved in attention tasks (Kastner and Ungerleider, 2000; Corbetta and Shulman, 2002). The prefrontal cortex is also suggested to be highly vulnerable to a wide variety of early developmental insults. For example, in utero exposure to nicotine can increase the risk of ADHD, lower IQ and conduct disorders later in life (Ernst et al., 2001; Wakschlag et al., 2002). Cortical nicotinic acetylcholine receptors are important for attention and are particularly important in the development of attention circuitry. The presence of functional nicotinic acetylcholine receptors in the prefrontal cortex make it a potential target for developmental changes caused by alterations in receptor function due to nicotine exposure. Inappropriate activation of these receptors may also alter normal cortical maturation since nicotinic receptors have been implicated in altering synaptic plasticity in the prefrontal cortex (Couey et al., 2007).

1.1.1 Development of the Prefrontal Cortex

The layered structure of the mature cerebral cortex is formed during development. The deep layers mature first, followed by middle and upper layers. The different cortical layers each contain a characteristic distribution of neuronal cell types and connections with other cortical and subcortical regions. Staining cross-sections of the cortex to reveal the position of neuronal cell bodies and the intracortical axon tracts allowed neuroanatomists in the early 20th century to produce a detailed description of the laminar structure of the cortex in different species. The neurons of the cerebral cortex are grouped into six main layers, from outside (pial surface) to inside (white matter), as described by Korbinian Brodmann in 1909.

The first pyramidal neurons generated migrate out of the ventricular zone and together with Cajal-Retzius cells form the preplate. A cohort of neurons migrating into
the middle of the preplate divides this transient layer into the superficial marginal zone, which will become layer one of the mature cortex. The subplate forms a middle layer called the cortical plate, which forms the deep layers of the mature cortex, layers five and six. Later born neurons, which will form layers two and three, migrate radially into the cortical plate past the deep layer neurons and become the upper layers. Thus, the layers of the cortex are created in an “inside-out” order.

### 1.1.2 Prefrontal Cortex Layer VI Connections

Layer VI of the prefrontal cortex contains interneurons and pyramidal neurons, which send efferent fibres to the thalamus, establishing reciprocal interconnections between the cortex and the thalamus (Creutzfeldt, 1995). Layer VI neurons in the prefrontal cortex are primarily corticothalamic. In addition, there are characteristic cortical connections mediated via local interneurons.

The cortex receives sensory input from the thalamus; however, corticothalamic projections to nuclei in the thalamus are more extensive than thalamocortical projections from the same thalamic nucleus as indicated in the schematic shown in **Figure 1.1** (Sherman, 2005). It is important to note that corticothalamic axons from layer VI target not only relay cells but also local (GABAergic) inhibitory neurons, which include cells of the thalamic reticular nucleus and local interneurons (Sherman et al., 2006). With this neural arrangement, layer VI corticothalamic neurons can gate thalamic inputs and engage in cortico-cortical communication via the thalamus (see Figure 1.1 C).
A) **Conventional View**

Cortex → Cortical area 1 → Cortical Area 2 → Cortical Area 3

Thalamus

Periphery

Motor Output

B) **Cortical communication via a corticothalamocortical pathway**

![Diagram of corticothalamocortical pathway]

C) **Alternative view**

Cortex → Cortical area 1 → Cortical Area 2 → Cortical Area 3

Thalamus (FO) → Thalamus (HO) → Thalamus (HO)

Periphery

Motor Output

**Figure 1.1:** Comparison of conventional and alternate views, inspired by Sherman, 2005. A) Conventional view showing peripheral input to the cortex via the thalamus staying within the cortex. B) An alternate view showing that cortical communication may occur
via the thalamus. C) Higher order relays are a key link in the corticothalamocortical route for cortical processing. (FO = First order; HO = Higher order)

1.1.3 Prefrontal Cortex role in Attention

The prefrontal cortex plays well-known roles in higher cognitive functions such as attention and working memory. When engaged in attention tasks, there is a reduction in the activation of the prefrontal cortex (Hugdahl et al., 2009). As attention tasks increase in difficulty, there is an increase in the deactivation of the prefrontal cortex (Fassbender et al., 2009). Studies have demonstrated attentional performance-associated increases in cortical acetylcholine release, which supports the hypothesis that the cortical cholinergic input system represents a core component of circuitry that mediates attention processes (Everitt and Robbins, 1997; McGaughy et al., 2000; Hasselmo and McGaughy, 2004; Kozak et al., 2006). However, assessing the synaptic activation of acetylcholine at cortical nicotinic acetylcholine receptors is difficult to demonstrate partly due to the nature of the cholinergic innervation of the cortex. The major source of cortical acetylcholine is the nucleus basalis in the basal forebrain, whose cholinergic neurons are mixed with noncholinergic neurons, and whose cholinergic projection is diffuse (Rye et al., 1984). The cortex also receives cholinergic inputs from cholinergic interneurons in the cortex (Engelhardt et al., 2007). These cholinergic actions in the cortex appear to involve tonic release via “volume transmission” where acetylcholine is released far from the postsynaptic target site (Umbriaco et al., 1994).

1.1.4 Rodent Prefrontal Cortex

The cerebral cortex of a rat is about a thousand times smaller than that of humans (Uylings et al., 1990). Yet, the rodent prefrontal cortex is used as a potential model for human disease states related to higher cognition. It is therefore important to compare cortical areas in the rat brain to cortical areas in primates.

In rats and primates, the entire neocortex receives cholinergic innervation from the basal forebrain (Uylings et al., 2003). In the rat, the medial prefrontal cortex is
divided into subregions with the most dorsal being the rostral portion of the anterior cingulate, the middle being the prelimbic and the most ventral being the infralimbic cortex. In the primate, these areas are termed the anterior cingulate cortex. The rat medial prefrontal cortex is agranular cortex lacking a layer IV, similar to the primate anterior cingulate cortex. With regards to the pattern of connections, the prefrontal cortex has been traditionally defined as the region rostral to motor and premotor areas and the prominent cortical projection area of the medial dorsal nucleus of the thalamus (Groenewegen et al., 1990; Uylings and van Eden, 1990; Uylings et al., 2003). The medial dorsal nucleus of the thalamus is no longer viewed as the defining connections and many thalamic nuclei project to the prefrontal cortex including the intralaminar and midline nuclei and the anterior medial nucleus (Uylings et al., 2003).

In humans, the anterior cingulate cortex is associated with selection of action or attention to response selection (Petersen et al., 1988; Frith et al., 1991; Paus et al., 1993). Damage to the medial prefrontal cortex in rodents has been shown to cause deficits in various types of attentional tasks (Muir et al., 1996) and tasks requiring a reversal or shift of attention from one set of cues to another (Birrel et al., 2000; De Bruin et al., 2000; Frysztak et al., 1994; Kolb et al., 1991). Thus, with regards to function, there are notable similarities in the primate and rodent prefrontal cortex.

1.2 Nicotinic Receptors

Abnormalities in binding of nicotinic receptors have been demonstrated in autism, epilepsy, schizophrenia, Alzheimer’s disease, Parkinson’s disease and Lewy Body Dementia (Perry et al., 2001; Martin-Ruiz et al., 2004; Picard et al., 2006; Breese et al., 2000; Marutle et al., 2001; Pimlott et al., 2004; Oishi et al., 2007). Mutations in one of the nicotinic acetylcholine subunit (β2) genes are responsible for a specific form of autosomal dominant frontal lobe epilepsy and predisposition to schizophrenia (Chini et al., 1994; Steinlein et al., 1995; Freedman et al., 1997). Neuronal nicotinic receptors are thus a potential therapeutic target in disorders such as Alzheimer’s disease, Parkinson’s disease and Tourette’s syndrome, where nicotine is observed to have protective effects.
and improvements in memory and attention (Quik et al., 2008; Jones et al., 1992; Vidal et al., 1996; Newhouse et al., 1997).

1.2.1 Background on Nicotinic Receptors

Nicotinic acetylcholine receptors are cholinergic receptors that form ligand-gated ion channels. These receptors are ionotropic and do not make use of a second messenger. Nicotinic receptors are made up of five subunits, arranged around a central pore and consist of various homomeric or heteromeric combinations of nicotinic receptor subunits.

In the rat, a total of eight ligand-binding α and three structural β subunits have been cloned, which in various combinations of α and β subunits, form distinct heteromeric or homomeric pentameric receptor subtypes (Couturier et al., 1990; Seguela et al., 1993; Sargent, 1999; Corringer et al., 2000). Two main subfamilies of neuronal nicotinic acetylcholine receptors subtypes have been identified: α-bungarotoxin-sensitive and α-bungarotoxin-insensitive nicotinic receptors. The α-bungarotoxin-sensitive receptors can be homopentameric (α7, α8 and α9) or heteropentameric (α7 α8 and α9 α10), whereas α-bungarotoxin-insensitive receptors are only heteropentameric and consist of α (α2–α6) and β (β2–β4) subunits (Gotti et al., 2004). In agreement with the data on mRNA distribution, receptor subtypes containing the α3, α6, β3 or β4 subunits have a relatively restricted distribution in the brain. α6 mRNA is restricted to a few nuclei throughout the brain: the locus coeruleus, ventral tegmental area, substantia nigra, reticular thalamic nucleus, supramamillary nucleus and the mesencephalic V nucleus (Novere et al., 1996). α9 is mainly found in olivocochlear neurons (Elgoyhen et al. 1994, 2001; Sgard et al. 2002). In neuronal populations in which these rare receptor subtypes are expressed, they can constitute the main subpopulations of nicotinic acetylcholine receptors with significant function.

In the cortex and hippocampus, α2 mRNA expression appears postnatally, exhibiting a rapid increase in intensity and numbers of α2-positive neurons during the first postnatal week, followed by a sharp decline (Son and Winzer-Serhan, 2006). This
transient developmental upregulation in cortical structures has also been reported for α3, β4, α5 and α7 subunits (Adams et al., 2002; Winzer-Serhan and Leslie, 1997, 2005). α2 mRNA expression is restricted to at least one subpopulation of non-pyramidal neurons in cortical layers V and VI (Son and Winzer-Serhan, 2006). α3 mRNA has been observed in the hippocampus, the medial habenula, the lateral geniculate, the granular layer of the cerebellum, as well as in the pineal gland; moderate levels were found in a few nuclei of the thalamus and in the deeper layers of the cerebral cortex in monkeys (Cimino et al., 1992). In the rodent brain, α3 mRNA was primarily found in layer IV (Cimino et al., 1992). Dihydro-β-erythroidine (DHβE) is an antagonist of high-affinity nicotinic receptors, with the α4β2 subunit combination being most sensitive (IC$_{50}$ = 4 nM) and the α3β2 subunit combination being least sensitive (IC$_{50}$ = 127 nM) (Luetje et al., 1989). DHβE is often identified as α4β2 nicotinic receptor since the α4β2 subtype constitutes the principal nicotinic acetylcholine receptor subtype in subregions such as the cortex, striatum, superior colliculus, lateral geniculate nucleus and cerebellum (Picciotto et al., 2005; Gotti et al., 2005; Turner et al., 2005).

In the most common form of nicotinic receptor, the α4β2 nicotinic receptor subtype, the acetylcholine-binding site is formed from amino acid residues of α and β subunits in the extracellular domain. Two acetylcholine-binding sites are thought to exist at the interface between α and β subunits of α4β2 nicotinic receptors (Alkondon and Albuquerque, 1993). Nicotinic receptors may exist in different conformational states. Binding of agonist stabilizes the open and desensitized states. Opening of this receptor near the resting potential of cortical neurons allows for net positive current influx, which causes the cell to depolarize. This current is predominantly sodium entering the cell with a small amount of potassium exiting.

The second most common form of nicotinic receptor is the α7-containing receptors, which are fast desensitizing in the presence of agonist and have high calcium permeability (Zhang et al., 1994; Castro and Albuquerque, 1995). α4β2 nicotinic receptors are high-affinity receptors with slower rates of desensitization compared to α7 nicotinic receptors (Fenster et al., 1997). Since nicotinic receptors activate and
desensitize, it is important to note that potential detrimental effects of nicotine exposure may occur through *activation and/or desensitization* of nicotinic receptors which disrupts the activation of the receptor by endogenous acetylcholine.

### 1.2.2 Nicotinic Receptors in Layer VI during Development

Previous studies have shown an increase in nicotinic receptor binding in layer VI of the cortex during development (Tribollet et al., 2004). A developmental study using whole cell recordings of prefrontal layer VI corticothalamic neurons has shown a developmental upregulation of nicotinic excitation (Kassam et al., 2008). I have contributed to experiments in this study and am a co-author of this paper. This study has also suggested that layer VI nicotinic currents may have unusual properties because of the potential incorporation of a rare accessory α5 nicotinic receptor subunit (Kassam et al., 2008). This subunit is expressed in layer VI (Marks et al., 1992; Winzer-Serhan and Leslie, 2005) and can substantially alter the properties of α4β2* nicotinic receptors (Ramirez-Latorre et al., 1996; Tapia et al., 2007; Kuryatov et al., 2008). My electrophysiology recordings in layer VI reveal dramatically lower nicotinic currents in α5 knockout mice compared to controls during development. Ongoing experiments on α5 knockout mice are mentioned in Section 3 of this Master’s dissertation.

### 1.2.3 Nicotine and Attention

Endogenous cholinergic signaling in the brain is important in mediating attention. Lesions of cortical cholinergic inputs to the cortex have deleterious effects on attentional performance and attentional performance is associated with increase in cortical acetylcholine release (Everitt and Robbins, 1997; McGaughy et al., 2000; Hasselmo and McGaughy, 2004; Sarter et al., 2005). Both muscarinic and nicotinic receptors are believed to be important at different stages of information processing in attention tasks: nicotinic receptors may be important in the early stages of stimulus evaluation, whereas muscarinic receptors may be important in later processing stages involving response selection (Mirza and Stolerman, 2000). Nicotinic receptors seem to play a particularly important role in attention under difficult conditions such as stress, fatigue, and
psychiatric and neurological illnesses. The nicotinic receptors that are expressed in the prefrontal cortex play a key role in these attentional processes. The binding of nicotine to nicotinic receptors is the presumed event that mediates the heightened attentional performance (Newhouse et al., 2004) and improved reaction times (Ernst et al., 2001) during smoking. Thus, both endogenous (acetylcholine) and exogenous (nicotine) agonists of nicotinic receptors play key roles in mediating enhanced attention performance.

1.2.4 The Developmental Role of Nicotinic Receptors in the Prefrontal Cortex

The fetal brain expresses nicotinic receptors at a very early stage (Cairns and Wonnacott, 1988; Sugiyama et al., 1985; Zoli et al., 1995) providing a window of vulnerability to developmental nicotine exposure during critical developmental processes. Understanding the neurobiological mechanisms involved in the consequences of early nicotine exposure merits exploration since developmental exposure to nicotine appears to have deleterious effects on cognitive and attentional processes. Epidemiology studies try to isolate the effects of in-utero nicotine by controlling for socio-economic status, maternal stress and other drug use. The use of rodents is critical in eliminating confounds related to maternal mental health, socio-economic status and education, the home environment, and genetic susceptibility of mother and child to psychiatric illness (Shenassa et al., 2003; Winzer-Serhan, 2008). This allows us to assess the effects of nicotine alone on attention circuitry in a homogenous population.

Due to developmental differences, the first three postnatal weeks in the rodent appear to correspond to the third trimester of human pregnancy, particularly for thalamic and cortical development (Dobbing and Sands, 1979; Eppolito and Smith, 2006). The β2 subunit, which is required for high-affinity nicotinic receptors and which is expressed on corticothalamic neurons, is seen to be important in establishing normal fear-associated learning (passive-avoidance) during the developmental period (King et al., 2003). This strongly suggests a developmental role in influencing cognitive differences manifested
later on. Nicotinic exposure during this time can alter the function of nicotinic receptors and have detrimental affects on cognitive functions later in life.

Cholinergic signaling through nicotinic receptors plays an important role in brain maturation. Disruption in cholinergic innervation during early postnatal development causes delayed cortical neuronal development and permanent changes in cortical cytoarchitecture and cognitive behaviours (Sherren et al., 2005; Goldman et al., 1974). The rat prefrontal cortex shows strong developmental changes in nicotinic signaling of pyramidal cells in layer VI, with nicotinic currents reaching its peak during postnatal week three (Kassam et al., 2008). In a similar pattern, nicotinic receptors in human brain also reach its highest density towards birth after which levels of nicotinic receptors start to decline gradually (Court et al., 2000). At this time in development, the prefrontal cortex receives cholinergic inputs from the nucleus basalis and local cholinergic interneurons (Mechawar et al., 2001). It is also important to note that, during this time, acetylcholinesterase is expressed in deep layers of cingulate cortex (Kristt et al., 1983; 1991). This suggests that acetylcholine stimulation of neurons in this region is tightly controlled during its postnatal development by acetylcholinesterase.

1.2.5 The Potential Function of Nicotinic Excitation during Development

The peak of nicotinic excitation of layer VI neurons during development coincides with the peak of maturation and synaptogenesis in the prefrontal cortex (Bourgeois, 1997). This maturation may involve the conversion of “silent synapses” which only contain NMDA receptors into “active synapses” which have AMPA and NMDA receptors. Since immature synapses only have NMDA receptors, they are electrophysiologically “silent” without depolarization to relieve the Mg$^{2+}$ block. The presence of nicotinic receptors could contribute to the maturation of synapses by providing a source of depolarization to help unsilence silent synapses. Since the activation or “AMPAfication” of silent synapses depends on calcium, the excitation of the depolarizing and highly calcium-permeable $\alpha 4\beta 2\alpha 5$ nicotinic receptors may play a key role in converting silent synapses to active synapses. Calcium entry through the nicotinic receptors may help with the recruitment of AMPA receptors in unsilenced synapses. The elimination of about 70% of silent synapses
in layer VI of the immature visual cortex is dramatic during the first four postnatal weeks (Rumpel et al., 2004). Interestingly, this time period in which these synapses become active coincides with the peak in nicotinic currents in layer VI (Kassam et al., 2008). Thus, nicotinic activation may affect neuronal circuitry involving corticothalamic and thalamocortical neurons, which are responsible for gating and relay of sensory information. It is likely that the developmental excitation of high-affinity nicotinic receptors during this period of development influences and fine-tunes the maturation of glutamatergic synapses. Further studies examining the effects of the developmental peak of nicotinic currents on unsilencing of synapses during this important period of cortical wiring are required.

1.3 Sex Differences

1.3.1 Sex Differences and Nicotinic Receptors

Previous studies have reported marked sex differences in sensitivity to nicotine. One example is the upregulation of nicotinic receptors in response to early nicotinic exposure, where males have more pronounced upregulation than females exposed to nicotine (Koylu et al., 1997). Calderone et al. (2008) have reported sex differences in the anxiolytic effects of nicotine. In their study, female rats were more sensitive to nicotine’s anxiolytic effects in the social interaction test but less sensitive in the elevated plus maze. Sex differences have also been reported in the developmental regulation of nicotinic receptors in midbrain dopamine neurons (Azam et al., 2007). This sex-specific regulation of nicotinic receptors during development will help understand sex differences in behaviours manifested early during development.

1.3.2 Sex Differences in the Brain

There are a number of sex differences in the brain and in the prevalence of psychiatric disorders such as depression and attention deficit disorders. The differences that begin early in development can occur due to a combination of genetic and hormonal events and
continue throughout the lifespan of an individual (Arnold et al., 2004; Becker et al., 2005). One example of this is during early critical periods of development, when the male brain is exposed to high levels of testosterone due to a testosterone surge at birth. This can cause the formation of neuronal cell groups and synaptic connections that control functions and behaviours that are more common in males than females. In other words, testosterone (and its metabolites such as 17β-estradiol) can contribute to the masculinization or feminization the brain permanently by acting during early critical periods of neuronal development.

The development of the cortex involves an overproduction of synapses followed by competitive elimination and pruning (Huttenlocher, 1979). These developmental processes may be under different control between the sexes and may also be involved in the emergence of anatomical differences in the cortex (Andersen et al., 2000). Bellis et al. (2001) have shown sex differences in cerebral maturation with males having more age-related grey matter decreases and white matter increases compared to females during childhood and adolescents in humans. Alonso-Nanclares et al. (2008) have looked at sex differences in the synaptic density of the temporal cortex and found that men have significantly higher synaptic density than women in all cortical layers of the temporal neocortex. This difference may represent an anatomical substrate contributing to the functional gender differences in brain activity.

Certain cognitive functions differ in men and women; however, the anatomical and functional substrates underlying these differences remain largely unknown. Cortical activity is generally related to higher brain function but only a few studies have focused on the cerebral cortex when searching for possible correlates of cognitive sex differences.

1.3.2 Sex Differences in Behaviours

There is a marked sex difference in the prevalence of conduct disorders and ADHD (Zahn-Waxler, 2008). It is believed that male gender is an independent risk factor in the development of ADHD (Romano et al., 2006). In addition, the male auditory attention
circuitry has been reported to have greater vulnerability to developmental nicotine exposure (Jacobsen et al., 2007). However, vast differences in the metabolizing rate of nicotine between males and females have been reported (Hatchell and Collins, 1980). Thus, male and female brains are exposed to different levels of nicotine in in-vivo studies. Our studies are able to remove these systemic confounds by applying nicotinic receptor agonists directly on brain slice to study the inherent sex differences between receptors under controlled pharmacological conditions. An understanding of the normal maturation and changes occurring in the brain during development and how these changes differ between sexes can bring us closer to elucidating the underlying pathophysiology of neurodevelopmental disorders with male or female preponderance.

1.3.3 Sex Differences in Nicotine and Acetylcholine Metabolism

Sex differences in in-vivo studies can arise through several potential mechanisms. Systemically-administered nicotine is metabolized by enzymes in the liver that differ between males and females. These pharmacokinetic variations in plasma and brain levels of nicotine can produce behavioural sex differences in responses to administered nicotine. One study showed that C57Bl/6 female mice eliminated nicotine faster than males (Hatchell and Collins, 1980). Similarly, studies in humans have found that women metabolize nicotine more rapidly, an effect that is related to estrogen (Benowitz et al., 2006).

There are also sex differences in the brain’s ability to metabolize acetylcholine. Akmaev et al. (1996), observed sex differences in acetylcholinesterase activity in the dorsal nucleus of the vagus in newborn rats during the critical period of sexual differentiation of the brain, when males had reliably greater amounts of acetylcholinesterase activity than females. Thus, brain cholinergic structures may be differently controlled between the sexes due to differing levels of acetylcholinesterase activity.
1.4. Sex Steroids in the Brain During Development

The early developmental period occurs before puberty and before the surge of gonadal hormones. The main source of steroid hormones in the brain can include the adrenal gland, the maternal ovary, the placenta and de novo synthesis of sex hormones within the developing brain itself (see review by Wagner, 2008). Steroid hormones are synthesized from cholesterol in the adrenal gland, gonads and placenta and their lipophilicity allows for passage across the blood-brain barrier. Thus, circulating systemic sex hormones can mediate its effects on the developing brain. Sex steroids can also be synthesized de novo in the developing brain from cholesterol.

Previous studies have suggested that neurosteroids can be synthesized by oligodendrocytes and released at concentrations of up to 0.1 μM (Baulieu et al., 1990). The developing rodent brain expresses all the enzymes necessary for the de-novo synthesis of progesterone from cholesterol (Compagnone et al., 1995; Kohchi et al., 1998; Sakamoto et al., 2001; Ukena et al., 1998, 1999; Zwain et al., 1999). Previous experiments have indicated that the presence or absence of steroid hormones during the critical period of brain differentiation may promote the development of sex differences in cognitive abilities. Goldman-Rakic and collaborators provided evidence that sex differences in the development of learning abilities are the result of the actions of steroid hormones on the maturation of specific brain areas related to learning abilities (Goldman et al., 1974).

1.4.1 Sex Steroid-related Sex Differences in the Brain During Development

During the early period of development, hormonal influences can permanently alter the development of the brain, producing effects which become apparent in later life (MacLusky et al., 1979). Estrogen and progesterone receptors are found in the cerebral cortex very early in development. The significance of their presence may allow for the cerebral cortex to be responsive to estrogen and progesterone during early postnatal development, thus allowing neurosteroids to modify cerebral cortical development. Studies indicate that mouse 8-day-old cortex and preoptic area in the female animal have
more progestin receptor cells than those in the males and demonstrate that progestin receptor cells are localized in a region of the cortex that are also known to contain few estrogen target cells (Shughrue et al., 1991). These results further suggest that a sexual dimorphism in progestin cell number may result in a differential effect of progestin on the cortex and preoptic area of the mouse, perhaps establishing a dimorphism in development and function (Shughrue et al., 1991). In relation to my studies, both estrogen and progesterone have also been suggested to be allosteric modulators of nicotinic acetylcholine receptors (Damaj 2000; Valera et al., 1992; Bertrand et al., 1991).

1.4.2 Sex Steroid Hormones and Relevance to Nicotinic Receptors

The function of neuronal nicotinic acetylcholine receptors is subject to modulation by steroids that do not bind to the classical acetylcholine sites. Steroids have the ability to desensitize nicotinic receptors by action at a site distinct from the acetylcholine site (Bertrand et al., 1991). The influence of sex steroids on cholinergic function in the cortex suggests that neurosteroids play an important role in the proper development and maintenance of the cortex and its connections to other brain regions. In addition to allosteric modulation of nicotinic receptors, sex steroids may also play a role in post-translational modification of nicotinic receptor subunits that affect receptor composition and permeability.

Several findings support the view that the perinatal hormonal environment can affect brain maturation by influencing neuronal connectivity at the cortical level. While steroid hormones have been suggested to activate and organize neural circuits during development (Romeo et al., 2002), sex differences very early in development may also arise due to differences in sex chromosomes (Arnold et al., 2004), without the need for any hormonal influence. Studies on the modulation of nicotinic receptor excitation of layer VI neurons will help us understand how attention circuitry develops differently between males and females and may provide insight to the reasons for the large male preponderance of attention deficit disorder. Studies that further examine the roles of these steroids during brain development are crucial in order to explore specific roles played by mediators of sex differences in cognitive abilities.
Objectives:

There is a large sex difference in the prevalence of attention deficit disorders; we, therefore, investigated sex differences during the development of neural circuitry involved in attention – layer VI of the prefrontal cortex.

1) We first examined sex differences in the function of nicotinic acetylcholine receptors in layer VI of the prefrontal cortex, which is a key component of the attention circuitry. Specifically, we examined sex differences in the inward currents elicited in layer VI pyramidal neurons by acetylcholine in early postnatal development.

Our slice electrophysiology approach allows us to examine functional receptors and estimate the effects of nicotine on nicotinic receptor desensitization without confounds that arise from different metabolizing rates between sexes. Studying this component of the prefrontal cortex is important in determining the specific site in the attention network that can cause sex differences to arise during development.

2) We investigated potential sex differences in:
   i) Acetylcholinesterase;
   ii) Distribution pattern of nicotinic receptor expressing cells; and
   iii) Modulation of nicotinic receptor function by sex steroid hormones.

Characterizing the sex differences of layer VI nicotinic acetylcholine receptor function and modulation during development can help us understand the role of nicotinic acetylcholine receptors in prefrontal cortex maturation and its role in sex differences in early-onset behaviours.
Section 2: Sex differences in nicotinic excitation of prefrontal cortical neurons during development

Abstract

There is a large sex difference in the prevalence of attention deficit disorder; yet, relatively little is known about sex differences in prefrontal attention circuitry. In male rats during development, nicotinic acetylcholine receptors excite corticothalamic neurons in layer VI, which play an important role in attention by gating the sensitivity of thalamic neurons to incoming stimuli. Here, we investigate sex differences in the developmental nicotinic excitation of these layer VI neurons. Using whole cell recording in prefrontal brain slices, we examined the inward currents elicited by nicotinic stimulation in rats and two strains of mice. We found a prominent sex difference in the currents during the first postnatal month when males had significantly greater nicotinic currents in layer VI neurons compared to females. Since the nicotinic currents in males and females were mediated by $\alpha 4\beta 2*$ nicotinic receptors, we examined potential sex differences in the distribution of the receptors using a line of knock-in mice in which all $\alpha 4$-containing nicotinic receptors are labeled with YFP. Immunohistochemical analysis revealed no sex difference in the pattern or proportion of YFP-positive neurons in layer VI. Further electrophysiological experiments tested the effects of sex steroids on nicotinic currents and found that progesterone is able to rapidly and significantly suppress nicotinic currents in layer VI neurons. This is the first illustration at a cellular level that prefrontal attention circuitry is differently excited by nicotinic stimulation in males and females during development.


2.1 Introduction

Attention deficit disorders are at least twice as prevalent in males than females (Brown et al., 2001; Cuffe et al., 2005; Smalley et al., 2007), yet the neurobiology behind this sex difference is not well understood. The normal development of the prefrontal cortex is critical for executive functions including attentional control (Shaw et al., 2007, Sullivan et al., 2003, Krain et al., 2006). Children with attention disorders appear to have higher activation of the prefrontal cortex at baseline and less change in its activation and synchronization with other cortical regions during the performance of attention tasks (Fassbender et al., 2009). Within the prefrontal cortex, the corticothalamic neurons of layer VI are thought to play a key role in this cortical synchronization and also play a role in the thalamic gating necessary for attention (Sherman et al., 2006). However, very little is known about sex differences in the development of layer VI.

Recent work has shown that layer VI corticothalamic neurons in male rats are prominently excited by nicotinic acetylcholine receptors during early postnatal development (Kassam et al., 2008). This time period is developmentally equivalent to the last trimester of human gestation (Romijn et al., 1991; Watson et al., 2006). Importantly, during this time, the prefrontal cortex is highly vulnerable to toxins and developmental insults (Sullivan et al., 2003), which predispose individuals to subsequent attention disorders. For example, prenatal exposure to the drug nicotine increases the risk of attention deficits (Ernst et al, 2001; Langley et al., 2005), particularly in males (Jacobsen et al., 2007). Interestingly, polymorphisms in the $\alpha_4$ nicotinic receptor subunit found in layer VI corticothalamic neurons have been associated with differences in performance on attention tasks (Epeseth et al., 2007; Todd et al., 2003; Rigbi et al., 2008). However, most of these studies have not compared attentional performance by sex.

Nicotinic receptor levels and function can be modulated by the sex steroid progesterone (Bertrand et al., 1991; Valera et al., 2001; Damaj et al., 2001), which is produced at higher levels in the developing female cortex (Kato et al., 1984). However, it is not known whether there are sex differences in the modulation of layer VI corticothalamic neurons by nicotinic acetylcholine receptors during development. Here, we address this question with whole cell recording in acute brain slices of rodent prefrontal cortex across early postnatal development. This technique allows us to assess
the function of nicotinic receptors on layer VI pyramidal neurons and the effects of nicotine on these cells, without the confound that would arise in vivo due to different rates of systemic metabolism of nicotine in male and female rodents (Hatchell and Collins, 1980). Our study also investigates the distribution of nicotinic receptor-expressing neurons in layer VI in males and females and the effects of progesterone on the excitation of layer VI pyramidal neurons by nicotinic acetylcholine receptors.

2.2 Materials and Methods

Brain Slice Preparation

The University of Toronto Animal Care and Use Committee approved the following protocols. These protocols conformed to international guidelines on the ethical use of animals. 400 μm-thick coronal slices of the medial prefrontal cortex were prepared from male and female FVB mice (postnatal (P)7-P34), C57Bl/6 mice (P7-P28), and Charles River Sprague-Dawley rats (P14-28). The brain was cooled as rapidly as possible with 4 °C oxygenated sucrose artificial cerebrospinal fluid (ACSF) (254 mM sucrose was substituted for NaCl). Prefrontal slices were cut from anterior to posterior using the appearance of white matter and the corpus callosum as anterior and posterior guides to target recording to the Cg1, Cg2 and PrL regions (Paxinos and Watson, 2004).

The slices were cut on a Dosaka Linear Slicer (SciMedia, Costa Mesa CA) and were transferred to room temperature oxygenated ACSF (128 mM NaCl, 10 mM D-glucose, 24 mM NaHCO3, 2 mM CaCl2, 2 mM MgSO4, 3 mM KCl, 1.25 mM NaH2PO4; pH 7.4) in a prechamber (Warner Instruments, Hamden CT) and allowed to recover for at least 1 hour prior to the beginning of an experiment. For whole cell recording, slices were placed in a modified superfusion chamber (Warner Instruments, Hamden CT) mounted on the stage of an Olympus BX50WI microscope (Olympus Canada, Markham ON). Regular ACSF at room temperature was bubbled with 95% oxygen and 5% carbon dioxide and flowed over the slice at 3–4 ml/minute.

Electrophysiology

Whole cell patch electrodes (2-3 MΩ) contained 120 mM potassium gluconate, 5 mM KCl, 2 mM MgCl, 4 mM K2-ATP, 0.4 mM Na2-GTP, 10 mM Na2-phosphocreatine, and
10 mM HEPES buffer (adjusted to pH 7.33 with KOH). Medial prefrontal cortex layer VI neurons were patched under visual control using infrared differential interference contrast microscopy. In current-clamp, neurons were recorded at their resting potentials. In voltage-clamp, neurons were held at –75 mV, near the equilibrium potential for chloride under our conditions, and currents were recorded using continuous single electrode voltage clamp mode with an EPC10 (HEKA Electronics, Mahone Bay NS), acquired and lowpass filtered at 3 kHz with Patchmaster 2.20 (HEKA Electronics, Mahone Bay NS).

Pharmacology
For most experiments, nicotinic currents were probed by adding 1 mM acetylcholine to the bath perfusion for a 15 s or 30 s interval after a one-minute baseline, followed by a five-minute washout period. Recordings were performed in the presence of atropine (200 nM) to block muscarinic receptors and methyllycaconitine (MLA) (10 nM) to block α7 nicotinic receptors. The peak current was measured in Clampfit (Molecular Devices) by subtracting the mean inward current at the peak of the acetylcholine response from the mean inward current at baseline. Subsequent applications of acetylcholine immediately following this protocol produced responses of similar magnitude to the initial acetylcholine response (See Figure 1A). Other drugs were also added to the bath in specific experiments: 10 nM MLA, 3 μM dihydro-β-erythroidine hydrobromide (DHβE), 1 mM carbachol, 300 nM nicotine hydrogen tartrate, 10 μM 2-hydroxypropyl β cyclodextrin, 10 μM water-soluble 17β-estradiol (cyclodextrin-conjugated form), and 10 μM water-soluble progesterone (cyclodextrin-conjugated form). All compounds were obtained from Sigma (Sigma Aldrich Canada, Oakville ON) or Tocris (Cedarlane Laboratories, Burlington ON) and stored in stock solutions at –20 °C before being diluted and applied to the slice in oxygenated ACSF.

Immunohistochemistry (This work was done by Dr. Craig Bailey)
A mouse line in which the nicotinic acetylcholine receptor α4 subunit has been labelled with the YFP motif has been generated and described previously (Nashmi et al., 2007). Immunohistochemistry for YFP was performed to identify the distribution pattern of neurons containing α4 subunits in layer VI of male and female medial prefrontal cortex.
Mouse brains were collected and 400 µm-thick coronal sections of the prefrontal cortex were made as described above for electrophysiology. For each mouse, immunohistochemistry for YFP was performed on a brain slice that was directly anterior to the corpus callosum, corresponding with approximately Bregma +1.34 mm to Bregma +1.74 mm (Paxinos and Franklin, 2001). Slices were incubated in oxygenated ACSF for one hour and were then fixed in a solution containing 4% (wt/vol) paraformaldehyde in 100 mM phosphate buffer (pH 7.5) overnight at 4 °C.

Free-floating sections were washed with Tris-buffered saline (TBS, 100 mM Tris and 150 mM NaCl, pH 7.5) and then incubated in 10% (wt/vol) bovine serum albumin (BSA), 0.25% (vol/vol) Triton X-100 and 4 drops/ml of a streptavidin solution (Vector Laboratories, Burlington ON) in TBS for 1 hour at room temperature. Sections were washed with TBS and incubated with a rabbit anti-GFP primary antibody (Invitrogen, Burlington ON, 1:200 dilution) with 3% (wt/vol) BSA, 0.25% (vol/vol) Triton X-100 and 4 drops/ml of a biotin solution (Vector Laboratories) in TBS for 72 hours at 4 °C. Sections were washed in TBS and incubated with a biotinylated goat anti-rabbit secondary antibody (Invitrogen, 1:500 dilution) with 3% (wt/vol) BSA and 0.25% (vol/vol) Triton X-100 in TBS for 24 hours at 4 °C. Sections were washed in TBS and then incubated with streptavidin labeled with Alexa Fluor 594 (Invitrogen, 1:500 dilution) with 3% (wt/vol) BSA and 0.25% (vol/vol) Triton X-100 in TBS for 24 hours at 4 °C. Sections were washed with TBS, mounted onto microscope slides and coverslipped using Fluoromount G (Southern Biotech, Birmingham, AL).

Imaging (This work was done by Dr. Craig Bailey)

Multiphoton imaging of the immunostained sections was performed using a Ti:sapphire laser (Mai Tai, Spectra Physics, Mountain View, CA) tuned to wavelength 780 nm and an Olympus Fluoview FV1000 microscope (Olympus, Markham, ON) with an Olympus XPlan N 25x, 1.05 NA water-immersion objective. Green and red fluorescence were separated with a dichroic mirror at 570 nm and filtered with green: 495-540 nm and red: 570-625 nm filters (Olympus), respectively. Multiphoton images containing green and red channels, measuring 500 µm x 500 µm (x,y), taken at equivalent depths from the top of the slice (approximately 12 µm deep), and having overlapping edges were captured.
with Olympus Fluoview FV10-ASW software. Six images were acquired per mouse covering the prelimbic and infralimbic areas of the medial prefrontal cortex from the pial surface to the white matter basal to layer VI. These images were then were stitched together to create one montage image (see Figure 6A and B for examples of red montages) using Image-Pro Plus software (Media Cybernetics, Bethesda, MD).

The proportion of neurons expressing the $\alpha_4\beta_2^*$ nicotinic receptor was measured by counting the total number YFP-immunoreactive neurons within a defined counting area of medial prefrontal layer VI in the red-channel montage and dividing by the total number of DAPI-positive neurons within that same counting area in the green-channel montage. The counting area was defined on the red-channel montages by first drawing a 750 µm-long basal line along the base of medial prefrontal layer VI (between layer VI and white matter) that generally extended along the base of the prelimbic and infralimbic areas. Next, a radial line was drawn at each end of the basal line that was perpendicular to the basal line and extended towards the pial surface, ending at the medial edge of the band of YFP-immunoreactive neurons. Last, a medial arc was drawn connecting the medial ends of the two radial lines and defining the curved medial length of the band of YFP-immunoreactive neurons. In addition to measuring the ratio of YFP: DAPI neurons within the counting area, the size of the area itself (as a function of the YFP-immunoreactive band thickness) was evaluated by measuring its area, and by measuring the mean distance between the basal line and the medial arc.

**Statistical Analysis**

We used parametric or non-parametric statistical tests respectively when the data under analysis passed or failed the Shapiro-Wilk test for normality ($p < 0.05$). The developmental changes in male and female nicotinic currents were assessed with Kruskal-Wallis nonparametric ANOVA and *post hoc* Mann-Whitney nonparametric $t$ tests. In order to test for gender and drug effects, DHβE, carbachol, nicotine and progesterone data were analyzed with two-way repeated measures ANOVA at a significance level of 0.05. *Post hoc* tests were performed to determine specific differences, when overall ANOVA results indicated significant effects of drug. Differences in acetylcholine response after nicotine exposure were determined using
Wilcoxon signed-rank nonparametric paired \( t \) tests, at a significance level of 0.05. Differences in intrinsic cell properties were examined with unpaired Student \( t \) tests, at a significance level of 0.05. All data are expressed as the mean ± SEM.

### 2.3 Results

**There are developmental differences in nicotinic currents in layer VI neurons in male and female FVB mice**

We find that layer VI pyramidal neurons in mouse prefrontal cortex are excited by nicotinic acetylcholine receptors. To observe and characterize the sex differences in nicotinic currents, we performed whole cell recordings at –75 mV in brain slices from male and female mice. The nicotinic inward currents were stimulated by bath application of acetylcholine (1 mM, 30 s) in the presence of atropine (200 nM) to block muscarinic receptors, the other subtype of acetylcholine receptors. Atropine is included in all subsequent experiments. Bath application of acetylcholine elicited inward currents in layer VI neurons as demonstrated in Figure 1 in males and females. We tested the reproducibility of the acetylcholine-elicited currents by our method of bath application. A similar response could be reproduced following a five-minute washout period in both males and females, indicating that this method of acetylcholine application does not alter the neurons’ responses to acetylcholine. The robustness of the peak response with the bath application makes it an ideal measure to compare across different pharmacological conditions to assess the properties of layer VI nicotinic currents.

In FVB mice, nicotinic inward currents in layer VI neurons showed significant developmental regulation as shown by Kruskal-Wallis ANOVA. Comparing the mean peak current amplitude across early postnatal weeks demonstrates the developmental upregulation of nicotinic excitation in male and female layer VI neurons as illustrated in Figure 1B. These findings are broadly consistent with earlier work in male rats showing that nicotinic currents in layer VI neurons are highest in the first postnatal month and then decline significantly and progressively to lower adolescent and adult levels (Kassam et al., 2008).
There are sex differences in the peak amplitude of layer VI nicotinic currents in FVB mice during development

Layer VI neurons from male and female FVB mice show nicotinic currents which are upregulated during the first postnatal month; however, we observed significant differences in the amplitude of the current elicited between the sexes. As illustrated in Figure 1C, there is a sex difference in acetylcholine-elicited nicotinic inward currents during postnatal weeks three and four. Two-way ANOVA showed an extremely significant effect of sex and postnatal week on nicotinic currents and a significant interaction between sex and postnatal week. Layer VI neurons from males had significantly higher currents than those from females during postnatal week three (males: 56 ± 5 pA, n = 41; females: 38 ± 5 pA, n = 43; Mann-Whitney test, P < 0.01). Similarly, layer VI neurons from males had significantly higher currents than those from females during postnatal week four (males: 66 ± 6 pA, n = 44; females: 25 ± 7 pA, n = 22; Mann-Whitney test, P < 0.01) During these weeks, there was no significant sex difference in the resting membrane potential (males: -77.4 ± 2.6 mV; females: -77.1 ± 2.4 mV; unpaired t test, P = NS) or input resistance (males: 246 ± 11 MΩ, females: 272 ± 12 MΩ; unpaired t test, P = NS). Spike amplitude was slightly greater in neurons from females than males during postnatal weeks three and four (males: 87.5 ± 5.4 mV, females: 94.4 ± 2.5 mV, unpaired t test, p < 0.05).
There is a developmental sex difference in nicotinic currents in layer VI neurons of prefrontal cortex in FVB mice. (A) Examples of voltage clamp traces from a P19 male and a P27 female showing nicotinic inward currents during bath application of acetylcholine (1 mM, 10 s). Line denotes acetylcholine application. Both, males and females have reproducible, non-desensitizing currents elicited by bath-applied acetylcholine, when given a five-minute washout duration. (B) Bar chart summarizing the mean amplitude of the peak inward current elicited by acetylcholine in FVB male (left panel) and female (right panel) mice in layer VI by postnatal week. The nicotinic inward current is present from postnatal weeks two to five. There is a highly significant developmental effect, in which the mean nicotinic current at postnatal week three is significantly higher than the mean inward current in postnatal weeks two and five in males. The mean nicotinic current at postnatal week four is significantly higher than the mean nicotinic current during postnatal week two in males. There is also a highly significant developmental effect in FVB females where the mean nicotinic current at postnatal week three is significantly higher than the mean inward current in postnatal weeks two, four and five. (C) Bar graph displays the sex difference in FVB mice in the average inward current caused by nicotinic receptor stimulation by 30 s bath-application of 1mM acetylcholine. Males have significantly greater currents than females during postnatal weeks three and four. All recordings are performed in the presence of atropine to block muscarinic receptors and MLA to block α7 nicotinic receptors in layer VI of FVB mouse prefrontal cortex. Males = black bars; females = open bars. (* P < 0.05, ** P < 0.01)
Layer VI nicotinic currents are mediated by $\alpha_4\beta_2^*$ nicotinic receptors in both male and female mice

To test our hypothesis that these nicotinic currents in layer VI neurons are mediated by $\alpha_4\beta_2^*$ nicotinic receptors, we investigated the effects of the competitive antagonist dihydro-β-erythroidine (DHβE) on the currents elicited by acetylcholine. We found that DHβE (3 μM, 10 min) almost completely suppressed the nicotinic currents in all layer VI neurons tested in both males and females, as illustrated in Figure 2. Two-way repeated measures ANOVA revealed a highly significant effect of DHβE ($p < 0.001$) and no significant interaction between sex and the effects of DHβE on nicotinic currents. Male currents were suppressed by 80% (control: 92 ± 9 pA, DHβE: 19 ± 5 pA; $n = 6$; paired $t$ test; $P < 0.001$; age-range examined: P16 - P22). Female currents were suppressed by 87% (control: 63 ± 9 pA; DHβE: 8 ± 3 pA, $n = 5$; paired $t$ test; $P < 0.01$; age-range examined: P16 - P23), as illustrated in Figure 2B. This pharmacological data suggests that DHβE-sensitive receptors are the primary contributors to the nicotinic currents in layer VI pyramidal neurons in both male and female FVB mice.
SECTION 2: SEX DIFFERENCES IN NICOTINIC EXCITATION

Figure 2. Acetylcholine elicits different a4b2* nicotinic acetylcholine receptor-mediated inward currents in layer VI neurons in the prefrontal cortex of males and females. (A) Voltage clamp traces showing acetylcholine-induced inward currents in a P21 male (above) and P16 female (below) layer VI neurons, before (1) and after (2) application of the competitive a4b2 antagonist DHβE (3 μM, 10 min). The inward current is significantly suppressed by DHβE. However, the inward current is not completely blocked as DHβE is applied at a much lower concentration of 3 μM than its competitive agonist, acetylcholine, which is applied at 1 mM. (B) Bar chart summarizing the amplitude of the nicotinic current before and after application of DHβE. This competitive antagonist of a4b2* nicotinic receptors significantly suppressed male and female nicotinic currents. (*** P < 0.001)
Sex differences in nicotinic currents are observed across mouse strains and species of rodent during development

To test if the developmental sex difference in nicotinic currents occurs across different strains of mice, we performed whole cell recordings on layer VI neurons from C57Bl/6 mice. Consistent with data from FVB mice, we found a sex difference in C57Bl/6 mice, as illustrated in Figure 3A. Layer VI neurons from males had significantly greater inward currents elicited by acetylcholine than those from females during postnatal week three (males: $61 \pm 9$ pA, $n = 18$; females: $32 \pm 7$ pA, $n = 17$; Mann-Whitney test, $P < 0.05$).

To test if this developmental sex difference occurs across different rodent species, we examined the nicotinic currents in layer VI neurons of male and female rats. Consistent with data from both strains of mice, we found a sex difference in rats as illustrated in voltage clamp traces in Figure 3B. Layer VI neurons from male rats had significantly greater inward currents elicited by acetylcholine than female rats during postnatal week three (males: $80 \pm 13$ pA, $n = 25$; females: $41 \pm 10$ pA, $n = 16$; Mann-Whitney test, $P < 0.001$). Similarly, males had greater inward acetylcholine-elicited currents than females during postnatal week four (males: $76 \pm 9$ pA, $n = 21$; females: $38 \pm 7$ pA, $n = 11$; Mann-Whitney test, $P < 0.01$) as shown in Figure 4C. Thus, the developmental sex differences in layer VI nicotinic currents are observed in FVB and C57Bl/6 mice, and Sprague Dawley rats, where male rodents have significantly greater inward currents elicited by acetylcholine than female rodents during an important period of cortical development.
Figure 3. Sex differences in nicotinic currents are observed across strains (FVB and C57Bl/6 mice) and species (rats) during development. (A) Bar chart summarizing the mean amplitude of the peak inward current elicited by 10 s bath application of 1 mM acetylcholine in layer VI neurons of prefrontal cortex in C57Bl/6 mice across postnatal week. Layer VI neurons of prefrontal cortex from male C57Bl/6 mice have significantly greater currents than female C57Bl/6 mice during postnatal week three. (B) Exemplary voltage clamp traces showing inward currents during the application of acetylcholine (1 mM, 30 s) from a P20 male rat (top) and P17 female rat (bottom). Line denotes acetylcholine application. (C) Bar chart summarizing the mean amplitude of the peak inward current elicited by acetylcholine (30 s, 1 mM) in prefrontal cortex layer VI neurons in rats from postnatal weeks three and four. Consistent with FVB and C57Bl/6 mice, male rats have significantly greater currents than female rats during postnatal weeks three and four. Males = black bars; female = open bars (*P < 0.05, ** P < 0.01, *** P < 0.001)
There is a prominent sex difference in the nicotinic currents elicited by carbachol, an analogue of acetylcholine, which is not broken down by acetylcholinesterase

Acetylcholinesterase, the enzyme which metabolizes acetylcholine, is expressed in the deep layers of cingulate cortex early in postnatal development (Kristt et al., 1983; 1991). Sex differences in acetylcholinesterase activity have been previously reported in the cerebral cortex of adult rodents (Das et al., 2001), suggesting that nicotinic currents elicited by acetylcholine might be under differential control by acetylcholinesterase in males and females during early postnatal development. To test whether different acetylcholinesterase activity accounts for the sex differences in nicotinic currents, we probed nicotinic currents using carbachol, a nicotinic acetylcholine receptor agonist that is not broken down by endogenous acetylcholinesterase. As expected, the inward currents elicited by carbachol (1 mM, 30 s) persisted for a longer duration compared to the inward currents elicited by acetylcholine (1 mM, 30 s) in both males and females, as seen in the voltage clamp traces in Figure 4A and 4B. This longer decay suggests that acetylcholinesterase normally contributes to the rapid removal of acetylcholine from the slice during the washout period. However, the peak current elicited by carbachol was very similar to that elicited by acetylcholine in both males \((n = 12)\) and females \((n = 13)\), as shown in Figure 4C. Two-way repeated measures ANOVA demonstrates a highly significant effect of sex \((P < 0.01)\), but no difference between acetylcholine and carbachol and no significant interaction between the effects of carbachol and sex. These results suggest that different levels of endogenous acetylcholinesterase do not account for our observed sex differences in nicotinic currents during development.
SECTION 2: SEX DIFFERENCES IN NICOTINIC EXCITATION

**Figure 4.** The sex difference in nicotinic currents is not explained by different levels of acetylcholinesterase activity. (A) Voltage-clamp traces showing inward current during bath application of nicotinic acetylcholine receptor agonists acetylcholine (1 mM, 30 s) (top trace) and carbachol (1 mM, 30 s) (bottom trace), an acetylcholine analogue that is not broken down by endogenous acetylcholinesterase in the same neuron from a brain slice from a P17 male FVB mouse. (B) Voltage-clamp trace showing inward current during bath application of nicotinic acetylcholine receptor agonists acetylcholine (1 mM; 30 s) (top trace) and carbachol (1 mM; 30 s) (bottom trace), in the same neuron from a brain slice from a P17 female FVB mouse. In both, males and females, the inward current persists longer with carbachol compared to acetylcholine, since the acetylcholinesterase in the brain slice metabolizes applied acetylcholine allowing the cell to return to baseline faster. (C) Bar chart summarizing the mean current amplitude elicited by 30 s application of 1 mM acetylcholine or carbachol. The sex difference in layer VI nicotinic currents persists even when the inward currents are elicited by 1 mM carbachol. Therefore, acetylcholinesterase levels do not account for the sex difference in nicotinic currents. (**P < 0.01)**
Nicotine elicits a greater inward current in males compared to females, and subsequent currents elicited by acetylcholine are suppressed in both sexes

A concentration of nicotine (300 nM), consistent with the peak blood level seen in smokers (Henningfield, 1993), elicited a larger inward current in layer VI neurons from male FVB mice than in those from females. The voltage clamp traces in Figure 5A illustrate the persistent inward currents elicited by nicotine (300 nM, 10 min) in male (top) and female (bottom) layer VI neurons. The bar chart illustrated in Figure 5B shows the mean currents elicited by nicotine in male and female layer VI neurons. The inward current elicited by nicotine is greater in layer VI pyramidal neurons from males than females in their third and fourth postnatal weeks (males: 23 ± 3 pA, n = 11; females: 12 ± 4 pA, n = 8; unpaired t test, P < 0.05). These results are consistent with the sex difference observed with nicotinic receptor stimulation with acetylcholine and carbachol and suggest that a potential sex difference in the density or distribution of nicotinic receptors on layer VI neurons during development should be investigated.

We investigated the extent of nicotine-induced desensitization of the currents elicited by acetylcholine in male and female layer VI neurons. At the time that the inward current elicited by nicotine had returned to baseline (~ 5 minutes washout; Kassam et al., 2008), the subsequent inward current in response to acetylcholine is suppressed in both male and female FVB mice. Two-way repeated measures ANOVA demonstrates a significant effect of sex (p < 0.05), an extremely significant effect of nicotine desensitization (p < 0.0001) and no significant interaction between nicotine desensitization and sex. Figure 5C illustrates a representative response to acetylcholine before and after a ten-minute application of nicotine, showing a significant suppression of the current elicited by acetylcholine. Figure 5D shows the mean inward currents before and after nicotine in males and females: (males: 82 ± 16 pA before, 43 ± 11 pA after, n = 10; Wilcoxon signed rank test, P < 0.01, age range examined: P14 - P26; females: 29 ± 7 pA before and 10 ± 4 pA after, n = 5; Wilcoxon signed rank test P < 0.05, age range examined: P15 - P26). Thus, while a physiological level of nicotine is able to elicit larger inward currents in male layer VI neurons than females, nicotinic receptors in layer VI neurons from both male and female mice appear to be substantially desensitized following nicotine exposure.
Figure 5. A greater inward current is elicited by nicotine in layer VI neurons of males compared to females; nicotine exposure desensitizes subsequent acetylcholine currents in both sexes. (A) Exemplary voltage-clamp traces showing a small, persistent inward current elicited by 300 nM nicotine in a layer VI neuron from a P21 male (top) and a P19 female (bottom). This concentration of nicotine is consistent with the peak blood level of nicotine seen in smokers (Henningfield, 1993). (B) Bar graph showing the mean inward current elicited by 300 nM nicotine in male and female layer VI neurons. Nicotine elicited greater inward currents in male neurons than females. (C1) A voltage-clamp trace from a P21 male shows a robust depolarization with acetylcholine (1 mM, 30 s) before application of nicotine. (C2) A voltage-clamp trace from the same neuron taken five minutes after the end of a ten minute application of nicotine (300 nM) shows that the depolarization elicited by acetylcholine (1 mM, 30 s) is significantly decreased. (D) Bar chart showing the highly significant suppression of the inward current elicited by acetylcholine (1 mM, 30 s) in males and females after the above nicotine exposure. The latter inward currents elicited by acetylcholine were examined five minutes after nicotine application when its inward current had returned to baseline. (*P < 0.05, ** P < 0.01)
The pattern and distribution of layer VI neurons containing the $\alpha_4$ nicotinic acetylcholine subunit is similar in males and females during development

During postnatal week three, 0% (0 of 41 neurons) of male and 7% (3 of 43 neurons) of female layer VI neurons had showed no inward current elicited by acetylcholine in FVB mice. In a parallel pattern, during postnatal week four, 7% (3 of 44 neurons) of male and 36% (8 of 22 neurons) of female layer VI neurons had no inward current elicited by acetylcholine in FVB mice. Therefore, we investigated whether male and female mice have different nicotinic receptor expression profiles in layer VI neurons. We examined layer VI neurons in a strain of knock-in mice expressing fluorescent $\alpha_4^*$ nicotinic receptors ($\alpha_4\text{YFP};$ Nashmi et al., 2007). These mice are deleted for the endogenous $\alpha_4$ nicotinic subunit and express the $\alpha_4$ subunit tagged with YFP. As demonstrated in Figure 6A, electrophysiological examination of prefrontal brain slices from these mice show nicotinic currents in layer VI neurons with a prominent sex difference (males: 57.04 ± 6.37 pA, $n = 34$; females: 28.02 ± 7.07 pA, $n = 15$; Mann Whitney test, $P < 0.01$). Therefore, this transgenic mouse is a suitable model for studying our observed sex differences. Furthermore, 0% (0 of 34 neurons) of male and 20% (3/15 neurons) of female layer VI neurons in these $\alpha_4\text{YFP}$ mice had no inward currents in response to acetylcholine. We, therefore, studied the proportion of cells within layer VI that express nicotinic receptors in males and females.

To study the distribution pattern and proportion of layer VI cells that express nicotinic receptors, we amplified the YFP signal with a 3-step immunohistochemistry protocol (detailed in Materials and Methods) in P15-16 mice. As demonstrated in Figure 6B, prefrontal cortex slices from male and female mice show prominent labeling of $\alpha_4\text{YFP}$-positive cells (shown in red) in layer VI. The average width of the band of YFP-immunopositive neurons within layer VI was not significantly different between males and females (males: 210 ± 17 um, $n = 5$ mice; females: 201 ± 18 µm, $n = 5$ mice; unpaired $t$ test, $P = \text{ns}$). To detect differences in the proportion of nicotinic receptor expressing cells in layer VI, we compared the number of cells expressing $\alpha_4\text{YFP}$ to those labeled by DAPI (shown in green), which stains the nuclei of all cells. Figure 6 D1 and E1 show high-magnification images of $\alpha_4\text{YFP}$ in layer VI neurons in male and female prefrontal cortex respectively. The same areas stained with DAPI are shown in Figure 6
D2 and E2. Exemplary YFP and DAPI merged images are demonstrated in Figure 6 D3 and Figure 6 E3 for males and females respectively. The ratio of α4YFP-expressing to DAPI-stained cells was not significantly different in males and females (males: 0.76 ± 0.02, n = 5 mice; females: 0.73 ± 0.03, n = 5 mice; unpaired t test, P = NS), nor were there significant differences in the number of either α4YFP-expressing cells or DAPI-stained cells. While we cannot distinguish between receptors inserted in the cell membrane and those in intracellular compartments, this data suggests that males and females do not differ in the pattern or proportion of neurons positive for α4YFP in layer VI of prefrontal cortex during development.
Figure 6. The density and distribution of nicotinic acetylcholine receptors in layer VI neurons are similar in males and females during development. (A) Bar graph showing larger inward currents in male a4YFP knock-in mice compared to age-matched female a4YFP knock-in mice during postnatal week three. Low-magnification image of a P15 male (B) and P15 female (C) prefrontal cortex slice with amplified YFP signal on the a4 subunits using a 3-step immunohistochemistry protocol described in Materials and Methods. Both sexes show a distinct neuronal band of staining in layer VI of the medial prefrontal cortex (bright red cells), showing the presence of the a4 nicotinic receptor subunit. The width of the band of YFP-immunopositive neurons within layer VI was not significantly different between males and females. Scale bar: 200 μm High-magnification of YFP immunostained neurons within layer VI of male (D1) and female (E1) prefrontal cortex. The same region shown in (D1) and (E1) stained with DAPI in (D2) (male) and (E2) (female), respectively. (D3) Merged image of (D1) and (D2) showing proportion of cells that express a4-YFP in males. (E3) Merged image of (E1) and (E2) showing proportion of cells that express a4YFP in females. Males and females did not differ in the proportion of cells that express a4YFP. Scale bar: 20 μm (**P < 0.01)
**Supplementary Figure 1.** A multiphoton image of neurons with a4YFP in layer VI of mouse prefrontal cortex. A high-resolution image of a small region in layer VI of a P15 a4YFP female mouse showing neuronal cell bodies and some dendrites with a4YFP imaged at 920nm. Note presence of autofluorescence under these imaging conditions. Scale bar: 20 µm.
Progesterone can modulate the function of nicotinic acetylcholine receptors and inhibit the response of prefrontal cortex layer VI neurons to acetylcholine.

Our experiments thus far suggest that the difference in nicotinic inward currents during development between males and females likely does not arise from sex differences in nicotinic receptor properties and α4 nicotinic receptor subunit expression profiles or from different levels of acetylcholinesterase. Previous studies have suggested that 17β-estradiol can potentiate α4β2* nicotinic receptors (Curtis et al., 2002) and progesterone has been suggested to be a negative allosteric modulator of α4β2* nicotinic receptors (Bertrand et al., 1991). To further investigate the mechanism involved in mediating the sex difference in nicotinic currents, we applied 17β-estradiol and progesterone to the slice to observe modulation of nicotinic currents through sex hormones. In all experiments, these lipophilic sex steroid hormones were conjugated with cyclodextrin to increase their solubility in water and thus allow for more efficient delivery to the brain slice through the perfusing ACSF. Each treatment (cyclodextrin control, 17β-estradiol and progesterone) was also co-applied with acetylcholine.

An exemplary neuron showing specific actions of progesterone on nicotinic currents is demonstrated in Figure 7A. Cyclodextrin (10 μM, 2 min) did not significantly alter the nicotinic currents in male and female layer VI neurons (Control: 81 ± 16 pA; cyclodextrin: 70 ± 12 pA, n = 7, paired t test, age range examined: P15 - P19, P = ns). Potentiating effects of nicotinic receptor modulators are not likely to be seen at the concentration of acetylcholine (1 mM), which produces maximal inward currents in layer VI neurons. In agreement, water-soluble 17β-estradiol (10 μM, 2 min) did not significantly alter nicotinic currents in male and female layer VI neurons (before: 80 ± 11 pA; with 17β-estradiol: 68 ± 7 pA; n = 5, paired t test, age-range examined: P18 - P26, P = ns) as shown in Figure 7A (bottom left). However, water-soluble progesterone (10 μM, 2 mins) significantly inhibited nicotinic currents in male and female layer VI neurons, as demonstrated in an exemplary neuron in Figure 7A and the bar graph shown in Figure 7B. Two-way ANOVA demonstrates a significant effect of gender, a highly significant effect of progesterone (P < 0.001) and a trend towards an interaction between gender and the effects of progesterone on nicotinic currents (P < 0.07) (males before: 78 ± 15 pA; males with progesterone: 45 ± 9 pA; n = 7; paired t test, age-range examined: P15 - P25,
p < 0.05; control females: 43 ± 5 pA; females after progesterone: 25 ± 5 pA, n = 7, paired $t$ test, age-range examined: P15 - P22, $P < 0.05$). Progesterone co-applied with acetylcholine suppressed inward currents and diminished the sex differences in inward currents in these cells (males: 45 ± 9 pA; females: 25 ± 5 pA, n = 7, unpaired $t$ test, $P = \text{ns}$). This data suggests that our observed sex difference in nicotinic currents may be due to differences in levels of progesterone in layer VI of the prefrontal cortex during this period of development, when females are thought to be able to synthesize higher levels of progesterone *de-novo* (Kato et al., 1984).
Figure 7. Progesterone can modulate the function of nicotinic acetylcholine receptors, reducing the currents elicited by acetylcholine in prefrontal layer VI neurons. (A) Examplary voltage clamp traces of the same layer VI neuron from a P19 male showing specific modulation of nicotinic inward current by progesterone: response during bath application of nicotinic acetylcholine receptor agonist acetylcholine (1 mM, 30 s) (top left) was similar to response during subsequent co-application with 10 µM cyclodextrin (vehicle for efficient delivery of lipophilic sex steroids to the brain slice), which does not alter inward currents by itself (top right). The sex steroid 17β estradiol did not alter inward currents (bottom left); whereas the sex steroid progesterone dramatically inhibited the inward currents elicited by acetylcholine (bottom right). (B) Bar graph showing that co-application of progesterone with acetylcholine suppresses inward currents in male and female layer VI neurons. There is a trend towards an interaction between sex and the effects of progesterone. Co-application of progesterone with acetylcholine reduces the sex differences in the inward currents elicited by acetylcholine in these cells. (*P < 0.05)
2.4 Discussion

In this study, we found a prominent developmental sex difference in the nicotinic currents activated by acetylcholine in layer VI pyramidal neurons of the prefrontal cortex. The specific $\alpha_4\beta_2*$ nicotinic receptor antagonist, DH$\beta$E, suppresses these currents in both sexes suggesting the currents are mediated by $\alpha_4\beta_2*$ nicotinic acetylcholine receptors. The sex difference persists when the nicotinic receptors were activated with carbachol, an analogue of acetylcholine that is not broken down by endogenous acetylcholinesterase. Consistent with this data, nicotine applied at the peak concentration of nicotine found in the blood of smokers (Henningfield, 1993) produced larger inward currents in male layer VI neurons when compared to female layer VI neurons. The prominent sex differences in nicotinic excitation seen with several different agonists prompted an anatomical investigation of nicotinic receptors in layer VI neurons during early postnatal development. We used a knock-in line of $\alpha_4$YFP mice and found a sex difference in the nicotinic currents in layer VI but no difference between males and females in the pattern or distribution of YFP-positive neurons. In subsequent experiments, we investigated whether sex steroids altered the nicotinic currents in layer VI pyramidal neurons. A brief application of progesterone significantly reduced the amplitude of nicotinic currents and diminished the sex difference in nicotinic currents of prefrontal cortex layer VI neurons. Our data suggests that progesterone may play a key role in mediating sex differences in the excitation and maturation of attention circuitry.

Sex differences in developmental nicotinic receptor regulation

Sex differences in the effects of developmental nicotine exposure on brain and behaviour have been reported in rodents and humans (Hatchell and Collins 1980; Fung 1988; Fung 1989; Ribary and Lichtensteiger 1989; Jacobsen et al., 2007). Work by Jacobsen showed that males exposed to nicotine during gestation had the most severe impairment in auditory attention tasks (Jacobsen et al., 2007). However, these findings are confounded by potential sex differences in the systemic metabolism of nicotine. In rodents and humans, nicotine is metabolized at a faster rate in females compared to males (Kyerematen et al., 1988; Gan et al., 2008) and thus, may be present at different concentrations in the brain in males and females (Rosecrans et al., 1972). An advantage
of our experimental approach is that slice electrophysiology allows nicotinic receptor agonists and modulators to be applied directly to the brain slice at known concentrations and under controlled pharmacological conditions. Yet very few in vitro studies have systematically examined the question of whether there are developmental sex differences in nicotinic excitation of the brain circuitry involved in attention. In midbrain dopamine neurons, nicotinic receptors are sex-specifically regulated during development (Azam et al., 2007). In that study, nicotine-elicited dopamine release in striatal brain slices in male rats had a greater sensitivity to nicotine when compared to female rats during the first few weeks of postnatal development. During this time, however, there were no apparent sex differences in expression of nicotinic receptor subunits in any brain region examined. These findings are broadly consistent with our results showing enhanced nicotinic currents in layer VI neurons in prefrontal brain slices from male rodents in the absence of an apparent sex difference in the anatomical distribution of key receptor subunits (α4).

**Progesterone as a mediator of sex differences in nicotinic excitation of layer VI neurons**

The sex differences we observed in the present study occur during the pre-pubertal period, before the surge of gonadal hormones. The rodent brain expresses all the enzymes necessary for the de-novo synthesis of progesterone from cholesterol (Collu et al., 1984; Compagnone et al., 1995; Kohchi et al., 1998; Sakamoto et al., 2001; Ukena et al., 1998; Ukena et al., 1999; Zwain et al., 1999). It is very likely that the developing cortex is exposed to significant amounts of progesterone. Interestingly, progestin binding is concentrated in layer VI cortical region early in development (Shughrue et al., 1992), suggesting that progesterone receptors are expressed in the deep layers of cortex. During early postnatal development, there is evidence that progesterone synthesis differs between males and females in the cerebral cortex (Kohchi et al., 1998). The enzyme 3β-hydroxysteroid dehydrogenase (3βHSD) which converts pregnenolone to progesterone is expressed in the cerebrum in male and female rats as early as P3 with a trend showing greater expression in females than males at P10 (Kohchi et al., 1998). The significant sex difference in nuclear progestin binding in the developing cortex (Kato et al., 1984) supports the suggestion that females are able to synthesize higher levels of progesterone...
de-novo in the cortex during a critical period of cortical maturation when compared to males. In agreement with the timing of the sex differences we found in nicotinic currents in the present study, male and female differences in nuclear progesterone receptor binding are evident at postnatal days 14 and 21, with females having significantly greater progesterone receptor activation (Kato et al., 1984), and thus, higher levels of progesterone in the cortex at postnatal days 14 and 21 when compared to age-matched males.

Progesterone has been reported to influence nicotinic receptor function through negative allosteric modulation of α4β2 nicotinic receptors (Bertrand et al., 1991; Valera et al., 1992; Damaj, 2000) and through interactions with a putative progesterone response element within the promoter for one of the nicotinic receptor subunits (Gangitano et al., 2009) found in layer VI pyramidal neurons (Salas et al., 2003; Kassam et al., 2008). Our examination of α4YFP receptors suggests that the number of neurons in layer VI expressing nicotinic receptors is similar in males and females. However, this approach cannot differentiate between intracellular and membrane receptors. There may be sex differences in the trafficking of nicotinic receptors to the cell membrane or sex differences in post-translational modification of nicotinic receptors that affect receptor function.

Our data show that progesterone can substantially inhibit nicotinic currents in layer VI prefrontal cortex and co-application of acetylcholine with progesterone reduces sex differences in nicotinic excitation of layer VI neurons. This inhibition appears to be specific to progesterone, as the nicotinic currents in layer VI neurons were not altered by estradiol, another sex steroid. We used water-soluble β-cyclodextrin-conjugated forms of these sex steroids to ensure they would permeate the thick slice preparation. The fast onset of the inhibition of nicotinic currents we observed with exogenous progesterone is consistent with negative allosteric modulation (Valera et al., 1992), but our experiments could not eliminate the possibility that the endogenous neurosteroid progesterone could involve slower genomic changes through activation of its receptors present in deep prefrontal cortex (Shughrue et al., 1992). Progesterone could affect nicotinic receptor function without changing its protein level by changing its post-translational modifications or levels of chaperone proteins. Alternatively, faster changes could occur if
an important chaperone or scaffolding protein that is associated with nicotinic receptors is physically disrupted by activation of a progesterone receptor.

**Role of nicotinic excitation in prefrontal cortex maturation**

Prenatal nicotine exposure is strongly associated with an increased incidence of attention deficit disorders (Langley, 2005; Schmitz, 2006). This suggests that during development, nicotinic excitation and subsequent desensitization of high-affinity nicotinic receptors influences the establishment and maturation of attention circuitry. The presence of functional nicotinic receptors in the prefrontal cortex makes it a potential target for developmental insults caused by alterations in receptor function due to nicotine exposure. Inappropriate activation and desensitization of these receptors may also alter normal cortical synaptic plasticity (Couey et al., 2007) with potential long-term consequences for cortical maturation. Previous work has demonstrated that cortical cholinergic neurotransmission is crucial for normal attention performance (Gill et al., 2000). Lesions of the cholinergic system during development alter cortical circuitry (Nishimura et al., 2002; Zhu et al., 2002; Kuczewski et al., 2005) and neuronal morphology (Robertson et al., 1998; Sherren et al., 2005), with implications for attention. It is likely that the developmental excitation of high-affinity nicotinic receptors by endogenous acetylcholine during this period of development would influence and fine-tune the maturation of synapses. The timing of the sex differences in nicotinic currents suggests that they may contribute to sex differences in the refinement and maturation of cortical projections to the inhibitory thalamic reticular neurons and excitatory thalamic projection neurons (Zhang and Jones, 2004; Sherman et al., 2006). These projections control the coordination of excitation and inhibition of the thalamus that underlies attention (Sherman et al., 2006). Under certain circumstances it is possible that greater nicotinic excitation of layer VI neurons in males during cortical maturation may lead to a greater number of stabilized synapses and thus a higher baseline activation of prefrontal cortex, a pattern which has been observed in human imaging studies of ADHD (Fassbender et al., 2009).
Relevance to attention deficit disorders

Increased distractibility is a prominent feature of ADHD that can result from the inability of the prefrontal cortex to sufficiently deactivate with increasing difficulty of attention tasks (Fassbender et al., 2009). The maturation of corticothalamic circuitry is influenced by nicotinic receptors during development and is likely to be under different control between males and females resulting in behavioural differences in tasks involving attention. In addition, our studies on the effects of progesterone on nicotinic currents in layer VI neurons may have important implications for the potential effects of progesterone on the developing cortex. High levels of gestational progesterone through maternal circulation may be an important factor contributing to healthy development of attention circuitry since prematurely born children who lack exposure to high levels of circulating maternal progesterone show persistent developmental impairments that include attentional disturbances (Dupin et al., 2000, Hall et al., 2008; Lawrence et al., 2009). Future studies on the modulation of nicotinic receptor excitation of layer VI neurons will help us understand how attention circuitry develops differently between males and females and may provide insight to the reasons for the large male preponderance of attention disorders.

Acknowledgements

We thank Dr. Sheena Josselyn and Dr. Richard Horner for their valuable comments in the preparation of this manuscript. This research was supported by CIHR (MOP 89825; EKL), the Canadian Foundation for Innovation (EKL), and the Canada Research Chairs program (EKL).
Section 3: Conclusions

An overview of the principle findings, functional significance and potential future directions will now follow and conclude this Master’s dissertation.

3.1 Summary of Contributions
In the opinion of the author, this thesis has made the following contributions:

1. Nicotinic receptors are developmentally regulated in layer VI medial prefrontal cortex in male and female FVB mice

2. There is a large sex difference in the peak amplitude of the nicotinic current elicited by acetylcholine during development

3. Developmental sex differences in nicotinic currents are observed in C57Bl/6 mice and rats

4. Differences in acetylcholinesterase levels do not appear to cause the observed sex differences in nicotinic currents in layer VI neurons

5. Nicotine elicits greater currents in males than females in layer VI neurons; however, the degree of desensitization of nicotinic receptors due to nicotine exposure is similar in both sexes

6. Males and females do not differ in the distribution pattern or number of layer VI neurons that express a key nicotinic receptor subunit (α4)

7. Progesterone can significantly reduce nicotinic currents elicited by acetylcholine in layer VI neurons in males and females
3.2 Ongoing work

In addition to progesterone being observed to influence nicotinic receptor function through negative allosteric modulation of α4β2 nicotinic receptors (Bertrand et al., 1991; Valera et al., 1992; Damaj, 2000), progesterone may also affect nicotinic receptor function through interactions with a putative progesterone response element on the promoter for the α5 nicotinic receptor subunit (Gangitano et al., 2009). This nicotinic receptor subunit is found in layer VI pyramidal neurons during development (Salas et al., 2003; Kassam et al., 2008). I have used an α5 knockout line of mice and found that in both males and females the α5 nicotinic subunit is important in mediating the developmental peak in nicotinic excitation of layer VI neurons. This dramatic reduction in nicotinic inward currents is demonstrated in Figure 3.1. This effect also observed in adulthood (data not shown).
Figure 3.1: Nicotinic inward currents of layer VI neurons are dramatically reduced across development in both male and female $\alpha_5$ knockout mice. **p < 0.01, ***p < 0.001
3.3 Future work

The underlying mechanisms mediating sex differences in nicotinic excitation of layer VI neurons is of high physiological relevance. The present study shows that progesterone can inhibit nicotinic currents in layer VI neurons; a plausible interpretation of this finding is that higher amounts of progesterone in female cortex during development (Kato et al., 1984) may account for the smaller nicotinic currents in female layer VI neurons when compared to males. An investigation into the mechanisms of progesterone’s actions on nicotinic receptors is a logical next step. Specifically, two lines of investigation may be undertaken:

1) Effects of the neurosteroid progesterone

In order to assess the inhibitory activity of endogenous progesterone in the brain slice, progesterone production in both male and female prefrontal cortex should be blocked. The enzyme 3β-hydroxysteroid dehydrogenase (3βHSD), which converts pregnenolone to progesterone, is expressed in the cerebrum in male and female rats as early as P3 (Kohchi et al., 1998). Trilostane can block 3βHSD, thereby halting the production of progesterone in the brain slice. If this manipulation removes the sex differences in layer VI nicotinic currents, then it would suggest that that higher amounts of endogenous progesterone in the female brain slice likely inhibits nicotinic currents to a greater degree in females when compared to males. However, if the sex difference remains in the presence of trilostane there are at least three possible explanations:

1) The neurosteroid progesterone does not account for sex differences in nicotinic currents in layer VI neurons
2) Existing neurosteroids in the slice remains in the slice due to its lipophilic nature
3) Trilostane does not reduce enzymatic activity of 3βHSD in the brain slice.

In-vivo and long-term manipulations to 3βHSD may be difficult to interpret. The neurosteroid biosynthetic pathway involves other sex steroid hormones, intermediate compounds, and steroid metabolites that can affect neurodevelopment (See Figure 3.2). In-vivo inhibition of 3βHSD using trilostane will also inhibit the conversion of
Figure 3.2: Schematic showing the biosynthesis of steroids (inspired by Cragun and Hopkin, 2005). Substrates = rectangles; enzymes = ovals
pregnenolone to progesterone in the adrenal gland. Cortisol, aldosterone and androstenedione are produced from progesterone and since trilostane inhibits the production of progesterone, it will also prevent the synthesis of its end products. This can result in Addison’s disease in healthy rodents. The inhibition of the conversion of pregnenolone to progesterone in the brain may cause an accumulation of its precursor pregnanalone and lower levels of the progesterone metabolite, allopregnanalone. Allopregnanolone is a potent endogenous modulator of GABA$_A$ function. Levels of neuroactive steroids vary across development and are regulated by pregnancy (Billiards et al., 2002) and are suggested to play a key role in maintaining the number of parvalbumin-positive GABA-ergic interneurons in the prefrontal cortex (Gizerian et al., 2004). Thus, a progesterone metabolite plays a crucial role in GABA signalling in the prefrontal cortex. Affecting levels of other neurosteroids in-vivo may severely hinder the proper neuronal development and maintenance of connectivity between prefrontal cortex and medial dorsal thalamus that is critical to normal development and function of the prefrontal cortex.

Endogenous progesterone may mediate its effects on nicotinic receptors through at least three potential mechanisms:

1) Negative allosteric modulation of nicotinic receptors (Bertrand et al., 1991; Valera et al., 1992; Damaj, 2000)

2) Activation of progesterone receptors to affect transcription of chaperones associated with nicotinic receptors

3) Progesterone binding to progesterone receptors activation can physically disrupt important chaperone or scaffolding proteins associated with nicotinic receptors.

To investigate the mechanism of action of endogenous progesterone, we will use RU-486, a progesterone receptor antagonist, to see if physiological levels of progesterone require its receptor to have inhibitory effects on nicotinic currents. If RU-486 does not interfere with progesterone’s inhibitory actions, progesterone is very likely affecting layer VI neurons nicotinic receptors through negative allosteric modulation.
II) Effects of exogeneous progesterone

Experiments in this thesis have shown that exogenous progesterone can significantly inhibit nicotinic currents in both, male and female prefrontal cortex. The next logical step to this finding is determining the mechanism by which this occurs. These studies have important physiological significance since they will help elucidate the effects of exogenous progesterone on brain development. Exogenous progestins are administered to women who are prone to preterm delivery. While this may produce immediate beneficial results, very little is known about the role of exogenous progesterone in brain development and the long-term effects of such exposure.

The brief onset (two minutes) of progesterone’s effects on nicotinic currents suggests that exogenous progesterone does not involve genomic mechanisms, which typically require at least thirty minutes to occur. Exogenous progesterone may mediate its effects through at least two potential mechanisms:

1) Negative allosteric modulation of nicotinic receptors (Bertrand et al., 1991; Valera et al., 1992; Damaj, 2000)

2) Progesterone binding to progesterone receptors activation can physically disrupt important chaperone or scaffolding proteins associated with nicotinic receptors.

Since we have used the water-soluble form of progesterone in our present study, progesterone may be more likely to exert its actions extracellularly, thus supporting the hypothesis that progesterone is allosterically modulating the nicotinic receptors on layer VI neurons. However, we did not see a reversal in the effects of progesterone after washing out progesterone for thirty minutes. This may be due to its high lipophilicity and/or slow dissociation of progesterone from its cyclodextrin molecule, which continues to affect nicotinic receptors and prevents us from observing a reversal of progesterone’s inhibitory effects on nicotinic receptors. Future studies may use progesterone that is not conjugated with cyclodextrin in lower concentrations.
To investigate the mechanism of action of exogenous progesterone, we will use RU-486, a progesterone receptor antagonist to see if progesterone requires its receptor to have inhibitory effects on nicotinic currents. If RU-486 blocks progesterone’s inhibitory actions on nicotinic receptors, it is likely that activation of progesterone receptors involves physical disruption of chaperone or scaffolding proteins that associate with nicotinic receptors that inhibit its function. If RU-486 does not interfere with progesterone’s inhibitory actions, progesterone is very likely affecting the receptors through negative allosteric modulation.

High levels of gestational progesterone may be an important factor contributing to healthy development of attention circuitry since prematurely born children, who lack progesterone exposure from maternal circulation, show persistent developmental impairments that include attentional disturbances (Dupin et al., 2000; Hall et al., 2008; Lawrence et al., 2009). The levels of progesterone circulating in females are extremely high during pregnancy and lactation (Peppe et al., 1974; Sanyal, 1978). Progesterone from maternal circulation has been shown to be able to bind in nuclear progesterone receptors in fetal male rat brain (Quadros et al., 1999). Similarly, in humans, progestins from contraceptive pills in breast milk can pass to breast-fed neonates (Betrabet et al., 1987; Toddywalla et al., 1995; Gainer et al., 2007). It is, therefore, highly likely that both the fetal and neonatal brain are exposed to significant concentrations of progesterone. This timing of exposure closely corresponds to important periods of brain maturation. Experiments in this Masters thesis have shown that the prefrontal cortex is sensitive to exogenous progesterone. Studies should further examine the roles of these steroids during brain development in order to explore specific roles played by mediators of sex differences in cognitive abilities.

To conclude, this thesis has shown there are sex differences in nicotinic currents in the prefrontal cortex during development. Future work will address whether a sex difference in endogenous levels of progesterone causes sex differences in nicotinic receptor currents and how exogenous progesterone affects nicotinic receptor function in the prefrontal cortex. This thesis has laid the groundwork for such exploration.
Section 4: Bibliography


Andersen SL, Teicher MH (2000) Sex differences in dopamine receptors and their relevance to ADHD *Neuroscience and Biobehavioral Reviews* **24**: 137–141


Couturier S, Bertrand D, Matter JM, Hernandez MC, Bertrand S, Millar N, Valera S, Barkas T, Ballivet M (1990). A neuronal nicotinic acetylcholine receptor subunit (alpha 7) is developmentally regulated and forms a homo-oligomeric channel blocked by alpha-BTX. *Neuron* **5**:847–856


Creutzfeldt, O. (1995) *Cortex Cerebri*. Springer-Verlag


Groenewegen HJ, HW Berendse, JG Wolters and AH Lohman (1990) The anatomical relationship of the prefrontal cortex with the striatopallidal system, the thalamus and


Hatchell PC, Collins AC (1980) The influence of genotype and sex on behavioral sensitivity to nicotine in mice *Psychopharmacology (Berl)* **71**: 45–49


Rosecrans JA, Schechter MD (1972). Brain area nicotine levels in male and female rats of two strains. *Arch Int Pharmacodyn Ther* **196**: 46-54


Sullivan RM, Brake WG (2003). What the rodent prefrontal cortex can teach us about attention-deficit/hyperactivity disorder: the critical role of early developmental events on prefrontal function. *Behav Brain Res* **146**: 43-55


