Nicotinic Signalling and Neurosteroid Modulation in Principal Neurons of the Hippocampal Formation and Prefrontal Cortex

by

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ABSTRACT

NICOTINIC SIGNALLING AND NEUROSTEROID MODULATION IN PRINCIPAL NEURONS OF THE HIPPOCAMPAL FORMATION AND PREFRONTAL CORTEX

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Nicotinic signalling plays an important role in coordinating the response of neuronal networks in many brain regions. During pre- and postnatal circuit formation, neurotransmission mediated by nicotinic acetylcholine receptors (nAChRs) influences neuronal survival and regulates neuronal excitability, synaptic transmission, and synaptic plasticity. Nicotinic signalling is also necessary for the proper function of the hippocampal formation (HF) and prefrontal cortex (PFC), which are anatomically and functionally connected and facilitate higher-order cognitive functions. The decline or dysfunction in nicotinic signalling and nAChR function has been observed in various neurological disorders, and the disruption or alteration of nicotinic signalling in the HF and/or PFC can impair learning and memory.

While the location and functional role of the α4β2* nAChR isoform has been well characterized in the medial portion of the PFC, this is not well-established in the HF. What is the role of α4β2* nAChRs in excitatory principal neurons of the HF during early development? Growing evidence suggests that the progesterone metabolite allopregnanolone (ALLO) plays a role in mediating the proper function of the HF and the PFC, and that it may also inhibit nAChR function. How might
ALLO influence α4β2* nAChR function during early development and/or affect neuronal excitation within a living system? This thesis aims to develop a foundation towards understanding the role of α4β2* nAChR-mediated neurotransmission in principal neurons of the HF during development, and the role of ALLO in modulating α4β2* nicotinic receptor function during this period.

In this thesis, I demonstrate that functional α4β2* nAChRs are present in principal neurons of the developing mouse HF. The function of these receptors is developmentally regulated, and nicotinic excitation differs between male and female mice. I also demonstrate that ALLO negatively modulates α4β2* nAChR function in living neurons. I show for the first time that crosstalk between the membrane progesterone receptor complex and the nAChR likely facilitates the actions of ALLO to modulate nAChR function. The findings in this thesis present new insights on α4β2* nAChR expression and function, while adding to our understanding of how these receptors may influence neuronal excitability, synaptic transmission, and synaptic plasticity during early development.
ACKNOWLEDGEMENTS

The work presented in this thesis not only reflects a milestone in my long-term interest in biomedical sciences, but also the product of those who have continuously supported me during the term of my candidature.

First and foremost, I wish to thank my advisor, Dr. Craig Bailey. I am most grateful for your guidance and support, your tremendous patience, and faith in my capabilities as I developed as a researcher. I would also like to thank Warren Bignell for giving me the strongest foundation I could ask for, and ‘members of the Bailey Lab’, for your friendship and wise advice on everything.

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I would like to thank my friends and family for your unconditional support despite my confusing role as a graduate student. To my mom and dad, I thank you for the opportunity that you have given me to unreservedly pursue and achieve my goals and aspirations. I hope that my work now and future milestones will continue to make you proud. To my sister, thank you for allowing me to exercise the perks of being the malicious older sibling and inspiring me to be a better person each day. Finally, thank you Calvin, for your unparalleled patience with my frenzy and the comfort that you have given me during the many long days and nights of this journey.
DECLARATION OF WORK PERFORMED

I declare that I have performed all the work presented in this thesis except for the following:

Electrophysiological recordings in Chapter 2 were in part completed by Warren Bignell and Derek Jacklin.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ACSF</td>
<td>Artificial Cerebral Spinal Fluid</td>
</tr>
<tr>
<td>ADHD</td>
<td>Attention-Deficit Hyperactivity Disorder</td>
</tr>
<tr>
<td>ALLO</td>
<td>Allopregnanolone</td>
</tr>
<tr>
<td>ASD</td>
<td>Autism Spectrum Disorder</td>
</tr>
<tr>
<td>BCC</td>
<td>Bicuculline</td>
</tr>
<tr>
<td>CA</td>
<td>Cornu Ammonis</td>
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<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>ChAT</td>
<td>Choline acetyltransferase</td>
</tr>
<tr>
<td>CNQX</td>
<td>6-Cyano-7-Nitroquinoxaline-2,3-Dione</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP Response Element-Binding Protein</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate Gyrus</td>
</tr>
<tr>
<td>dlPFC</td>
<td>Dorsolateral PFC</td>
</tr>
<tr>
<td>DHβE</td>
<td>Dihydro-β-Erythroidine Hydrobromide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>EC</td>
<td>Entorhinal Cortex</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-Regulated Kinase</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma Aminobutyric Acid</td>
</tr>
<tr>
<td>GDP</td>
<td>Giant Depolarizing Potentials</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein Coupled Receptor</td>
</tr>
<tr>
<td>HF</td>
<td>Hippocampal Formation</td>
</tr>
<tr>
<td>HSD</td>
<td>Hydroxysteroid Dehydrogenase</td>
</tr>
<tr>
<td>Jnk1</td>
<td>c-Jun N-Terminal Kinase-1</td>
</tr>
<tr>
<td>mAChR</td>
<td>Muscarinic Acetylcholine Receptor</td>
</tr>
<tr>
<td>MLA</td>
<td>Methyllycaconitine</td>
</tr>
<tr>
<td>mPFC</td>
<td>Medial Prefrontal Cortex</td>
</tr>
<tr>
<td>mPR</td>
<td>Membrane Progesterone Receptor</td>
</tr>
<tr>
<td>MSDB</td>
<td>Medial Septum / Diagonal Band of Broca</td>
</tr>
<tr>
<td>nAChR</td>
<td>Nicotinic Acetylcholine Receptor</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-Aspartate</td>
</tr>
<tr>
<td>P</td>
<td>Postnatal Day</td>
</tr>
<tr>
<td>PAQR</td>
<td>Progestin and adipQ receptors</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal Cortex</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PGRMC</td>
<td>Progesterone Membrane Receptor Component</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone Receptor</td>
</tr>
<tr>
<td>SK</td>
<td>Small Conductance Calcium Activated-Potassium</td>
</tr>
<tr>
<td>SUB</td>
<td>Subiculum</td>
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<tr>
<td>3α-HSD</td>
<td>3alpha-Dehydrogenase</td>
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<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>3β-HSD</td>
<td>3beta-Dehydrogenase</td>
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<tr>
<td>5α-DHP</td>
<td>5alpha-Dihydroprogesterone</td>
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CHAPTER 1

General Introduction
1.1 Role of the hippocampal formation and prefrontal cortex in higher-order cognitive functions

Higher-order cognitive function refers to brain processes that require conscious and voluntary control. These processes include intellectual function, such as learning and memory, speech and language production, emotional control, and executive function. While broadly defined, it is generally accepted that executive function includes attention, the process of focusing on relevant information and ignoring distractors; working memory, the process of maintaining information until execution; goal-oriented planning, the process of organizing, regulating, and monitoring actions; and motor planning, the processing of spatial and contextual cues (Baddeley 1992; Barkley 1997; Miyake et al. 2000). Both the hippocampal formation (HF) and prefrontal cortex (PFC) have long been implicated in mediating these higher-order cognitive functions.

Early studies of primates and rodents suggested that the HF is involved in emotional control, attention, and spatial orientation, but its role in mediating memory processes was highlighted by the patient H.M., who demonstrated severe memory impairment after surgical removal of the HF and neighbouring regions (Scoville and Milner 1957). It is now recognized that the HF is involved in various memory processes (Eichenbaum et al. 1992; Fanselow and Dong 2010; Kim 2015; Scoville and Milner 1957; Squire and Zola 1996). Imaging studies in humans demonstrated that the HF is involved in short-term memory, where the process of retaining novel information across brief delays activates the HF (Ranganath and D'Esposito 2001), and the encoding and retrieval of memories, where distinct subregions of the HF are found to be differentially activated during memory formation (Eldridge et al. 2000), and recollection of a learning episode (Ji and Maren 2008). Furthermore, studies in mice demonstrated that the HF is involved in contextual processing
and emotional processing, and lesions of the HF reduced the capacity for retrieval of detailed contextual fear memories (Mizumori et al. 2009; Wiltgen et al. 2010).

The PFC is thought to exert top-down influence, whereby it acts as a mediator of executive function (Goldman-Rakic 1988; Miller and Cohen 2001). Early behavioral studies of monkeys demonstrated that damage to the frontal cortex disrupts goal-directed behaviours, and subsequent studies of humans demonstrated that lesions in the PFC impairs executive function, with particular deficits in attention (Rossi et al. 2009). Lesion studies in rodents have further demonstrated the role of the PFC in decision making, working memory, and temporal processing. Specifically, lesions in the PFC increases preference for small immediate rewards in rats (Mobini et al. 2002; Rudebeck et al. 2006), impairs visual object information or spatial information processing (Kesner and Churchwell 2011; Ragozzino and Kesner 2001), and impairs performance on tasks that require the completion of several behavioural responses in sequential order (Fuster and Bressler 2015; Luchicchi et al. 2016), respectively.

While it is recognized that both the HF and PFC independently contribute to mediating higher-order cognitive functions, recent studies have also begun to elucidate the interaction between the HF and PFC. These studies suggest that the HF and PFC may not only independently mediate these higher-order cognitive functions, but may also modulate these processes together (Benchenane et al. 2010; Churchwell and Kesner 2011; Guise and Shapiro 2017). For example, Churchwell and Kesner (2011) demonstrated that during performance of tasks that require working memory over longer delays, both the HF and the PFC interact to coordinate memory processes in anticipation of obtaining a remote goal, whereas over short delays, either structure may independently represent
spatial information that is sufficient for the successful completion of the task. The following sections will examine the neuroanatomy of the HF and PFC in the rodent brain, as well as further elucidate the functional mechanisms of the interaction between these two regions.

1.1.1 Anatomical organization of the hippocampal formation

The HF is composed of five cytoarchitecturally distinct but adjoining regions that form the basis for information flow. These areas include the dentate gyrus (DG), cornu ammonis area 3 (CA3), cornu ammonis area 1 (CA1), subiculum (SUB) and entorhinal cortex (EC) subregions, and are linked by a functional, unidirectional and excitatory neuronal circuit (Andersen 2007). It is now widely agreed upon that the HF circuit begins with an excitatory efferent signal from the superficial layers of the EC (EC layer II and EC layer III), which give rise to axons that project to the DG via the major input pathway called the perforant path. The granule cells of the DG then project to the CA3 via mossy fiber projections, followed by the projection of CA3 pyramidal neurons to the CA1 via Schaffer collaterals. Pyramidal neurons in the CA1 then project to the SUB, and axons of the pyramidal neurons of the SUB then terminate in the deep layers of the EC, including layer V (ECV) and VI (ECVI) (Andersen 2007). The circuitry has now expanded to include additional projections extending from or terminating in each subregion, as shown in Fig. 1.1. There is now accumulating evidence to suggest that the CA2, which is situated between the CA1 and CA3, has unique characteristics and plays an active role in facilitating the HF circuit. The subregions of the HF are thought to serve specific functions. The neurons of CA3 are specialized to perform pattern completion and may function as an autoassociative network involved in memory, storage and recall (Ji and Maren 2008; Mishra et al. 2016; Tort et al. 2009). The DG supports pattern separation (Knierim and Neunuebel 2016), and CA1 has been linked to spatial and temporal processing
(Carasatorre et al. 2015; Churchwell and Kesner 2011; Isgor and Sengelaub 1998). The SUB receives convergent input from numerous sources and acts as a major source of output from the hippocampus (Naber et al. 2001a; Witter et al. 2000b), and similarly, the deep layers of the EC are thought to provide output from the HF to the rest of the brain (Canto et al. 2008; Witter et al. 1986).

Whereas the pyramidal neurons of the CA3, CA1, SUB, and EC generally consist of a large triangular cell body with one long apical dendrite, granule neurons of the DG comprise a small, ovoid cell body with a single, conical dendritic tree (Schultz and Engelhardt 2014). However, both pyramidal and granule neurons are glutamatergic excitatory neurons that have also been referred to as principal neurons (Andersen 2007; Schultz and Engelhardt 2014); therefore, these terms will be used interchangeably within this thesis. The HF also consists of a highly diverse population of gamma-aminobutyric acid (GABA)-producing local circuit inhibitory interneurons. While interneurons only represent approximately 10-15% of the total hippocampal neuronal population, they are scattered throughout almost all subfields of the HF and play an important role in mediating the HF circuitry (Jones and Yakel 1997; Pelkey et al. 2017; Rosato-Siri et al. 2006). The CA and DG are structured in layers or strata. Beginning with the most superficial layer in the CA is the stratum moleculare, followed by the stratum lacunosum moleculare, stratum radiatum, stratum lucidum, stratum pyramidale, stratum oriens and the alveus. In the DG, the most superficial stratum is the polymorphic layer, followed by the stratum granulosum and stratum moleculare. The principal layers of the CA and DG are the stratum granulosum and pyramidale, which contain the cell bodies of principal neurons (Andersen 2007), and interneurons are scattered throughout all strata. The HF has also been anatomically divided into dorsal and ventral regions based on anatomical studies that have demonstrated that the input and output connections of the HF are...
regionally distinct (Fanselow and Dong 2010; Swanson and Cowan 1977). For example, the perirhinal cortex receives more input from the ventral HF and the postrhinal cortex receives more input from the dorsal HF (Agster and Burwell 2013). Similarly, the postrhinal cortex typically directs output connections to the dorsal CA1 and SUB (Naber et al. 2001b), whereas the perirhinal cortex directs output connections to the ventral SUB (Agster and Burwell 2013; Swanson and Cowan 1977). EC neurons that project to the dorsal DG are also distinct from EC neurons that project to the ventral DG (Dolorfo and Amaral 1998a; b). Additionally, the amygdala, which plays a general role mediating emotion and fear memory, only receives direct input from the ventral HF and not the dorsal HF (Maren and Fanselow 1995).
Fig. 1.1: The neural circuitry in the rodent hippocampus. A. An illustration of the hippocampal circuitry. B. Diagram of the hippocampal neural network. The excitatory hippocampal formation circuit (entorhinal cortex (EC layer II and III) → dentate gyrus (DG) → cornu ammonis area (CA) 3 → CA1 → EC layer V and VI) is depicted by solid arrows. CA3 also receives direct projections from EC layer II neurons through the perforant pathway. CA1 receives direct input from EC layer III neurons through the temporoammonic pathway. Adapted from (Deng et al. 2010).
1.1.2 Anatomical organization of the prefrontal cortex

The PFC was originally defined based on cytoarchitectural features described by Brodmann, where in the human brain, the frontal region or the PFC is defined by the granular cortex anterior to the motor strip (Brodmann, 1909). Due to species differences in homology however, where the granular zone or layer IV is absent in the rodent PFC, this area is also defined based on its subcortical projection. Therefore, the PFC is recognized as the region of the frontal lobe which exhibits dense reciprocal projections with the mediodorsal nucleus of the thalamus (Fuster 2001; Leonard 1969; Rose and Woolsey 1948; Sesack et al. 1989; Uylings and van Eden 1990). In rodents, the PFC contains several distinct regions, including the lateral PFC, the medial PFC (mPFC), and the ventral PFC. The PFC is thought to exert top-down influence on a variety of cognitive and executive control functions (Miller and Cohen 2001; Poorthuis and Mansvelder 2013; Rossi et al. 2009), and the mPFC has been of particular interest due to its contribution to these processes. For example, a number of studies have emphasized the mPFC role in attentional processes in rats. These studies demonstrate that the mPFC is critical in tasks that require rats to shift attention among competing stimulus features that require distinct behavioral responses (Luchicchi et al. 2016; Ng et al. 2007; Passetti et al. 2000). These findings are found in non-human primates as well as humans (Dias et al. 1996). Additionally, studies investigating the role of the prefrontal cortex with working memory processes in rodents demonstrate that lesions of the mPFC cause profound impairment of working memory tasks when delays were imposed on task performance. For example, lesions of the mPFC impaired performance in mice even when the animal was well trained, suggesting that the mPFC is critical not only for acquisition but also for successful performance (Rossi et al. 2012).
While the homology of PFC regions in different species has been extensively debated, it has been suggested that the rat mPFC roughly correspond to the dlPFC in primates (Hoover and Vertes 2007; Kesner and Churchwell 2011; Uylings and van Eden 1990). The mPFC is further subdivided into distinct areas and categorized into the dorsal mPFC and ventral mPFC based on functional criteria and connectivity with other brain areas (Heidbreder and Groenewegen 2003). Specifically, the dorsal mPFC includes the medial precentral area and the anterior cingulate cortex; the ventral mPFC includes the prelimbic cortex, infralimbic cortex and medial orbital cortex; and the ventral PFC, which contains the ventral orbital and ventral lateral orbital cortices (Heidbreder and Groenewegen 2003). The mPFC is also separated into cortical layers I, II, III, V and VI in rodents (Heidbreder and Groenewegen 2003; Luchicchi et al. 2016; Ragozzino et al. 1998; Riga et al. 2014; Van Eden and Uylings 1985; Vertes 2004) and neural information processed by the mPFC is relayed to subcortical sites through populations of excitatory pyramidal neurons mainly situated in layers V and VI. Specifically, layer VI neurons act as a modulatory output layer by sending projections to a number of regions, with 37% of layer VI pyramidal neurons in the mPFC projecting to the mediodorsal thalamus (DeFelipe and Farinas 1992). Layer VI neurons also send and receive cortico-cortical projections within the cortex itself (Ongur and Price 2000; Thomson 2010; Uylings and van Eden 1990; Zarrinpar and Callaway 2006). Together with thalamic feedback, these neurons mediate cortical excitation and exert complex effects on higher cognitive functions (Gabbott et al. 2005; Kang et al. 2015; Lam and Sherman 2010; Proulx et al. 2014; Thomson 2010). Over 80% of neurons within the mPFC are glutamatergic excitatory pyramidal neurons and are found in layers II, III, V, and VI, while 10-20% of all mPFC neurons are GABAergic interneurons and are scattered throughout the mPFC (Riga et al. 2014).
1.1.3 *Functional interaction between the hippocampal formation and prefrontal cortex*

Recent research in both experimental animals and humans has considerably advanced our understanding of the relationship between the HF and PFC. Several studies provide evidence of neuronal connections between the HF and the mPFC (Ferino et al. 1987; Hoover and Vertes 2007; Swanson 1981). In rats, neurons in the HF directly project to the prelimbic and infralimbic regions of the mPFC (Swanson and Kohler 1986; Thierry et al. 2000; Varela et al. 2014), and this is referred to as the hippocampal-prefrontal pathway. In monkeys and humans, it was also determined that this pathway originates from the CA1 and SUB (with the strongest projections from the ventral HF), projects via the fimbria fornix, and terminates at the medial orbital cortex of the mPFC (Cavada et al. 2000; Cenquizca and Swanson 2007; Croxson et al. 2005; Hoover and Vertes 2007; Jay et al. 1992; Swanson et al. 1981). This pathway consists of excitatory glutamatergic pyramidal neurons that terminate at both pyramidal and interneurons of the mPFC (Carr and Sesack 1996; Gabbott et al. 2002; Jay et al. 1992; Tierney et al. 2004). While less investigated than the hippocampal-prefrontal pathway, there are also multi-synaptic efferent projections from the mPFC to the HF. In studies of both rodents and humans, the mPFC can either send projections to the perirhinal cortex, which then projects to the lateral EC, or, project to the parahippocampal cortex which then projects to the medial EC. Both of these pathways can then converge at the dorsal HF (Cenquizca and Swanson 2007; Eichenbaum et al. 2007; Navawongse and Eichenbaum 2013; Vertes 2004). Together, the circuitry between these two regions has been suggested to act as a functional loop that facilitates a bidirectional flow of information to mediate higher-order cognitive functions that were initially believed to be independently modulated by the HF and/or mPFC. The function of this coordinated signalling loop is described next.
While both the HF and mPFC have independently been implicated in working memory and reward-based learning, growing evidence suggests that communication originating from the HF and projecting to the mPFC, or the hippocampal-prefrontal pathway, is critical for these processes (Benchenane et al. 2010; Churchwell and Kesner 2011; Izaki et al. 2000; Siapas et al. 2005). In a set of cross-lesion experiments, compromising the hippocampal–prefrontal pathway in the rat brain by bilaterally disrupts the CA1/SUB region in one hemisphere and the mPFC in the other hemisphere led to disrupted performance in the win-shift radial arm maze task that tests for multiple-item working memory and decision making. Particularly, in comparison to unilaterally lesioned rodents, cross-lesioned rodents demonstrate deficits when faced with longer delays (or the “delayed condition”) before they were allowed to revisit the arm maze to retrieve a food reward (Floresco et al. 1997). This finding suggests that both the HF and the mPFC play a role in mediating working memory, but shorter versus longer delays may differentially evoke PFC-dependent or HF-dependent working memory processes. Other cross-lesion studies in rats in the HF and PFC have reported impairments to operant learning as well (Izaki et al. 2000), and lesions in the HF have been shown to disrupt anticipatory activity of mPFC neurons in reward tasks (Burton et al. 2009; Kyd and Bilkey 2003). Further characterization of the role for the hippocampal-prefrontal pathway in working memory comes from experiments that demonstrate an emergence of correlated neural activity between the HF and mPFC during behavioral tasks in rats. Simultaneous recordings in the HF and mPFC during working memory tasks demonstrate synchronized activity, as well as phase-locking behavior, where hippocampal theta activity precedes the activity of mPFC neurons and is believed to entrain mPFC neurons to hippocampal theta activity (Benchenane et al. 2010; Fujisawa and Buzsaki 2011; Jones and Wilson 2005; Siapas et al. 2005). In a study by Benchenane et al., (2010) there is also evidence to support the view that the HF and mPFC interact during reward
learning, as coherence of theta-oscillations in these two regions is strongest when rats are at a choice point of the maze and acquire a new learning rule. Together, these findings support the idea that the HF and mPFC may facilitate higher-order cognitive functions through the hippocampal prefrontal pathway, and collaboratively mediate these processes.

While the studies described above provide evidence that the hippocampal-prefrontal pathway is critical for learning and memory, there is also considerable evidence suggesting that the PFC to HF pathway plays an important role in memory retrieval. Damage to the mPFC has been associated with impairments in the capacity for rats, monkeys and humans to switch between remembering different stimuli during task-switching tasks (Dias et al. 1996; Navawongse and Eichenbaum 2013; Ragozzino et al. 2003; Rich and Shapiro 2007). In functional imaging studies, it has been demonstrated that, when rats use either of two spatial contexts to guide retrieval of otherwise contradictory object-reward associations, neurons in the dorsal HF encode these memories as selective firing to specific objects in particular places in each spatial context. However, when the mPFC is inactivated, dorsal HF neurons indiscriminately retrieve both appropriate and inappropriate object memory representations (Komorowski et al. 2009; Navawongse and Eichenbaum 2013). These findings suggest that the HF is capable of retrieving memories even in the absence of mPFC input, but that the mPFC is important in selecting the appropriate memory for that particular context. Additional imaging studies further contribute to this model. In humans, it has been demonstrated that the dorsal-ventral axis of the HF may contain a topographical representation of memories. While the anterior HF in humans (ventral HF in rodents) creates more general representations of the salience of events, the posterior HF in humans (dorsal HF in rodents) represents the information content presented within individual events and is differentially activated
during retrieval of specific events in memories (Liang et al. 2013). These combined findings suggest for a bidirectional flow of information between the HF and mPFC, wherein ventral HF signals carry general contextual information to the mPFC and the activation of the mPFC can then initiate its control over the retrieval of specific memories from the dorsal HF.

The importance of the proper development and establishment of the circuitry within and between the HF and mPFC is also evident in studies investigating mechanisms underlying neurodevelopmental and neurological disorders that involve compromised cognitive functioning. While schizophrenia, depression and anxiety disorders appear to be distinct disorders, there is substantial comorbidity and overlap of negative symptoms, including cognitive impairment and emotional dysregulation (Anticevic et al. 2015; Braga et al. 2005; Buckley et al. 2009; Castaneda et al. 2008; Elvevag and Goldberg 2000; Esslinger et al. 2009). Moreover, there are reports of shared risk factors among these disorders that are found in the HF and mPFC, and there is evidence that these risk factors share a genetic basis. For example, abnormal coupling between the dlPFC (the primate equivalent of the mPFC) and HF have been implicated in schizophrenia. Through a genome-wide association study, it was found that the single nucleotide polymorphisms in the ZNF804A and CACNA1C genes, rs1344706 and rs100637, respectively, are associated with schizophrenia. Specifically, in comparison to non-carriers, carriers of the rs1344706 risk-allele demonstrate reduced ipsilateral and contralateral connectivity in the dlPFC, and increased connectivity between the HF and dlPFC. Additionally, functional connectivity analysis reveals that individuals with the rs1006737 risk-allele expression have altered right-hemispheric dlPFC activation and increased fronto-hippocampal connectivity (Esslinger et al. 2009; Paulus et al. 2014; Rasetti et al. 2011). Since the HF and mPFC functional loop facilitates bidirectional flow of
information between these regions to mediate higher-order cognitive functions, and these functions are commonly impaired in a number of neurodevelopmental and neurological disorders, this loop may be an important site when considering the basis for these disorders. It is important to understand the mechanisms that contribute to the proper development and function of the HF and mPFC within cognitive brain circuits, both separately and together, as this will improve our understanding of processes that underlie normal and aberrant cognitive behaviours.

1.2. Nicotinic acetylcholine receptors in the hippocampal formation and prefrontal cortex

Neurotransmission mediated by the neurotransmitter acetylcholine (ACh) plays an important role in coordinating the response of neuronal networks in many brain regions. ACh is synthesized, stored, and released by cholinergic neurons, which are found in the pedunculopontine and laterodorsal tegmental areas, the medial habenula, and the basal forebrain complex, including the medial septum. This local release of ACh alters neuronal excitability, influences synaptic transmission, positively modulates plasticity and coordinates the firing of groups of neurons (Picciotto et al. 2012). Within the brain, ACh acts through two major receptor subtypes: the metabotropic muscarinic acetylcholine receptor (mAChR) and the ionotropic nicotinic acetylcholine receptor (nAChR).

There are five distinct mAChRs, denoted M₁-M₅. One group of mAChRs contains the M₁, M₃, and M₅ receptors, which are Gαq coupled and mediate cellular changes by activating phospholipase C (PLC)-dependent signalling cascades leading to Ca²⁺ release from intracellular stores (Felder 1995). The other group of mAChRs contains the M₂ and M₄ receptors, which are Gαi/o-coupled and lead to the inhibition of adenylyl cyclase activity, thereby decreasing the production of intracellular
cyclic adenosine monophosphate (cAMP) (Bubser et al. 2012; Felder 1995; Hulme et al. 1999). Modulation of mAChRs is involved in normal cognitive processes and altered function of this system may contribute to functional deficits. For example, mAChR knockout mice have shown deficits in tasks involving both the mPFC and HF, including working memory and cognitive flexibility, as well as robust impairments in contextual fear conditioning (Poulin et al. 2010; Seeger et al. 2004; Tzavara et al. 2003).

The nAChRs respond to both endogenous ACh and exogenous nicotine, and like mAChRs, are implicated in a variety of physiological processes in the brain (Hurst et al. 2013). These receptors are particularly important during pre- and postnatal circuit formation, where signalling mediated by nAChRs influences neuronal survival (Bunker and Nishi 2002; Dajas-Bailador and Wonnacott 2004; Meriney et al. 1987), promotes neurite elongation (Coronas et al. 2000), determines dendritic shaft and spine synapses (Lozada et al. 2012a; b), and coordinates synchronized neuronal activity (Myers et al. 2005). They also play an important role in age-related cell degeneration (Aubert et al. 1992; Bowen et al. 1983; Gotti et al. 2006; Levin and Rezvani 2002; Perry et al. 1995). Changes to nAChR-mediated neurotransmission improves or disrupts mechanisms which facilitate learning and memory processes (Deutsch 1971; Mansvelder et al. 2006). For instance, several studies have together demonstrated that nAChRs play a role in facilitating the release of a wide range of neurotransmitters, including ACh, serotonin, dopamine, norepinephrine, GABA, and glutamate (McGehee et al. 1995; Wonnacott 1997), suggesting that nAChR may contribute to the ability of ACh and nicotine to modulate learning and memory. In a study of ketamine-treated rats presenting multisensory integration impairment, it was found that activation of presynaptic nAChRs restores GABAergic function which was significantly reduced in ketamine-treated rats (Cloke et al. 2016).
Conversely however, disruption of nAChR-mediated signalling can prove to be detrimental as the decline or dysfunction in ACh signalling and nAChR function has been observed in a number of neuropathological disorders that occur during development and adulthood, such as epilepsy, neuropathic pain, anxiety, depression, addiction and schizophrenia (Changeux 2010; Drenan and Lester 2012; Gotti and Clementi 2004; Hurst et al. 2013; Picciotto et al. 2012; Picciotto and Zoli 2008) as well as in old age, such as Alzheimer’s disease and Parkinson’s disease (Picciotto and Zoli 2008).

1.2.1 Nicotinic receptor composition and isoforms

The nAChRs belong to the cys-loop ligand-gated ion channel superfamily of homologous receptors, which also includes receptors for GABA, serotonin and glycine (Gotti et al. 2009). Like other ligand-gated ion channels of this superfamily, distinct regions of the transmembrane proteins that form nAChRs are responsive to and regulated by the presence of molecules such as activators (agonists), inhibitors (antagonists), or other agents that modify the efficacy of these molecules (Gotti et al. 2009; Taly et al. 2009). Upon binding of an extracellular agonist such as ACh, the nAChR changes its conformation from a closed to an open state. While open, general physiological conditions will favour an inward movement of cations such as Na\(^+\) and Ca\(^{2+}\), resulting in the depolarization of the cell. The open state is rapidly followed by either deactivation, which includes current decay and a closed state of the receptor after removal of the agonist, or desensitization, which includes current decay and a closed state of the receptor with the bound agonist. While much is still unknown about the desensitization process, it functions as a mechanism that limits both amplitude and duration of nAChR activation by cholinergic signalling. The return of the receptor from the desensitized to the resting state is referred to as ‘recovery from
desensitization’ (Miyazawa et al. 2003; Quick and Lester 2002; Taly et al. 2009). In addition to regulation at the extracellular domain by ligands, nAChRs are also subject to modifications at their cytoplasmic domains. For example, accumulating evidence suggests that phosphorylation of the nAChR may act to ensure proper receptor expression, distribution, and signalling that is consistent with cellular demands (Fenster et al. 1999; Lee et al. 2015; Nakayama et al. 1993; Nishizaki and Sumikawa 1998).

Twelve nAChR subunit genes have so far been cloned and classified into two subfamilies which include nine α (α2–α10) and three β subunits (β2–β4) (Hurst 2013; Gotti 2009). These subunits arrange in specific pentameric combinations to form the two nAChR subtypes: (i) the α-bungarotoxin-sensitive homomeric or heteromeric receptors, which comprise α7-α10 subunits, and (ii) the α-bungarotoxin-insensitive heteromeric receptors, which comprise α2–α6 and β2–β4 subunits (Gotti et al. 2009). Both the α and β subunits contribute towards the pharmacological properties of ligand binding sites, with principal agonist binding sites situated at the interface between two identical subunits in homomeric receptors, and at the interface between α (α2, α3, α4 or α6) and β (β2 or β4) subunits in heteromeric receptors. While the α5 and β3 subunits may be incorporated into heteromeric nAChRs, these subunits do not appear to carry the primary or the complementary components that are required to form an agonist binding site (Groot-Kormelink et al. 1998; Ramirez-Latorre et al. 1996). Accordingly, it is recognized that homomeric receptors have five identical ACh-binding sites per receptor molecule (one on each subunit interface), and heteromeric receptors have two to three binding sites per receptor (one per α/β or α/α subunit interface) (Moroni et al. 2008; Palma et al. 1996; Taly et al. 2009). In addition to the primary
agonist binding sites, multiple distinct allosteric sites may allow for distinct non-agonist ligands to bind to and modulate receptor activity (Pandya and Yakel 2011).

The two predominant nAChR isoforms in the CNS are the homomeric α7 nAChR and the heteromeric α4β2* nAChR (Lindstrom 1996; Marks and Collins 1982; Morley et al. 1979; Perry et al. 2002; Wu and Lukas 2011). Homomeric receptors are composed of five α7 subunits and are known to desensitize rapidly (Fenster et al. 1997). They have a relatively-high Ca\(^{2+}\) permeability compared to most other nAChR isoforms, and thus, can impact several Ca\(^{2+}\)-dependent mechanisms including the activation of second messenger pathways (Cheng and Yakel 2014; Nordman and Kabbani 2014; Peng et al. 1994; Quik et al. 1997; Seguela et al. 1993). The α4β2* nAChR is composed of two α4 subunits, two β2 subunits, and a fifth accessory subunit that is denoted by the asterisk. The α4 subunit mRNA is present in the majority of brain areas, and α4 and β2 subunits co-localize in most brain areas of both rodents and primates (Gotti et al. 2007; Picciotto et al. 2001; Wada et al. 1989; Zoli et al. 1998). The α4β2* receptor isoform binds with high affinity to nicotinic agonists: it comprises 90% of the high-affinity nicotine binding sites in the brain and knocking down the α4 or β2 subunit abolishes high-affinity nicotinic agonist binding in most brain regions (Colombo et al. 2013; Dani and Bertrand 2007; Perry et al. 2002; Wu and Lukas 2011).

The presence of the distinct accessory subunit, α4, α5, β2 or β3, influences the function of the α4β2* nAChR in a specific manner (Moroni et al. 2006; Ramirez-Latorre et al. 1996; Salminen et al. 2004). Specifically, the (α4)\(_2\)(β2)\(_3\) stoichiometry is activated at much lower ACh concentrations (EC\(_{50}\) = ~1μM ACh) than the (α4)\(_3\)(β2)\(_2\) stoichiometry (EC\(_{50}\) = ~100 μM ACh), and has different affinity for nicotinic ligands (Moroni et al. 2006; Zwart et al. 2008). Additionally, the (α4)\(_2\)(β2)\(_3\)
stoichiometry is more sensitive to nicotine-induced up-regulation and more sensitive to desensitization induced by low concentration of agonists, whereas the (α4)3(β2)2 stoichiometry has a higher permeability to Ca\(^{2+}\) and more rapid desensitization kinetics than the (α4)2(β2)3 stoichiometry (Albuquerque et al. 2009; Gotti et al. 2009; Kuryatov et al. 2008; Lopez-Hernandez et al. 2004; Tapia et al. 2007). The (α4β2)α5 receptor isoform is also likely to be an important regulator of neurotransmission. It is estimated that the α5 subunit is associated with α4 and β2 subunits in 11-37% of total α4β2* nAChRs, depending on the brain region (Gotti et al. 2007; Ramirez-Latorre et al. 1996). Of the different α4β2* nAChR isoforms, that with the (α4β2)2α5 stoichiometry has the greatest Ca\(^{2+}\) permeability (Kuryatov et al. 2008), and the inclusion of the α5 subunit appears to enhance receptor assembly and expression, reduce the relative magnitude of ligand-mediated upregulation, and facilitate receptor channel closure (Bailey et al. 2010; Gotti et al. 2007; Mao et al. 2008; Moroni et al. 2006; Ramirez-Latorre et al. 1996). The presence of the α5 subunit also confers sensitivity to the allosteric modulator galantamine (Kuryatov et al. 2008). The diverse combinations of subunit assemblies in the brain lead to nAChR isoforms with different basic functional properties and varied contributions to neurophysiological outcomes, depending on their location within the neuronal circuitry.

1.2.2 Nicotinic receptor signalling in the hippocampal formation

The HF receives afferent cholinergic neurotransmission from the medial septum / diagonal band of Broca (MSDB) within the basal forebrain (Yakel 2013). Numerous findings suggest that cholinergic signalling is necessary for the proper function of the HF. For instance, ACh levels increase in the HF of animals exploring novel objects and environments or, while performing memory tasks (Aloisi et al. 1997; Anzalone et al. 2009; Ceccarelli et al. 1999; Stanley et al. 2012).
Conversely, the reduction of cholinergic activity in the HF by septohippocampal lesions impairs spatial learning and object recognition memory (Berger-Sweeney et al. 2001; Cai et al. 2012; Easton et al. 2011). Additionally, the HF is widely populated with nAChRs, and recent studies demonstrate that following spatial memory task acquisition, nAChR expression is upregulated in the HF (Kutlu et al. 2016; Shanmugasundaram et al. 2015; Subramaniyan et al. 2014). Within the HF, the α7 and α4β2* nAChRs act as the predominant nAChR isoforms. They are present on both GABAergic interneurons (Alkondon et al. 1997; Bell et al. 2015; Frazier et al. 1998a; Jones and Yakel 1997; Maggi et al. 2001; Rosato-Siri et al. 2006) and glutamatergic pyramidal neurons (Griguoli and Cherubini 2012; Grybko et al. 2011; He et al. 2013; Ji et al. 2001; Kalappa et al. 2010; Tu et al. 2009), and can be found either presynaptically or postsynaptically (Bell et al. 2011; Cheng and Yakel 2014; Grybko et al. 2011; Gu et al. 2012; Zarei et al. 1999) (See Fig. 1.2). Recent work has demonstrated that functional α7 nAChRs on glutamatergic pyramidal neurons play an important role in mediating long-term potentiation (LTP) and plasticity at HF synapses, and may therefore provide a mechanism by which ACh mediates memory processes (Cheng and Yakel 2015a; Gu et al. 2012; Gu and Yakel 2011). However, the role of α4β2* on pyramidal neurons in HF is not as clear. Particularly, while previous electrophysiological work in adolescent-equivalent and older rodents failed to demonstrate the presence of functional α4β2* nAChRs within these principal output neurons (Placzek et al. 2009), in situ studies have shown that mRNA for the nicotinic α4, β2 and α5 subunits is expressed within the CA1 pyramidal neuron layer of mice (Heath et al. 2010; Hsu et al. 2013; Marks et al. 1992; Salas et al. 2003), rats (Sudweeks and Yakel 2000), and humans (Machaalani et al. 2010). Given the importance of nAChR-mediated signalling in the proper function of the HF, understanding the expression of α4β2* nAChRs within principal output neurons of the HF will be significant because this will allow us to better understand the
potential cellular mechanisms that contribute to the normal higher-order cognitive functions of the HF.

The subcellular location of an nAChR isoform determines its influence on neurotransmission. Activation of presynaptic nAChRs can lead to neurotransmitter release (Engelman and MacDermott 2004; Gray et al. 1996; Placzek et al. 2009; Wonnacott 1997) and the activation of postsynaptic nAChRs can lead to depolarization that can activate second messenger cascades (Alkondon et al. 1997; Fabian-Fine et al. 2001; Kenney and Gould 2008; McKay et al. 2007). The activation of second messenger cascades in HF neurons by nAChRs has been proposed to induce synaptic plasticity in the form of LTP. Much like the N-methyl-D-aspartic acid (NMDA) receptor, activation of nAChRs can induce Ca\(^{2+}\) flow into the cell. Therefore, the nAChR and NMDA receptor have been suggested to jointly modulate cell-signalling via synaptic plasticity. Specifically, Ca\(^{2+}\) influx due to nAChR and NMDA receptor activation may induce protein kinase A (PKA) activation, which, in turn, can activate the cAMP response element-binding protein (CREB) via extracellular signal-regulated kinase (ERK) 1/2 phosphorylation (Impey et al. 1998a; Impey et al. 1998b). Importantly, recent studies have linked PKA and ERK1/2 activation, and phosphorylated CREB, to HF-dependent learning (Abel and Nguyen 2008; Atkins et al. 1998; Bernabeu et al. 1997). Activation of nAChRs in the HF by the exogenous agonist nicotine has also been found to induce second messenger signalling cascades that are not typically activated during normal memory formation. Kenney et al. (2010) found that nicotine administered while learning increased c-Jun N-terminal kinase-1 (Jnk1) expression in the dorsal HF, and that this process was mediated by β2 subunit-containing nAChRs (Kenney et al. 2010). The involvement of Jnk1 is of particular interest because it activates transcription factors such as the JUN family, ATF-2, and
ELK-1, which are known to modulate and strengthen synaptic signalling (Bogoyevitch and Kobe 2006; Sananbenesi et al. 2002; Strekalova et al. 2003). The importance of β2 subunit-containing nAChRs in modulating synaptic signalling is further highlighted, as both Jnk1 activation and CREB phosphorylation at the Jnk1 promoter region are absent in β2 subunit knockout mice (Kenney et al. 2012a), which suggests that the specific effects of nicotine on synaptic signalling are mediated by β2 subunit-containing nAChRs such as the α4β2* isoform.

Besides mediating cell signalling cascades to elicit changes in neuron function, nAChR activation also modulates dendrite and neurite development in the HF. In adult-born neurons, or those which are formed in the adult HF, nAChR activation regulates both the maturation and integration of these neurons into HF networks by promoting neurite/dendrite elongation (Campbell et al. 2010; Coronas et al. 2000). In experiments with genetically-modified mice, nAChRs containing the β2 subunit contribute to the production of dendrite spines in CA1 pyramidal neurons through a Ca\(^{2+}\)-dependent pathway requiring calmodulin-dependent protein kinase II (Ballesteros-Yanez et al. 2010; Lozada et al. 2012b). These findings suggest that nAChR function in the HF serves as an important mechanism by which neuronal networks are modulated, and may have direct consequences on HF functional output.
Fig. 1.2. Simplified overview of the expression of α7 and α4β2* nAChR isoforms on glutamatergic principal neurons (black) and GABAergic interneurons (grey) in the HF CA1 region. Line and dot represent a dendrite and synaptic terminal, respectively. The symbol “?” depicts uncertainty of the functional presence of the α4β2* nAChR on HF principal neurons. Functional α7 and α4β2* nAChRs are expressed presynaptically and/or postsynaptically on neurons situated within the region, or present on synaptic terminals of neurons projecting from outside the CA1 region. Adapted from (Griguoli and Cherubini 2012).
1.2.3 Nicotinic signalling in the prefrontal cortex

The mPFC receives dense cholinergic innervation from the basal forebrain, a region which contains numerous cholinergic nuclei such as the nucleus basalis, septum, diagonal band of Broca, and substantia innominata (Bloem et al. 2014; Mechawar and Descarries 2001; Mesulam 1995; Zaborszky et al. 1999). Like the HF, the proper function of the mPFC appears to be modulated by cholinergic signalling. Particularly, during attention tasks, there is an increase in ACh that is proportional to the attentional demands of the task (Kozak et al. 2006; Parikh et al. 2007; Passetti et al. 2000). Additionally, in studies of rhesus monkeys with selective lesions of cholinergic input to the primate dlPFC, depletion of cholinergic input impairs spatial working memory, whereas application of α4β2* nAChR agonists enhances the task-related firing of delay and fixation cells in the dlPFC of monkeys performing a working memory task (Croxson et al. 2011; Sun et al. 2017).

Nicotinic signalling is layer specific in the mPFC and each layer has a different pattern of nAChR expression (Dajas-Bailador and Wonnacott 2004; Gullidge et al. 2007; Yakel 2013). In layer I of the mPFC, both α7 and α4β2* nAChRs are present in interneurons, and stimulation of these nAChRs enhances synchronous activity of inhibitory cortical networks in the superficial cortex (Alitto and Dan 2012; Bandyopadhyay et al. 2006; Christophe et al. 2002). The majority of pyramidal neurons within layer II/III of the mPFC do not express nAChRs or receive glutamatergic inputs influenced by nicotinic modulation (Poorthuis et al. 2013b). However, both α7 and α4β2* nAChRs are found on interneurons within layer II/III, where they exert a feedforward inhibition onto local pyramidal neurons (Bloem et al. 2014). Although cholinergic afferent fibers are dense throughout the mPFC, they are most prominently located within the deep layers V and VI (Bloem et al. 2014). In layer V, interneurons contain both α7 and α4β2* nAChRs (Alkondon et al. 2000;
Poorthuis et al. 2013b), which exert inhibitory postsynaptic signals onto neighbouring pyramidal neurons (Poorthuis et al. 2013b). Pyramidal neurons within layer V are predominantly modulated directly by postsynaptic mAChRs, but not nAChRs (Gulledge et al. 2009). However, activation of presynaptic $\alpha 4\beta 2^*$ nAChRs on glutamatergic inputs from the thalamus strongly enhances glutamate release and postsynaptic activity of layer V pyramidal neurons, and postsynaptic $\alpha 7$ nAChR-mediated nicotinic response has been documented in prefrontal cortex layer V pyramidal neurons of juvenile mice (Poorthuis et al. 2013a). In layer VI, interneurons express $\alpha 7$ nAChRs that mediate cholinergic excitation by thalamocortical axon terminals (Dominguez del Toro et al. 1994; Kassam et al. 2008; West et al. 2006). Immunohistochemistry for the YFP-tagged $\alpha 4$ subunit suggests that high-affinity nAChRs are also densely expressed in mPFC layer VI pyramidal neurons (Alves et al. 2010; Poorthuis et al. 2013a), and that these neurons show a robust excitatory response to ACh that is mediated by postsynaptic $\alpha 4\beta 2^*$ nicotinic receptors (Bailey et al. 2010; Guillem et al. 2011) (See Fig. 1.3).

The specific expression of $\alpha 4\beta 2^*$ nAChRs in mPFC layer VI pyramidal neurons may be important in shaping layer-specific output that underlies the production of cognitive behaviours that are related to the mPFC. Previous studies have demonstrated in cultured neurons that the stimulation of nAChRs limits neurite outgrowth (Lipton et al. 1988; Nordman and Kabbani 2012), a process which is mediated in a $Ca^{2+}$-dependent manner (Pugh and Berg 1994). In addition, it has been demonstrated that the $(\alpha 4\beta 2)\alpha 5$ nAChR isoform is highly $Ca^{2+}$-permeable and prominently-expressed in layer VI of the mPFC (Salas et al. 2003; Wada et al. 1990). Therefore, the specific expression of the $(\alpha 4\beta 2)\alpha 5$ isoform in mPFC layer VI pyramidal neurons may play an important role in modulating neurite development. Consistent with this idea, it has
been demonstrated that mPFC layer VI pyramidal neurons are directly mediated by postsynaptic $(\alpha_4\beta_2)\alpha_5$ nAChRs (Bailey et al. 2010; Kassam et al. 2008; Poorthuis et al. 2013b; Tian et al. 2011), and that the stimulation of $(\alpha_4\beta_2)\alpha_5$ nAChRs appears to mediate the retraction of apical dendrites in these neurons during postnatal maturation (Bailey et al. 2012). Furthermore, as mice lacking the $\beta_2$ nAChR subunit exhibit decreased attention performance and the re-expression of this subunit specifically within mPFC layer VI pyramidal neurons restores this effect (Guillem et al. 2011), $\alpha_4\beta_2^*$ nAChRs may serve as an important mechanism that facilitates the proper function of the mPFC circuitry.

Studies investigating the influence of nAChRs within the mPFC circuitry using intracranial injection of receptor agonists and antagonists into the mPFC have further demonstrated their role in mPFC-dependent higher-order cognitive functions. Using the 5-choice serial reaction time task (5-CSRTT), an operant task used to assess attention and impulsivity, it was demonstrated in rats that while acute and chronic nicotine treatment improves performance in this test, cessation of chronic nicotine treatment decreases performance in this test as well (Semenova and Markou 2007; Shoaib and Bizarro 2005; Stolerman et al. 2009). This change in performance is likely to be mediated by $\alpha_4\beta_2^*$ nAChRs located within the prelimbic PFC, as infusion of the $\alpha_4\beta_2^*$ nAChR isoform-selective antagonist dihydro-β-erythroidine (DHβE) in this region block the effects of nicotine on the task (Blondel et al. 2000; Hahn et al. 2003). In another study comparing standard cue fear conditioning (a form of learning which does not critically involve the hippocampus) and trace fear conditioning (a form of learning which is hippocampus dependent), it was found that the infusion of DHβE into the mPFC enhances trace but not context fear conditioning (Raybuck and Gould 2010). These findings demonstrate that while learning may involve both the mPFC and HF,
learning is in part subjected to modulation by heteromeric α4β2* nAChRs of the mPFC specifically. Together, these findings suggest that nAChR function in the mPFC serves as an important point through which neuronal networks are modulated to affect mPFC functional output.
Fig. 1.3. Simplified overview of the expression of α7 and α4β2* nAChR isoforms on glutamatergic pyramidal neurons (black) and GABAergic interneurons (grey) in the mPFC. Line and dot present a dendrite and synaptic terminal, respectively. Functional nAChRs of both isoforms have been identified in both neurons. Thalamic terminal expressing the α4β2* nAChR is presented (purple). Adapted from (Bloem et al. 2014).
1.2.4 Sex differences in nicotinic signalling

Males and females are different not only in reproductive function but also in brain structure and function (Pogun 2001). This is true for nicotinic excitation as well, which is known to differ based on sex during brain during development. In a study that examined the developmental regulation of nAChRs within midbrain dopamine neurons of rats, it was reported that nAChRs on dopamine terminals are developmentally regulated and that there are sex differences in nAChR function in these neurons. Particularly, in both males and females, there is a decrease in nAChR function during the first postnatal week, followed by an increase in function during the second and third postnatal weeks. However, during the transition from adolescence to adulthood, there is a complex pattern of functional maturation of nAChRs, with significant changes in both nicotine potency and efficacy in males but not females (Azam et al. 2007). Similarly, in a study that examined nicotinic currents of prefrontal layer VI neurons in both mice and rats, it was found that there is a prominent sex difference in the currents during the first postnatal month of development. Specifically, while nicotinic currents in mPFC layer VI pyramidal neurons peak in magnitude around the fourth week of postnatal life in males, this occurs during the third week of postnatal life in females, and additionally, nicotinic currents are larger and observed in a greater proportion of cells in male than in female mice (Alves et al. 2010). Differences in cholinergic input may also explain for sex differences in nicotinic signalling. Previous studies have demonstrated that the regulation of choline acetyltransferase (the enzyme responsible for the synthesis of ACh) in the basal forebrain of rats is different in males and females (Luine et al. 1975). Particularly, estradiol in ovariectomized female rats has been shown to increase ChAT immunoreactivity in the nucleus basalis (Gibbs 1997), and the number of ChAT immunoreactive cells in the nucleus basalis is greater in females than in males (Jitsuki et al. 2009). In addition, destruction of cholinergic
afferents from the MSDB has also been found to inhibit the effects of estradiol on CA1 and DG cell morphology and development, respectively (Lam and Leranth 2003; Mohapel et al. 2005). Together, these findings suggest that sex steroids may be involved in regulating afferent cholinergic input to the mPFC and HF and also in contributing to sexual differences in nicotinic signalling in these two brain regions.

Studies on the effects of nicotine further demonstrate differences in nAChR signalling between males and females. For example, in a study examining whether behavioural sensitivity to chronic nicotine varies across sex, it was demonstrated that female mice are less sensitive to the locomotor activating effects of chronic nicotine, and nicotine produces an anxiogenic-like response in females, but no effect in males (Caldarone et al. 2008). Another study also demonstrated that female mice show a greater response to the conditioned rewarding properties of nicotine. While females maintain consistent levels of intake over several doses of nicotine, males respond to a greater degree to the pharmacological aspects of nicotine and reduce levels of nicotine drinking at higher concentrations (Isiegas et al. 2009). Additionally, studies in both rodents and humans demonstrated that females take a shorter time to become dependent on nicotine, make fewer quit attempts, stay abstinent for shorter periods and have a higher rate of relapse (Donny et al. 2000; Perkins et al. 2001; Pogun et al. 2017).

Sex differences observed in in vivo studies can arise through several mechanisms. For example, pharmacokinetic variations in plasma and brain levels of nicotine can produce behavioural differences between males and females, as one study demonstrated that female C57BL/6 mice eliminate nicotine faster than males (Hatchell and Collins 1980). As well, sex steroid hormones
can modulate responses to nicotine, where both progesterone and estradiol block nicotine-induced analgesia (Marubio et al. 1999). However, numerous studies suggest that the observed sex differences in responsiveness to nicotine specifically arise from differences in nAChR expression. First, studies that examined the association of allelic variants of the nAChR α4 subunit gene (CHRNA4) suggest the existence of gender specificity in the association of CHRNA4 with nicotine dependence (Li et al. 2005). Second, although chronic nicotine administration increases nicotinic receptor content in cultured neurons and several brain regions of rodents (Besson et al. 2007; Buisson and Bertrand 2002; Flores et al. 1997; Lomazzo et al. 2011; Marks et al. 1985), this effect is found in male, but not female rats. Specifically, it was found that while female rats had higher baseline densities of nAChRs, only male rats had increased nAChR binding in the whole brain in the HF and cortex after chronic nicotine administration (Koylu et al. 1997). Additionally, using a specific in vivo radioligand for nAChRs, it was found that after chronic nicotine administration, binding was increased in all brain regions studied for male and female animals, but males showed a much more pronounced increase than females (Mochizuki et al. 1998). Unfortunately, the molecular mechanisms underlying these observed sex differences in the central actions of nicotine are not fully understood. However, one study demonstrated that although daily nicotine injections increase the number of binding sites in the rat brain, there is no parallel increase in the mRNA content for genes encoding nAChR subunits, suggesting a post-translational effect of nicotine on nAChR function (Rowell and Li 1997). There is strong evidence to suggest that many of the effects of nicotine are specifically mediated by nAChRs containing the β2 subunit (Brody et al. 2006; Brunzell et al. 2006; King et al. 2004; Picciotto et al. 1998), as β2-containing nAChRs are substantially upregulated by nicotine treatment (Besson et al. 2007; Marks et al. 1985; McCallum et al. 2006). Interestingly, the presence of the α5 subunit, which associates with the
α4β2* nAChR receptor, appears to prevent in vivo upregulation of β2-containing nAChRs (Tuesta et al. 2011). Sex differences in the expression and function of the α4β2* nAChR receptor may therefore lead to differences in neuronal excitation, and underlie the differential development and incidence of aberrant brain pathologies, and therefore warrants further investigation.

1.3. Modulation of brain function by the progesterone metabolite allopregnanolone

Steroids were typically thought to be secreted from peripheral endocrine glands and released into the circulation, where they can then exert effects at distant target sites including the brain. The brain however, is now recognized as an endocrine organ as well. Not only does it require coordinated actions of steroidogenic enzymes in neurons and glia in order to metabolize steroids that have crossed the blood-brain barrier, but it is also capable of endogenous de novo production of steroids in a manner that is independent of peripheral endocrine glands (Frye 2009a; Paul and Purdy 1992). The demonstration that steroids can be synthesized de novo in the brain came initially from observations made in the 1980s by Baulieu and colleagues, who found that steroids such as pregnenolone and their sulfate and lipoidal esters were present in higher concentrations in tissue from the nervous system than those found in circulation, and that the concentration of these steroids remained high in the brain after adrenalectomy and orchietomy (Corpechot et al. 1981; Corpechot et al. 1983). Steroids that are synthesized in the brain are now widely studied and referred to as neurosteroids (Paul and Purdy 1992).

The classic mechanism of steroid action involves the binding of steroid molecules to the classic intracellular nuclear steroid receptors, which leads to changes in transcription and translation of target genes and proteins, respectively (Joels 1997; Reddy 2003; Rupprecht et al. 1993). While
this classic process may take place on the scale of hours or days, steroid hormones have also been shown to exert rapid signalling actions within minutes to seconds (Frye 2009b). It is now recognized that neurosteroids can produce rapid effects on neuronal excitability and synaptic function by direct or indirect modulation of membrane receptors, such as neurotransmitter-gated ion channels, or other neurotransmitter receptors and transporters (Majewska 1992; Reddy 2010; Rupprecht 2003; Wu et al. 1991). Emerging findings regarding the source, mechanisms of action, and effects of neurosteroids have challenged and redefined the traditional view of their capacity to influence these processes in the brain and warrant further investigation. The following sections of this thesis will further discuss the current understanding of neurosteroid action, with particular focus on the progesterone metabolite allopregnanolone (ALLO), which is one of the most widely studied compounds among the neurosteroids.

1.3.1 The de novo production of progesterone and its metabolites within the brain

Neurosteroids are synthesized de novo from cholesterol by neurons and glial cells (Melcangi et al. 2008; Melcangi and Panzica 2014; Mensah-Nyagan et al. 1999). Cholesterol in the CNS is transported to the inner mitochondrial membrane of these cells by the steroidogenic acute regulatory protein, and the first enzymatic reaction of steroidogenesis is the transformation of cholesterol to pregnenolone by the cytochrome P450 cholesterol side-chain cleavage enzyme (Rossetti et al. 2016). Progesterone can then be synthesized from pregnenolone by the enzymatic action of 3β-hydroxysteroid dehydrogenase (HSD) (Rossetti et al. 2016). The 3β-HSD enzyme is found in both neurons and in glia of rat brains (Akwa et al. 1993; Brown et al. 2000; Ibanez et al. 2003; Sanne and Krueger 1995). Progesterone may be further metabolized by the enzyme 5α-reductase to become 5α–dihydroprogesterone (5α-DHP). The formation of 5α-DHP largely takes
place in neurons, but type 2 astrocytes and oligodendrocytes also possess considerable 5α-
reductase activity (Melcangi et al. 1993). The enzyme 3α-HSD can further convert 5α-DHP to the
neurosteroid ALLO, and back-convert ALLO to 5α-DHP (Li et al. 1997; Rossetti et al. 2016;
Rupprecht et al. 1993). The full ALLO biosynthesis pathway is shown in Fig. 1.4.

Rodents have one single 3α-HSD isozyme that is expressed in the cerebral cortex and HF, as well
as in the olfactory bulb, basal ganglia, hypothalamus, thalamus and cerebellum (Compagnone and
Mellon 2000). The enzyme is also developmentally regulated, where 3α-HSD mRNA expression
is two-fold greater during the second week than at the twelfth week of postnatal development in
the rat HF (Higo et al. 2009). 3α-HSD activity has been reported in neurons, astrocytes, and
oligodendrocytes of the adult rat spinal cord, suggesting that ALLO may also be synthesized by
glial cells (Patte-Mensah et al. 2004). More recent findings have also demonstrated that 3α-HSD
and 5α–reductase mRNA co-localize in principal glutamatergic neurons of the cerebral cortex,
hippocampus, olfactory bulbs, and in glutamatergic output neurons of the thalamus and amygdala
(Agis-Balboa et al. 2006). These findings suggest that the de novo production of the progesterone
metabolites 5α–DHP and ALLO may be regulated by the regional- and developmental-specific
expression of metabolizing enzymes, and therefore, changes to this dynamic and regulated process
may have important implications for the function of cognitive brain regions such as the HF and
PFC.
Fig. 1.4. Biosynthesis pathway for allopregnanolone in the brain.
1.3.2 Allopregnanolone in the hippocampal formation and prefrontal cortex

The steroid progesterone, like estrogen, was historically largely studied within the confines of reproductive function. Consequently, the effects of progesterone and its metabolites on the brain were focused on the hypothalamus. Growing evidence suggests that additional brain regions including the HF and cerebral cortex are equally regulated by neurosteroids (Djebaili et al. 2004; Pibiri et al. 2008; Wang et al. 2005). Furthermore, ALLO has emerged as an important neurosteroid metabolite that targets both neurons and glial cells in the central and peripheral nervous systems (Noorbakhsh et al. 2014). ALLO is also involved in numerous aspects of brain function, such as neuroprotection and neuroplasticity, and has been affiliated with the regulation of emotions and higher-order cognitive functions such as learning and memory. Therefore, the role of ALLO in the HF and PFC should not be overlooked.

First, ALLO influences diverse aspects neural structure and activity in the HF and PFC, with an overall effect that appears to be neuroprotective. In both the rat and human HF, ALLO increases neurogenesis and neuron survival by promoting neural progenitor cell proliferation (Wang et al. 2005). Additionally, ALLO reduces apoptosis. Specifically, by blocking the metabolism of progesterone to ALLO using co-administration of the 5α-reductase inhibitor finasteride, the protective effects of progesterone on cell death in the DG are attenuated in female rats (Rhodes et al. 2004). Within the PFC, administration of ALLO also decreases neuronal loss after traumatic brain injury. Following the application of controlled physical blunt trauma to the PFC, rats were injected with ALLO every day for 5 consecutive days after the injury. One day after injury, both progesterone-treated and ALLO-treated rats showed less caspase-3 activity, an effector caspase that executes apoptosis, and rats treated with higher doses of ALLO showed less DNA
fragmentation in the lesion area, suggesting reduced apoptosis (Djebaili et al. 2004). ALLO also plays a role in neuroplasticity. In primary cultures of HF neurons that were either transfected with green fluorescent protein (GFP) or immunostained against drebrin (an actin-binding protein that forms a unique stable actin structure in dendritic spines), morphological analysis of neurons revealed that exposure to ALLO for 24 hours significantly increases dendritic spine and drebrin cluster density (Shimizu et al. 2015). In a study evaluating the role of ALLO on synaptic pruning at the onset of puberty, ALLO also increases spine density two-fold in adolescent female mice, with its greatest effects on mushroom spine expression (Afroz et al. 2017).

Second, ALLO also appears to influence higher-order cognitive functions. During pro-estrus and late pregnancy, when cerebral cortical ALLO content is greatest, young rats exhibit better performance on the object recognition task than di-estrous rats or rats in early pregnancy (Frye 2009a). Additionally, administration of ALLO in the HF prevents memory impairment in inhibitory avoidance tasks (Escudero et al. 2012). Studies investigating neuropsychiatric disorders also suggest that ALLO plays a role in modulating normal cognitive functions. For example, executive function is a significant area of impairment in individuals with Tourette’s syndrome (Channon et al. 2003). In a study of D1CT-7 mice, which is the best validated model of Tourette’s syndrome, ALLO content increased in the PFC during exposure to moderate environmental stressors. Separate administration of ALLO in the PFC exacerbated Tourette’s syndrome-like manifestations in D1CT-7, but not wild-type littermates, and these effects were suppressed by finasteride. These findings suggest that ALLO in the PFC may augment cognitive symptoms of Tourette’s syndrome (Mosher et al. 2017). In a study investigating the efficacy of ALLO to promote neurogenesis in the hippocampal subgranular zone to reverse learning and memory
deficits in 3-month-old male triple transgenic mouse model of Alzheimer's, it was also demonstrated that ALLO reverses the neurogenic and cognitive deficits associated with these mice (Wang et al. 2010).

Finally, ALLO serves as a neuroendocrine factor that acts as an anxiolytic to protect an animal from stress and to enable appropriate behavioural responses (Engel and Grant 2001; Purdy et al. 1991). In support of this, ALLO is present during prenatal development and its content increases in the brain in response to stressors such as maternal separation (Kehoe et al. 2000; Kellogg and Frye 1999; McCormick et al. 2002). Conversely, mice subjected to three-to-four weeks of social isolation exhibit enhanced contextual fear responses and impaired fear extinction. These findings correspond with decreased 5α-reductase mRNA expression and ALLO content in neurons of the HF, mPFC and basolateral amygdala, areas that have been previously implicated in the control of anxiety in animal models (Pibiri et al. 2008). The effect of ALLO microinfused into each of these three regions was evaluated in Sprague-Dawley rats tested on the elevated plus-maze and the shock-probe burying test. In these studies, ALLO in the amygdala produces anxiolytic-like effects in both tests, ALLO in the mPFC produces anxiolytic-like effects only on the plus-maze test, and ALLO in the HF is ineffective in both tests (Engin and Treit 2007). These findings suggest that the anxiolytic effects of ALLO may vary depending on its region of action.

1.3.3 Non-classical progesterone receptors: membrane progesterone receptor and the progesterone membrane receptor component

The classic genomic mechanism of progesterone action involves the activation of the nuclear progesterone receptor (PR). While progesterone and the downstream metabolite 5α–DHP can
mediate effects through the PR, it is reported that ALLO does not activate the PR (Belelli and Lambert 2005; Rupprecht et al. 1993). Nevertheless, the PR undergoes a conformational change upon neurosteroid binding; they dissociate from chaperone proteins, dimerize, and directly interact with specific response elements in the promotor regions of target genes to regulate gene expression, a process which typically occurs over a time scale of hours (DeMarzo et al. 1991; Edwards et al. 1991; Evans 1988). It has also been reported that like other neurosteroids, progesterone metabolites also exert rapid non-genomic effects, and that these actions can occur within minutes by activating membrane receptors and their intracellular transduction pathways (Frye 2001; Hwang et al. 2009; Meyerson 1972). These data support the concept that progesterone and its metabolites likely act through both classical PRs and also through non-classical mechanisms.

The membrane PRs (mPRs) belong to a larger class of membrane receptors called progestin and adipoQ receptors (PAQR) (Tang et al. 2005). There are five mPR isoforms, including the mPRα (PAQR7), mPRβ (PAQR8), mPRγ (PAQR5), mPRδ (PAQR6) and mPRε (PAQR9), which have been found in a wide variety of tissues (Dressing et al. 2012; Intlekofer and Petersen 2011; Pang et al. 2013; Zhu et al. 2003; Zuloaga et al. 2012). Both the mPRα and mPRβ have been detected in rat cerebral cortex (Intlekofer and Petersen 2011; Meffre et al. 2013), and their expression in the brain varies with hormonal changes during estrus with the highest expression during proestrus, suggesting receptor down regulation by progesterone (Liu and Arbogast 2009). While mapped expression for mPRδ and mPRε in the rat forebrain found no specific signal for either of these mRNAs (Intlekofer and Petersen 2011), they have been detected within the human brain (Pang et al. 2013). Of the five mPR subtypes, the mPRα is the most extensively characterized (Thomas 2008). The neurosteroid ALLO is an effective ligand for recombinant mPRα with a relative binding
affinity of 7.6% that of progesterone (Kelder et al. 2010). Analysis of mPR sequences suggest that these peptides contain seven trans-membrane domains and therefore have been characterized as a novel group of G protein-coupled receptors (GPCRs) that mediate rapid progesterone actions through intracellular signalling pathways (Tang et al. 2005; Thomas and Pang 2012). The mPRα, mPRβ and mPRγ are thought to decrease membrane-bound adenylyl cyclase cAMP-producing activity through the inhibitory Gαi/o signalling pathway, as pertussis toxin (an inhibitor of Gαi/o activity), suppresses progesterone-dependent cAMP repression in neuronal cells (Thomas and Pang 2012). Conversely, mPRδ and mPRϵ appear to activate a stimulatory Gs signalling pathway (Pang et al. 2013). There also is conflicting evidence that mPRs may not function as GPCRs. Following heterologous expression of human mPRα and mPRγ in yeast, progesterone-dependent signalling did not appear to require the heterotrimeric Gα protein (Smith et al. 2008). As well, while the promotion of ERK phosphorylation via mPRβ leads to progesterone dependent neurite outgrowth, the process did not exhibit typical GPCR signalling either (Kasubuchi et al. 2017). Therefore, while the topology of mPRs remains uncertain, these findings suggest that mPRs may control different aspects of cell signalling and function through various signalling cascades.

Another family of membrane-bound proteins that has been recognized as a candidate for the mediation of non-classical effects of progesterone is the progesterone receptor membrane component (PGRMC) family, which consists of PGRMC1 and PGRMC2. These peptides have one single transmembrane domain and contain a heme/steroid-binding domain similar to that of cytochrome b5 (Peluso et al. 2008; Thomas 2008). PGRMC1 preferentially binds to progesterone, and the knockdown of PGRMC1 by 60% has been found to reduce progesterone binding by the
same amount in spontaneously immortalized granulosa cells (Meyer et al. 1996; Peluso et al. 2008). There are currently no published reports demonstrating a direct effect of ALLO at either PGRMC subtype. PGRMC1 is expressed in many brain regions (Intlekofer and Petersen 2011; Meffre et al. 2013). While PGRMC2 often co-localizes with PGRMC1, PGRMC2 does not bind to progesterone and its role in progesterone signalling is not clear (Petersen et al. 2013). PGRMC1 has recently been suggested to be the same protein as the sigma-2 receptor: two peptides have similar steroid hormone-binding profiles (Meyer et al. 1996; Peluso et al. 2008); they exhibit co-localized expression in the endoplasmic reticulum and mitochondria; changes in PGRMC1 content correlate with changes in sigma-2 ligand binding (Xu et al. 2011); and functionally, both receptors regulate intracellular Ca\(^{2+}\) with PGRMC1 signalling through the PKG pathway (Bashour and Wray 2012; Peluso et al. 2008; Vilner and Bowen 2000). It has not been fully determined whether PGRMC1 binds progesterone directly or requires a binding partner. For example, Glutathione S-transferase-tagged PGRMC1 expressed in E. coli does not bind to progesterone in pull-down assays (Min et al. 2005), and PGRMC1 has been shown to require a binding partner known as serpine mRNA binding protein 1 before it can bind progesterone (Peluso et al. 2006). One possible explanation of these findings is that a protein present in eukaryotes but lacking in E. coli complexes with PGRMC1 and is required for progesterone binding.

Recent evidence suggests that the mPRs and PGRMC1 may form one membrane progesterone receptor complex. In support of this idea, mPR\(\alpha\) and PGRMC1 exhibit overlapping distributions in specific brain regions such as the cerebral cortex (Intlekofer and Petersen 2011; Meffre et al. 2013); they both show increased expression in ovarian tumours (Peluso 2011); and they both mediate similar anti-apoptotic functions of progestins in vertebrate granulosa and cancer cells
In a study by Thomas et al. (2014), immunocytochemical and co-immunoprecipitation experiments showed a close association of mPRα with PGRMC1 in cell membranes of nuclear PR-negative breast cancer cell lines and in rat spontaneously immortalized granulosa cells. This study demonstrated that overexpression of PGRMC1 increases plasma localization of mPRα, as well as the amount of mPRα associated with PGRMC1 in these cells. Additionally, co-transfection with small interfering (si) RNA for mPRα abolished the anti-apoptotic effects of progestins. Specifically, progesterone binding to membranes of spontaneously immortalized granulosa cells overexpressing PGRMC1 decreased to near background levels when mPRα expression is down-regulated by treatment with mPRα siRNA (Thomas et al. 2014). These findings provide strong evidence that functions previously ascribed to PGRMC1 are dependent on cell surface expression of mPRα, and that the mPRs and PGRMC1 are components of the same membrane progesterone receptor complex that modulate non-classical progesterone metabolite signalling. Understanding how mPRs and PGRMC1 mediate the non-classical effects of progesterone and its metabolites will be important in our understanding of how neurosteroids can influence neurophysiology underlying cognitive functions.

1.3.4 Neurosteroid modulation of ligand-gated ion channel receptors

Beside the mPRs and PGRMC1, the most extensively investigated non-classical actions of neurosteroids are those at synaptic and extrasynaptic GABA\textsubscript{A} receptors (Purdy et al. 1991). Unlike progesterone, ALLO is inactive at nuclear PRs (Borowicz et al. 2011). Instead, ALLO mediates its rapid effects through positive allosteric modulation of GABA\textsubscript{A} receptors at nanomolar concentrations (Fodor et al. 2005; Harrison and Simmonds 1984; Majewska et al. 1986; Schmid et al. 1998). ALLO enhances GABA\textsubscript{A} receptor function when bound with GABA by increasing
receptor opening frequency and duration, through the modification of the kinetics of entry and exit from desensitized states of the receptor (Harrison and Simmonds 1984; Hosie et al. 2006; Majewska 1992; Puia et al. 2003; Puia et al. 1990; Zhu and Vicini 1997). Many of the neurophysiological effects of ALLO are similar to those exhibited by other pharmacological positive allosteric modulators of the GABA<sub>A</sub> receptor, although it has been reported that ALLO increases chloride channel currents by extending the opening time of the channel and lowers postsynaptic neuronal excitability by prolonging inhibitory currents, with 20- and 200-fold higher efficacy than benzodiazepines or barbiturates, respectively (Allan et al. 1992; Belelli and Lambert 2005; Majewska 1992; Purdy et al. 1992; Roca et al. 1990). Through its interaction with GABA<sub>A</sub> receptors, ALLO affects diverse aspects of neural cell physiology (Stromberg et al. 2006), modulates stress (Gunn et al. 2015; Purdy et al. 1991), and appears to contribute to the development of neurological disorders such as epilepsy (Smith et al. 2007) and Alzheimer’s disease (Irwin et al. 2011).

In addition to their actions at GABA<sub>A</sub> receptors, progesterone and its metabolites have also been reported to modulate nAChR function. In a study that assessed the effects of several neurosteroids on the function of α4β2* nAChRs expressed in Xenopus oocytes, Valera et al. (1992) reported that progesterone acts as a noncompetitive inhibitor of receptor function (Valera et al. 1992). In a separate study by Ke and Lukas (1996), it was demonstrated that progesterone also inhibits nicotinic receptor function in TE671 and SH-SY5Y cells (Ke and Lukas 1996). While TE671 cells express the muscle-type nAChR and SH-SY5Y cells express α3β4 nAChRs, progesterone acts as an allosteric inhibitor of both of these nAChR isoforms. Using <i>ex vivo</i> mouse striatal and thalamic synaptosomes, Bullock et al. (1997) examined the effects of progesterone, and its metabolites 5α-
DHP and ALLO, on $\alpha_4\beta_2^*$ nAChR-mediated function using an $^{86}\text{Rb}$-efflux assay. This study reported that both 5$\alpha$-DHP and ALLO function as non-competitive inhibitors of brain nAChRs. Interestingly, progesterone, 5$\alpha$-DHP and ALLO do not appear to act, or have little action, on the other cys-loop ligand gated ion channel superfamily of homologous receptors, which include the glycine and serotonin receptors (Mellon 2007).

Whereas the modulatory effects of ALLO on GABA$_\text{A}$ receptor function have been studied extensively over the past three decades at the molecular, cellular and physiological levels, the effects of ALLO on nAChR function have only been reported in in vitro and ex vivo reduced preparations. The influence of ALLO on nAChRs function has not been investigated in living mammalian neurons. Understanding the role of ALLO in intact native neurons is important to our understanding of how neurosteroids may modulate nicotinic signalling in the brain. This information will have strong implications for the function of cognitive brain regions such as HF and mPFC, which depend on nicotinic signalling for the regulation of neuron excitability, synaptic transmission, and synaptic plasticity during early development.

1.4 Thesis Rationale and Approach

Rationale

The HF and PFC have long been implicated in mediating higher-order cognitive functions such as learning, memory and attention. The proper development and function of HF and PFC neurocircuitry depends on the interaction between the neurotransmitter ACh and nAChRs. The nAChR $\alpha_4\beta_2^*$ isoform is one of the primary mediators of such ACh signalling within the brain. Dysregulation of ACh signalling and nAChR function has been observed in a number of
neurological disorders during development and adulthood that involve deficits to learning, memory or attention (Changeux 2010; Drenan and Lester 2012; Gotti and Clementi 2004; Hurst et al. 2013; Picciotto et al. 2012; Picciotto and Zoli 2008), and dysregulation of ACh/nAChR neurotransmission within the HF and/or mPFC can lead to a disruption of these cognitive functions (Deutsch 1971; Mansvelder et al. 2006).

While the location and functional role of α4β2* nAChRs have been well characterized within the mPFC, the location, function and developmental regulation of these receptors are not well known within the HF. Previous electrophysiological work in adolescent-equivalent and older rodents failed to demonstrate the presence of functional α4β2* nAChRs within CA1 principal neurons (Placzek et al. 2009), but in situ expression analysis suggested that α4β2* nAChRs are present within HF DG, CA3, CA1 and SUB principal neurons of the HF during the first two weeks of postnatal life. Understanding the expression and function of α4β2* nAChRs within principal neurons of the HF will be significant, as this will allow us to better understand the role that nAChRs plays in regulating neuronal excitability, synaptic transmission, and synaptic plasticity in the HF during early development, as well as how this may influence the contribution of the HF toward higher-order cognitive functions such as learning and memory.

There is growing evidence to suggest that the progesterone metabolite ALLO plays a role in mediating the proper function of neurons within the HF and PFC, whereby it influences neuron physiology, higher-order cognitive functions, and the brain’s stress responses (Djebaili et al. 2004; Pibiri et al. 2008; Wang et al. 2005). While it is well known that ALLO can mediate these effects through interactions through the mPR and/or PGRMC1, and by potentiating GABA<sub>A</sub> receptor
function, studies in reduced preparations suggest that ALLO also negatively modulates α4β2* nAChR function. Since the development of the HF and mPFC, and the contribution of these two regions toward cognitive functions depend on local α4β2* nAChR signalling, negative modulation of α4β2* nAChR function by ALLO may impart a significant negative impact on the development and mature function of these two regions, and their contributions to higher-order cognitive functions.

It should be noted, as described above, that there are known sex differences in the magnitude and developmental pattern for α4β2* nAChR function within the brain (Alves et al. 2010). Studies conducted in both the male and female brain will be important to help improve our understanding of how sex differences in nicotinic signalling within the brain may influence cognitive behaviour and the risk for brain disorders.

Approach

Given the importance of nicotinic signalling in the normal development of the HF and mPFC, and the combined contribution of these regions towards normal cognitive function, the overall aim of this thesis is to contribute novel fundamental knowledge towards the complete understanding of the function of α4β2* nAChR function within these regions. This aim was met by completing the following three objectives:

Objective 1: To determine whether functional heteromeric α4β2* nAChRs are present in CA1 principal neurons of the developing mouse hippocampal formation. Since previous *in situ* expression analysis suggested the α4β2* nAChR component α5 subunit is expressed in these
neurons during early postnatal life, I hypothesized that functional α4β2* nAChRs are present in hippocampal CA1 pyramidal neurons and their function is developmentally regulated to peak during the first two weeks of postnatal life. Experiments described in Chapter 2 were performed in male mice.

**Objective 2: To characterize α4β2* nAChR function across the primary regions of the hippocampal formation in young postnatal mice.** Since the expression of subunits comprising heteromeric nAChRs varies in magnitude across regions of the young postnatal HF, I hypothesized that α4β2* nAChRs are present in principal neurons of each primary region of the young postnatal HF, and that the magnitude of this function varies across these regions. Experiments described in Chapter 3 measured α4β2* nAChR function in CA1, CA3, DG, SUB and ECVI principal neurons in young postnatal male mice. Experiments described in Chapter 4 measured α4β2* nAChR function in CA1, CA3, DG, SUB and ECVI principal neurons in young postnatal female mice, and compare results with those observed in males.

**Objective 3: To determine the effect of allopregnanolone on α4β2* nAChR function within living neurons of the medial prefrontal cortex of young postnatal mice.** Since ALLO negatively modulates α4β2* nAChR function in reduced synaptosome and oocyte preparations, I hypothesized that ALLO also negatively modulates α4β2* nAChR function in living principal neurons of the mouse brain. Results from Objectives 1 and 2 demonstrate that α4β2* nAChR function is present at low levels in principal neurons of the HF. Since it would not be reliable to measure the inhibition of small nicotinic responses using my whole-cell electrophysiology technique in HF neurons, I tested this hypothesis using mPFC layer VI pyramidal neurons because
they are known to exhibit robust $\alpha 4\beta 2^*\ nAChR$-mediated responses. Experiments are described in Chapter 5, and were conducted in young postnatal male and female mice. I determined: (i) the overall effect of ALLO on $\alpha 4\beta 2^*\ nAChR$ function, (ii) the effect of pharmacologically-isolated progesterone metabolites on $\alpha 4\beta 2^*\ nAChR$ function, and (iii) whether progesterone metabolites act via the mPR/PGRMC1 complex to affect $\alpha 4\beta 2^*\ nAChR$ function.
CHAPTER 2

Functional heteromeric α4β2* nicotinic receptors in hippocampal formation CA1 principal neurons of the developing male mouse

2.1 Abstract

The hippocampus plays a key role in learning and memory. The normal development and mature function of hippocampal networks supporting these cognitive functions depends on afferent cholinergic neurotransmission mediated by nicotinic acetylcholine receptors. While it is well-established that nicotinic receptors are present on GABAergic interneurons and on glutamatergic presynaptic terminals within the hippocampus, the ability of these receptors to mediate postsynaptic signalling in pyramidal neurons is not well understood. Here, we use whole-cell electrophysiology to show that heteromeric nicotinic receptors mediate direct inward currents, depolarization from rest and enhanced excitability in hippocampus CA1 pyramidal neurons of male mice. Measurements made throughout postnatal development provide a thorough developmental profile for these heteromeric nicotinic responses, which are greatest during the first two weeks of postnatal life and decrease to low adult levels shortly thereafter. Pharmacological experiments find that responses are blocked by a competitive antagonist of α4β2* nicotinic receptors and augmented by a positive allosteric modulator of α5 subunit-containing receptors, which is consistent with expression studies suggesting the presence of α4β2 and α4β2α5 nicotinic receptors within the developing CA1 pyramidal cell layer. These findings demonstrate that functional heteromeric nicotinic receptors are present on CA1 pyramidal neurons during a period of major hippocampal development, placing these receptors in a prime position to play an important role in the establishment of hippocampal cognitive networks.

2.2 Introduction

The hippocampus is a specialized component of the cortico-limbic system that plays an important role in higher-order cognitive processes such as learning, memory and attention (Eichenbaum et
Alterations to the morphology and/or function of the hippocampus are implicated in the etiology of a number of neurodevelopmental disorders that involve deficits in these cognitive processes, including Autism Spectrum Disorder (ASD) (Loveland et al. 2008; Schumann et al. 2004), Attention Deficit Hyperactivity Disorder (ADHD) (Li et al. 2014; Plessen et al. 2006), and the teratogenic effects of developmental exposure to ethanol (McGoey et al. 2003; Sutherland et al. 1997) and nicotine (Damborsky et al. 2012). Proper hippocampal function depends on afferent cholinergic neurotransmission originating in the medial septum / diagonal band of Broca (MSDB) within the basal forebrain, as hippocampal acetylcholine (ACh) levels increase in animals exploring novel objects and environments (Aloisi et al. 1997; Anzalone et al. 2009; Ceccarelli et al. 1999; Stanley et al. 2012), and selective lesions of the septohippocampal cholinergic neurons decrease markers of cholinergic activity within the hippocampus and also impair spatial learning and object recognition memory (Berger-Sweeney et al. 2001; Cai et al. 2012; Easton et al. 2011).

Pharmacological studies demonstrate that ACh acts at the nicotinic class of acetylcholine receptor (nAChR) within the hippocampus to support learning and memory (Davis et al. 1997; Felix and Levin 1997; Kenney et al. 2012b; Nott and Levin 2006). These nAChRs are predominantly of the homomeric α7 and heteromeric α4β2* sub-classes, and it is now understood that their modulation of both GABAergic and glutamatergic signalling influences the net impact of ACh on hippocampal networks (McQuiston 2014; Placzek et al. 2009; Yakel 2012). Functional nAChRs of both sub-classes have been identified in hippocampal GABAergic interneurons (Alkondon and Albuquerque 2001; Bell et al. 2011; Frazier et al. 1998b; Ji and Dani 2000; Jones and Yakel 1997; Khiroug et al. 2003; McQuiston and Madison 1999b; Sudweeks and Yakel 2000), and their
activation can mediate local inhibition or disinhibition responses (Alkondon et al. 1999; Bell et al. 2015; Ji and Dani 2000). The role of nAChRs in hippocampal pyramidal neurons is not as well understood. Electrophysiological experiments in rodents age postnatal day (P) 11 and older show no direct nicotinic responses (Frazier et al. 1998b; Jones and Yakel 1997; Khiroug et al. 2003; Sudweeks and Yakel 2000) or nicotinic responses that occur in a low proportion of pyramidal neurons (Hefft et al. 1999; McQuiston and Madison 1999b), and there is conflicting evidence for the contribution of heteromeric nAChRs toward responses that are observed (He et al. 2013; Hefft et al. 1999; McQuiston and Madison 1999b; Tu et al. 2009). Expression analyses of whole hippocampal tissue (Shacka and Robinson 1998b; Zhang et al. 1998) and specifically of the cornu ammonis area 1 (CA1) pyramidal neuron layer (Didier et al. 1995b; Machaalani et al. 2010; Winzer-Serhan and Leslie 2005) suggest that the quantity of heteromeric nAChR subunits is developmentally regulated with a peak during the first two weeks of postnatal life followed by a significant decline shortly thereafter. However, to the best of our knowledge the function of heteromeric nAChRs has not been measured in developing hippocampal pyramidal neurons prior to P11.

The objective of this current study was to determine whether functional heteromeric nAChRs are present in developing mouse hippocampus CA1 pyramidal neurons. Whole-cell electrophysiological recording of visually-identified CA1 pyramidal neurons located within acute hippocampal slices of mice aged P5-10 consistently identified postsynaptic current responses to bath-applied ACh (in the presence of blockers of both muscarinic ACh receptors and α7 subunit-containing nAChRs) that also mediated depolarization from rest and increased the rate of action potential firing in these same neurons. Nicotinic current responses were resistant to the inhibition
of synaptic transmission and were significantly reduced by a selective antagonist of α4β2* nAChRs, suggesting that they were mediated by this class of receptors located directly on recorded CA1 pyramidal neurons. The measurement of nicotinic responses throughout postnatal development revealed that the developmental regulation of heteromeric nAChR function is consistent with previous subunit expression analyses, with nicotinic responses being significantly greater during the early postnatal P5-10 period than at later maturational stages of P15-20 (juvenile), P25-30 (adolescent) and P60-100 (young adult). Since the rodent early postnatal period is characterized by significant positive neurotransmission that drives hippocampal neuron development, these findings suggest that postsynaptic heteromeric nAChRs may contribute to these processes in CA1 pyramidal neurons to influence the development and maturation of cortico-limbic learning and memory networks.

2.3 Materials and Methods

2.3.1 Experimental Animals

Timed-pregnant female CD1 strain mice were purchased from Charles River Canada (Saint-Constant, QC, Canada) and housed individually in plastic cages measuring 47 cm X 25 cm X 15 cm with ad libitum access to water and food (Tekland Global 14% Protein Rodent Maintenance Diet, Harlan Laboratories, Mississauga, ON, Canada). Mice were kept in a secure vivarium with an ambient temperature of 21-24°C and a 12-hour reverse light cycle with lights on at 8:00 pm. The day of birth was considered to be postnatal day (P) 0 for that litter. Litters were left in the same cage until weaning on P30, at which time offspring were separated based on sex and housed in groups of up to five mice per cage in plastic cages measuring 29 cm X 19 cm X 13 cm and provided with ad libitum access to water and food. Male offspring were analyzed at four
developmental ages: P5-10 (young postnatal), P15-20 (juvenile), P25-30 (adolescent) and P60-100 (young adult). Experimental animals were cared for according to the principles and guidelines of the Canadian Council on Animal Care, and the experimental protocol was approved by the University of Guelph Animal Care Committee.

2.3.2 Electrophysiology

Mice were killed by decapitation under isoflurane anaesthesia. Brains were rapidly excised from the skull in 4°C oxygenated sucrose artificial cerebrospinal fluid (ACSF) (254 mM sucrose, 10 mM D-glucose, 26 mM NaHCO$_3$, 2 mM CaCl$_2$, 2 mM MgSO$_4$, 3 mM KCl, 1.25 mM NaH$_2$PO$_4$, pH 7.4). Coronal slices containing the rostral/dorsal hippocampus were cut at 400 μm thickness using a Leica VT1200 vibrating microtome (Leica Microsystems, Richmond Hill, ON, Canada) from approximately Bregma -1.46 mm to Bregma -2.18 mm (Paxinos and Franklin 2001). Slices were transferred to oxygenated ACSF (128 mM NaCl, 10 mM D-glucose, 26 mM NaHCO$_3$, 2 mM CaCl$_2$, 2 mM MgSO$_4$, 3 mM KCl, 1.25 mM NaH$_2$PO$_4$, pH 7.4), maintained at 30°C in a prechamber and allowed to recover for at least two hours before being used for whole-cell recordings. Slices were transferred to a modified chamber (Warner Instruments, Hamden, CT, USA) mounted on the stage of an Axioskop FS2 microscope (Carl Zeiss Canada, Toronto, ON, Canada). ACSF was oxygenated with carbogen (95% O$_2$ and 5% CO$_2$) and superfused over the slice at room temperature at a rate of 3-4 mL/min. Whole-cell recording of hippocampal CA1 pyramidal neurons was performed using glass pipette electrodes (2–5 MΩ) containing 120 mM K-glucuronate, 5 mM KCl, 2 mM MgCl$_2$, 4 mM K$_2$-ATP, 400 μM Na$_2$-GTP, 10 mM Na$_2$-phosphocreatine, 33 μM Alexa Fluor 488 hydrazide (Life Technologies, Burlington, ON, Canada) and 10 mM HEPES buffer (adjusted to pH 7.3 with KOH). Individual CA1 pyramidal neurons
were visualized using infrared differential interference contrast microscopy and identified based on location within the pyramidal cell layer and the presence of a prominent proximal apical dendrite. Pyramidal neuron cell type was confirmed visually once the Alexa Fluor 488 hydrazide from the intracellular recording electrode solution had diffused throughout the neuron, allowing for the epifluorescent visualization of the full apical dendrite morphology. All recordings were made using a Multiclamp 700B amplifier, acquired at 20 kHz and lowpass filtered at 2 kHz using a Digidata 1440A data acquisition system (Molecular Devices, Sunnyvale, CA, USA), and corrected for the liquid junction potential.

Neuron electrophysiological properties were first recorded in current clamp mode by measuring responses to positive and negative current steps, and are shown in Table 2.1. All experiments were performed in the continuous presence of 200 nM atropine and 10 nM methyllycaconitine (MLA) in order to block muscarinic receptors and α7 subunit-containing nicotinic receptors, respectively. Nicotinic receptor-mediated responses were probed by the addition of 1 mM ACh for 15 s in the ACSF bath following a constant baseline of recording. ACh was allowed to wash out of the slices for at least five minutes between applications. All responses were measured using Clampfit 10.3 software (Molecular Devices). For the measurement of depolarizing and current responses, the mean membrane potential or holding current value for each one-second period of an experiment was calculated as the average of the 20,000 data values recorded during that period. Depolarizing responses were measured in current clamp mode by subtracting the mean membrane potential at the peak of the response from the mean resting membrane potential at baseline. Acceleration of action potential firing was measured in current clamp mode by first injecting a positive current to elicit an approximately 1 Hz baseline firing frequency. The rate of firing was measured over a 15
s period during the peak ACh response and reported as a percent increase in frequency from the baseline frequency for each cell. Current responses were assessed in voltage clamp mode with neurons held at -75 mV. This approach was employed to mitigate the potential for nicotinic-driven presynaptic GABA release and subsequent activation of postsynaptic GABA<sub>A</sub> receptors to induce chloride currents in recorded neurons. As -75 mV is near the equilibrium potential for chloride in this preparation, there is minimal flux of chloride ions and there are no observable chloride currents under these recording conditions. Inward current responses were measured by subtracting the mean holding current at the peak of the ACh response from the mean holding current at baseline.

Additional pharmacological experiments in Fig. 2.2 were performed using a cocktail of synaptic blockers applied to the bath comprising 2 μM tetrodotoxin (TTX) to block voltage-gated sodium channels, 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) to block AMPA and kainate glutamate receptors, 50 μM D(-)-2-amino-5-phosphonopentanoic acid (APV) to block NMDA glutamate receptors and 10 μM bicuculline to block GABA<sub>A</sub> receptors. The α4β2* nAChR competitive antagonist dihydro-β-erythroidine (DHβE) was applied to the bath at 3 μM for a subset of experiments in Fig. 2.2. Nicotine desensitization of nAChR function was assessed in Fig. 2.3 by measuring the inward current response to 1 mM ACh, allowing for five minutes of washout and then applying either 100 nM, 300 nM or 500 nM nicotine in the bath for 10 min. Following a 10 min washout, the 1 mM ACh response was measured again and the percent desensitization was calculated for that cell as: [(ACh current before nicotine - ACh current after nicotine) / ACh current before nicotine] x 100. Developmental effects of acetylcholinesterase and extracellular matrix density were assessed in Fig. 2.5 using 1 mM carbamoylcholine chloride (carbachol). The incorporation of α5 subunits into nAChRs was assessed by first inactivating acetylcholinesterase
in the slice by 10 min pre-exposure to 20 μM diisopropyl fluorophosphate (DFP), and then measuring the inward current response to 10 μM ACh before and after 10 min application of 0.1 μM galanthamine. The percent potentiation was calculated as: 
\[
\frac{(ACh \text{ current after galanthamine} - \text{ACh current before galanthamine})}{\text{ACh current before galanthamine}} \times 100.
\]
TTX citrate was obtained from Alomone Labs (Jerusalem, Israel). ACh chloride, carbachol chloride, atropine and nicotine hydrogen bitartrate were purchased from Sigma Aldrich (Oakville, ON, Canada). CNQX disodium, APV, bicuculline methiodide, DHβE, and galanthamine hydrobromide were purchased from Tocris Bioscience / Bio-Techne (Minneapolis, MN, USA). DFP was purchased from BioShop Canada (Burlington, ON, Canada). All drugs were stored in stock solutions at -20°C.

2.3.3 Neuron Morphology

A subset of all recorded hippocampus CA1 pyramidal neurons were patched and electrophysiological recordings were made in acute brain slices as described above, with the exception that the intracellular recording electrode also contained 0.3% (wt/vol) Biocytin (Tocris Bioscience). Neurons were held for at least 40 minutes to allow for Biocytin to diffuse into the cell, and recording electrodes were then withdrawn slowly to allow resealing of neuronal cell membranes. Slices were fixed overnight at 4°C in a solution containing 4% (wt/vol) paraformaldehyde in 100 mM phosphate buffer (pH 7.5). Free-floating slices were washed 3 x 10 min with Tris-buffered saline (TBS; 100 mM Tris and 150 mM NaCl, pH 7.5) and then treated with 0.5 % (vol/vol) H₂O₂ and 0.25% (vol/vol) Triton X-100 in TBS for 15 min to suppress endogenous peroxidase activity. Slices were washed 3 x 10 min with TBS and then incubated in a solution made from Vectastain Elite ABC avidin / biotinylated horseradish peroxidase complexes (Vector Laboratories, Burlington, ON, Canada; prepared according to the manufacturer’s
recommendations) and 0.25% (vol/vol) Triton X-100 in TBS overnight at room temperature. Slices were washed 3 x 10 min with TBS and incubated in a solution containing 0.05% (wt/vol) 3,3-diaminobenzidine (DAB), 0.04% (wt/vol) nickel chloride and 0.25% (vol/vol) Triton X-100 in TBS for 5 min, and then in this same solution that also contained 0.001% (vol/vol) H₂O₂ for 15 min. Slices were washed with TBS for 3 x 10 min, mounted onto microscope slides and allowed to dry overnight. They were then dehydrated using a series of ethanol gradients, cleared and coverslipped using Permount (Fisher Scientific, Ottawa, ON, Canada).

Neurons were imaged in brightfield using an Olympus BX53 upright microscope equipped with an Olympus UPlanSApo 4X 0.16 NA objective and an Olympus UPlanSApo 30X, 1.05 NA silicone-immersion objective (Olympus, Richmond Hill, ON, Canada). For high-resolution photomicrographs, image stacks that overlapped in the x and y axes and separated in the z axis by 1 μm per image were captured using the 30X objective and a three-axis motorized stage under the control of Neurolucida software (MicroBrightField, Williston, VT, USA). Image stacks were collapsed into two dimensional minimum-intensity images that were then stitched together in the x and y axes.

2.3.4 Statistical Analysis

All data are reported as mean ± SEM values for individual neurons within each experimental group. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) and a level of $p < 0.05$ was required to indicate statistical significance. Effects of synaptic blockers and receptor antagonists on ACh responses were analyzed using two-tailed paired $t$ tests. Effects of age on nicotinic current, depolarization and excitability responses, and effects of
nicotine concentration on receptor desensitization were analyzed by one-way ANOVA followed by the Tukey’s post hoc test. Comparisons between regular firing and burst firing pyramidal neurons, and effects of age and application time on carbachol responses were performed using two-way ANOVA followed by the Bonferroni’s post hoc test. The effect of age on galanthamine potentiation of ACh currents was measured by two-tailed unpaired t test.

2.4 Results

2.4.1 Excitation of young postnatal CA1 pyramidal neurons by postsynaptic heteromeric nicotinic receptors

Several in situ studies have shown that mRNA for the nicotinic α4, β2 and α5 subunits is expressed within the CA1 pyramidal neuron layer of mice (Heath et al. 2010; Hsu et al. 2013; Marks et al. 1992; Salas et al. 2003), rats (Sudweeks and Yakel 2000) and humans (Machaalani et al. 2010), and that subunit expression is greater during early postnatal periods than in adulthood (Didier et al. 1995b; Machaalani et al. 2010; Winzer-Serhan and Leslie 2005). We first sought to determine whether functional heteromeric nAChRs are present on young postnatal CA1 pyramidal neurons using whole-cell recording within acute brain slices from mice aged P5-10. The pyramidal morphology of each recorded neuron was confirmed by the addition of Alexa Fluor 488 hydrazide in the pipette solution and live epifluorescent visualization of its prominent apical dendrite, and a subset of neurons were also analyzed in greater detail using Biocytin in the pipette solution and post hoc chromogenic visualization. Photomicrographs of a typical recorded neuron are shown in Fig. 2.1A. Its response to the injection of positive and negative current steps is shown in Fig. 2.1B, which is typical of responses in regular-firing CA1 pyramidal neurons (Graves et al. 2012). Heteromeric nAChRs were isolated pharmacologically in this preparation by the continuous bath
application of atropine (200 nM, to block muscarinic acetylcholine receptors) and MLA (10 nM, to block α7 subunit-containing nAChRs). Bath application of 1 mM ACh for 15 s resulted in positive inward currents in neurons held at -75 mV for all neurons examined (108/108 neurons), and positive depolarization responses from rest for all neurons examined (25/25 neurons). Typical responses are shown in Fig. 2.1C (current response) and 1D (depolarization response). This nAChR activation has a profound impact on the function of active neurons, as the same application of 1 mM ACh increased the action potential firing frequency by approximately 250% in all neurons tested (39/39 neurons). A typical recording of ACh acceleration of action potential firing is shown in Fig. 2.1E. We applied ACh at 1 mM because it is actively metabolized by endogenous acetylcholinesterase while washing into the slice preparation (Bailey et al. 2010). To facilitate comparison of our data with that from cell culture and synaptosome studies, we verified in a separate set of experiments that current responses to 1 mM ACh in our slice preparation (9.0 ± 1.6 pA, n = 4) were not different than current responses to 100 μM ACh measured after acetylcholinesterase had been inactivated by 10 min exposure to 20 μM DFP (7.6 ± 1.0 pA, n = 7; two-tailed unpaired t test, p = 0.4).
Fig. 2.1. Activation of heteromeric nicotinic acetylcholine receptors facilitates excitation of young postnatal mouse CA1 pyramidal neurons. A1: Representative photomicrograph of the dorsal hippocampus from a mouse aged postnatal day 10 showing two CA1 pyramidal neurons that were analyzed for electrophysiology and visualized post hoc using Biocytin. Scale bar is 500 μm. A2: A magnified view of the neuron on the left of A1 shows its characteristic CA1 pyramidal neuron morphology. Scale bar is 100 μm. B: Current-clamp recording from the neuron in A2 showing changes to membrane potential following the injection of 500 ms depolarizing and hyperpolarizing current steps, which are consistent with typical responses from CA1 pyramidal neurons. Application of 1 mM ACh for 15 s (grey bars) results in an inward current response (C), depolarization from rest (D) and an acceleration of action potential firing (E). All recordings were made in the continuous presence of 200 nM atropine to block muscarinic acetylcholine receptors and 10 nM MLA to block α7 subunit-containing nicotinic acetylcholine receptors.
We next sought to characterize these nicotinic responses in CA1 neurons from young postnatal mice. Nicotinic receptors did not desensitize to repeated application of 1 mM ACh, which is consistent with heteromeric β2 subunit-containing receptors (Fig. 2.2A; first application: 7.6 ± 1.4 pA; second application: 7.7 ± 1.5 pA; n = 8; two-tailed paired t test: p = 0.8). Nicotinic current responses to ACh were not affected by the inhibition of synaptic transmission using a cocktail of blockers for voltage-gated sodium channels (2 μM TTX), ionotropic glutamate receptors (20 μM CNQX and 50 μM APV) and GABA_A receptors (10 μM bicuculline) (Fig. 2.2B; baseline: 10.8 ± 1.4 pA; after 10 min exposure to synaptic blockers: 10.6 ± 1.0 pA; n = 7; p = 0.8). However, the α4β2* nAChR competitive antagonist DHβE significantly suppressed ACh-induced nicotinic currents (Fig. 2.2C; baseline: 10.0 ± 1.6 pA; after 10 min exposure to 3 μM DHβE: 1.7 ± 1.2 pA; n = 5; p = 0.002). Moreover, although the ACh-induced nicotinic currents before exposure to DHβE were significantly different from the root mean square (RMS) current noise in these neurons of 2.5 ± 0.3 pA (two-tailed unpaired t-test, p = 0.002), currents measured after exposure to DHβE were not significantly different from the baseline RMS current noise (p = 0.5). Exposure to DHβE also significantly suppressed the ACh-induced increase in action potential firing frequency for neurons already firing action potentials at approximately 1 Hz (Fig. 2.2D; baseline: 311.4 ± 33.5 percent increase; after 10 min exposure to 3 μM DHβE: 78.8 ± 15.3 percent increase; n = 9; p < 0.0001). The results from this set of experiments are consistent with the activation of α4β2*nAChRs located directly on CA1 pyramidal neurons of young postnatal mice.
In young postnatal mice, excitation of CA1 pyramidal neurons by acetylcholine is mediated by postsynaptic α4β2* nicotinic receptors. All recordings were made using mice aged postnatal day 5-10 in the continuous presence of 200 nM atropine to block muscarinic acetylcholine receptors and 10 nM MLA to block α7 subunit-containing nicotinic acetylcholine receptors. 

A: Current responses were not desensitized by repeated application of 1 mM ACh for 15 s (paired t test: p = 0.8). Similarly in B, current responses to 1 mM ACh (15 s) were not significantly affected by 10 min pretreatment with the following synaptic blockers: 2 μM TTX, 20 μM CNQX, 50 μM APV and 10 μM bicuculline (p = 0.8). C: Current responses to 1 mM ACh (15 s) were significantly reduced by 10 min pretreatment with the α4β2* nicotinic receptor competitive antagonist dihydro-β-erythroidine (DHβE; 3 μM) (p = 0.002). D: For neurons induced to fire action potentials at 1 Hz by positive current injection, the increase in firing frequency following application of 1 mM ACh
(15 s) was significantly reduced by 10 min pretreatment with 3 μM DHβE (p < 0.0001). All values are mean ± SEM. Representative voltage-clamp or current-clamp traces are shown on the right for one neuron from each pharmacological experiment with ACh application marked by grey bars.

2.4.2 Nicotine desensitization of nicotinic receptors in young postnatal CA1 pyramidal neurons.

Nicotine binds with high affinity to α4β2* nAChRs in the human brain (Brody et al. 2006) and also within the mouse hippocampus (Horti et al. 1997; Lomazzo et al. 2010). It possesses both agonist and desensitizing abilities at nAChRs, and this complex pharmacological profile is influenced by the specific subunit composition and posttranslational modification of the heteromeric nAChR pentamer (Bailey et al. 2010; Bailey et al. 2014; Grady et al. 2012; Quick and Lester 2002; Wageman et al. 2014). We tested the ability of multiple nicotine concentrations to desensitize nAChRs in CA1 pyramidal neurons from mice aged P5-10 by measuring inward current responses to 1 mM ACh (15 s application) before and after a 10 min exposure to 100 nM, 300 nM, or 500 nM nicotine. Nicotine was allowed to wash out of the brain slice for 10 min before ACh was re-applied, and nicotine was applied only once to each brain slice. Nicotine at 100 nM did not significantly reduce the ACh response (initial response: 5.5 ± 0.6 pA; after 100 nM nicotine: 4.6 ± 0.5 pA; n = 9; paired t test: p = 0.1). However, nicotine at 300 nM and 500 nM, which are concentrations similar to those expected in the blood and brain after smoking a cigarette (Henningfield et al. 1993; Rose et al. 2010), both significantly reduced ACh current responses at the nAChR. Values were 9.1 ± 2.2 pA (initial response) versus 6.6 ± 1.7 pA (after 300 nM nicotine) (n = 7; p = 0.02) and 8.5 ± 1.0 pA (initial response) versus 4.6 ± 0.6 pA (after 500 nM nicotine) (n = 10; p = 0.001). These values are expressed in Fig. 2.3 as the percent by which nicotine desensitized the initial ACh in each neuron, and were significantly affected by nicotine concentration (one-way ANOVA: p = 0.04). Tukey’s post hoc test found that percent
desensitization following 500 nM nicotine was greater than that following 100 nM nicotine (p = 0.03).
Fig. 2.3. Acute nicotine exposure desensitizes nicotinic receptors in young postnatal CA1 pyramidal neurons in a concentration-dependent manner. A: Receptor desensitization by nicotine was calculated in neurons of mice aged postnatal day 5-10 by measuring the peak inward current response to 1 mM ACh (15 s, in the presence of 200 nM atropine and 10 nM MLA) before and after a 10 min exposure to either 100 nM, 300 nM or 500 nM nicotine. Nicotine was allowed to wash out of the brain slice for 10 min before ACh was re-applied, and nicotine was applied only once to each brain slice. The percent decrease/desensitization for the ACh response was significantly affected by nicotine concentration (one-way ANOVA: p = 0.04), with percent desensitization following 500 nM nicotine application being greater than that following 100 nM nicotine application (Tukey’s post hoc test: p = 0.03). All values are mean ± SEM and data sets marked with different letters indicate a statistically significant difference. B: Representative voltage-clamp traces showing inward current responses to ACh (grey bars) before and after 10 min exposure to 100 nM, 300 nM or 500 nM nicotine.
2.4.3 Developmental changes to nicotinic receptor function.

Since developmental analyses find the expression of heteromeric nAChR subunits to be greater in the CA1 pyramidal neuron layer during young postnatal life than at later ages (Didier et al. 1995b; Machaalani et al. 2010; Winzer-Serhan and Leslie 2005), we next sought to determine whether the electrophysiological function of these receptors is developmentally regulated within CA1 pyramidal neurons. This was done by measuring whole-cell current and depolarization responses to ACh in mice aged P5-10 (young postnatal), P15-20 (juvenile), P25-30 (adolescent) and P60-100 (young adult). Heteromeric nAChRs were isolated pharmacologically in all experiments by the bath application of 200 nM atropine and 10 nM MLA. Inward current responses to 1 mM ACh (15 s) showed a significant effect of age (Fig. 2.4A; one-way ANOVA: \( p < 0.0001 \)), where currents in neurons from mice aged P5-10 (9.5 ± 1.0 pA, \( n = 59 \)) were more than three times greater than the RMS baseline current noise of 2.6 ± 0.2 pA and significantly greater than currents in neurons from mice aged P15-20 (4.1 ± 0.4 pA, \( n = 26 \)), P25-30 (3.3 ± 0.6 pA, \( n = 28 \)) and P60-100 (2.2 ± 0.5 pA, \( n = 30 \)) (Tukey’s post hoc test, \( p \leq 0.0003 \) for each comparison). A similar effect of age was observed on the ability of 1 mM ACh (15 s) to depolarize neurons from rest (\( p < 0.0001 \)), as shown in Fig. 2.4B. Here, nicotinic depolarization responses were significantly greater in neurons from mice aged P5-10 (3.5 ± 0.8 mV, \( n = 25 \)) than neurons from mice aged P25-30 (1.0 ± 0.2 mV, \( n = 23 \)) and P60-100 (0.6 ± 0.1 mV, \( n = 32 \)) (\( p \leq 0.0009 \) for each comparison). The influence of these nAChR responses on neuron excitability was measured by applying 1 mM ACh (15 s) to neurons that had been previously induced to fire action potentials at a frequency of 1 Hz by positive current injection. The amount of positive current injection required to induce the 1 Hz firing frequency was recorded for the majority of the neurons in this experiment, and was found to vary across development with values of 48.7 ± 7.7 pA (\( n = 20 \)) at P5-10, 22.2 ± 2.3 pA (\( n = 22 \)) at
P15-20, 26.9 ± 4.6 pA (n = 24) at P25-30 and 47.2 ± 11.1 pA (n = 23) at P60-100 (Kruskal-Wallis test, p = 0.002). This amount of current was significantly greater at P5-10 than at P15-20 and P25-30 (Dunn’s post hoc test, p < 0.02 for each comparison). There was an effect of age on the percent increase in firing frequency following ACh application that was similar to that seen for current and depolarization responses (Fig. 2.4C; p < 0.0001), where the ACh response was significantly greater in neurons from mice aged P5-10 (256.9 ± 38.5 percent increase, n = 30) than from mice aged P15-20 (105.2 ± 12.9 percent increase, n = 32), P25-30 (53.9 ± 9.3 percent increase, n = 30) and P60-100 (41.2 ± 8.1 percent increase, n = 29) (p < 0.0001 for each comparison). Basic electrophysiological properties for neurons in this experiment are shown in Table 2.1. During postnatal development, resting membrane potential did not change (one-way ANOVA, p = 0.6), input resistance decreased (p < 0.0001), and spike amplitude increased (p < 0.0001).

Two distinct classes of CA1 pyramidal neurons have been identified that display either regular-firing or burst-firing patterns of action potential activity (Graves et al. 2012). Neurons from this experiment were classified based on threshold level current injection, where regular-firing neurons were identified based on their initial action potentials having an instantaneous frequency less than 100 Hz and burst-firing neurons were identified by the presence of two or more initial action potentials having an instantaneous frequency greater than 100 Hz riding on a distinct slow depolarizing envelope (Graves et al. 2012; Su et al. 2001). There were more regular-firing neurons than burst-firing neurons at each age, and the proportion of each neuron type was not affected by age. Numbers were: P5-10 (regular: 55, burst: 7); P15-20 (regular: 24, burst: 11); P25-30 (regular: 26, burst: 7); and P60-100 (regular: 26, burst: 9) (Chi-square test: p = 0.1). Two-factor analysis of the developmental data for effects of age and neuron type showed that nicotinic receptor function
was not different, and also showed similar developmental changes among the two neuron types. Inward currents were affected by age (two-way ANOVA: F(3,135) = 7.62, p < 0.0001) but not by neuron type (F(1,135) = 0.008, p = 0.9) and there was no interaction between these two factors (F(3,135) = 0.05, p = 1.0). Depolarization responses from rest showed a strong trend toward an effect of age (F(3,97) = 2.64, p = 0.05) but no effect of neuron type (F(1,97) = 1.52, p = 0.2) or interaction between the two factors (F(3,97) = 1.62, p = 0.2). Nicotinic acceleration of action potential firing in active neurons showed an effect of age (F(3,111) = 11.82, p < 0.0001), but no effect of neuron type (F(1,111) = 0.00003, p = 1.0) or interaction between these two factors (F(3,111) = 0.40, p = 0.8). Resting membrane potential (F(1,157) = 1.02, p = 0.3) and input resistance (F(1,152) = 0.32, p = 0.6) were not affected by neuron type. Spike amplitude was significantly greater in regular-firing neurons than burst-firing neurons (F(1,156) = 5.16, p = 0.02).
Fig. 2.4. Nicotinic stimulation of CA1 pyramidal neurons is developmentally regulated and greatest in young postnatal mice. A: Inward current responses to 1 mM ACh (15 s) were significantly affected by age (one-way ANOVA: p < 0.0001), where currents were greater at postnatal day (P) 5-10 than at each of P15-20, P25-30 and P60-100 (Tukey’s post hoc test: all p ≤ 0.0003). Typical voltage-clamp traces at each age are shown below the histogram plot. B: Depolarization from rest following 1 mM ACh application (15 s) was significantly affected by age (p < 0.0001), where the response at P5-10 was significantly greater than that at P25-30 and P60-100 (both p ≤ 0.0009). Typical current-clamp traces are located below the histogram plot and show typical depolarization responses at each age. C: In neurons that had been induced to fire action potentials at 1 Hz, acceleration of firing frequency following application of 1 mM ACh (15 s) was significantly affected by age (p < 0.0001), where the response was greater at P5-10 than at P15-20, P25-30 and P60-100 (all p < 0.0001). Typical current-clamp traces showing the typical changes in firing frequency at each age are shown below the histogram plot. All recordings were made in the continuous presence of 200 nM atropine and 10 nM MLA. All values are mean ± SEM. Data sets marked with different letters indicate a statistically significant difference.
Table 2.1. Electrophysiological properties for developing CA1 pyramidal neurons.

<table>
<thead>
<tr>
<th></th>
<th>P5-10 (n = 62)</th>
<th>P15-20 (n = 35)</th>
<th>P25-30 (n = 33)</th>
<th>P60-100 (n = 35)</th>
<th>p-value (one-way ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting membrane potential (mV)</td>
<td>-73.4 ± 0.5</td>
<td>-72.7 ± 0.5</td>
<td>-73.3 ± 0.4</td>
<td>-73.8 ± 0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Input resistance (MΩ)</td>
<td>318.3 ± 11.1a</td>
<td>222.9 ± 8.9b</td>
<td>160.7 ± 7.6c</td>
<td>156.2 ± 4.1c</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Spike amplitude (mV)</td>
<td>75.5 ± 1.3a</td>
<td>83.0 ± 2.5b</td>
<td>91.8 ± 1.9c</td>
<td>82.5 ± 1.6b</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

Values are mean ± 1 SEM. Data sets marked with different letters indicate a statistically significant difference (Tukey’s post hoc test).
2.4.4 Influence of endogenous acetylcholinesterase and extracellular matrix density on observed developmental changes to CA1 nicotinic receptor function.

Acetylcholinesterase is active in the early postnatal mouse hippocampus (Virgili et al. 1991) and endogenous acetylcholinesterase can metabolize exogenous ACh as it washes into brain slices (Bailey et al. 2010). Since hippocampus acetylcholinesterase activity increases during rodent postnatal development (Cermak et al. 1999; Virgili et al. 1991), we sought to determine whether this underlies the observed developmental changes to CA1 nAChR function using the ACh analogue carbachol that is not rapidly metabolized by acetylcholinesterase, or to the best of our knowledge by another enzyme present within brain slices (Goodman et al. 1985; Molitor 1936). Current responses to 1 mM carbachol (in the presence of 200 nM atropine and 10 nM MLA) were measured at P5-10 and P60-100. Carbachol was applied in the bath for either 15 s or 60 s, in order to also determine whether the kinetics of drug wash-in to the receptor are affected by potential developmental changes to extracellular matrix density. Similar to the experiment in Fig. 2.4A using ACh, the magnitude of nicotinic currents elicited by carbachol was significantly affected by age (Fig. 2.5A; two-way ANOVA: F (1, 14) = 7.11; p = 0.02) with currents being greater at P5-10 than at P60-100 (Bonferroni’s post hoc test: all p < 0.05). However, the magnitude of nicotinic currents was not affected by the length of carbachol application F(1,14) = 1.28; p = 0.3) and there was no interaction between effects of age and length of carbachol application (F(1,14 = 1.04; p = 0.3), suggesting that postnatal age does not affect the ability of drug to wash into the slice in our preparation. The kinetics of drug wash-in for this experiment were further investigated by measuring the time required for carbachol to elicit a peak current response in each neuron. The time to peak response was not affected by age (Fig. 2.5B: two-way ANOVA: F (1, 14) = 0.42; p = 0.5) or length of carbachol application (F(1,14) = 0.70; p = 0.4), and there was no interaction
between effects of these two factors \( F(1,14) = 0.54; \ p = 0.5 \). These results suggest that observed developmental changes to nAChR function do not depend on altered acetylcholinesterase activity or extracellular matrix density.
Fig. 2.5. Developmental changes to CA1 nicotinic responses do not result from altered acetylcholinesterase activity or extracellular matrix density. Inward current responses were measured at P5-10 and P60-100 following application of carbachol, which is a nicotinic receptor agonist that is not metabolized by endogenous acetylcholinesterase present within brain slices. 

A: The magnitude of current responses to 1 mM carbachol (applied for 15 s or 60 s in the presence of 200 nM atropine and 10 nM MLA) was significantly affected by age (two-way ANOVA: $F(1,14) = 7.11; \ p = 0.02$), where currents were greater at postnatal day (P) 5-10 than at P60-100 (*Bonferroni’s post hoc test: all $p < 0.05$). Response magnitude was not affected by length of carbachol application ($F(1,14) = 1.28; \ p = 0.3$) and there was no interaction between effects of age and application length ($F(1,14) = 1.04; \ p = 0.3$). 

B: In this experiment, the time required for
carbachol to wash into the slice and elicit a maximal current response was not affected by age (F(1,14) = 0.42; p = 0.5) or length of carbachol application (F(1,14) = 0.70; p = 0.4), and there was no interaction between effects of these two factors (F(1,14) = 0.54; p = 0.5). C: Representative voltage-clamp traces showing inward current responses to 1 mM carbachol at each age. All values are mean ± SEM.

2.4.4 Influence of α5 subunit incorporation toward developmental changes to CA1 nicotinic receptor function

The nicotinic α5 subunit is present in approximately 35 percent of heteromeric nAChRs within hippocampus tissue samples (Mao et al. 2008) and its incorporation is known to augment nAChR function (Kuryatov et al. 2008; Tapia et al. 2007). Since the expression of α5 subunit mRNA within the rat CA1 layer peaks during the first postnatal week and decreases thereafter, similar to our observed developmental pattern for nAChR function in CA1 pyramidal neurons, we sought to determine pharmacologically whether the proportion of nAChRs containing the α5 subunit changes during development. Incorporation of α5 subunits was probed at P5-10 and P60-100 using the α5 subunit-selective positive allosteric modulator galanthamine (Kuryatov et al. 2008). Peak current responses to 10 μM ACh (15 s, in the presence of 200 nM atropine and 10 nM MLA) were measured before and after a 10 min exposure to 0.1 μM galanthamine. Experiments were performed after endogenous acetylcholinesterase within the slice was inactivated by a 10 min exposure to 20 μM DFP because galanthamine inhibits this enzyme at high concentrations. Although current responses were greater at P5-10 than at P60-100, the percentage by which galanthamine potentiated ACh responses was not different between these two ages (Fig. 2.6; two-tailed unpaired t test: p = 0.8). These data suggest that the α5 subunit is incorporated into a similar proportion of heteromeric nAChRs in both young postnatal and adult CA1 pyramidal neurons.
Fig. 2.6. Potentiation of CA1 nicotinic responses by galanthamine, a positive modulator of α5 subunit-containing α4β2* nicotinic receptors, is not affected by postnatal age. Nicotinic current responses to 10 μM ACh (15 s) were measured before and after a 10 min exposure to 0.1 μM galanthamine. All recordings were made following the inactivation of acetylcholinesterase by diisopropyl fluorophosphate, and in the continuous presence of 200 nM atropine and 10 nM MLA. The percent increase for ACh responses was not affected by age (unpaired t test: p = 0.8). All values are mean ± SEM. Typical voltage-clamp traces for current responses to ACh before and after galanthamine exposure are shown on the right.
2.5 Discussion

Our findings provide evidence that CA1 pyramidal neurons of the mouse hippocampus are transiently excited by postsynaptic heteromeric nAChRs during early postnatal development. This conclusion is based on a series of physiological and pharmacological experiments performed using whole-cell electrophysiological recording of visually-identified CA1 pyramidal neurons within acute hippocampal brain slices collected at distinct developmental ages. Nicotinic stimulation in young postnatal animals produced direct inward currents and increased neuron excitability in a manner that is consistent with the presence of functional α4β2* nAChRs located directly on recorded neurons. By characterizing these responses across postnatal development, we now present a cohesive developmental profile for heteromeric nAChR function in mouse CA1 pyramidal neurons from P5 to P100, with the greatest function occurring during the first two weeks of postnatal life. This strong developmental regulation suggests that heteromeric, putative α4β2* nAChRs are present and active selectively during a period when they may contribute to the cholinergic regulation of CA1 pyramidal neuron development within hippocampal learning and memory networks.

2.5.1 Heteromeric nicotinic receptors within the hippocampus.

The proper development and mature function of the hippocampus depends on cholinergic innervation from the MSDB (Aloisi et al. 1997; Anzalone et al. 2009; Berger-Sweeney et al. 2001; Cai et al. 2012; Ceccarelli et al. 1999; Easton et al. 2011; Liu et al. 2006; Lozada et al. 2012b; Stanley et al. 2012), with ACh activating nAChRs located on local GABAergic interneurons and glutamatergic excitatory neurons (McQuiston 2014; Placzek et al. 2009; Yakel 2012). It is well-
established that both α7 and α4β2* nAChRs mediate nicotinic currents in GABAergic interneurons to regulate the excitability of hippocampal circuits (Adams et al. 2002; Alkondon and Albuquerque 2001; Alkondon et al. 1999; Bell et al. 2011; Bell et al. 2015; Frazier et al. 1998b; Ji and Dani 2000; Jones and Yakel 1997; Khiroug et al. 2003; McQuiston and Madison 1999b; Sudweeks and Yakel 2000). Conversely, it is only more recently that studies have shown evidence for the presence of functional α7 (Grybko et al. 2011; Ji et al. 2001; Kalappa et al. 2010) and α4β2* (He et al. 2013; Tu et al. 2009) nAChRs located directly on hippocampus pyramidal neurons. We provide in this study comprehensive in situ evidence that heteromeric, putative α4β2* nAChRs are present on CA1 pyramidal neurons, with their greatest function occurring during hippocampal development. In our slice preparation, nicotinic current responses to bath-applied ACh were maintained following the blockade of synaptic transmission using inhibitors of voltage-gated sodium channels (TTX), glutamatergic signalling (CNQX and APV) and GABAergic signalling (bicuculline), suggesting that they were mediated by postsynaptic nAChRs. Currents were likely not mediated by α7 homomeric nAChRs because of their rapid desensitization kinetics (Quick and Lester 2002), and also because we conducted all experiments with α7 nAChRs blocked using the selective antagonist MLA. MLA has also been demonstrated to block the more recently-discovered α7β2 heteromeric nAChR that could potentially form in pyramidal neurons (Liu et al. 2012; Murray et al. 2012). We found that ACh-induced postsynaptic currents and enhanced neuron excitability were both inhibited significantly by the α4β2* nAChR selective antagonist DHβE, suggesting that responses were mediated by this class of nAChR located directly on recorded pyramidal neurons. Further evidence to support our findings may be generated in future studies using the complementary electrophysiological approach of recording responses to the stimulation of heteromeric nicotinic receptors in acutely dissociated CA1 pyramidal neurons, and labeling
receptor subunit protein on CA1 pyramidal neurons within high-resolution immunohistochemical experiments.

Analysis of whole hippocampus tissue finds expression of the α2, α3, α4, α5, β2 and β4 heteromeric nAChR subunits, and that the predominant assembled isoforms are those containing α4 and β2 subunits only (comprising 40 percent of heteromeric nAChRs) and those containing α4 and β2 subunits along with the α5 accessory subunit (comprising 35 percent of heteromeric nAChRs) (Lomazzo et al. 2010; Mao et al. 2008). In situ analysis confirms that the α4, β2 and α5 subunits are expressed in the CA1 pyramidal neuron layer (Heath et al. 2010; Marks et al. 1992; Salas et al. 2003; Winzer-Serhan and Leslie 2005) and within the pyramidal neurons themselves (Sudweeks and Yakel 2000). Since the developmental profile for heteromeric nAChR function in this current study is consistent with that for expression of the α4 (Didier et al. 1995b) and α5 (Winzer-Serhan and Leslie 2005) subunits within the CA1 pyramidal neuron layer, it is likely that our measured responses were mediated by α4β2 and α4β2α5 receptors. Further evidence for the incorporation of α5 subunits into α4β2* nAChRs comes from the nicotine desensitization and galanthamine potentiation experiments. The degree by which nicotine desensitized nAChR function in this current study was similar to that reported previously for prefrontal cortex pyramidal neurons that express α4, β2 and α5 subunits, but not for prefrontal cortex pyramidal neurons that lack the α5 subunit (Bailey et al., 2010). CA1 neuron nAChR currents were also potentiated by the α5 subunit-selective postitive allosteric modulator galanthamine. The degree by which galanthamine potentiated nAChR currents was similar at P5-10 and P60-100, suggesting that a similar proportion of nAChRs contain the α5 subunit at each age. Given that the presence of an α5 subunit augments nAChR activity (Bailey et al. 2010; Kuryatov et al. 2008; McClure-Begley
et al. 2009; Tapia et al. 2007), these data further suggest that our observed developmental decrease in nAChR function does not result from a selective decrease in the number of α4β2α5 nAChRs only.

We also investigated whether factors in our preparation other than receptor number could explain the observed developmental decrease in nAChR function. Increased acetylcholinesterase activity in the slices of older mice (Virgili et al. 1991) could have metabolized exogenous ACh as it washed into the slice toward nAChRs on the recorded neuron. However, we found that the developmental decrease to nAChR function was also present for CA1 neurons when we used the receptor agonist carbachol that is not rapidly metabolized by acetylcholinesterase. The interpretation that this result confirms a developmental decrease in nAChR function for CA1 pyramidal neurons depends on carbachol not being rapidly metabolized by enzymes other than acetylcholinesterase within brain slices, which is consistent with available literature (Goodman et al. 1985; Molitor 1936). It was also possible that increased extracellular matrix density in older mice impeded the ability of exogenous ACh to wash in and maximally activate nAChRs when applied for 15 s. We found in the carbachol experiment that 15 s and 60 s application of carbachol elicited similar nAChR currents, and that the same developmental decrease in nAChR function was observed using both lengths of application. Moreover, the time required for carbachol to wash in and elicit a maximal current response was not affected by application length or age of the animal. The input resistance for CA1 pyramidal neurons decreased significantly as mice aged, which may have contributed to the observed developmental profiles for excitability responses measured in current-clamp mode. This is especially true for the depolarizing responses from rest (Fig. 2.4B) that show a relative developmental profile similar to that observed for input resistance. Our findings also suggest
however, that while the augmentation of excitability in firing neurons (Fig. 2.4C) may be influenced by input resistance and active electrophysiological properties (e.g. afterhyperpolarization), nicotinic currents appear to play a greater role here. For example, neurons at P5-10 required approximately 2.5 times greater injected current than neurons at P15-20 to achieve the 1 Hz baseline firing (see Results for data), and also received approximately 2.5 times greater inward current than neurons at P15-20 following ACh application (Fig. 2.4A). However, instead of providing similar effects on neuron firing, nicotinic stimulation increased firing frequency at P5-10 by an amount that was approximately 2.5 times greater than that measured at P15-20.

2.5.2 Nicotinic signalling in normal and aberrant hippocampal development

Markers for cholinergic neurotransmission appear within the rat hippocampus during late gestation and are widespread by postnatal day 3-5 (Happe and Murrin 1992; Milner et al. 1983). Expression of heteromeric nAChR subunits follows a similar early profile to peak during the first two weeks before declining shortly thereafter (Didier et al. 1995b; Shacka and Robinson 1998b; Winzer-Serhan and Leslie 2005; Zoli et al. 1995). The ontogenic profile for these neurochemical markers and for the observed peak in CA1 neuron heteromeric nAChR function in this current study coincides with a period of local GABA-mediated excitatory neurotransmission resulting from high intracellular concentrations of Cl⁻ ions flowing through GABAₐ receptor channels to form primitive network oscillations known as giant depolarizing potentials (Ben-Ari et al. 1989; Garaschuk et al. 1998). Nicotinic signalling at α7 and β2* nAChRs contributes to the switch in GABAergic signalling from excitation to inhibition by mediating increased expression of the KCC2 Cl⁻ transporter that maintains a low intracellular concentration of Cl⁻ (Liu et al. 2006). Since
this switch occurs in rat hippocampus during the first two weeks of postnatal life (Garaschuk et al. 1998; Tyzio et al. 2006; Tyzio et al. 2007) and depends on postsynaptic Ca$^{2+}$ transients (Ganguly et al. 2001), nicotinic signalling at the more Ca$^{2+}$-permeable α4β2α5 nAChR is positioned spatially and temporally to contribute toward this developmental phenomenon in CA1 pyramidal neurons.

Nicotinic signalling plays an important role in neuron morphological development. Experiments in genetically-modified mice demonstrate that nAChRs containing the β2 subunit contribute to the production of dendritic spines in vivo for pyramidal neurons of the cerebral cortex and CA1 area of the hippocampus (Ballesteros-Yanez et al. 2010; Lozada et al. 2012b). Nicotinic signalling regulates dendrite growth during neuron development, although its net effect may depend on cell type and timing. In cultured neurons, signalling at nAChRs limits neurite outgrowth (Lipton et al. 1988; Nordman and Kabbani 2012; Pugh and Berg 1994) and mediates neurite retraction in a Ca$^{2+}$-dependent manner (Pugh and Berg 1994). Consistent with these effects, heteromeric nAChRs containing the α5 subunit appear to mediate the retraction of apical dendrites for medial prefrontal Layer VI pyramidal neurons during postnatal maturation (Bailey et al. 2012). Genetic deletion of the β2 nAChR subunit leads to either increased or decreased dendritic fields for Layer II/III pyramidal neurons depending on the cortical region examined (Ballesteros-Yanez et al. 2010). Moreover, for cultured rat olfactory bulb neurons and for hippocampal adult-born neurons, nAChR activation has been found to promote neurite/dendrite elongation (Campbell et al. 2010; Coronas et al. 2000). The postnatal development of CA1 pyramidal neurons is characterized by increased dendrite complexity and length over the first two-to-four weeks for basal dendrites (Nishimura et al. 2011) and over at least the first five weeks for apical dendrites (Jacobson et al. 1988). However, to the best of our knowledge the influence of nAChRs on hippocampal dendrite complexity has
The presence of functional heteromeric nAChRs on CA1 pyramidal neurons may have important implications for our understanding of aberrant hippocampus development. Several studies have demonstrated that perinatal exposure to nicotine in rodents and humans impairs cognitive functions that are supported by the hippocampus, such as learning, memory and attention (Dwyer et al. 2009; Ernst et al. 2001; Fried and Watkinson 2001; Johns et al. 1982; Levin et al. 1999; Li et al. 2015; Pauly and Slotkin 2008; Poorthuis et al. 2013b; Portugal et al. 2012; Schneider et al. 2011), and alters neurotransmission in the CA1 region (Damborsky et al. 2012; Parameshwaran et al. 2013; Parameshwaran et al. 2012). We show in this current study that nicotine at concentrations relevant to those seen in the brain of smokers desensitizes heteromeric nAChRs in young postnatal CA1 pyramidal neurons. This functional inhibition may alter the role of nicotinic signalling in the maturation of CA1 pyramidal neurons, as it does for medial prefrontal pyramidal neurons (Bailey et al. 2014), to alter their function within mature hippocampal networks. Autism Spectrum Disorder (ASD) and Attention Deficit Hyperactivity Disorder (ADHD) are associated with deficits in learning, memory and attention (Allen and Courchesne 2001; Loveland et al. 2008; Mayes and Calhoun 2007; Sowerby et al. 2011), and also with increased hippocampus size (Plessen et al. 2006; Schumann et al. 2004). Both nicotine and α4β2* nAChR agonists can improve attention and working memory in ADHD (Plessen et al. 2006; Wilens and Decker 2007; Wilens et al. 2006) and the augmentation of nicotinic neurotransmission is a proposed therapeutic strategy for ASD (Deutsch et al. 2010; Ghaleiha et al. 2013). Moreover, the α4β2* nAChR currently is a target of interest for a number of neurological disorders that involve deficits to hippocampus-dependent cognitive functions, including the neurodevelopmental disorders ASD, ADHD and depression, and
the neurodegenerative disorder Alzheimer’s disease (Arneric et al. 2007; Dineley et al. 2015; Hurst et al. 2013; Taly et al. 2009).

2.5.3 Conclusion
Our results demonstrate that heteromeric nAChRs mediate postsynaptic nicotinic signalling in developing CA1 pyramidal neurons. This places heteromeric nAChRs in a position to directly influence the physiological and morphological maturation of CA1 pyramidal neurons and determine their function within mature hippocampal cognitive circuits. This work provides insight into the normal development of the hippocampus and may also inform efforts for pharmaceutical development aimed to mitigate or treat multiple neurodevelopmental disorders involving the hippocampal nicotinic system.
CHAPTER 3

Characterization of heteromeric α4β2* nicotinic receptor function across regions of the hippocampal formation in young postnatal male mice

Based on the publication: Chung B.Y.T. and Bailey C.D.C. (2018) Similar nicotinic excitability responses across the developing hippocampal formation are regulated by small conductance calcium-activated potassium channels. Journal of Neurophysiology, 10.1152/jn.00426.2017
3.1 Abstract

The hippocampal formation forms a cognitive circuit that is critical for learning and memory. Cholinergic input to nicotinic acetylcholine receptors plays an important role in the normal development of principal neurons within the hippocampal formation. However, the ability of nicotinic receptors to stimulate principal neurons across all regions of the developing hippocampal formation has not been determined. We show here that heteromeric nicotinic receptors mediate direct inward current and depolarization responses in principal neurons across the hippocampal formation of the young postnatal mouse. These responses were found in principal neurons of the CA1, CA3, dentate gyrus, subiculum and entorhinal cortex layer VI, and varied in magnitude across region with the greatest responses occurring in the subiculum and entorhinal cortex. Despite this regional variation in the magnitude of passive responses, heteromeric nicotinic receptor stimulation increased the excitability of active principal neurons by a similar amount in all regions. Pharmacological experiments found this similar excitability response to be regulated by small conductance calcium-activated potassium (SK) channels, which exhibited regional differences in their influence on neuron activity that offset the observed regional differences in passive nicotinic responses. These findings demonstrate that SK channels play a role to coordinate the magnitude of heteromeric nicotinic excitability responses across the hippocampal formation at a time when nicotinic signalling drives the development of this cognitive brain region. This coordinated input may contribute to the normal development, synchrony and maturation of the hippocampal formation learning and memory network.
3.2 Introduction

The hippocampal formation (HF) forms a cognitive circuit that is critical for learning and memory (Eichenbaum 1992; Kesner et al. 1993; Scoville and Milner 1957; Squire and Zola 1996). The classical view of information flow through the constituent regions of the HF involves a series of excitatory glutamatergic synapses beginning in the entorhinal cortex layer II/III, and connecting through principal neurons of the dentate gyrus (DG), the cornu ammonis area 3 (CA3), the cornu ammonis area 1 (CA1), the subiculum (SUB), and deep layers of the entorhinal cortex (Andersen 2007). Abnormal changes to the structure and function of the HF have been linked with several neurodevelopmental disorders that involve learning and memory, including autism spectrum disorders (ASD) (Nicolson et al. 2006; Schumann et al. 2004), attention-deficit hyperactivity disorder (ADHD) (Li et al. 2014; Plessen et al. 2006), epilepsy (Seidenberg et al. 2005; Squier et al. 2003; Sun and Goodkin 2016) and Down syndrome (Belichenko et al. 2004; Pinter et al. 2001; Witton et al. 2015). Cholinergic input from the medial septum / diagonal band of Broca (MSDB) to the HF plays an important role in modulating normal development of the HF (Chang and Berg 1999; Dutar et al. 1995; Frotscher and Leranth 1985; Liu and Wu 2006; Lozada et al. 2012b). This is mediated in part by acetylcholine (ACh) activation of nonselective ligand-gated cation channel nicotinic acetylcholine receptors (nAChRs), which modulate neurotransmission by γ-aminobutyric acid (GABA) (Alkondon et al. 1997; Jones and Yakel 1997; Maggi et al. 2001; McQuiston and Madison 1999a; Sudweeks and Yakel 2000) and glutamate (Cheng and Yakel 2015b; Ge and Dani 2005) to shape the formation of the HF cognitive network.

The two major isoforms of nAChR in the HF belong to the homomeric α7 and heteromeric α4β2* families (Alkondon and Albuquerque 2004; Jones and Yakel 1997; Seguela et al. 1993; Sudweeks
and Yakel 2000; Wada et al. 1989; Zarei et al. 1999). Previous work in rodents age postnatal day (P) 2 and older demonstrates that functional nAChRs of both isoforms are present on hippocampal interneurons (Alkondon and Albuquerque 2004; Bell et al. 2011; Bell et al. 2015; Frazier et al. 1998a; Ji and Dani 2000; Jones and Yakel 1997; Khiroug et al. 2003; Maggi et al. 2001; McQuiston and Madison 1999b; Sudweeks and Yakel 2000). Although homomeric nAChRs have been demonstrated at P10 or older to mediate inward currents in glutamatergic principal neurons of the CA1, CA3, DG, and EC layer VI (ECVI) regions (Alkondon et al. 1997; Alkondon et al. 2007; Cheng and Yakel 2015b; Grybko et al. 2011; John et al. 2015; Kalappa et al. 2010), the function of heteromeric nAChRs in principal neurons across the HF is not well understood. These receptors mediate currents in principal neurons of ECVI (Tu et al. 2009) as they do in layer VI principal neurons of other cortical regions (Tian et al. 2014), but studies in principal neurons of other HF regions such as CA1 report conflicting results (He et al. 2013; Hefft et al. 1999; Tu et al. 2009). We have recently demonstrated that heteromeric nAChR-mediated inward current and excitability responses in principal neurons of the mouse CA1 follow a distinct developmental pattern in which responses are greatest in magnitude during the first two weeks of postnatal life and decline to low adult magnitudes shortly thereafter (Chung et al. 2016). This finding is consistent with molecular studies in which expression for heteromeric nAChR subunits also peaks in the hippocampus during the first two weeks of postnatal life and declines significantly shortly thereafter (Didier et al. 1995a; Machaalani et al. 2010; Shacka and Robinson 1998b; Winzer-Serhan and Leslie 2005). This early postnatal expression of heteromeric nAChR subunits varies in magnitude across regions of the HF (Didier et al. 1995a; Machaalani et al. 2010; Winzer-Serhan and Leslie 2005), however to the best of our knowledge, the function of heteromeric nAChRs during early postnatal life has not been characterized for principal neurons across all regions of the HF.
The objective of this current study was to determine whether functional heteromeric nAChRs are present in principal neurons located within each region of the young postnatal mouse HF. Whole-cell electrophysiological recordings were made of visually-identified principal neurons located within the CA1, CA3, DG, SUB and ECVI regions in acute brain slices of mice aged P5-10. Heteromeric nAChR inward current and depolarization responses were identified in all regions of the HF, and the magnitude of these responses varied across the HF with greater responses in the SUB and ECVI than in the hippocampus proper (CA1, CA3 and DG). Interestingly, although this same heteromeric nAChR stimulation increased the action potential firing frequency in active neurons, the magnitude of this response was similar across all HF regions. Mechanistic experiments focused on small conductance calcium-activated potassium (SK) channels, which generate a medium afterhyperpolarization potential (mAHP) to modulate action potential firing frequency (Gustafsson and Wigstrom 1983; Hotson and Prince 1980; Sah 1996). These experiments employed the SK channel blocker apamin (Hugues et al. 1982) to show that SK channels differentially influence the mAHP in principal neurons and mediate the similar heteromeric nAChR excitability responses observed in active principal neurons across regions of the HF. This finding demonstrates that SK channels play a role to coordinate the magnitude of nicotinic excitability responses across the HF at a time when nicotinic signalling drives HF neuron development, suggesting that this coordination contributes to the normal development, synchrony and maturation of the HF learning and memory network.
3.3 Materials and Methods

3.3.1 Experimental Animals

All animals were housed in a secure vivarium with an ambient temperature of 21-24°C and a 12-hour reverse light cycle with lights on at 8:00 pm. Pregnant female CD1-strain mice were purchased from Charles River Canada (Saint-Constant, QC, Canada) and housed with *ad libitum* access to water and food as described previously (Chung et al. 2016). The day of birth for each litter was considered to be postnatal day (P) 0 and male offspring were analyzed at P5-10, which falls within the human third trimester-equivalent for brain development. All efforts were made to minimize animal suffering and to limit the number of mice used in this study. Experimental animals were cared for according to the principles and guidelines of the Canadian Council on Animal Care, and the experimental protocol was approved by the University of Guelph Animal Care Committee.

3.3.2 Electrophysiology

Mice were anesthetized using isoflurane and killed by decapitation. Brains were immediately removed from the skull while submerged in 4°C sucrose artificial cerebrospinal fluid (ACSF) (254 mM sucrose, 10 mM D-glucose, 26 mM NaHCO₃, 2 mM CaCl₂, 2 mM MgSO₄, 3 mM KCl, 1.25 mM NaH₂PO₄, pH 7.4) that had been oxygenated using carbogen (95% O₂ and 5% CO₂). Coronal slices containing the rostral/dorsal hippocampus or entorhinal cortex were cut at 400 um thickness from approximately Bregma -1.46 mm to -2.18 mm or Bregma -2.92 to -3.52 mm, respectively, using a Leica VT1200 vibrating microtome (Leica Microsystems, Richmond Hill, ON, Canada) (Paxinos and Franklin 2001). Slices were transferred to oxygenated regular ACSF (128 mM NaCl,
10 mM D-glucose, 26 mM NaHCO$_3$, 2 mM CaCl$_2$, 2 mM MgSO$_4$, 3 mM KCl, 1.25 mM NaH$_2$PO$_4$, pH 7.4) and maintained at 30°C in a recovery chamber for at least two hours before use.

Slices were transferred to a modified recording chamber (Warner Instruments, Hamden, CT, USA) that was mounted on the stage of an Axioskop FS2 Microscope (Carl Zeiss Canada, Toronto, ON, Canada) and superfused with oxygenated ACSF at a rate of 3-4 mL/min. Whole-cell recording of CA1, CA3, SUB, DG and ECVI principal neurons was performed at room temperature (21-24°C) using borosilicate glass pipette electrodes (resistance of 2–5 MΩ) filled with an internal solution containing 120 mM K-gluconate, 5 mM KCl, 2 mM MgCl$_2$, 4 mM K$_2$-ATP, 400 µM Na$_2$-GTP, 10 mM Na$_2$-phosphocreatine, 33 µM Alexa Fluor 488 hydrazide (Life Technologies, Burlington, ON, Canada) and 10 mM HEPES buffer (adjusted to pH 7.3 with KOH). Individual principal neurons were visualized using infrared differential interference contrast microscopy and selected for recording based on the location of the soma within the hippocampal principal cell layer or cerebral cortical layer. Specifically, CA1 and SUB regions were differentiated by the tightly-packed principal cell layer of the CA1 compared to the diffuse principal cell layer of the SUB. CA3 neurons were located within principal cell layer prior to the hilus and DG neurons were located within the suprapyramidal blade. ECVI principal neurons were located in the deep layer VI immediately lateral to the hippocampus. The morphology/type of each recorded neuron was verified during recordings using epifluorescent visualization of the Alexa Fluor 488 that had diffused throughout the dendrite arbor from the recording pipette. All recordings were made using a Multiclamp 700B amplifier. Signals were acquired at 20 kHz and lowpass filtered at 2 kHz using a Digidata 1440A data acquisition system (Molecular Devices, Sunnyvale, CA, USA). The liquid
junction potential was corrected at the time of recording and all data was analyzed \textit{post-hoc} using Clampfit 10.3 software (Molecular Devices).

All experiments were performed in the continuous presence of 200 nM atropine to block muscarinic acetylcholine receptors and 10 nM methyllycaconitine (MLA) to block \(\alpha_7\) subunit-containing nAChRs. Basic electrophysiological properties were assessed in current clamp mode by measuring responses to the injection of positive and negative current steps for 500 ms each. Nicotinic receptor-mediated responses were probed by the addition of ACh to the ACSF superfusion bath after a baseline recording period and were followed by a 5-min washout period. Inward current responses were assessed in voltage clamp mode with neurons held at -75 mV by subtracting the mean holding current at the peak of the ACh response from the mean holding current at baseline. Depolarizing responses from rest were measured in current clamp mode by subtracting the mean membrane potential at the peak of the ACh response from the resting membrane potential at baseline. Acceleration of action potential firing was measured in current clamp mode by first injecting a positive current to elicit an approximately 1 Hz baseline firing frequency. The rate of firing was measured over a 30 s period during the peak ACh response and reported as a percent increase in frequency from the \(\sim 1\) Hz baseline frequency for each neuron, using the equation of: \(\frac{\text{firing frequency at the peak ACh response} - \text{firing frequency at baseline}}{\text{firing frequency at baseline}} \times 100\). Additional pharmacological experiments were performed using the \(\alpha_4\beta_2^*\) nAChR competitive antagonist dihydro-\(\beta\)-erythroidine (DH\(\beta\)E) which was applied to the bath at 3 \(\mu\)M for 10 min prior to ACh application. For a subset of neurons, input–output curves were generated by applying 500 ms depolarizing current steps from rest in 50 pA increments. The magnitude of postburst mAHP responses was assessed following trains of action potentials that
were elicited by injecting brief depolarizing current pulses (2 nA, 2 ms) at 50 Hz. Peak mAHP magnitude and timing were measured at the lowest membrane potential observed during the 50 to 1000 ms mAHP period after of the last action potential was elicited. Peak mAHP magnitude was calculated for each neuron as the difference between its resting membrane potential and this lowest membrane potential measured during the mAHP period. For a subset of experiments, the SK channel blocker apamin was applied to slices at 100 nM for 10 min before measuring nicotinic or mAHP responses. ACh chloride and atropine were purchased from Sigma Aldrich (Oakville, ON, Canada). MLA citrate, DHβE and apamin were purchased from Tocris Bioscience / Bio-Techne (Minneapolis, MN, USA). All drugs were stored in stock solutions at -20°C.

3.3.3 Statistical Analysis

All data are reported as mean ± SEM values for neurons within each experimental group. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) and a level of \( p < 0.05 \) was required to indicate statistical significance. Basic electrophysiological properties and ACh-induced nicotinic current, depolarization and excitability responses were assessed across regions using one-way ANOVA followed by the Tukey’s post hoc test. The effect of DHβE on ACh-induced nicotinic excitability responses was assessed within each region using two-tailed paired \( t \) tests. Comparison of input–output curves was assessed using two-way ANOVA followed by the Tukey’s post hoc test at each magnitude of current injection. Effects of apamin on mAHP magnitude were determined using the two-tailed paired \( t \) test within each region, and assessment of mAHP timing across HF regions was performed using one-way ANOVA followed by the Tukey’s post hoc test. The effect of apamin on ACh-induced nicotinic excitability responses was assessed using two-way ANOVA followed by the Bonferroni post hoc test within each region.
3.4 Results

3.4.1 Basic electrophysiological properties of principal neurons across the hippocampal formation.

Basic electrophysiological properties for principal neurons in the HF regions are presented in Table 3.1. To the best of our knowledge, a comparison of these properties across regions of the young postnatal HF has not been reported previously. There was a significant effect of region on resting membrane potential (one-way ANOVA, F(4,125) = 7.0, p < 0.0001), input resistance (F(4,113) = 36.7, p < 0.0001), and spike amplitude (F(4,125) = 3.3, p = 0.01). Specifically, resting membrane potential was lower in the DG than in each of the other HF regions (Tukey’s post hoc test, p ≤ 0.04 for each pairwise comparison), input resistance in the DG was higher than in each of the other HF regions (p < 0.0001 for each pairwise comparison), and spike amplitude was higher in the ECVI than in DG (p = 0.04).
### Table 3.1. Electrophysiological properties for principal neurons of the young postnatal hippocampal formation.

<table>
<thead>
<tr>
<th></th>
<th>CA1</th>
<th>CA3</th>
<th>DG</th>
<th>SUB</th>
<th>ECVI</th>
<th>p-value (one-way ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 21 mice = 13</td>
<td>n = 30 mice = 12</td>
<td>n = 27 mice = 9</td>
<td>n = 23 mice = 10</td>
<td>n = 28 mice = 11</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Resting membrane potential (mV)</td>
<td>-70.8 ± 0.8</td>
<td>-74.5 ± 1.1</td>
<td>-79.0 ± 1.7 *</td>
<td>-72.0 ± 1.0</td>
<td>-74.3 ± 0.8</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Input resistance (MΩ)</td>
<td>343.4 ± 18.6</td>
<td>353.1 ± 37.2</td>
<td>826.4 ± 79.1 *</td>
<td>186.4 ± 14.5</td>
<td>215.0 ± 22.2</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Spike amplitude (mV)</td>
<td>86.9 ± 1.7</td>
<td>83.1 ± 1.4</td>
<td>82.4 ± 2.7 †</td>
<td>89.0 ± 2.1</td>
<td>89.8 ± 1.2 †</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Values are mean ± 1 SEM. For resting membrane potential and input resistance, * indicates a statistical difference compared with each of the other regions (Tukey’s post hoc test, each p < 0.04). For spike amplitude, † indicates a statistical difference between the DG and ECVI only (Tukey’s post hoc test, p = 0.04).
3.4.2 Nicotinic inward currents in principal neurons of the hippocampal formation

The subunits that comprise heteromeric $\alpha 4\beta 2*$ nAChRs are expressed in the rodent brain as early as embryonic day 18, and several studies suggest that the content of these subunits peaks within the hippocampus during early postnatal life before declining to lower adulthood levels shortly thereafter (Shacka and Robinson 1998b; Winzer-Serhan and Leslie 2005; Zhang et al. 1998). Consistent with these expression studies, we have recently demonstrated that heteromeric, putative $\alpha 4\beta 2*$ nAChRs mediate excitatory responses in hippocampus CA1 principal neurons, with the greatest magnitude of responses also occurring during early postnatal life (Chung et al. 2016). In this present study, we first sought to determine whether functional heteromeric nAChRs are present on additional principal neurons of the HF during early postnatal life, by probing for ACh-induced inward current responses in principal neurons sampled from the CA1, CA3, DG, SUB and ECVI regions of the HF. Acute brain slices containing these regions were prepared from mice aged P5-10, and heteromeric nAChRs were isolated pharmacologically in all experiments by the continuous bath application of 200 nM atropine (to block muscarinic acetylcholine receptors), and 10 nM MLA (to block $\alpha 7$ subunit-containing nAChRs). Endogenous acetylcholinesterase in mouse cortical and hippocampal brain slices metabolizes ACh as it washes into the slice preparation, such that the effective ACh concentration reaching nAChRs is approximately 10- to 100-fold lower than the concentration applied in the ACSF bath (Bailey et al. 2010; Chung et al. 2016). We therefore applied ACh at 1 mM to facilitate comparison of our data with that from cell culture and synaptosome studies. Application of 1 mM ACh (15 s) resulted in a positive inward current response in every principal neuron that was recorded, although there was a significant effect of HF region on the magnitude responses observed (Fig. 3.1A, one-way ANOVA, $F(4,97) = 16.2$, $p < 0.0001$). Nicotinic current responses were significantly greater in SUB ($30.0 \pm 9.1$ pA, $n = 18$
neurons from 8 mice) and ECVI (37.5 ± 2.9 pA, n = 24 neurons from 11 mice) than in CA1 (8.6 ± 1.1 pA, n = 18 neurons from 12 mice), CA3 (5.5 ± 0.9 pA, n = 25 neurons from 12 mice) and DG (3.2 ± 0.9 pA, n = 17 neurons from 9 mice) (Tukey’s post hoc test, p ≤ 0.005 for each pairwise comparison). Previous investigation into the role of nAChRs in learning and memory has focused primarily within the hippocampus proper, where glutamatergic principal neurons of the CA1, CA3 and DG form the trisynaptic circuit (Andersen 2007). Analysis only within these three subregions of the hippocampus proper demonstrated that the magnitude of nicotinic inward currents is significantly affected by subregion (Fig. 3.1B, F(2,57) = 6.7, p = 0.002), with currents in CA1 being greater than those in DG (p = 0.002). Typical inward current responses are shown for one neuron from each region of the HF in Fig. 3.1C.
Fig. 3.1. The magnitude of nicotinic inward current responses varies across regions of the hippocampal formation. A: Inward current responses to 1 mM ACh (15 s) were significantly affected by region (one-way ANOVA, $p < 0.0001$), where currents were greater in SUB and ECVI compared with CA1, CA3 and DG (Tukey’s post hoc test, ***$p \leq 0.005$ for each pairwise comparison). B: Analysis of neurons within the hippocampus proper, which form the trisynaptic circuit, also demonstrated a significant effect of subregion ($p = 0.002$), with nicotinic inward current responses in CA1 being greater than those in DG (Tukey’s post hoc test, ***$p = 0.002$). C: Typical voltage-clamp traces are shown for one neuron in each region with the 15 s ACh application indicated by the gray horizontal bar. All recordings were made in the continuous presence of 200 nM atropine and 10 nM MLA. All values are displayed as mean ± SEM.
3.4.3 Nicotinic depolarization and excitability in principal neurons of the hippocampal formation

We next sought to assess consequences of the observed nicotinic inward currents on a passive neuronal response in principal neurons of the HF by measuring depolarization from rest. Application of 1 mM ACh (15 s) resulted in current clamp depolarization responses in every neuron tested, and the magnitude of these responses was significantly affected by HF region (Fig. 3.2A, one-way ANOVA, $F(4,78) = 18.2, p < 0.0001$). Tukey’s post hoc test demonstrated that the magnitude of depolarization was significantly greater in principal neurons of ECVI ($17.2 \pm 1.4$ mV, $n = 15$ neurons from 9 mice) than CA1 ($6.4 \pm 0.9$ mV, $n = 17$ neurons from 12 mice), CA3 ($5.3 \pm 1.1$ mV, $n = 19$ neurons from 9 mice), DG ($2.8 \pm 0.5$ mV, $n = 12$ neurons from 7 mice) and SUB ($8.9 \pm 1.5$ mV, $n = 20$ neurons from 9 mice) ($p \leq 0.0001$ for each comparison), and significantly greater in principal neurons of SUB than in DG ($p = 0.01$). Analysis of responses within the hippocampus proper (CA1, CA3 and DG) revealed that although there was no significant effect of subregion (Fig. 3.2B, $F(2,45) = 3.0, p = 0.06$), the nicotinic depolarization response was greater in CA1 than in DG ($p = 0.0496$). Typical depolarization responses are shown for one neuron from each region of the HF in Fig. 3.2C. It should be noted that the regional pattern for measured nicotinic depolarization responses across the HF in Fig. 3.2A matches the anticipated regional pattern calculated using Ohm’s Law ($\Delta V = \Delta I \times R$), using the measured inward current responses from Fig. 3.1A and the measured input resistance for each neuron from Table 3.1. The passive response to heteromeric nAChR stimulation of principal neurons across the HF thus relates directly to the inward current elicited in each neuron.
Fig. 3.2. The magnitude of nicotinic depolarization from rest varies across regions of the hippocampal formation. A: Depolarization responses to 1 mM ACh (15 s) were significantly affected by region (one-way ANOVA, $p < 0.0001$), where depolarization was greater in ECVI than in all other regions (Tukey’s *post hoc* test, ***$p \leq 0.005$ for each pairwise comparison) and greater in SUB than in DG (Tukey’s *post hoc* test, **$p \leq 0.01$). B: Analysis of neurons within the hippocampus proper, which form the trisynaptic circuit, did not demonstrate a significant effect of subregion ($p = 0.06$), although the depolarization response was greater in CA1 than in DG (Tukey’s *post hoc* test, *$p = 0.0496$). C: Typical current-clamp traces are shown for one neuron in each region with the 15 s ACh application indicated by the gray horizontal bar. All recordings were made in the continuous presence of 200 nM atropine and 10 nM MLA. All values are displayed as mean + SEM.
Our next line of experiments aimed to determine whether an active excitability response to heteromeric nAChR stimulation in these neurons was proportional to inward current and/or depolarization responses. We measured excitability responses of active neurons in current clamp mode by applying 1 mM ACh (15 s) to neurons that had been induced previously to fire action potentials at a frequency of 1 Hz by positive current injection, and measuring the percent increase in action potential firing frequency at the peak of the ACh response relative to the ~1Hz baseline for each neuron. In stark contrast with the regional differences observed above for inward current and passive depolarization responses, neurons in all regions showed similar active excitability responses to ACh that were not significantly affected by HF region (Fig. 3.3A, one-way ANOVA, F(4,78) = 0.5, p = 0.7). The percent by which ACh accelerated firing over baseline (Fig. 3.3A, right y axis) was: CA1 (196.7 ± 33.9, n = 12 neurons from 8 mice), CA3 (281.2 ± 48.5, n = 21 neurons from 10 mice), DG (230.7 ± 43.4, n = 18 neurons from 7 mice), SUB (257.3 ± 37.4, n = 17 neurons from 6 mice) and ECVI (261.0 ± 33.0, n = 15 neurons from 10 mice). This same data expressed for each neuron relative to the mean for CA1 principal neurons (Fig. 3.3A, left y axis) shows that the ACh-induced acceleration of action potential firing was 1.4 ± 0.2 times as strong in CA3 versus CA1, 1.2 ± 0.2 times as strong in DG versus CA1, 1.3 ± 0.2 times as strong in SUB versus CA1 and 1.3 ± 0.2 times in ECVI versus CA1. Typical traces showing the ACh-induced increase of action potential firing are shown for one neuron from each region of the HF in Fig. 3.3B. We also sought to determine whether these ACh-induced excitatory responses are mediated by α4β2* nAChRs, by repeating this experiment in the absence and presence of the α4β2* nAChR competitive antagonist DHβE (3 μM, 10 min pre-exposure). Data presented in Table 3.2 demonstrates that DHβE decreased the nicotinic excitability response in neurons from each region of the HF (paired t test, p = 0.03 to 0.0006 for each region). Moreover, one-way ANOVA
confirmed no regional difference in the magnitude of responses either at baseline (F(4,20) = 1.0, 
p = 0.4) or in the presence of DHβE (F(4,20) = 1.2, p = 0.3). These results demonstrate that, in 
contrast with the varied inward current and passive depolarization responses to heteromeric 
nAChR stimulation across regions of the HF, the active response to this same stimulation in firing 
neurons is quite similar across all regions of the HF.
Fig. 3.3. The ability of nicotinic stimulation to increase excitability in active neurons is similar across regions of the hippocampal formation. 

**A**: For principal neurons that had been induced to fire action potentials at 1 Hz by positive current injection, the increase of firing frequency from the 1 Hz baseline following application of 1 mM ACh (15s) was not significantly affected by region (one-way ANOVA, p = 0.7).

**B**: Typical current-clamp traces are shown for one neuron in each region, which represent typical changes in firing frequency following application of ACh (indicated by the gray horizontal bar). All recordings were made in the continuous presence of 200 nM atropine and 10 nM MLA. All values are displayed as mean + SEM.
Table 3.2. Effect of DHβE on nicotinic stimulation of active principal neurons of the young postnatal hippocampal formation.

<table>
<thead>
<tr>
<th>Region</th>
<th>Before (% increase from baseline)</th>
<th>After DHβE (% increase from baseline)</th>
<th>p-value (paired t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA1</td>
<td>273.5 ± 48.4</td>
<td>66.6 ± 21.6</td>
<td>0.03</td>
</tr>
<tr>
<td>(n = 5 neurons from 4 mice)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA3</td>
<td>356.4 ± 93.3</td>
<td>151.2 ± 57.4</td>
<td>0.02</td>
</tr>
<tr>
<td>(n = 5 neurons from 3 mice)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DG</td>
<td>223.1 ± 35.4</td>
<td>70.2 ± 25.1</td>
<td>0.004</td>
</tr>
<tr>
<td>(n = 5 neurons from 4 mice)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUB</td>
<td>364.4 ± 89.9</td>
<td>183.0 ± 73.0</td>
<td>0.03</td>
</tr>
<tr>
<td>(n = 5 neurons from 4 mice)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECVI</td>
<td>373.3 ± 44.1</td>
<td>157.9 ± 47.5</td>
<td>0.0006</td>
</tr>
<tr>
<td>(n = 5 neurons from 4 mice)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The ability of ACh (1 mM) to increase action potential firing frequency was measured in principal neurons before and after exposure to DHβE (3 μM, 10 min). All recordings were made in the continuous presence of 200 nM atropine and 10 nM MLA. Values are mean ± 1 SEM.
3.4.4 Capacity for excitation in principal neurons of the young postnatal hippocampal formation

Given that principal neurons experience varying magnitude of nicotinic inward current and depolarization responses across the young postnatal HF, yet show very similar nicotinic increases to action potential firing, we generated input-output curves to determine whether this resulted from similar upper excitation limits for these neurons that were attained by the nicotinic inward currents. Input-output curves were generated by injecting positive current steps from 50 pA to 200 pA, in 50 pA increments and for 500 ms each. The resulting curves for each HF region are shown in Fig. 3.4A (left side) and typical traces for one neuron from each HF region in response to 50 pA and 150 pA current steps are shown in Fig. 3.4B (right side). Comparing all regions together, two-way ANOVA revealed significant main effects of current injected (F(4,415) = 140.4, p < 0.0001) and HF region (F(4,415) = 13.8, p < 0.0001), and a significant interaction between these two factors (F(16,415) = 2.1, p = 0.008). The lowest 50 pA current step resulted in a greater action potential firing frequency in principal neurons in CA1 (17.5 ± 2.7 Hz, n = 13 neurons from 6 mice) than in CA3 (8.8 ± 1.1 Hz, n = 17 neurons from 7 mice), SUB (8.6 ± 1.3 Hz, n = 32 neurons from 9 mice) and ECVI (8.2 ± 1.7 Hz, n = 12 neurons from 7 mice) (Tukey’s post hoc test, p ≤ 0.009 for each pairwise comparison). At the 200 pA current step, which resulted in maximal firing frequency for each neuron type, principal neurons in both CA1 (27.4 ± 3.7 Hz, n = 13 neurons from 6 mice) and SUB (26.6 ± 1.5 Hz, n = 32 neurons from 9 mice) showed a greater action potential firing frequency than in ECVI (18.5 ± 2.9 Hz, n = 12 neurons from 7 mice) (p ≤ 0.02 for each pairwise comparison). The application of depolarizing current steps beyond 200 pA elicited decreasing mean action potential frequencies across all HF regions, with some neurons presenting over-excitation depolarization block. These data clearly show that, although principal neurons in all
regions of the early postnatal HF are capable of firing at frequencies well beyond the 2.7 to 3.8 Hz absolute frequency observed at the peak of the nicotinic response in Fig. 3.3, the maximum firing frequency is lower in ECVI compared with other HF regions.

We also performed a theoretical experiment by approximating the total amount of inward current received by neurons at the peak of the nicotinic response in Fig. 3.3 by adding (i) the amount of current required to elicit baseline firing at approximately 1 Hz in the experiment from Fig. 3.3 plus (ii) the mean nicotinic inward current received by each neuron type in Fig. 3.1. This resulted in theoretical total inward currents of: CA1 (25.2 ± 2.0 pA, n = 11 neurons from 3 mice), CA3: (19.2 ± 1.1 pA, n = 14 neurons from 2 mice), DG: (12.9 ± 1.7 pA, n = 12 neurons from 2 mice), SUB: (49.2 ± 9.9, n = 11 neurons from 3 mice) and ECVI: (66.7 ± 4.1 pA, n = 11 neurons from 4 mice).

Using this theoretical total current and extrapolating from the input-output curves in Fig. 3.4, this experiment suggests that ACh-induced nicotinic currents applied in a 500 ms step to neurons at rest would result in varying firing frequencies between 15 and 60 percent of the maximum firing frequency for each principal neuron type across the HF. This contrasts with results from Fig. 3.3 demonstrating that actual ACh-induced nicotinic currents received by active neurons leads to very similar firing frequencies between 10 and 20 percent of the maximum firing frequency for each principal neuron type across the HF. We next focused on mechanisms that regulate excitability in active neurons.
Fig. 3.4. Input-output curves for principal neurons of the young postnatal hippocampal formation. 

A (left side): Input-output curves were generated by measuring action potential firing frequency in response to the injection of positive current steps from 50 pA to 200 pA, in 50 pA increments and for 500 ms each. The resulting pattern of firing frequency was significantly affected by the amount of current injected (two-way ANOVA, $p < 0.0001$) and region (two-way ANOVA, $p < 0.0001$), and there was a significant interaction between these two main effects (two-way ANOVA, $p = 0.008$). Following 50 pA injection, firing frequency was higher in CA1 than in CA3, SUB and ECVI (Tukey’s post hoc test, $p \leq 0.009$ for each pairwise comparison). Maximal firing for each region was observed following 200 pA injection, where firing frequency was higher in both CA1 and SUB than in ECVI ($p \leq 0.02$ for each pairwise comparison). 

B (right side): Typical current-clamp traces demonstrating action potentials elicited following injection of 50 pA and 150 pA are displayed for one neuron from each region of the hippocampal formation. All values are displayed as mean ± SEM.

SK channels are widely expressed throughout the brain and regulate neuron excitability following the firing one or more action potentials (Sah 1996). They are activated by a rise in intracellular calcium during the action potential and mediate potassium efflux contributing to the generation of an afterhyperpolarization of medium duration (mAHP) (Nicoll 1988; Schwindt and Crill 1998). The mAHP activates within milliseconds and decays with a time constant in the range of hundreds of milliseconds, until the onset of the slow AHP which is active from one to several seconds (Faber 2009; Lancaster and Adams 1986; Lancaster and Zucker 1994; Matthews et al. 2009). Since SK channels are expressed in HF neurons that receive glutamatergic neurotransmission (Babiec et al. 2017; Lappin et al. 2005; Mateos-Aparicio et al. 2014; Ngo-Anh et al. 2005) and we have observed regional differences across the HF for action potential firing frequency in response to low and high current injection, we next determined whether SK channels differentially regulate the mAHP in principal neurons of the young postnatal HF. This was accomplished by measuring mAHP magnitude within single neurons before and after application of the SK channel blocker apamin at 100 nM for 10 min. Action potentials were elicited from rest by applying brief depolarizing current pulses (trains of 8 or 16 pulses of 2 nA for 2 ms each at 50 Hz) and measuring peak mAHP.
magnitude and timing at the lowest membrane potential observed during the 50 to 1000 ms mAHP period after the last action potential was elicited. Peak mAHP magnitude was calculated for each neuron as the difference between its resting membrane potential and this lowest membrane potential measured during the mAHP period. Data for peak mAHP magnitude and representative current-clamp traces are shown in Fig. 3.5 for each neuron type before (baseline) and after apamin treatment following trains of 8 pulses (Fig. 3.5A) and 16 pulses (Fig. 3.5B). In CA1 principal neurons, peak mAHP magnitude for 8 current pulses was -0.6 ± 0.6 mV at baseline and 0.2 ± 0.8 mV after apamin treatment (two-tailed paired t-test, n = 6 neurons from 3 mice, p = 0.4), and for 16 current pulses was -1.1 ± 0.5 mV at baseline and -0.9 ± 0.6 mV after apamin treatment (n = 6 neurons from 3 mice, p = 0.8). Apamin also did not affect peak mAHP magnitude in SUB principal neurons, which for 8 current pulses was -0.004 ± 0.4 mV at baseline and 1.3 ± 0.8 mV after apamin treatment (n = 6 neurons from 3 mice, p = 0.2), and for 16 current pulses was -0.7 ± 0.4 mV at baseline and 0.4 ± 0.2 mV after apamin treatment (n = 6 neurons from 3 mice, p = 0.07). In ECVI principal neurons, however, apamin treatment did significantly reduce peak mAHP amplitude. Peak mAHP was reduced for 8 current pulses from -0.6 ± 0.2 mV at baseline to 0.3 ± 0.4 mV after apamin treatment (n = 6 neurons from 4 mice, p = 0.007) and was reduced for 16 current pulses from -1.3 ± 0.4 mV at baseline to 0.2 ± 0.2 mV after apamin treatment (n = 6 neurons from 4 mice, p = 0.01).

We found that in young postnatal CA3 and DG neurons, membrane potential following multiple action potentials did not return to rest within the 50 to 1000 ms timeframe for the mAHP. As such, measured peak mAHP magnitudes at baseline were positive relative to the resting membrane potential. Apamin did not affect peak mAHP magnitude in CA3 principal neurons, which for 8
current pulses was $2.6 \pm 1.2$ mV at baseline and $1.9 \pm 0.6$ mV after apamin treatment ($n = 5$ neurons from 2 mice, $p = 0.6$), and for 16 current pulses was $1.3 \pm 1.1$ mV at baseline and $2.0 \pm 0.7$ mV after apamin treatment ($n = 5$ neurons from 2 mice, $p = 0.6$). Interestingly, apamin treatment increased peak mAHP magnitude in DG principal neurons. Peak mAHP was increased for 8 current pulses from $3.4 \pm 0.7$ mV at baseline to $0.4 \pm 1.24$ mV after apamin treatment ($n = 6$ neurons from 2 mice, $p = 0.05$) and was increased for 16 current pulses from $1.9 \pm 0.7$ mV at baseline to $0.3 \pm 1.0$ mV after apamin treatment ($n = 6$ neurons from 2 mice, $p = 0.07$).

The timing for the peak mAHP magnitude at baseline varied across regions of the HF, as indicated using arrows in Fig. 3.5 with peaks generally occurring earlier for CA1 and SUB than in the CA3, DG and ECVI. One-way ANOVA for peak times after 8 current pulses reveals a significant effect of region ($F(4,24) = 8.1$, $p = 0.0003$). Here, mAHP peak time occurred earlier in CA1 and ECVI than in CA3 (Tukey’s post hoc test, $p = 0.02$ for each comparison), and earlier in CA1, SUB and ECVI than in DG ($p < 0.01$ for each comparison). The peak mAHP time was also affected by region following 16 current pulses ($F(4,24) = 5.7$, $p = 0.002$), with the peak occurring earlier in CA1, SUB and ECVI than in DG ($p < 0.05$ for each comparison). Results from this experiment using apamin suggest that SK channels increase mAHP magnitude in ECVI neurons and decrease mAHP magnitude in DG neurons to differentially regulate firing frequencies for principal neurons of the young postnatal HF.
Fig. 3.5. The medium afterhyperpolarization (mAHP) is differentially affected by the SK channel blocker apamin in principal neurons of the young postnatal hippocampal formation. Trains of 8 (A) or 16 (B) action potentials were elicited from rest by applying brief depolarizing current pulses (2 nA, 2 ms) at 50 Hz and measuring the peak mAHP magnitude during the 50 to 1000 ms mAHP period after the last action potential was elicited, relative to resting membrane potential. Data for this mAHP magnitude is shown in histogram plots for each neuron type at baseline (white bars) and following application of 100 nM apamin for 10 min (red bars). Current-clamp traces are shown below each histogram plot for one typical neuron at baseline (black trace) and after apamin administration (red trace), with the timing for the peak mAHP magnitude at baseline indicated by the arrow. Apamin treatment increased mAHP magnitude in DG neurons following 8 current pulses (two-tailed paired t test, *p = 0.05) and decreased mAHP magnitude in ECVI neurons following both 8 (two-tailed paired t test, **p = 0.007) and 16 current pulses (two-tailed paired t test, *p = 0.01). There was a significant effect of HF region on peak mAHP time at baseline following 8 (one-way ANOVA, p = 0.0003) and 16 (one-way ANOVA, p = 0.002) current pulses. All values are displayed as mean ± SEM.
3.4.5 SK channels differentially regulate nicotinic excitability in active principal neurons of the young postnatal hippocampal formation

Given that SK channels differentially regulate the mAHP across regions of the young postnatal HF, primarily to increase mAHP magnitude in ECVI, we next sought to determine whether SK channels influence the magnitude of ACh-induced nicotinic excitation responses in active neurons within this cognitive circuit. The experiment from Fig. 3.3 was repeated by measuring the percent increase in firing frequency following application of 1 mM ACh (15s) to neurons that had been induced by positive current injection to fire action potentials at a baseline of approximately 1 Hz. We compared results from Fig. 3.3 with those from this current experiment, which was performed following blockade of SK channels by exposing slices to 100 nM apamin for 10 min. In order to directly compare results from this new experiment and results from Fig. 3.3, the percent by which ACh increased firing frequency was normalized relative to the mean for CA1 neurons within each experiment. Results are shown in Fig. 3.6A, with data from Fig. 3.3A presented using white bars and new data following apamin treatment presented using red bars. There was a significant main effect of region (two-way ANOVA, F(4, 104) = 8.0, p < 0.0001) but no main effect of apamin treatment (F (1, 104) = 0.3, p = 0.6) on relative ACh-induced acceleration of firing, although there was a significant interaction between these two variables (F (4, 104) = 7.0, p < 0.0001). Bonferroni’s post hoc test revealed a significant effect of apamin treatment to increase the ACh-induced acceleration of firing in ECVI neurons compared with untreated ECVI neurons (p < 0.0001). However, apamin treatment did not significantly affect the magnitude of the ACh response in neurons of CA1 (p = 1.0), CA3 (p = 1.0), DG (p = 0.1) or SUB (p = 1.0). One-way ANOVA within each treatment group shows that although there was no effect of HF region on ACh-induced acceleration of firing in untreated neurons (as per data for Fig. 3.3 described above),
a significant effect of HF region emerged following apamin treatment (F(4,26) = 6.6, p = 0.0009). Tukey’s post hoc test demonstrated that after apamin treatment, relative ACh-induced acceleration of firing was significantly greater in principal neurons of ECVI than CA1 (p = 0.02), CA3 (p = 0.007), DG (p = 0.0004) and SUB (p = 0.02). One-way ANOVA within the hippocampus proper after apamin treatment showed an effect of subregion (F(2,16) = 3.9, p = 0.04) with relative ACh-induced acceleration of firing being greater in CA1 than DG (p = 0.04). It is most interesting that this pattern of regional differences for the magnitude of ACh-induced acceleration of firing after apamin treatment in this experiment (red bars in Fig. 3.6A) is strikingly similar to the pattern of regional differences for ACh-induced depolarization from rest as shown in Fig. 3.2. These findings suggest that SK channel regulation of neuron excitability normalizes nicotinic responses in active neurons by increasing the relative response in DG and limiting the relative response in ECVI, with an end result to facilitate similar nicotinic excitability responses in active principal neurons across the young postnatal HF.
Fig. 3.6. The ability of nicotinic stimulation to increase excitability in active neurons is differentially regulated by SK channels across regions of the hippocampal formation. A: Principal neurons were induced to fire action potentials at 1 Hz by positive current injection and the increase in firing frequency in response to 1 mM ACh (15 s) was measured. The magnitude of this nicotinic response was normalized relative to CA1 for neurons that were not treated (white bars) and for
neurons that had been treated with 100 nM apamin for 10 min (red bars). ACh-induced acceleration of firing frequency was affected by HF region (two-way ANOVA, \( p < 0.0001 \)) but not by apamin treatment (two-way ANOVA, \( p = 0.6 \)), and there was a significant interaction between these two variables (two-way ANOVA, \( p < 0.0001 \)). Apamin treatment increased the relative response to ACh in ECVI only (Bonferroni’s *post hoc* test, \( \dagger p < 0.0001 \)). Analysis of each treatment group separately showed no effect of HF region in untreated neurons (One-way ANOVA, \( p = 0.7 \)) and a significant effect of HF region after apamin treatment (One-way ANOVA, \( p = 0.0009 \)), where the nicotinic response was greater in ECVI than in CA1, CA3, DG and SUB (Tukey’s *post hoc* test, \( p \leq 0.02 \) for each comparison). Analysis of apamin-treated neurons within the hippocampus proper also demonstrated a significant effect of subregion (\( p = 0.04 \)) with greater nicotinic responses in CA1 than in DG (Tukey’s *post hoc* test, \( p = 0.04 \)).

**B:** Typical current-clamp traces are shown for one neuron from each region after apamin treatment, which represent typical changes in firing frequency following application of ACh (indicated by the gray bar). All recordings were made in the continuous presence of 200 nM atropine and 10 nM MLA. All values are displayed as mean ± SEM.

### 3.5 Discussion

This study provides evidence that glutamatergic principal neurons in the CA1, CA3, DG, SUB and ECVI regions of the HF are excited by heteromeric nAChRs during early postnatal development. SK channels regulate this excitability response, resulting in similar changes to action potential firing frequency elicited by nicotinic input across regions of the HF. These findings were obtained through a series of physiological and pharmacological experiments performed using whole-cell electrophysiological recording of visually-identified principal neurons of each HF region within acute hippocampal brain slices collected from mice age P5-10. We first found that heteromeric nAChR stimulation elicits direct inward current and depolarization responses in principal neurons of all HF regions investigated, and that the magnitude of these passive responses is greater in the SUB and EC layer IV than in the hippocampus proper regions CA1, CA3 and DG. Interestingly, this same heteromeric nAChR stimulation applied to active principal neurons increased action potential firing frequency in a manner that was similar across all HF regions. Mechanistic experiments demonstrated that SK channels differentially influence mAHP magnitude in principal
neurons of the HF, which appears to offset the varied nicotinic inputs to produce the similar excitability responses observed across regions of the HF. The comprehensive characterization of heteromeric nAChR function presented in this study demonstrates that SK channels regulate the magnitude of nicotinic excitability responses in active principal neurons during a developmental period when nicotinic signalling drives HF neuron development. This regulation of excitability by SK channels may therefore contribute to normal development, synchrony and maturation of the HF learning and memory network.

3.5.1 Heteromeric nicotinic receptor signalling within the developing hippocampal formation

Afferent cholinergic neurotransmission from the MSDB plays an important role in the normal development and mature function of the HF (Berger-Sweeney et al. 2001; Chang and Berg 1999; Dutar et al. 1995; Frotscher and Leranth 1985; Mitsushima et al. 2013; Nott and Levin 2006; Placzek et al. 2009). Functional nAChRs have been identified on GABAergic interneurons (Alkondon and Albuquerque 2004; Bell et al. 2011; Bell et al. 2015; Frazier et al. 1998a; Ji and Dani 2000; Jones and Yakel 1997; Khiroug et al. 2003; McQuiston and Madison 1999b; Sudweeks and Yakel 2000) and on glutamatergic principal neurons (Alkondon et al. 1997; Alkondon et al. 2007; Cheng and Yakel 2015b; Grybko et al. 2011; He et al. 2013; Kalappa et al. 2010; Tu et al. 2009) of the HF. We provide evidence in this current study that functional heteromeric, putative α4β2* nAChRs are present on principal neurons within the CA1, CA3, DG, SUB and ECVI regions of the HF during a period equivalent to the human third trimester for brain development (Clancy et al. 2001; Clancy et al. 2007; Dobbing and Sands 1979; Workman et al. 2013). This is consistent with previous in situ reports that α4, β2 and α5 subunits are expressed within hippocampus principal neurons (Heath et al. 2010; Marks et al. 1992; Salas et al. 2003; Sudweeks
Nicotinic responses to bath-applied ACh in our slice preparation were likely not mediated by homomeric α7 nAChRs because this isoform desensitizes rapidly (Quick and Lester 2002) and all experiments were conducted in the continuous presence of the α7 subunit-containing nAChR antagonist MLA (Macallan et al. 1988; Murray et al. 2012). We have recently reported, using the same experimental design as this current study, that heteromeric nAChRs mediate postsynaptic nicotinic signalling in young postnatal CA1 principal neurons, and that these responses are inhibited by the α4β2* nAChR-selective antagonist DHβE (Chung et al. 2016). This is consistent with findings from this current study, which show statistically-significant inhibition of nicotinic responses by DHβE in principal neurons of all HF regions examined. These previous findings, in combination with this current study, suggest that heteromeric nAChRs of the α4β2* isoforms mediate excitation of principal neurons within CA1, CA3, DG, SUB and ECVI during early postnatal development.

Previous electrophysiological studies in rodents aged P10 and older demonstrate nicotinic responses in ECVI principal neurons (Tu et al. 2009), and either no direct nicotinic responses or nicotinic responses that occur in a low proportion of CA1, CA3, DG and SUB principal neurons (He et al. 2013; Hefft et al. 1999; McQuiston and Madison 1999b; Tu et al. 2009). Our findings in younger mice aged P5-10 demonstrate the greatest heteromeric nAChR function in SUB and ECVI principal neurons, but that heteromeric nAChRs also mediate direct inward currents and depolarization from rest in principal neurons of the CA1, CA3 and DG at this age. Previous work suggests that endogenous cholinergic signalling plays a role in determining the pattern of glutamatergic synapse and network formation during development. For example, nicotinic stimulation of immature CA3 principal neurons or local astrocytes in young rats can convert silent
synapses to a functional status, resulting in enhanced glutamatergic synaptic transmission through the CA3-CA1 Schaffer collateral pathway (Maggi et al. 2001; Wang et al. 2013). Nicotinic signalling also influences the morphological growth and maturation of neurons within the central nervous system (Campbell et al. 2010; Lipton et al. 1988; Liu and Wu 2006; Pugh and Berg 1994) and β2 subunit-containing nAChRs specifically are important for the normal development of dendritic spines in principal neurons of the cerebral cortex (Ballesteros-Yanez et al. 2010) and hippocampus (Lozada et al. 2012b). This body of work suggests that nicotinic signalling not only regulates glutamatergic transmission throughout the mature HF, but may also play an important role in the formation of integrated and stabilized network flow through this cognitive brain region.

3.5.2 Coordinated activity in the developing hippocampal formation

Spontaneous coordinated activity of immature neuronal networks appears in the rodent hippocampus during the first postnatal week (Ben-Ari et al. 1989; Crepel et al. 2007; Garaschuk et al. 1998). These oscillations are mediated by excitatory GABAergic neurotransmission to form giant depolarizing potentials (GDPs) in the HF (Nardou et al. 2009; Sipila et al. 2005), which are a primordial form of synchrony between neurons that precedes more organized coordinated activity such as theta and gamma rhythms (Bragin et al. 1995; Buzsaki and Draguhn 2004; Fellous and Sejnowski 2000; Kasyanov et al. 2004; Mohajerani and Cherubini 2006). Nicotinic signalling appears to play an important modulatory role in hippocampus GDP function during this developmental period. Both α7 and β2 subunit-containing nAChRs exert regulatory actions on network-driven GDPs, where stimulation of these nAChRs increases GDP frequency (Le Magueresse et al. 2006; Maggi et al. 2001), and conversely, blockade of these nAChRs decreases GDP frequency (Maggi et al. 2001). Since it is well-established that α7 and α4β2* nAChRs are
present on interneurons throughout regions of the hippocampus, it is proposed that the activation of these receptors on interneurons synapsing directly onto principal cells leads to an increase in GABA release and increased GDP formation. Our present finding that functional heteromeric nAChRs are present on all principal neuron types of the young postnatal HF, and that their activation leads to similar-magnitude excitability responses in active neurons across all regions of the HF, suggests that these postsynaptic nAChRs may also be involved in the formation of coordinated and organized rhythms within the developing HF. Slightly later during postnatal development, nicotinic signalling at α7 and β2 subunit-containing nAChRs also facilitates the normal switch of GABAergic neurotransmission from excitation to inhibition by mediating increased expression of the KCC2 chloride transporter to establish the mature low intracellular chloride concentration (Liu et al. 2006). Because this switch occurs in rodent HF during the first two weeks of postnatal life, when we observed the greatest nAChR function (Garaschuk et al. 1998; Tyzio et al. 2007), and requires calcium transients (Ganguly et al. 2001), nAChRs located directly on principal neurons during this developmental period may also aid to shape the development of mature HF neuronal networks through this mechanism.

The generation of somatic mAHPs by SK channels contributes to the excitability and firing frequency of neurons by regulating the speed and pattern of instantaneous firing frequency (Hallworth et al. 2003; Stocker et al. 1999), tonic firing frequency (Sah 1996; Wolfart et al. 2001; Zhang and McBain 1995), rhythmic burst activity and oscillatory activity (Cingolani et al. 2002; Cueni et al. 2008; Wolfart and Roeper 2002). Our results show that during early postnatal development, SK channels mediate mAHPs of varying magnitude and timing across regions of the HF, which likely underlies their ability to differentially modulate and coordinate excitability
responses to nAChR stimulation of varying magnitudes in active principal neurons across regions of the HF. The end result is quite striking, as the nAChR-mediated inward current and depolarization responses are much lower in the CA1, CA3 and DG regions of the hippocampus proper than in ECVI, yet this same stimulation leads to near-identical acceleration of action potential firing in active neurons across all HF regions. This coordinated nicotinic response may be important to the development of synchronized activity during development and the establishment of mature HF networks.

We found that principal neurons in young postnatal CA1 and SUB presented hyperpolarizing mAHPs following trains of action potentials that were not significantly affected by apamin treatment. Previous reports using older rodents demonstrate that the two SK channel subtypes K\textsubscript{Ca}2.1 and K\textsubscript{Ca}2.2 are co-expressed at high levels in principal neurons of CA1, CA3, DG, SUB and ECVI (Stocker and Pedarzani 2000) and that K\textsubscript{Ca}2.2 channels are necessary and sufficient to generate a mAHP in CA1 principal neurons (Bond et al. 2004). The discrepancy between our results and this previous literature may be a factor of the young postnatal age of mice examined in this current study. In contrast with extrinsic afferents, intrinsic associational connections of the rodent hippocampus develop relatively late. Entorhinal axons innervate the hippocampus proper at embryonic day 15 (Skutella et al. 1999; Super and Soriano 1994), while principal neurons are generated in the second half of gestation and granule cells are generated postnatally (Altman and Bayer 1990; Jabes et al. 2011). To the best of our knowledge, the \textit{in situ} expression of SK channels has not been reported previously for rodent HF principal neurons during P5-10. More-recent literature has provided evidence that is consistent with our finding that SK channels likely do not contribute to the mAHP in CA1 principal neurons. This work employed pharmacological channel
blockers to show that the mAHP and neuronal excitability are regulated by voltage-gated Kv7/KCNQ/M potassium channels and hyperpolarization-activated cyclic nucleotide-gated (HCN) potassium channels, with little or no contribution from SK channels (Gu et al. 2008; Gu et al. 2005; Yue and Yaari 2004).

While principal neurons in CA3 and DG did not present true hyperpolarization responses during the mAHP period following trains of action potentials, apamin treatment surprisingly decreased the membrane potential of DG neurons during the period, suggesting that SK channels influence the hyperexcitable nature of DG neurons during early postnatal development. Synchronized activity in immature networks may depend on the hyperexcitability of developing circuits in the hippocampus proper (Ben-Ari et al. 1989; Ben-Ari et al. 2007; Rivera et al. 1999), as depolarizing GABA supports episodes of bursting activity underlying waves of depolarization that precede more organized forms of HF activity (Blankenship and Feller 2010; Bragin et al. 1995; Buzsaki and Draguhn 2004; Fellous and Sejnowski 2000; Kasyanov et al. 2004; Mohajerani and Cherubini 2006). The effect of apamin to decrease excitability in DG neurons seems counterintuitive to the nature of SK potassium channels, although this is not without precedent. In approximately half of neurons tested within the nucleus tractus solitarii, apamin decreased neuron excitability in a bicuculline-dependent manner, suggesting an indirect effect of apamin to increase the excitability of local interneurons and the resulting tonic GABAergic input to recorded neurons (Butcher et al. 1999). A similar mechanism may underlie apamin effects on young postnatal DG neurons in our study, given the extensive interneuron network already present at this age (Holter et al. 2007; Szabo et al. 2017). Alternatively, blockade of SK channels may unmask the function of additional potassium channels that are present in recorded DG neurons. Similar unmasking occurs in CA1
pyramidal neurons: While Kv7/KCNQ/M channels are major contributors to the mAHP in these neurons, as described above, blockade of Kv7/KCNQ/M channels unmasks a previously-shunted influence of SK channels to enhance the mAHP (Chen et al. 2014). Given that the channel contributions toward excitability are reversed in mature DG neurons compared with CA1 neurons, such that SK channels are the major contributor to the mAHP and Kv7/KCNQ/M channels reduce excitation through decreased input resistance and increased firing threshold (Mateos-Aparicio et al. 2014), it is possible that SK channel blockade in young DG neurons removes a source of shunting and allows Kv7/KCNQ/M channels to powerfully lower membrane potential following action potential firing. Additional voltage-gated potassium channels that may be unmasked in young DG neurons include the Kv1/KCNA channel, for which Kv1.1 subunit expression is greatest during the first two postnatal weeks in mice (Pruss et al. 2010) and function can strongly regulate action potential firing at a relatively-low threshold (Kirchheim et al. 2013; Ovsepian et al. 2016), and Kv3/KCNC voltage-gated potassium channels, for which Kv3.4 subunit expression and function are normally present at low levels during the first postnatal week in rat (Riazanski et al. 2001).

Principal neurons in ECVI exhibited relatively slower-onset mAHPs that were significantly inhibited by apamin, suggesting that SK channels act to decrease the excitability of these neurons. The regional differences in peak mAHP kinetics across the HF may results from SK channels being found in different subcellular compartments and coupled to a variety of calcium sources across different neuron types (Bennett et al. 2000; Hallworth et al. 2003; Stocker et al. 1999; Umemiya and Berger 1994; Williams et al. 1997). Regardless, the regional differences in SK channel regulation of principal neuron excitability are consistent with the disconnect between the strength
of nicotinic passive depolarization responses from rest and nicotinic active excitability responses in firing neurons, for example, as SK channels appear to augment excitability of DG neurons and limit excitability of ECVI neurons, resulting in similar excitability responses to nicotinic stimulation in active neurons.

This study measured the overall influence of SK channel activation on an ACh-induced nicotinic excitability response when SK channels were activated by all sources of calcium. It should be noted that although this was not measured in this current study, calcium-permeable ligand-gated ion channels such as the nAChR may form a direct source of calcium influx to activate SK channels. For the α4β2* nAChRs that most-likely mediated ACh responses in recorded neurons, receptors with the (α4)3(β2)2 stoichiometry display greater calcium permeability than receptors with the (α4)2(β2)3 stoichiometry (Fucile 2004; Tapia et al. 2007). Receptors with the (α4)2(β2)2(α5) stoichiometry, which are likely present in CA1 and ECVI principal neurons (Chung et al. 2016, Wada et al. 1990), exhibit greater calcium permeability than those containing α4 and β2 subunits only (Tapia et al. 2007). In addition, nicotinic receptor activation may indirectly increase SK channel function through elevations in calcium from intracellular calcium stores (Dajas-Bailador et al. 2002; Sharma and Vijayaraghavan 2001), and may facilitate the activation of voltage-dependent calcium channels in immature neurons (Dajas-Bailador et al. 2002; Rathouz and Berg 1994). One example occurs in cochlear outer hair cells, where SK channels are tightly coupled with heteromeric nAChRs such that receptor activation by ACh induces an influx of calcium that activates SK channels, followed by the consequent efflux of potassium. The resulting fast inhibitory signal modulates the frequency of action potential trains elicited by cholinergic signalling (Oliver et al. 2000). The potential contribution of nAChR stimulation toward SK
channel activation, and its role in coordinated activity in principal neurons across the developing HF, are important areas for future research.

3.5.3 Implications for aberrant development of the hippocampal formation

The presence of functional heteromeric nAChRs on principal neurons during early postnatal HF development has implications for the understanding of neurodevelopmental disorders affecting HF structure and HF-dependent higher-order cognitive functions such as learning, memory and attention. For example, children diagnosed with ASD and ADHD have presented with abnormal postnatal development of the HF leading to increased hippocampus size (Plessen et al. 2006; Schumann et al. 2004). Dysfunction specifically to hippocampal nAChRs has also been linked with specific neurodevelopmental disorders including epilepsy and schizophrenia (Labate et al. 2013; Roshan-Milani et al. 2003; Tizabi 2007; Tregellas et al. 2010). It will be important to determine whether heteromeric nAChRs present on young postnatal HF principal neurons contribute to regulating HF network activity and synchrony during this time because some forms of epilepsy involves dysregulation of normal oscillations and/or neuron loss in the CA1, CA3 and entorhinal cortex (Bragin et al. 1999; Coras et al. 2014; Lega et al. 2015; Schmeiser et al. 2017), and schizophrenia is linked with alterations to the morphology of dentate granule principal neurons (Lauer et al. 2003; Senitz and Beckmann 2003), the synaptic organization of CA3 principal neurons (Kobayashi 2009; Kolomeets et al. 2007), and markers of synaptic transmission for glutamatergic and GABAergic systems (Reynolds et al. 1990; Talbot et al. 2004). Heteromeric nAChRs are currently targeted for the treatment of neurodevelopmental disorders such as ASD, ADHD and schizophrenia, which each involve abnormal HF-dependent cognitive functions (Arneric et al. 2007; Dineley et al. 2015; Taly et al. 2009).
3.5.4 Conclusion

Results from this study demonstrate that heteromeric nAChRs mediate nicotinic signalling in CA1, CA3, DG, SUB and ECVI principal neurons of the young postnatal HF. Differential regulation of excitability by SK channels in these neurons normalizes the differential magnitude of their passive nAChR responses, leading to active nAChR responses in firing neurons that are similar in magnitude across all HF regions. These findings provide novel insight into normal HF development, and place heteromeric nAChRs in a position to directly influence the development of coordinated, synchronous neurotransmission within the developing HF cognitive network.
CHAPTER 4

Characterization of heteromeric α4β2* nicotinic receptor function in principal neurons of the hippocampal formation in young postnatal female mice

Based on the publication: Chung B.Y.T. and Bailey C.D.C. Heteromeric nicotinic acetylcholine receptors in the female mouse hippocampal formation: excitation of principal neurons and comparison with male mice. Manuscript in preparation.
4.1 Abstract

The hippocampal formation (HF) mediates higher-order cognitive functions and is composed of a network of excitatory principal neurons. Cholinergic activation of nicotinic acetylcholine receptors (nAChRs) plays an important role in the normal development of principal neurons within the HF. However, previous studies investigating the ability of nAChRs to stimulate principal neurons across regions of the developing HF have been limited to males. We show here that glutamatergic principal neurons in the HF CA1 of female mice are excited by heteromeric nAChRs during early postnatal development, with the greatest function occurring during the first two postnatal weeks. Pyramidal neurons of the CA3, dentate gyrus, subiculum and entorhinal cortex layer VI (ECVI) in female mice are also excited by heteromeric nAChRs during early postnatal development, with the greatest responses occurring in the ECVI. This nAChR action leads to proportional passive and active excitability responses in neurons across all HF regions, which contrasts with the non-proportional responses observed previously in male mice. This sex difference appears to result from a medium afterhyperpolarization component in ECVI principal neurons that is smaller in female mice than in male mice, leading to larger active responses in females. These findings demonstrate that nAChR excitation of HF principal neurons differ between male and female mice during a period of major HF circuitry development. These differences in nicotinic responses may contribute to differences observed in the normal development, synchrony and maturation of the male and female HF.

4.2 Introduction

The hippocampal formation (HF) is associated with important higher-order cognitive functions such as learning and memory, as well as neurodevelopmental disorders involving these processes,
such as autism, attention-deficit hyperactivity disorder (ADHD) (Li et al. 2014; Plessen et al. 2006), and epilepsy (Seidenberg et al. 2005; Squier et al. 2003; Sun and Goodkin 2016). The HF is composed of the hippocampus proper (which consists of the dentate gyrus (DG), cornu ammonis area 3 (CA3), and cornu ammonis area 1 (CA1)), subiculum (SUB), and entorhinal cortex (EC) (Andersen 2007; Eichenbaum et al. 1992; Kesner et al. 1993; Scoville and Milner 1957; Squire and Zola 1996). One of the distinguishing features of the HF is the largely unidirectional connection between these regions. This network of glutamatergic excitatory principal neurons starts from the superficial layers of the EC, which then, sequentially, projects to the principal neurons of the dentate gyrus (DG), the CA3, the CA1, the SUB, and finally, back to the deep layers of the EC (Andersen 2007). Subsequent projections from the deep layers of the EC not only project to other cortical areas, but they also project back to the superficial layers of the EC to complete the closed-loop structure of the HF (Agster and Burwell 2013; van Groen et al. 2003).

The proper development of the HF depends on afferent cholinergic signalling (Chang and Berg 1999; Dutar et al. 1995; Frotscher and Leranth 1985; Liu et al. 2006; Lozada et al. 2012b). Particularly, acetylcholine activation of the ligand-gated cation channel nicotinic acetylcholine receptor (nAChR) can modulate both glutamate neurotransmission (Cheng and Yakel 2015b; Ge and Dani 2005) and gamma-aminobutyric acid (GABA) neurotransmission (Alkondon et al. 1997; Jones and Yakel 1997; Maggi et al. 2001; McQuiston and Madison 1999b; Sudweeks and Yakel 2000) to influence excitability and plasticity within the developing HF (Hasselmo 1999; Levin 2002; Yakel 2012).
One major isoform of nAChR in the mammalian HF is the heteromeric α4β2* nAChR (Alkondon and Albuquerque 2004; Jones and Yakel 1997; Sudweeks and Yakel 2000; Wada et al. 1989; Zarei et al. 1999). Molecular studies have demonstrated the expression of α4, β2 and α5 heteromeric nAChR subunits in HF principal neurons during early postnatal development (Didier et al. 1995b; Machaalani et al. 2010; Shacka and Robinson 1998b; Winzer-Serhan and Leslie 2005), and consistent with these reports, we recently demonstrated that α4β2* nAChRs mediate inward current and excitability responses in DG, CA1, CA3, SUB and EC layer IV principal glutamatergic principal neurons of male mice during this period (see Chapters 2 and 3 of this thesis). Interestingly, previous electrophysiological studies in older rodents have only reported the presence of direct heteromeric α4β2* nAChR-mediated responses in EC layer VI (ECVI) principal neurons (Tu et al. 2009). These findings raise an interesting question for the role of α4β2* nAChRs on HF principal neurons during early development, and suggest that the presence of these receptors may play an important role in mediating early HF neural circuitry formation.

Multiple studies have reported sex differences within the HF of both rodents and humans, demonstrating that DG, CA1, CA3, SUB and EC principal neurons in the male and female have both similar and distinct characteristics in their structure, plasticity and function (Andrade et al. 2000; Giedd et al. 1996; Huang and Woolley 2012; McEwen 2010; McLaughlin et al. 2010; Monfort et al. 2015; Roof et al. 1993; Scharfman and MacLusky 2017; Tabatadze et al. 2015). There are also known sex differences in heteromeric nAChR stimulation of principal neurons within the mouse prefrontal cortex during early development (Alves et al. 2010). Determining the presence of sex differences in nicotinic modulation of HF principal neuron physiology during early development will further our understanding of normal HF development. Previous reports on α4β2*
nAChR expression and function in HF principal neurons have not isolated for sex differences (Didier et al. 1995b; Machaalani et al. 2010; Shacka and Robinson 1998a; Winzer-Serhan and Leslie 2005) (also see Chapters 2 and 3 of this thesis) and to the best of our knowledge, the function of heteromeric nAChRs in principal neurons of the developing HF has not been investigated in female mice.

The objective of this current study was to determine whether functional heteromeric α4β2* nAChRs are present in HF principal neurons of young postnatal female mice. Whole-cell electrophysiological recording was performed in visually-identified principal neurons located within the CA1, CA3, DG, SUB and ECVI regions in acute brain slices of female mice aged P5-10 (young postnatal), P15-20 (juvenile) or P60-100 (adult). CA1 principal neurons consistently produced postsynaptic current responses to bath-applied ACh (in the presence of blockers to both muscarinic ACh receptors and α7 subunit-containing nAChRs). These currents also mediated passive depolarization responses from rest, and increased the rate of action potential firing in these same neurons. Heteromeric nAChR-mediated inward current and passive depolarization responses were also identified in all other subregions of the HF in young postnatal female mice, where the magnitude of responses was greater in ECVI than in CA1, CA3, DG and SUB. Unlike ECVI principal neurons in male mice, which show a blunted active response to nicotinic stimulation, ECVI principal neurons in female mice responded to heteromeric nAChR stimulation with a robust increase in action potential firing frequency that was proportional to passive inward current and depolarization responses. Mechanistic experiments found that the medium afterhyperpolarization potential (mAHP), which modulates action potential firing frequency (Gustafsson and Wigstrom 1983; Hotson and Prince 1980; Sah 1996), did not limit ECVI neuron firing frequency in female mice.
mice to the same degree that it does in male mice. These findings suggest that heteromeric nicotinic responses in the HF differ between the two sexes during early postnatal development and that the mAHP plays a role in this sex difference.

4.3 Methods

4.3.1 Experimental Animals

Experimental animals were cared for as per the principles and guidelines of the Canadian Council on Animal Care, and the experimental protocol was approved by the University of Guelph Animal Care Committee. All efforts were made to minimize animal suffering and to limit the number of mice used in this study. CD1 strain mice were kept in a secure vivarium as described in Chapter 2 of this thesis. Mice were bred in-house with ad libitum access to water and food. The day of birth was considered to be postnatal day (P) 0 for each litter. Litters were weaned at P21 and separated based on sex and housed in groups of up to four mice per cage. Female offspring were analyzed at three developmental ages: P5-10 (young postnatal), P15-20 (juvenile), and P60-100 (young adult), and for comparison, male offspring were sampled from the same litters as females at P5-10.

4.3.2 Electrophysiology

Mice were anesthetized using isoflurane and killed by decapitation. Brains were rapidly dissected while submerged in 4°C oxygenated (carbogen, 95% O₂ and 5% CO₂) sucrose artificial cerebrospinal fluid (ACSF) (254 mM sucrose, 10 mM D-glucose, 26 mM NaHCO₃, 2 mM CaCl₂, 2 mM MgSO₄, 3 mM KCl, 1.25 mM NaH₂PO₄, pH 7.4). Coronal brain slices (400 μm) containing the rostral/dorsal hippocampus or entorhinal cortex were obtained from approximately Bregma -1.46 mm to -2.18 mm or Bregma -2.92 to -3.52 mm, respectively, using a Leica VT1200 vibrating
microtome (Leica Microsystems, Richmond Hill, ON, Canada) (Paxinos and Franklin 2001). Slices recovered in 30°C oxygenated regular ACSF (128 mM NaCl, 10 mM D-glucose, 26 mM NaHCO₃, 2 mM CaCl₂, 2 mM MgSO₄, 3 mM KCl, 1.25 mM NaH₂PO₄, pH 7.4) for a minimum of two hours before use.

Slices were transferred to a modified recording chamber (Warner Instruments, Hamden, CT, USA) that was mounted on the stage of an Axioskop FS2 Microscope (Carl Zeiss Canada, Toronto, ON, Canada). Whole-cell recordings of principal neurons were made at room temperature (21-24°C), and slices were perfused with oxygenated ACSF at a rate of 3-4 mL/min. Recordings were made using borosilicate glass pipette electrodes (resistance of 2–5 MΩ) that were filled with an internal solution containing 120 mM K-gluconate, 5 mM KCl, 2 mM MgCl₂, 4 mM K₂-ATP, 400 μM Na₂-GTP, 10 mM Na₂-phosphocreatine, 33 μM and 10 mM HEPES buffer (adjusted to pH 7.3 with KOH). Individual principal neurons were visualized using infrared differential interference contrast microscopy and selected for recording based on the location of the soma within the hippocampal principal cell layer or cerebral cortical layer, as described in Chapter 3 of this thesis. Signals were acquired at 20 kHz and lowpass filtered at 2 kHz using a Digidata 1440A data acquisition system (Molecular Devices, Sunnyvale, CA, USA). The liquid junction potential was corrected at the time of recording and all data was analyzed post-hoc using Clampfit 10.3 software (Molecular Devices).

All experiments were performed in the continuous presence of 200 nM atropine to block muscarinic acetylcholine receptors and 10 nM methyllycaconitine (MLA) to block α7 subunit-containing nAChRs. Basic electrophysiological properties were assessed in current clamp mode.
by measuring responses to the injection of positive and negative current steps for 500 ms each. Nicotinic receptor-mediated responses were probed by the addition of ACh to the ACSF superfusion bath after a baseline recording period and were followed by a 5-min washout period. Inward current responses were assessed in voltage clamp mode with neurons held at -75 mV (to mitigate the potential for nicotinic-driven presynaptic GABA release and subsequent activation of postsynaptic GABA<sub>A</sub> receptors) by subtracting the mean holding current at the peak of the ACh response from the mean holding current at baseline. Depolarization responses from rest were measured in current clamp mode by subtracting the mean membrane potential at the peak of the ACh response from the resting membrane potential at baseline. Acceleration of action potential firing was measured in current clamp mode by first injecting positive current to elicit a baseline firing frequency at approximately 1 Hz. The rate of firing was measured over a 30 s period at baseline and a 15s period during the peak of the ACh response. The ACh-induced acceleration of action potential firing was reported as the percent increase in firing frequency from the ~1 Hz baseline for each neuron, using the equation of: (firing frequency at the peak ACh response – firing frequency at baseline) / firing frequency at baseline x 100. Input–output curves were generated in current clamp mode by applying 500 ms depolarizing current steps from rest in 50 pA increments. The magnitude of postburst mAHP responses was assessed at resting membrane potential in current clamp mode following a train of 16 of action potentials that were elicited by injecting brief depolarizing current pulses (2 nA, 2 ms) at 50 Hz. The magnitude and timing of the peak mAHP were measured for each neuron at the lowest membrane potential observed during the 50 to 1000 ms mAHP time period after of the last action potential was elicited, where peak mAHP magnitude was calculated as the difference between the neuron’s resting membrane potential and the lowest membrane potential measured during the mAHP period. ACh chloride and atropine were
purchased from Sigma Aldrich (Oakville, ON, Canada). MLA citrate and apamin were purchased from Tocris Bioscience / Bio-Technne (Minneapolis, MN, USA). All drugs were stored in stock solutions at -20°C.

4.3.3 Statistical Analysis

All data are reported as mean ± SEM values for neurons within each experimental group. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) and a level of $p < 0.05$ was required to indicate statistical significance. ACh-induced nicotinic current, depolarization and excitability responses were assessed using one-way ANOVA or Kruskal-Wallis test, followed by the Tukey’s post hoc tests or Dunn’s post hoc tests, and/or two-tailed unpaired $t$ test, as well as Fisher’s exact test. Comparison of input–output curves was assessed using two-way ANOVA followed by the Bonferroni’s post hoc test at each magnitude of current injection. Comparison of mAHP magnitude was assessed using one-way ANOVA followed by the Tukey’s post hoc tests and one-sample $t$ test.

4.4 Results

4.4.1 Excitation of CA1 principal neurons by heteromeric nicotinic receptors in female mice

The nAChR $\alpha 4$, $\alpha 5$, and $\beta 2$ subunits are expressed in the hippocampus during early development and the content of these subunits appear to peak during early postnatal life before declining to lower adulthood levels shortly thereafter (Shacka and Robinson 1998b; Winzer-Serhan and Leslie 2005; Zhang et al. 1998). Consistent with these studies, I demonstrated in Chapter 2 of this thesis that heteromeric, putative $\alpha 4\beta 2^*$ nAChRs mediate excitatory responses in HF CA1 principal neurons in male mice, with the greatest magnitude of responses also occurring during early
postnatal life and declining to adult levels by the second week of age. In this study, we sought to
determine whether functional heteromeric nAChRs are also present on CA1 principal neurons of
the female mice during early postnatal life, and whether the electrophysiological function of these
receptors is developmentally regulated. We conducted whole-cell recordings in acute brain slices
and probed for ACh-induced inward current response from CA1 principal neurons sampled from
female mice aged P5-10 (young postnatal), P15-20 (juvenile) and P60-100 (young adult). ACh
was applied at 1 mM to facilitate comparison of our data with that from cell culture and
synaptosome studies. Heteromeric nAChRs were isolated pharmacologically by the continuous
bath application of atropine (200 nM, to block muscarinic acetylcholine receptors) and MLA (10
nM, to block α7 subunit-containing nAChRs).

Basic electrophysiological properties for CA1 principal neurons are presented in Table 4.1. To the
best of our knowledge, a comparison of these properties of female CA1 principal neurons across
distinct developmental age groups has not been reported previously. Resting membrane potential
was not affected by age (one-way ANOVA, F(2,21) = 0.1, p = 0.9). Input resistance was affected
by age (Kruskal-Wallis test, p = 0.001), where input resistance was higher in CA1 principal
neurons at P5-10 than at P60-100 (Dunn’s post hoc test, p < 0.0008). Spike amplitude was also
affected by age (one-way ANOVA, F(2,21) = 3.5, p < 0.05), and this value was higher in CA1
principal neurons at P5-10 than at P60-100 (Tukey’s post hoc test = p < 0.04). Last, rheobase was
affected by age as well (Kruskal-Wallis test, p = 0.01), and this was higher at P60-100 than at P15-20
(Dunn’s post hoc test, p = 0.01).
Table 4.1. Electrophysiological properties for developing CA1 principal neurons of the hippocampal formation in the female mouse.

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Resting Membrane Potential (mV)</th>
<th>Input Resistance (MΩ)</th>
<th>Spike Amplitude (mV)</th>
<th>Rheobase (pA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P5-10</td>
<td>-75.2 ± 0.9</td>
<td>293.2 ± 133.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>80.2 ± 2.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>58.2 ± 7.2&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>(11 neurons from 4 mice)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P15-20</td>
<td>-75.1 ± 2.4</td>
<td>248.3 ± 82.7&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>83.6 ± 2.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>47.1 ± 2.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(7 neurons from 2 mice)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P60-100</td>
<td>-76.2 ± 2.4</td>
<td>116.2 ± 13.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>88.7 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>105 ± 18.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(6 neurons from 2 mice)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SEM. Data sets marked with different letters denote significant difference (p < 0.05).
Inward current responses to 1 mM ACh (15 s) showed a significant effect of age (Fig. 4.1A, one-way ANOVA, F (2, 15) = 24.0, p < 0.0001), where currents in neurons from female mice age P5-10 (8.1 ± 0.8 pA, n = 6 neurons from 2 mice) were significantly greater than currents in neurons from female mice aged P15-20 (4.3 ± 0.6 pA, n = 6 neurons from 2 mice) and P60-100 (2.1 ± 0.5 pA, n = 6 neurons from 2 mice) (Tukey’s post hoc test, p ≤ 0.002 for each comparison). A similar effect of age was observed for the ability of 1 mM ACh (15 s) to depolarize neurons from rest (Fig. 4.1B, one-way ANOVA, F (2, 15) = 4.3, p = 0.03), where nicotinic depolarization responses were significantly greater in neurons from female mice aged P5-10 (3.6 ± 1.0 mV, n = 7 neurons from 3 mice) than neurons from those aged P60-100 (0.5 ± 0.1 mV, n = 5 neurons from 2 mice) (Tukey’s post hoc test, p = 0.03). The influence of these passive nAChR responses on neuron excitability was measured by applying 1 mM ACh (15 s) to neurons that had been previously induced to fire action potentials at a frequency of 1 Hz by positive current injection. The amount of positive current injection required to induce the 1 Hz firing frequency was recorded for the majority of the neurons in this experiment and showed a significant effect of age (one-way ANOVA, F (2, 13) = 4.9, p = 0.03), with values of 23.1 ± 6.0 pA (n = 5 neurons from 2 mice) at P5-10, 19.1 ± 2.2 pA (n = 6 neurons from 2 mice) at P15-20, and 37.2 ± 4.5 pA (n = 6 neurons from 2 mice) at P60-100. The amount of current injected was significantly greater at P15-20 than at P60-100 (Tukey’s post hoc test, p = 0.02). There was an effect of age on the percent increase in firing frequency following ACh application that was similar to that seen for current and depolarization responses (Fig. 4.1C, one-way ANOVA, F (2, 16) = 10.77, p = 0.001), where the ACh response was not different between neurons from female mice aged P5-10 (291.9 ± 55.8 percent increase, n = 7 neurons from 4 mice) and female mice aged P15-20 (134.6 ± 44.7 percent increase, n = 6 neurons from 2 mice).
(Tukey’s post hoc test, p = 0.05), but was significantly greater at P5-10 than at P60-100 (10.8 ± 6.6 percent increase, n = 6 neurons from 2 mice) (Tukey’s post hoc test, p < 0.0008).
Fig. 4.1. Nicotinic stimulation of CA1 pyramidal neurons is developmentally regulated and greatest in young postnatal female mice. A: Inward current responses to 1 mM ACh (15 s) were significantly affected by age (one-way ANOVA, p < 0.0001), where currents were greater at postnatal day (P) 5-10 than at each of P15-20 and P60-100 (Tukey’s post hoc test: all p ≤ 0.002). Typical voltage-clamp traces at each age are shown to the right of the histogram plot. B: Depolarization from rest following 1 mM ACh application (15 s) was significantly affected by age (one-way ANOVA, p = 0.03), where the response at P5-10 was significantly greater than at P60-100 (one-way ANOVA, p = 0.03). Typical current-clamp traces are located to the right the histogram plot and show typical depolarization responses at each age. C: In neurons that had been induced to fire action potentials at 1 Hz, acceleration of firing frequency following application of 1 mM ACh (15 s) was significantly affected by age (one-way ANOVA, p = 0.001), where the response was greater at P5-10 than at P60-100 (Tukey’s post hoc test, p < 0.0008). Typical current-clamp traces showing the typical changes in firing frequency at each age are shown to the right of the histogram plot. All recordings were made in the continuous presence of 200 nM atropine and 10 nM MLA. All values are mean ± SEM. Data sets marked with different letters indicate a statistically significant difference.
4.4.2 Nicotinic inward currents and nicotinic depolarization in principal neurons across the female hippocampal formation

Data presented in Chapter 3 demonstrate that heteromeric α4β2* nAChR responses are present in principal neurons of the CA1, CA3, DG, SUB and ECVI in the male mouse during early postnatal development (P5-10). Previous electrophysiological experiments reported that rodents P11 and older show no direct heteromeric nicotinic responses, or nicotinic responses that occur in a low proportion of principal neurons of the CA1, CA3, DG and SUB principal neurons (He et al. 2013; Hefft et al. 1999; McQuiston and Madison 1999b), while principal neurons of the ECVI do exhibit nicotinic responses beyond P11 (Tu et al. 2009). Our next line of experiments sought to determine whether functional heteromeric nAChRs are similarly present in principal neurons of the female HF during early postnatal life, by probing for ACh-induced inward current responses in principal neurons sampled from the CA1, CA3, DG, SUB and ECVI regions during P5-10. Basic electrophysiological properties for HF principal neurons are presented in Table 4.2. Resting membrane potential was not affected by region (Kruskal-Wallis test, p = 0.3). Spike amplitude was similarly not affected by region (one-way ANOVA, F(4,39) = 1.3, p = 0.3). Input resistance was affected by region (one-way ANOVA, F(4,37) = 5.7, p = 0.001), and this value was higher in DG principal neurons than CA1, CA3, SUB and ECVI principal neurons (Tukey’s post hoc test, p < 0.03 for each comparison). Rheobase is not significantly affected by region (one-way ANOVA, F (4, 24) = 0.4, p = 0.8).
Table 4.2. Electrophysiological properties of principal neurons of the young postnatal hippocampal formation in the female mouse sampled at postnatal days 5-10.

<table>
<thead>
<tr>
<th>Region</th>
<th>Resting membrane potential (mV)</th>
<th>Input resistance (MΩ)</th>
<th>Spike amplitude (mV)</th>
<th>Rheobase (pA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA1</td>
<td>-75.2 ± 0.9</td>
<td>293.2 ± 133.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>80.2 ± 2.3</td>
<td>58.2 ± 7.2</td>
</tr>
<tr>
<td>CA3</td>
<td>-77.9 ± 1.2</td>
<td>435.5 ± 43.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>80.5 ± 2.2</td>
<td>58.3 ± 10.5</td>
</tr>
<tr>
<td>DG</td>
<td>-79.1 ± 2.4</td>
<td>1121 ± 332.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>75.5 ± 2.3</td>
<td>46.7 ± 8.8</td>
</tr>
<tr>
<td>SUB</td>
<td>-73.6 ± 1.2</td>
<td>262.1 ± 47.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79.3 ± 3.3</td>
<td>67.5 ± 7.5</td>
</tr>
<tr>
<td>ECVI</td>
<td>-73.3 ± 2.2</td>
<td>414.2 ± 43.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>84.3 ± 2.5</td>
<td>55.0 ± 6.2</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. Data sets marked with different letters denote significant difference (p < 0.05).
Application of 1 mM ACh (15 s) resulted in a positive inward current response in every principal neuron that was recorded, and there was a significant effect of HF region on the magnitude of responses measured (Fig. 4.2A, Kruskal-Wallis test, p < 0.0004). Nicotinic current responses were significantly greater in ECVI (43.0 ± 7.6 pA, n = 5 neurons from 3 mice) than in CA3 (4.0 ± 1.0 pA, n = 7 neurons from 4 mice) and DG (1.4 ± 0.6 pA, n = 7 neurons from 2 mice) (Dunn’s post hoc test, p < 0.03 for each comparison). Analysis within the hippocampus proper, where glutamatergic principal neurons of the CA1, CA3 and DG form the trisynaptic circuit (Andersen 2007), demonstrated that the magnitude of nicotinic inward currents is significantly affected by subregion (Fig. 4.2A inset, one-way ANOVA, F (2, 17) = 17.45, p < 0.0001), with currents in CA1 (8.1 ± 0.8 pA, n = 6 neurons from 2 mice) being greater than those in CA3 and DG (Tukey’s post hoc test, p < 0.007 for each comparison). Typical inward current responses are shown for one neuron from each region of the HF in Fig. 4.2A. We next sought to assess consequences of the observed nicotinic inward currents on a passive neuronal response in principal neurons of the HF by measuring depolarization from rest. Application of 1 mM ACh (15 s) resulted in current clamp depolarization responses in every neuron tested, and the magnitude of these responses was significantly affected by HF region (Fig. 4.2B, one-way ANOVA, F(4,28) = 29.3, p < 0.0001). The magnitude of depolarization was significantly greater in principal neurons of ECVI (19.4 ± 2.4 mV, n = 6 neurons from 3 mice) than CA1 (3.6 ± 1.0 mV, n = 7 neurons from 3 mice), CA3 (3.7 ± 0.8 mV, n = 7 neurons from 4 mice), DG (2.5 ± 0.4 mV, n = 7 neurons from 2 mice) and SUB (3.5 ± 1.4 mV, n = 6 neurons from 4 mice) (Tukey’s post hoc test, p ≤ 0.0001 for each comparison). Analysis of nicotinic depolarization responses within the hippocampus proper revealed that there was no significant effect of subregion (one-way ANOVA, F(2,18) = 0.7, p = 0.5). Typical depolarization responses are shown for one neuron from each region of the HF in
Fig. 4.2B. Using the measured inward current responses from Fig. 4.2A and the measured input resistance for each neuron from Table 4.2, the measured nicotinic depolarization responses in Fig. 4.2B match the anticipated depolarization calculated using Ohm’s Law ($\Delta V = \Delta I \times R$) for neurons in CA1, CA3, DG, and SUB. This calculation could not be made for ECVI principal neurons because the majority of neurons reached the maximal threshold depolarization that elicited action potential firing.
Fig. 4.2. The magnitude of nicotinic inward current and depolarization from rest varies across regions of the female hippocampal formation. A: Inward current responses to 1 mM ACh (15 s) were significantly affected by region (Kruskal-Wallis test, p < 0.0004), where currents were greater in ECVI than in CA3 and DG (Dunn’s post hoc test, p < 0.03 for each comparison). Inset: Analysis of neurons within the hippocampus proper also demonstrated a significant effect of subregion (one-way ANOVA, p < 0.0001), with nicotinic inward current responses in CA1 being greater than those in CA3 and DG (Tukey’s post hoc test, p < 0.007 for each comparison). Typical voltage-clamp traces are shown to the right of the histogram plot for one neuron in each region with the 15 s ACh application indicated by the grey horizontal bar. B: Depolarization responses to 1 mM ACh (15 s) were significantly affected by region (one-way ANOVA, p < 0.0001), where depolarization was greater in ECVI than in all other regions (Tukey’s post hoc test, p < 0.0001 for each comparison). Typical current-clamp traces are shown to the right of the histogram plot for one neuron in each region with the 15 s ACh application indicated by the grey horizontal bar. All recordings were made in the continuous presence of 200 nM atropine and 10 nM MLA. All values are displayed as mean ± SEM.
4.4.3 Nicotinic excitability in principal neurons across the female hippocampal formation

We next sought to determine whether an active excitability response to heteromeric nAChR stimulation in these female neurons is proportional to inward current and/or depolarization responses. We measured excitability responses of active neurons in current clamp mode by applying 1 mM ACh (15 s) to neurons that had been induced to fire action potentials at a frequency of 1 Hz, and measuring the percent increase in action potential firing frequency from baseline within each neuron. Application of 1 mM ACh (15 s) resulted in acceleration of action potential firing in every neuron tested, and the magnitude of this response was significantly affected by HF region (Fig. 4.3, one-way ANOVA, F(4,29) = 5.4, p = 0.002). Tukey’s post hoc test demonstrated that the magnitude of the active response was significantly greater in principal neurons of ECVI (737.1 ± 70.1 percent increase, n = 7 neurons from 3 mice) than CA1 (291.9 ± 55.8 percent increase, n = 7 neurons from 4 mice), CA3 (335 ± 123.5 percent increase, n = 6 neurons from 3 mice), DG (305.1 ± 74.7 percent increase, n = 6 neurons from 2 mice) and SUB (372.3 ± 74.9 percent increase, n = 8 neurons from 4 mice) (p ≤ 0.02 for each comparison). Analysis of responses within the hippocampus proper revealed no significant effect of subregion (one-way ANOVA, F(2,16) = 0.07, p = 0.9). The active response to heteromeric nAChR stimulation of principal neurons across the HF of female mice leads to active excitability responses that relate directly to the passive response elicited in each neuron.
Fig. 4.3. Nicotinic receptor-mediated excitability responses in active neurons vary across region of the hippocampal formation in female mice. A: For principal neurons that had been induced by positive current injection to fire action potentials at 1 Hz, the application of 1 mM ACh (15s) increased firing frequency in a manner that was significantly affected by region (one-way ANOVA, p = 0.002). The magnitude of this excitability response was greater in ECVI than in CA1, CA3, DG and SUB (Tukey’s post hoc test, p ≤ 0.02 for each comparison). B: Typical current-clamp traces are shown for one neuron in each region, with the 15 s ACh application indicated by the grey horizontal bar. All recordings were made in the continuous presence of 200 nM atropine and 10 nM MLA. All values are displayed as mean + SEM.
4.4.4 Sex differences in nicotinic responses in entorhinal cortex layer VI principal neurons

In contrast to our current findings in female mice, data in Chapter 3 demonstrate that, for male mice, varied passive inward current and depolarization responses to heteromeric nAChR stimulation resulted in similar excitability responses in active neurons across all regions of the HF. This normalization of active nicotinic responses was most prominent in male ECVI principal neurons. To facilitate the direct comparison of active nicotinic responses between sexes, experiments were repeated in ECVI neurons using male mice obtained from the same litters as the female mice. Application of 1 mM ACh (15 s) to ECVI principal neurons at rest resulted in a positive depolarization response in every neuron that was recorded in male mice. Nicotinic depolarization responses in ECVI principal neurons were significantly greater in females (20.2 ± 2.1 mV, n = 7 neurons from 4 mice) than in males (12.9 ± 1.4 mV, n = 7 neurons from 3 mice) (Fig. 4.4A, two-tailed unpaired t test, p = 0.01). This nicotinic stimulation elicited action potential firing in 85.7% of ECVI principal neurons from female mice (n = 7 neurons from 4 mice). This finding was significantly different from male mice, where nicotinic stimulation elicited action potential firing in 14.3% of ECVI principal neurons (n = 7 neurons from 3 mice) (Fisher’s exact test, p <0.03). Typical responses are shown in Fig. 4.4B. In active ECVI principal neurons from male mice that had been induced to fire action potentials at a frequency of 1 Hz, application of 1 mM ACh (15 s) resulted in the acceleration of firing frequency in every neuron tested, and the magnitude of these responses were significantly greater in ECVI principal neurons of females (737.1 ± 70.1 percent increase, n = 7 neurons from 3 mice) than of males (249.3± 49.6 percent increase, n = 8 neurons from 2 mice) (Fig. 4.4C, two-tailed unpaired t test, p < 0.0001). Typical responses are shown in Fig. 4.4C. These findings demonstrate differences in both passive and active nicotinic responses in ECVI principal neurons of female and male mice.
Fig. 4.4. Nicotinic depolarization and excitability responses in ECVI principal neurons of the postnatal hippocampal formation differs between males and females. All recordings were made using male and female mice aged postnatal day 5-10 in the continuous presence of 200 nM atropine to block muscarinic acetylcholine receptors and 10 nM MLA to block α7 subunit-containing nicotinic acetylcholine receptors. A: Depolarization responses to 1 mM ACh (15 s) were significantly greater in female than male ECVI principal neurons at rest (unpaired t test: *p < 0.01). B: A majority of ECVI principal neurons at rest in the female mice elicited firing in response to 1 mM ACh (15 s), whereas a majority of ECVI principal neurons at rest in male mice did not elicit firing in response to 1 mM ACh (15s) (Fisher’s exact test, *p < 0.03). Typical current-clamp traces are shown for one neuron in each sex with the 15 s ACh application indicated by the grey horizontal bar. C: In neurons induced to fire action potentials at 1 Hz by positive current injection current, the increase in firing frequency in response to 1 mM ACh (15 s) was significantly greater in female than male ECVI principal neurons (unpaired t test: p < 0.0001). Typical current-clamp traces are shown to the right of the histogram plot, with the 15 s ACh application indicated by the grey horizontal bar. All values are mean + SEM.
4.4.5 Sex differences in the capacity for excitation in young postnatal entorhinal cortex layer VI principal neurons

To understand the capacity for excitation in female mice, we characterized the upper excitation limits in HF principal neurons by generating input-output curves. This was performed by injecting positive current steps from 50 pA to 200 pA, in 50 pA increments for 500 ms each in principal neurons of the DG, CA1, CA3, SUB and ECVI in female mice. The resulting curves for each HF region are shown in Fig. 4.5A. Comparing all regions together, two-way ANOVA revealed significant main effects of current injected \( F(4,200) = 64.7, p < 0.0001 \) and HF region \( F(4,200) = 4.6, p = 0.001 \), and no significant interaction between these two factors \( F(16,200) = 0.8, p = 0.6 \). The 100 pA and 150 pA current step resulted in maximal firing frequency for ECVI and SUB principal neurons, respectively, and the 200 pA current step resulted in maximal firing frequency for principal neurons within the hippocampus proper. Specifically, principal neurons of the CA1 \( (11.7 \pm 1.2 \text{ Hz}, n = 10 \text{ neurons from 4 mice}) \), CA3 \( (10.2 \pm 1.1 \text{ Hz}, n = 9 \text{ neurons from 4 mice}) \) and DG \( (10.6 \pm 2.0 \text{ Hz}, n = 8 \text{ neurons from 2 mice}) \) showed a greater maximal action potential firing frequency than those in ECVI \( (8.6 \pm 0.4 \text{ Hz}, n = 7 \text{ neurons from 3 mice}) \) (Tukey’s post hoc test, \( p \leq 0.04 \) for each comparison). This is in contrast to our findings for active nicotinic responses (Fig. 4.3), where peak nicotinic response was greatest in ECVI principal neurons in the HF. To test for a sex difference in the capacity for excitation in ECVI principal neurons specifically, we conducted the same experiments as above using male mice obtained from the same litter as the female subjects. Comparison of data for ECVI neurons by two-way ANOVA revealed significant main effects of current injected \( F(4,70) = 50.9, p < 0.0001 \) and sex \( F(4,70) = 4.5, p = 0.04 \), and a significant interaction between these two factors \( F(4,70) = 11.57, p < 0.0001 \). The 100 pA current step resulted in maximal firing frequency for ECVI principal neurons in the female mice, whereas the
200 pA current step resulted in maximal firing frequency for ECVI principal neurons of male mice. The application of depolarizing current steps beyond 100 pA in female mice and 200 pA in male mice elicited decreasing action potential frequencies with neurons presenting over-excitation depolarization block. Current injection at 50 pA elicited a significantly greater action potential firing frequency in female neurons than in male neurons, where 50 pA elicited 5.6 ± 0.3 Hz (n = 7 neurons from 3 mice) in female mice and 1.9 ± 0.8 Hz (n = 9 neurons from 2 mice) in male mice (Bonferroni’s post hoc test, p < 0.002). Injections of 150 pA and 200 pA elicited significantly greater action potential firing frequency in male ECVI principal neurons than in female neurons, where 150 pA elicited 10.6 ± 0.6 Hz (n = 9 neurons from 2 mice) in male mice and 7.6 ± 1.4 Hz (n = 7 neurons from 3 mice) in female mice, and 200 pA elicited 11.8 ± 0.6 Hz (n = 9 neurons from 2 mice) in male mice and 5.4 ± 1.6 Hz (n = 7 neurons from 3 mice) in female mice (Bonferroni’s post hoc test: p < 0.03 for each comparison). We also compared basic electrophysiological properties of ECVI principal neurons. Resting membrane potential was significantly higher in ECVI principal neurons of females (-73.71 ± 2.0 mV, n = 7 neurons in 3 mice) than males (-81.0 ± 1.1 mV, n = 9 neurons in 2 mice) (two-tailed unpaired t test p < 0.005). Input resistance was also significantly higher in females (414.2 ± 43.5 mV, n = 6 cells from 3 mice) than in males (162.4 ± 25.0 mV, n = 9 cells from 2 mice) (two-tailed unpaired t test, p = 0.0001). Spike amplitude was not different between females (84.26 ± 2.5 mV) and males (84.5 ± 2.5 mV) (two-tailed unpaired t test, p = 1.0). These findings demonstrate that, in comparison with male mice, early postnatal ECVI principal neurons in female mice are hyperexcitable in response to low current injection and active ECVI principal neurons in female mice exhibit an earlier onset of depolarization block.
The study presented in Chapter 3 demonstrates that the small conductance calcium-activated potassium channel (SK channel), which can contribute to the afterhyperpolarization of medium duration (mAHP) (Nicoll 1988; Schwindt and Crill 1998), plays a role in regulating the excitability of active principal neurons within the HF of young postnatal male mice. To investigate the contribution of the mAHP in regulating excitability in active principal neurons within the HF of young postnatal female mice, mAHP was measured following the generation of action potentials from rest through the application of brief depolarizing current pulses (trains of 16 pulses of 2 nA for 2 ms each at 50 Hz). The peak mAHP magnitude and timing was calculated for each neuron as the difference between its resting membrane potential and the lowest membrane potential measured during the 50 to 1000 ms mAHP period after the last action potential was elicited. There was a significant effect of HF region on the magnitude of mAHP (Fig. 4.5B, one-way ANOVA, F(4,39) = 6.8, p = 0.0003). Tukey’s post hoc test demonstrated that the magnitude of mAHP was significantly greater in principal neurons of CA1 (-2.0 ± 0.4 mV, n = 11 neurons from 4 mice) and SUB (-2.2 ± 0.7 mV, n = 9 neurons from 4 mice) than in CA3 (0.6 ± 0.8 mV, n = 9 neurons from 4 mice) and DG (1.3 ± 0.7 mV, n = 8 neurons from 2 mice) (p ≤ 0.02 for each pairwise comparison). One-sample t test also demonstrates that the magnitude of mAHP is significantly different from zero in CA1 (t(10)=5.1, p = 0.0004) and SUB (t(8)=3.2, p = 0.01) principal neurons only. These findings in female DG, CA1, CA3, and SUB principal neurons are similar to those found in Chapter 3 for male mice. However, findings in female ECVI principal neurons differ from those found in Chapter 3 for male mice. We repeated mAHP measurements using male mice obtained from the same litters as the female mice in this current study, and confirmed that the magnitude of mAHP in ECVI principal neurons of female mice (0.1 ± 0.2 mV, n = 7 neurons from 3 mice) is
significantly less hyperpolarized than that in male mice (-1.2 ± 0.2 mV, n = 9 neurons from 2 mice) (Fig. 4.5C, two-tailed unpaired t test, p = 0.01). Results from this experiment suggest that the magnitude of the mAHP in ECVI neurons of female and male mice differentially regulate firing frequencies for principal neurons of the young postnatal HF. The timing for the peak mAHP magnitude varied across regions of the HF. Peak times were 290.6 ± 114 ms in CA1, 794.6 ± 49.2 ms in CA3, 794.5 ± 76.1 in DG, 260.2 ± 83.17 in SUB, and 789.3±74.1 in ECVI. One-way ANOVA for peak times reveals a significant effect of region (F(4,38) = 11.3, p < 0.0001), with peaks occurring earlier for CA1 and SUB than in the CA3, DG and ECVI (Tukey’s post hoc test, p < 0.002 for each comparison). There was no significant difference in peak times in the ECVI when comparing females to males (unpaired t test, p = 0.3). Examples of the peak mAHP are indicated using arrows for one neuron from each region in Fig. 4.5D.
Fig. 4.5. The capacity for excitation in entorhinal cortex layer VI principal neurons of the young postnatal hippocampal formation differs between males and females. A: Input-output curves were generated with positive current steps from 50 pA to 200 pA, in 50 pA increments and for 500 ms in principal neurons of the young female postnatal hippocampal formation. The resulting pattern of firing frequency was significantly affected by the amount of current injected (two-way ANOVA, p < 0.0001) and region (two-way ANOVA, p = 0.001), and there was no significant interaction between these two main effects (two-way ANOVA, p = 0.6). Current injections of 50 pA elicited a significantly greater action potential firing frequency in female ECVI principal neurons than in male neurons (Bonferroni’s post hoc test, *p < 0.002). Injections of 150 pA and 200 pA elicited significantly greater action potential firing frequency in male ECVI principal neurons than in female neurons (Bonferroni’s post hoc test, *p < 0.03 for each comparison). Input-output curves for females are presented in black and males are presented in red. B: Trains of 16 action potentials were elicited from rest by applying brief depolarizing current pulses (2 nA, 2 ms) at 50 Hz to elicit mAHP in HF principal cells. Data for mAHP magnitude is shown in histogram plots for each neuron type. The magnitude of mAHP is significantly different from zero in CA1 and SUB (One-sample t test, t(10)=5.1, *p = 0.0004 and t(8)=3.2, *p = 0.01, respectively. C: The magnitude of mAHP in ECVI principal neurons of female mice is significantly lower than in male mice (two-tailed unpaired t test, *p = 0.01). D: Current-clamp traces are shown for one typical neuron of each region. Traces for females are presented in black and males are presented in red, with the timing for the peak mAHP magnitude at baseline indicated by the arrow.
4.5 Discussion

This study provides evidence that glutamatergic principal neurons in the CA1 of female mice are excited by heteromeric nAChRs during early postnatal development, with the greatest function occurring during the first two weeks of postnatal life. Principal neurons within the CA3, DG, SUB and ECVI of female mice are also excited by heteromeric nAChRs during early postnatal development. The active response to heteromeric nAChR stimulation is directly proportional to the passive response observed in principal neurons across all regions of the HF in young postnatal female mice, which contrasts with findings in male mice observed in this current study and in the study described in Chapter 3. This sex difference appears to be mediated by the presence of a mAHP component in ECVI principal neurons of male, but not female mice. These findings were obtained through a series of neurophysiological experiments performed using whole-cell electrophysiological recording of visually-identified principal neurons of each HF region within acute brain slices collected from female and male mice aged P5-10, and from female mice aged P15-20 and P60-100 for a subset of developmental characterization studies.

4.5.1 Cholinergic innervation and heteromeric nicotinic receptor signalling within the developing hippocampal formation

Cholinergic innervation of the HF is proposed to play an important role in modulating the development and normal function of the hippocampal network. (Aloisi et al. 1997; Anzalone et al. 2009; Berger-Sweeney et al. 2001; Cai et al. 2012; Ceccarelli et al. 1999; Easton et al. 2011; Liu et al. 2006; Lozada et al. 2012b; Stanley et al. 2012). This afferent signalling by ACh can activate nAChRs located on local GABAergic interneurons and glutamatergic excitatory neurons of the HF, causing a subsequent modulation of cellular excitability and synaptic transmission
While it is well-established that α4β2* nAChRs mediate nicotinic currents in GABAergic interneurons (Adams et al. 2002; Alkondon and Albuquerque 2001; Alkondon et al. 1999; Bell et al. 2011; Bell et al. 2015; Frazier et al. 1998b; Ji and Dani 2000; Jones and Yakel 1997; Khiroug et al. 2003; McQuiston and Madison 1999b; Sudweeks and Yakel 2000), recent evidence suggests the presence of functional α4β2* nAChRs located directly on glutamatergic principal neurons of the male HF (He et al. 2013; Tu et al. 2009), in addition to the findings presented in Chapters 2 and 3 of this thesis. In this chapter, we also provide in situ evidence that heteromeric, putative α4β2* nAChRs are present on CA1 principal neurons in female mice during early postnatal development. And similar to male mice, the α4β2* nAChR-mediated signalling is greatest in CA1 principal neurons during the first two weeks of postnatal development. Furthermore, we provide evidence that functional heteromeric, putative α4β2* nAChRs are present on principal neurons within the CA3, DG, SUB and ECVI regions of the female HF during P5-10.

The septohippocampal pathway originates from the medial septal nucleus / diagonal band complex (MDSB) and is the main source of cholinergic innervation to the HF (Dutar et al. 1995; Lewis and Shute 1967). This pathway undergoes major development during the first 10 days of postnatal life. Specifically, while cholinergic fibers remain extremely sparse in the dorsal HF at P1, by P3, all CA and DG regions become diffusely innervated by cholinergic fibers, and by P10, these fibers are widespread and demonstrate fiber patterns that resemble those found at adult ages (Nyakas et al. 1994). It has been suggested that the development of the septohippocampal pathway depends on the reciprocal hippocamposeptal pathway, which consists of neurons originating from the HF that project to the lateral septum instead. Previous studies have demonstrated that this
hippocamposeptal fibre serves as a template by which septohippocampal fibers use to find their way to the hippocampus (Linke and Frotscher 1993). The EC layer VI principal neurons are principal output neurons of the HF and project efferents to the lateral septum (Alonso and Kohler 1984). Our current findings demonstrate that α4β2* nAChRs are present on principal neurons within the CA1, CA3, DG, SUB and ECVI regions of the female HF during P5-10, which places them in a prime position to respond to cholinergic signalling by the developing septohippocampal pathway. Possibly, nicotinic excitation of ECVI principal neurons by developing septohippocampal pathways may elicit a feedback circuit which assists the development of the septohippocampal pathway itself during early development. It is possible that this early synchrony between the two pathways may serve to develop adult forms of neural circuits and synchrony. For example, while cholinergic neurons play a role in regulating, hippocampal theta oscillations (Apartis et al. 1998; Bassant et al. 1995), inputs from the EC, activity of the recurrent network of CA3 pyramidal cells and intrinsic resonant properties of hippocampal neurons have also been demonstrated to contribute significantly to hippocampal theta oscillations (Buzsaki 2002; Goutagny et al. 2009).

Early postnatal development is characterized by rapid brain growth involving dendritic arborization, axonal growth, increased synaptogenesis, and maturation of neurotransmission (Clancy et al. 2001; Clancy et al. 2007; Cowan 1979; Dobbing and Sands 1979; Khazipov et al. 2001; Levitt 2003; Pressler and Auvin 2013; Semple et al. 2013; Workman et al. 2013; Zagon and McLaughlin 1977). This transiently-increased level of brain plasticity during early postnatal development is also shaped by endogenous cholinergic signalling. For example, nicotinic stimulation of immature CA3 principal neurons in young rats can convert silent synapses to a
functional status, resulting in enhanced glutamatergic synaptic transmission through the CA3-CA1 Schaffer collateral pathway (Maggi et al. 2001; Wang et al. 2013). Additionally, nicotinic signalling can influence the morphological growth and maturation of neurons within the central nervous system (Campbell et al. 2010; Lipton et al. 1988; Liu and Wu 2006; Pugh and Berg 1994), where it has been demonstrated that β2 subunit-containing nAChRs are specifically important for the normal development of dendritic spines in principal neurons of the cerebral cortex (Ballesteros-Yanez et al. 2010) and hippocampus (Lozada et al. 2012b). Our findings suggest that nicotinic signalling not only regulates glutamatergic transmission throughout the HF, but may also play an important role in the formation of integrated network flow during early postnatal development. Our previous findings described in Chapters 2 and 3 for male mice, in combination with those from this current study, suggest that heteromeric nAChRs of the α4β2* isoform mediate excitation of principal neurons within CA1, CA3, DG, SUB and ECVI during at a time when nicotinic signalling drives the development of this cognitive brain region.

4.5.2 Sex differences in the developing hippocampal formation

A number of studies have demonstrated sex differences in the HF, suggesting that DG, CA1, CA3, SUB and EC neurons in males and females have distinct characteristics in their structure, plasticity and function (Andrade et al. 2000; Giedd et al. 1996; McEwen 2010; McLaughlin et al. 2010; Monfort et al. 2015; Roof et al. 1993; Scharfman and MacLusky 2017; Tabatadze et al. 2015). The presence of steroid hormones in the brain may account for these differences, as steroid actions during early development can produce long term neurodevelopmental consequences and sex differences in cognitive functions (Goldman et al. 1974; MacLusky et al. 1979). In rodents, these effects may be mediated by 17beta-estradiol (E2), the downstream derivative of testosterone
(Bowers et al. 2010; Higo et al. 2009); while in nonhuman primates these effects may be mediated by testosterone or its nonaromatizable metabolite dihydrotestosterone (Wallen 2005). For example, in a classic study examining the role of sex steroids on working and reference memory in rats, it was demonstrated that hippocampal function can be permanently altered by sex steroids by early postnatal development, or within the first 10 days after birth. Specifically, castration of males prior to P10 produced impaired task performance, whereas treatment of gonadally intact females with estradiol from P5-10 produced enhanced task performance (Williams et al. 1990). Therefore, understanding the implications of sex differences during early development will be important as this will guide our understanding of differences in neural function, including hippocampal neuron morphology, synaptic plasticity, and cell signalling.

### 4.5.3 Sex differences in the entorhinal cortex of the hippocampal formation

While our current study reports a generally similar presence of functional heteromeric α4β2* nAChRs in glutamatergic principal neurons of the HF in both male and female mice, we also report a sex difference in nicotinic excitation of young postnatal ECVI principal neurons.

First, our findings demonstrate that nicotinic stimulation of ECVI principal neurons elicit a significantly greater change in depolarization from rest in female mice than in male mice, and a significantly greater proportion of resting ECVI principal neurons in female mice were induced to fire action potentials upon nicotinic stimulation. These findings may be explained for by our observations that there are significant differences in the basic electrophysiological properties of ECVI principal neurons from female and male mice, where ECVI principal neurons from female mice may be intrinsically more excitable at rest. Particularly, we report a higher resting membrane
potential in females that may increase the likelihood that the neuron will reach the threshold for firing action potentials, as well as a greater input resistance that likely leads to the greater passive depolarization response (based on Ohm’s Law \( V = IR \)). While not conclusive from these data, a greater input resistance suggests for a smaller surface area of female ECVI neurons. Interestingly, previous studies in male rats report greater volumes of the HF principal cell layers in CA1 and CA3, as well as longer dendrites in CA3 neurons in comparison to female rats. However, early developmental exposure to testosterone or 17beta-estradiol can reduce both volume and dendritic lengths to measurements that are similar to those found in females (Isgor and Sengelaub 2003; 1998).

Second, nicotinic stimulation of ECVI principal neurons that had been induced previously to fire at 1 Hz generated an approximately three-fold greater increase in action potential firing in female mice than in male mice. Excitatory neurotransmission facilitated by \( \alpha 4\beta 2^* \) nAChRs during early development may be important in developing and forming an integrated cognitive circuit between regions of the HF. Particularly, excitation by \( \alpha 4\beta 2^* \) nAChRs may contribute to the maturation of giant depolarizations (GDPs), which are a primordial form of synchrony that is facilitated by gamma-aminobutyric acid (GABA) neurotransmission. During early development, activation of GABA\(_A\) receptors results in chloride efflux (rather than chloride influx), which leads to a membrane depolarization that opens voltage-sensitive calcium channels (Kasyanov et al. 2004; Nardou et al. 2009; Sipila et al. 2005). Interestingly, there is evidence for a sex difference in the duration of GABA\(_A\) receptor-mediated excitation during normal brain development (Galanopoulou 2008). Within the substantia nigra pars reticulata, the age of the switch for GABA\(_A\) receptor-mediated excitation to inhibition occurs in males at around P17, while this occurs
in female rats at around P10 (Kyrozis et al. 2006). Within the hippocampus, there are also reports of an extended duration of GABA-mediated excitation in the developing male versus female hippocampus, as at P7 only males were susceptible to GABA_A receptor agonist-induced damage and exhibit GABA_A receptor-mediated elevation of intracellular Ca^{2+} (Nunez and McCarthy 2007). These findings suggest that sex differences observed in nAChR signing in ECVI principal neurons may be complementary to the sex differences observed in GABA_A receptor-mediated excitation within the developing HF (Garaschuk et al. 1998; Tyzio et al. 2007). Coordination of these two excitatory mechanisms may consequently facilitate the development of more organized synchronous activity such as theta and gamma rhythms (Bragin et al. 1995; Fellous and Sejnowski 2000; Mohajerani and Cherubini 2006).

The current pulse experiment in this study demonstrates that ECVI principal neurons of female mice do not exhibit a mAHP, and that this lack of mAHP differs significantly from the pronounced mAHP that is present in ECVI principal neurons of male mice. Interestingly, in rodents, prepubertal female striatal medium spiny neurons exhibit a smaller mAHP than males (Dorris et al. 2015), and although not reported as significant, female nucleus accumbens shell medium spiny neurons also exhibit a smaller mAHP than males (Yu et al. 2017). This current work in combination with that in Chapter 3 demonstrates that ECVI neurons in young postnatal male mice display a prominent mAHP that is significantly inhibited by apamin, suggesting that small-conductance calcium-activated potassium (SK) channels act to decrease the excitability of these neurons in male mice (Hallworth et al. 2003; Stocker et al. 1999). Previous reports demonstrate that the mAHP plays a role in modulating firing patterns, for example, by regulating the switch from tonic to burst discharge (Goldberg and Wilson 2005), firing precision (Deister et al. 2009).
and frequency adaptation (Vatanparast and Janahmadi 2009). For example, the mAHP has been demonstrated to play a role in modulating firing pattern in the interneurons within the stratum radiatum of the HF (Savic et al. 2001). The absence of a mAHP in female mice in this current study may provide an explanation for the increased capacity of ECVI principal neurons to fire action potentials rapidly when excited by nicotinic stimulation or low-level positive current injection. This property of young postnatal female neurons may be important for their development into an overall integrated neuronal network that involves the EC. Anatomical studies of the EC layers suggest that layer-specific modulation of ECVI principal neurons is likely important for the proper coordination of the HF neuronal network (Canto et al. 2008; van Groen et al. 2003; Witter et al. 2000a). Particularly, excitatory input originates in the superficial layers of the EC, and projects via the DG, CA3, CA1, and SUB, to the deep layers of the EC (Canto et al. 2008; Witter 2007; Witter et al. 2000b). Subsequent corticocortical axon collaterals back to the superficial layers of the EC may form a critical anatomical feedback loop that allows the hippocampal output to directly affect the neural processing of hippocampal inputs. The EC also receives input from many cortical association areas, and most of the projections from the association cortices to the EC are reciprocal (Canto et al. 2008). Therefore, coordination and integration of signalling via heteromeric nAChRs in ECVI principal neurons, along with CA1, CA3, DG and SUB principal neurons, may be important in developing and forming an integrated cognitive circuit during a period of rapid brain growth and when synchronized brain activity assists the development of proper brain function.
4.5.4 Conclusion

Results from this study demonstrate that heteromeric nAChRs mediate nicotinic signalling in CA1, CA3, DG, SUB and ECVI principal neurons of the young female postnatal HF. ECVI principal neurons of young female mice demonstrate a hyperexcitable nature compared with ECVI principal neurons of young male mice, both intrinsically and to nicotinic input. The greater passive responses likely result from the greater input resistance in female ECVI neurons, and the greater active responses are likely influenced by the absence of firing frequency modulation by the mAHP. These findings provide novel insight into the normal development of the female HF and place heteromeric nAChRs in a position to directly influence the development of this cognitive brain region. The presence of functional heteromeric nAChRs on HF principal neurons and the observed sex difference in heteromeric nAChR responses in ECVI principal neurons during early postnatal development also have direct implications for the understanding of neurodevelopmental disorders. Dysfunction of hippocampal nAChRs has been linked with specific neurodevelopmental and neurological disorders, such as attention-deficit hyperactivity disorder (Levin 2002), epilepsy (Dani 2000; Labate et al. 2013; Roshan-Milani et al. 2003), schizophrenia (Tizabi 2007; Tregellas et al. 2010), and depression (Mineur et al. 2017). Moreover, there are known sex differences in incidence, clinical manifestations, and therapeutic response for a number of neurodevelopmental disorders involving the HF and/or cognitive functions (Savic and Engel 2014). In this context, it will be important to determine mechanisms by which heteromeric nAChRs on young postnatal HF principal neurons contribute to regulating HF network activity, synchrony and development, in order to inform efforts for pharmaceutical development aimed at treating neurodevelopmental and neurological disorders involving the hippocampal nicotinic system.
CHAPTER 5

Modulation of heteromeric $\alpha 4\beta 2^*$ nicotinic acetylcholine receptor function by allopregnanolone in the young postnatal mouse

Based on the publication: Chung B.Y.T. and Bailey C.D.C. Inhibition of $\alpha 4\beta 2^*$ nicotinic acetylcholine receptors by allopregnanolone and the membrane progesterone receptor complex in the mouse medial prefrontal cortex. Manuscript in preparation.
5.1 Abstract

Progesterone and its neuroactive metabolite allopregnanolone (ALLO) have been reported to inhibit nicotinic acetylcholine receptor (nAChR) function in oocyte and *ex vivo* preparations (Bullock et al. 1997; Ke and Lukas 1996; Valera et al. 1992). To date, the influence of ALLO on nAChR function has not been demonstrated in live neurons. This is an important gap in our knowledge as nAChR signalling plays a critical role in the normal development and function of the HF and mPFC. Findings from Chapters 2 to 4 demonstrate that, although functional α4β2* nAChRs are present on pyramidal neurons of the HF, the inward current responses mediated by these receptors are relatively small. Therefore, neurons of the HF are not technically suitable to serve as *in situ* models to investigate ALLO inhibition of these α4β2* nAChR-mediated responses, or to compare this inhibition with that observed previously in reduced preparations.Pyramidal neurons located in layer VI of the mPFC were therefore used as a model system to test ALLO inhibition of α4β2* nAChR function because this neuronal population exhibits the most robust α4β2* nAChR-mediated response of any neuron type tested (Bailey et al. 2010; Kassam et al. 2008; Tian et al. 2014). We show here that ALLO inhibits α4β2* nAChR function in mPFC layer VI pyramidal neurons sampled from young postnatal male and female mice. This inhibition was present at nanomolar and micromolar concentrations of ALLO, and was evident within minutes of ALLO exposure. Pharmacological experiments using an agonist (ORG OD-02) and antagonist (AG-205) of the membrane progesterone receptor (mPR) / progesterone receptor membrane component 1 (PGRMC1) complex suggest that ALLO may inhibit nAChR function through this complex, followed by downstream PKC kinase action. Together, these findings confirm that ALLO inhibits α4β2* nAChR function in living neurons and describes a novel interaction between the mPR complex and nAChR function. These results add to our understanding of neurosteroid
modulation of these two systems, sex differences in nAChR signalling, and how this modulation may regulate neurotransmission within brain regions that support higher-order cognitive functions.

5.2 Introduction

The prefrontal cortex (PFC) is a central mediator of higher-order cognitive functions, with distinct subregions mediating specific aspects of cognition. In rodents, the medial part of the PFC (mPFC) is important for goal-orientation, learning, working memory, and attention (Euston et al. 2012; Killcross and Coutureau 2003; Muir et al. 1996; Passetti et al. 2000; Proulx et al. 2014; Rossi et al. 2012). The mPFC receives dense cholinergic innervation from the nucleus basalis of basal forebrain, which also contains other cholinergic nuclei such as the septum, the diagonal band of Broca, and the substantia innominata (Bloem et al. 2014; Mechawar and Descarries 2001; Mesulam 1995; Zaborszky et al. 1999). Cholinergic signalling is mediated within the mPFC through the muscarinic (mAChR) and nicotinic (nAChR) class of acetylcholine receptor. The nAChRs are pentameric ligand-gated cation channels that, when bound to the agonist acetylcholine (ACh), are permeable to Na⁺, K⁺, and Ca²⁺ ions ((Gotti et al. 2009). The most widely-expressed type of nAChR throughout the cerebral cortex is the α4β2* isoform (Gotti et al. 2009; Hill et al. 1993; Lena and Changeux 1999; Zoli et al. 1995). Within the mPFC, there is a strong presence of α4β2* nAChRs on layer VI pyramidal neurons (Poorthuis et al. 2013b), and these neurons show a robust excitatory response to ACh that is mediated by α4β2* nAChRs (Bailey et al. 2010; Kassam et al. 2008).

A growing number of ligands have been discovered to selectively alter the function of neuronal nAChRs. Particularly, the neurosteroid progesterone and its metabolites, 5α-dihydroprogesterone
(5α-DHP) and ALLO, modulate nAChRs in oocyte expression systems and in reduced ex vivo preparations (Bullock et al. 1997; Ke and Lukas 1996; Valera et al. 1992). However, the actions of ALLO at nAChRs within live neuronal systems in situ or in vivo have not been demonstrated. ALLO affects multiple aspects of neural cell physiology (Stromberg et al. 2006) by modulating neuroprotection and neuroplasticity, regulating higher-order cognition such as learning and memory (Channon et al. 2003; Escudero et al. 2012), modulating response to stress (Gunn et al. 2015; Pibiri et al. 2008; Purdy et al. 1991), and modulating neurological disorders such as epilepsy (Smith et al. 2007) and Alzheimer’s disease (Irwin et al. 2011; Wang et al. 2010). Typically, it is reported that ALLO mediates its rapid non-genomic effects through positive allosteric modulation of the GABA_A receptor (Fodor et al. 2005; Harrison and Simmonds 1984; Majewska et al. 1986; Schmid et al. 1998). Otherwise, it is also reported that ALLO acts through the membrane progesterone receptor (mPR) and the progesterone membrane receptor component (PGRMC), which more-recently have been proposed to function together as the mPR complex (Thomas et al. 2014). It has been suggested that the binding of ALLO at the mPR complex, or its components, elicits a cellular response through downstream second messenger cascades and altered intracellular Ca^{2+} levels to produce rapid changes in neuron function (Charles et al. 2010; Petersen et al. 2013; Rekawiecki et al. 2008; Thomas et al. 2014). Both the mPR and PGRMC demonstrate overlapping distribution in the cerebral cortex (Intlekofer and Petersen 2011; Meffre et al. 2013), where the α4β2* nAChR is also widely expressed (Hill et al. 1993; Zoli et al. 1995). However, it is not yet known whether the mPR complex and α4β2* nAChR interacts, or what the implications of such an interaction may be regarding the modulation of principal neuron excitability. Understanding the role of ALLO in modulating nicotinic signalling in a functioning in situ mPFC neuronal population will be important to our understanding of how neurosteroids may regulate normal cognitive
functions. As previously mentioned, the mPFC serves as an ideal model to complete this objective not only because is it involved in higher-cognitive processes that are known to be mediated by ALLO, but also because mPFC layer VI pyramidal neurons also demonstrate a robust excitatory response to nicotinic signalling at the α4β2* nAChR (Bailey et al. 2010; Kassam et al. 2008; Tian et al. 2014).

In this study, we determine whether the neurosteroid ALLO modulates α4β2* nAChR function in live mPFC layer VI pyramidal neurons. Whole-cell electrophysiological recordings were performed in visually-identified layer VI pyramidal neurons located within acute mPFC slices of male and female mice aged P14-21. Results demonstrate that ALLO inhibits whole-cell responses to α4β2* nAChR stimulation in both male and female mice. This inhibitory effect was present at nanomolar and micromolar concentrations of ALLO, and although it was evident after 5 min and 20 min ALLO exposure, inhibition was significantly stronger at 20 min. Pharmacological experiments suggested that ALLO inhibition was not mediated via direct action at the α4β2* nAChR, but rather through the activation of the mPR complex. Specifically, the mPR complex antagonist AG-205 blocked the inhibitory effect of ALLO, and the mPR complex agonist ORG OD 02-0 mimicked the inhibitory effect of ALLO. Additional experiments investigating mPR complex downstream signalling suggest that the effect of this complex to inhibit α4β2* nAChR function depends on PKC kinase activity. This study is the first to demonstrate the inhibition of nAChR function by ALLO in live neurons, and is also the first to demonstrate crosstalk between the mPR complex and nAChR function. These findings may improve our understanding of how these two systems act to regulate neuron excitability and neurotransmission that supports higher-order cognitive functions.
5.3 Methods

5.3.1 Experimental Animals

Experimental animals were sourced from a colony of wildtype CD1-strain mice that were originally purchased from Charles River Canada (Saint-Constant, QC, Canada) and bred in-house. Mice were kept in a secure vivarium with an ambient temperature of 21-24°C and a 12-hour reverse light cycle with lights on at 8:00 pm, and had *ad libitum* access to water and food. The day of birth was considered to be postnatal day (P) 0 for each litter. Male and female pups were analyzed at P14-21, which is the equivalent of the human juvenile period and a time when α4β2* nAChR function is greatest in mPFC layer VI pyramidal neurons (Alves et al. 2010). Experimental animals were cared for as per the principles and guidelines of the Canadian Council on Animal Care, and the experimental protocol was approved by the University of Guelph Animal Care Committee. All efforts were made to minimize animal suffering and to limit the number of mice used in this study.

5.3.2 Electrophysiology

Mice were anesthetized using isoflurane and decapitated. Brains were quickly removed while submerged in 4°C sucrose artificial cerebrospinal fluid (ACSF) (254 mM sucrose, 10 mM D-glucose, 26 mM NaHCO₃, 2 mM CaCl₂, 2 mM MgSO₄, 3 mM KCl, 1.25 mM NaH₂PO₄, pH 7.4) that had been oxygenated using carbogen (95% O₂ and 5% CO₂). Coronal slices of 400 µm thickness containing the medial prefrontal cortex (approximately Bregma +1.34 mm to Bregma +1.74 mm) (Paxinos and Franklin 2001) were cut using a Leica VT1200 vibrating microtome (Leica Microsystems, Richmond Hill, ON, Canada). Slices were left to recover at 30°C in a recovery chamber with oxygenated ACSF (128 mM NaCl, 10 mM D-glucose, 26 mM NaHCO₃, 2 mM CaCl₂, 2 mM MgSO₄, 3 mM KCl, 1.25 mM NaH₂PO₄, pH 7.4) for at least two hours before
Slices were transferred to a modified recording chamber (Warner Instruments, Hamden, CT, USA) that was mounted on the stage of an Axioskop FS2 Microscope (Carl Zeiss Canada, Toronto, ON, Canada). Slices were continuously perfused with oxygenated ACSF at a rate of 3-4 mL/min and maintained at room temperature (21-24°C). Individual pyramidal neurons were visualized using infrared differential interference contrast microscopy and selected for recording based on the location immediately adjacent to the white matter and a typical pyramidal morphology including a prominent apical dendrite extending toward the medial pial surface. Patches were made using borosilicate glass pipette electrodes (resistance of 2–5 MΩ) that were filled with an internal solution containing 120 mM K-glucuronate, 5 mM KCl, 2 mM MgCl2, 4 mM K2-ATP, 400 μM Na2-GTP, 10 mM Na2-phosphocreatine, 33 μM and 10 mM HEPES buffer (adjusted to pH 7.3 with KOH). All recordings were made using a Multiclamp 700B amplifier. Signals were acquired at 20 kHz and lowpass filtered at 2 kHz using a Digidata 1440A data acquisition system (Molecular Devices, Sunnyvale, CA, USA). The liquid junction potential was corrected at the time of recording and all data were analyzed post-hoc using Clampfit 10.3 software (Molecular Devices).

All experiments were performed in the continuous presence of 200 nM atropine to block mAChRs and 10 nM methyllycaconitine (MLA) to block α7 subunit-containing nAChRs. All heteromeric nicotinic receptor-mediated responses were probed by the addition of ACh to the ACSF superfusion bath after a baseline recording period and were followed by a 5-min washout period. Inward current responses were assessed in voltage clamp mode with neurons held at -75 mV. This
approach was employed to mitigate the potential for nicotinic-driven presynaptic GABA release and subsequent activation of postsynaptic GABA_A receptors to induce chloride currents in recorded neurons. As -75 mV is near the equilibrium potential for chloride in this preparation, there is minimal flux of chloride ions and there are no observable chloride currents under these recording conditions. Inward current responses were measured by subtracting the mean holding current at the peak of the ACh response from the mean holding current at baseline. For the measurement of current responses, the mean holding current value for each one-second period of an experiment was calculated as the average of the 20,000 data values recorded during that period. The modulation of the current response by ALLO was assessed by measuring the inward current response to 1 mM ACh (Before ALLO), allowing for 5 min of washout, and then applying either DMSO vehicle, 1 nM ALLO, 100 nM ALLO, or 10 μM ALLO in the bath for 20 min. After a 20-min application, resting membrane and spike amplitude were assessed in current clamp-mode by measuring baseline current noise and responses to positive current steps, respectively, to ensure good access to the cell was maintained. Neurons presenting ± 20% change in resting membrane or spike amplitude from baseline were discarded. Then, the 1 mM ACh response was measured again in the presence of ALLO (After ALLO), and the percent change in current response was calculated for that cell as [(ACh current after ALLO – ACh current before ALLO) / ACh current before ALLO] x 100]. Pharmacological agents that were applied in the superfusion bath were diluted to final concentrations in ACSF and were delivered to the slice through a gravity-fed perfusion system. Pharmacological agents that were applied intracellularly were diluted at final concentrations in the internal electrode solution and dialyzed through the tip of the patch pipette after obtaining the whole-cell configuration. Dimethyl sulfoxide (DMSO) was used at concentrations of 0.1% and applied to bath ACSF and/or pipette electrode solutions. This
concentration of DMSO did not affect any measure for basic electrophysiological properties or nAChR function in this study.

DMSO was purchased from Thermo Fisher Scientific (Mississauga, ON, Canada). ACh chloride, AG-205, atropine, and 5α-dihydroprogesterone were purchased from Sigma Aldrich (Oakville, ON, Canada). Apamin, CNQX disodium, bicuculline methiodide, PKC-I (19-36) and MLA citrate were purchased from Tocris Bioscience / Bio-Techne (Minneapolis, MN, USA). Allopregnanolone, allopregnanediol and ganaxolone were purchased from Steraloids (Newport, Rhode Island, USA). ORG OD 02-0 was purchased from Axon Medchem (Reston, VA, USA). All drugs were made in stock solutions and stored at -20°C.

5.3.3 Statistical Analysis.

All data are reported as mean ± SEM values for neurons within each experimental group. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) and a level of $p < 0.05$ was required to indicate statistical significance. ACh-induced nicotinic current responses across treatment group were first assessed for normality by the Shapiro-Wilk normality test. These responses were then assessed by one-way ANOVA followed by the Tukey’s or Dunnett’s post hoc tests; or, the non-parametric Kruskal-Wallis test followed by the Dunn’s post hoc test if non-normal distribution were detected and/or inhomogeneity of variance was not correctable by transformation.
5.4 Results

5.4.1 Allopregnanolone decreases the magnitude of α4β2* nAChR-mediated responses in medial prefrontal cortex layer VI pyramidal neurons

Neurosteroids such as ALLO may be synthesized in the brain independently from peripheral sources and reach physiologically relevant levels that modulate gene expression and/or neurotransmission (Baulieu 2001; Cheney et al. 1995; Lambert et al. 2009; Puia et al. 2003; Stoffel-Wagner 2001). It is well established that ALLO acts directly at the GABA_A receptor in a concentration-dependent manner, where ALLO at low nanomolar concentration acts allosterically to enhance the action of the natural ligand GABA, while ALLO at high micromolar concentrations directly gates the opening of the GABA_A receptor (Callachan et al. 1987; Puia et al. 2003). We first sought to determine whether ALLO influences α4β2* nAChR responses in mPFC layer VI principal neurons using whole-cell recording within acute brain slices from male and female mice aged P14-21. ALLO was tested in this experiment at two physiologically relevant concentrations of 1 nM and 100 nM (Gunn et al. 2011; Herbison 2001; Paul and Purdy 1992), and at a supraphysiological concentration of 10 μM. Neurons were held at -75 mV in voltage clamp mode. Peak inward current responses to 1 mM ACh (30 s application) were measured before and after a 5 min or 20 min exposure to DMSO vehicle, 1 nM ALLO, 100 nM ALLO, or 10 μM ALLO, and the effect of treatment was calculated by expressing the post-treatment nAChR response as a percent change from the baseline pre-treatment nAChR response within each neuron. DMSO or ALLO treatments were applied only once to each brain slice. One-sample t test demonstrates that the nicotinic inward current response was not affected by the 5 min or 20 min treatment period or application of DMSO in both males and females (p > 0.2 for each comparison).
In males, α4β2* nAChR-mediated inward current responses to 1 mM ACh (30 s) were not significantly affected by 5 min exposure to ALLO (Fig. 5.1A, one-way ANOVA, F (3, 32) = 0.2, p = 0.8). Changes in peak inward current responses following 5 min exposure to 1 nM, 100 nM, and 10 μM ALLO were not significantly different from DMSO control, with the average change in nicotinic response of +4.7 ± 5.7% from baseline for 1 nM ALLO (Dunnett’s post hoc test, p = 0.9; n = 9 cells from 5 mice), -0.3 ± 4.2% from baseline for 100 nM ALLO (p = 1.0; 9 cells from 5 mice) and -0.7 ± 3.9% from baseline for 10 μM ALLO (p = 1.0; 9 cells from 5 mice). In females, α4β2* nAChR-mediated inward current responses to 1 mM ACh (30 s) were not significantly affected by 5 min exposure to ALLO (Fig. 5.1B, one-way ANOVA, F (3, 40) = 2.6, p = 0.06). However, while changes in inward current responses were not significantly different from DMSO control at 1 nM ALLO (-6.4 ± 4.3% from baseline; Dunnett’s post hoc test, p = 0.9; n = 9 cells from 7 mice) and 100 nM ALLO (-13.0 ± 4.7% from baseline; p = 0.3; n =12 cells from 6 mice), the change in inward current responses was significantly different from DMSO at 10 μM ALLO (-19.1 ± 4.5% from baseline; p = 0.03; n = 12 cells from 6 mice). In male mice with neurons exposed to 20 min of ALLO, the change to α4β2* nAChR-mediated responses was significantly affected by treatment (Fig. 5.1C, one-way ANOVA, F (3, 33) = 5.4, p = 0.004). Here, 20 min exposure changed the nicotinic inward current response by -4.0 ± 6.3% percent from baseline for 1 nM ALLO (Dunnett’s post hoc test, p = 0.5; n = 12 cells from 5 mice), -4.4 ± 7.7% from baseline for 100 nM ALLO (p = 0.5; n = 7 cells from 5 mice) and -27.7 ± 3.9% from baseline for 10 μM ALLO (p = 0.001; n = 9 cells from 5 mice). In female mice with neurons exposed to 20 min of ALLO, the Shapiro-Wilk normality test indicated that the distribution for data following exposure to 1 nM ALLO is non-normal (p = 0.03; n = 12 cells from 7 mice). Transformation of data values to create a Gaussian distribution prevents accurate representation of this data set, which contains
both positive and negative percent changes in values. We have therefore excluded the 1 nM ALLO data set from this analysis, which included the DMSO, 100 nM ALLO and 10 μM ALLO groups, each of which did meet the assumption of a normal distribution (Shapiro-Wilk normality test, p > 0.1 for each comparison). For female neurons exposed to 20 min of ALLO, the change to α4β2* nAChR-mediated responses was significantly affected by treatment (Fig. 5.1D, one-way ANOVA, F (2, 24) = 13.37, p = 0.0001). The change in nAChR-mediated responses was significantly different than DMSO following exposure to 100 nM ALLO (-17.4 ± 3.0% from baseline; Dunnett’s post hoc test, p = 0.02; n = 9 cells from 6 mice) and following exposure to 10 μM ALLO (-35.8 ± 6.1% from baseline; p = 0.0001; n = 9 cells from 5 mice). One sample t test also confirmed that 20 min exposure to DMSO (t(8)=0.59, p = 0.6) did not affect nAChR currents, and that 20 min exposure to 100 nM ALLO (t(8)=5.8, p = 0.0004) and 10 μM ALLO (t(8)=5.9, p = 0.0004) significantly decreased nAChR responses.

All nAChR-mediated current responses for this experiment were measured in voltage clamp mode with neurons held at -75 mV, which is near the equilibrium potential for chloride in this preparation. This approach was taken to minimize the potential for GABA_A receptor-mediated signalling to influence measured nicotinic currents because ALLO is known to modulate GABA_A receptor function. Similarly, α4β2* nAChRs located on local presynaptic terminals could enhance the release of neurotransmitters such as glutamate or GABA (Wonnacott 1997; Wonnacott et al. 2000). To verify that the observed action of ALLO to decrease α4β2* nAChR-mediated currents was not influenced by glutamate or GABA, we repeated the experiment to test effects of 100 nM ALLO application on ACh-induced nicotinic currents following the inhibition of AMPA glutamate receptors (using 20 μM CNQX) and GABA_A receptors (using 10 μM bicuculline). We compared
the percent change from baseline in cells exposed to DMSO control, 100 nM ALLO by itself or 100 nM ALLO in the presence of CNQX and bicuculline (ALLO+CNQX+BCC). Blockade of AMPA and GABA_A receptors did not change the inhibition of α4β2* nicotinic responses by 100 nM ALLO. In male mice with neurons exposed to 20 min of treatment, the change to α4β2* nAChR-mediated responses to 1 mM ACh (30s) was not significantly affected by treatment (one-way ANOVA, F (2, 23) = 1.7, p = 0.2). Following the exposure to 100 nM ALLO+CNQX+BCC, there was a change in nicotinic response by -9.6 ± 6.9% from baseline (n = 10 cells from 3 mice). This was not significantly different from the change in nAChR-mediated response following exposure to DMSO or 100 nM ALLO (Tukey’s post hoc test, p ≥ 0.2 for each comparison). In female mice with neurons exposed to 20 min of treatment, the change to α4β2* nAChR-mediated responses was significantly affected by treatment (Kruskal-Wallis test, p = 0.01). Following the exposure to 100 nM ALLO+CNQX+BCC, there was a change in nicotinic response by -17.1 ± 2.5% from baseline (n = 9 cells from 3 mice). This was significantly different from the change in nAChR-mediated response following exposure to DMSO (Dunn’s test, p = 0.03), but not significantly different from the change in response after exposure to 100 nM ALLO (Dunn’s test, p < 1.0).
Fig. 5.1. Allopregnanolone influences α4β2* nAChR responses in medial prefrontal cortex layer VI pyramidal neurons. A: In males, inward current responses to 1 mM ACh (30 s) were not significantly affected by 5 min ALLO treatment (one-way ANOVA, p = 0.8). B: In females, inward current responses to ACh were not significantly affected by 5 min ALLO treatment (one-way ANOVA, p = 0.06), although the current response following exposure to 10 μM ALLO was significantly different than that following exposure to DMSO control (Dunnett’s post hoc test, *p
In males, inward current responses to ACh were significantly affected by 20 min ALLO treatment (one-way ANOVA, p = 0.004), where the current response following exposure to 10 μM ALLO was significantly different than that following exposure to DMSO control (Dunnett’s post hoc test, **p = 0.004). D: In females, inward current responses to ACh were significantly affected by 20 min ALLO treatment (one-way ANOVA, p = 0.0001), where the current response following exposure to 100 nM and 10 μM ALLO was significantly different than that following exposure to DMSO control (Dunnett’s post hoc test, *p = 0.02 and ****p = 0.0001, respectively). Typical current-clamp traces for ACh responses before and after 20 min application of ALLO are shown below the histogram plot, with the 30 s ACh application indicated by the grey horizontal bar. All recordings were made in the continuous presence of 200 nM atropine and 10 nM MLA. All values are displayed as mean ± SEM.

5.4.2 Isolating for the effect of allopregnanolone on α4β2* nAChR function using the synthetic analogue ganaxolone

The de novo production of ALLO within the brain is catalyzed by the endogenous enzyme 3α-hydroxysteroid dehydrogenase (3α-HSD), which can also convert ALLO back into its precursor 5α-dihydroprogesterone (5α-DHP) (Li et al. 1997; Rossetti et al. 2016; Rupprecht et al. 1993), or forward convert ALLO to its downstream metabolite allopregnanediol. It is possible, in our brain slice preparation, that the observed inhibitory effects of ALLO results from its conversion to one of these potentially active metabolites. Ganaxolone is the synthetic analog of ALLO that is known to exert similar positive allosteric effects at the GABA_A receptor. Unlike ALLO, ganaxolone does not biotransform or undergo back-conversion due to a 3β-methyl substituent in its chemical structure (Monaghan et al. 1999). Therefore, to isolate for the effect of ALLO on α4β2* nAChR signalling, we repeated the set of experiments described above using ganaxolone as it cannot be metabolized by 3α-HSD. Again, ganaxolone was applied only once to each brain slice. In males, α4β2* nAChR-mediated inward current responses to 1 mM ACh (30 s) were significantly affected by 5 min exposure to ganaxolone (Fig. 5.2A, one-way ANOVA, F (3, 25) = 3.4, p = 0.03). Individual changes to peak inward current response following ganaxolone administration were not
significantly different from DMSO control following 1 nM ganaxolone exposure (-14.2 ± 6.9 percent from baseline; Dunnett’s post hoc test, p = 0.2; n = 5 cells from 3 mice), 100 nM ganaxolone exposure (-15.4 ± 5.3% from baseline; p = 0.1; n = 7 cells from 4 mice), and 10 μM ganaxolone exposure (+4.7 ±7.5% from baseline; p = 0.7; n = 7 cells from 2 mice). Inward current responses were not significantly affected in male mice by 20 min exposure to ganaxolone (Fig. 5.2B; one-way ANOVA, F (3, 24) = 0.9, p = 0.4). Following 20 min exposure to ganaxolone, changes in peak inward current responses were not significantly different from DMSO control, where the average change in nicotinic response for 1nM ganaxolone exposure was -2.1 ± 3.6% from baseline (Dunnett’s post hoc test, p = 0.6; n = 5 cells from 4 mice), for 100 nM ganaxolone exposure was -4.9 ± 5.0% from baseline (p = 0.3; n = 8 cells from 4 mice) and for 10 μM ganaxolone exposure was -0.6 ± 8.5% from baseline (p = 0.3; n = 6 cells from 2 mice). In females, peak inward current responses to ACh were also not significantly affected by 5 min or 20 min exposure to ganaxolone (Fig. 5.2C and 5.2D; one-way ANOVA, F (3, 23) = 1.0, p = 0.4 and F (3, 25) = 0.7, p = 0.6, respectively). The change to inward current responses following ganaxolone exposure were not significantly different from DMSO control at 1 nM (5 min: -7.0 ± 3.6% from baseline; Dunnett’s post hoc test, p = 0.2, n = 5 cells from 3 mice / 20 min: +15.05 ± 8.0% from baseline; p = 0.7; n = 5 cells from 3 mice), 100 nM (5 min: +1.6 ± 4.2% from baseline; p = 1.0; n = 5 cells from 3 mice/ 20 min: +10.4 ± 12.3% from baseline; p = 0.9; n = 8 cells from 4 mice ) or 10 μM ALLO (5 min: -0.6 ± 2.1% from baseline; p = 0.9; n = 6 cells from 3 mice / 20 min: +2.1 ± 6.8% from baseline; p = 0.9; n = 7 cells from 3 mice). Additionally, one-sample t test demonstrates that changes in inward current response after 5 min or 20 min exposure of DMSO control was not significantly different from zero for all measurements (p > 0.2 for each comparison). It is important to note however, that while ganaxolone acts in a similar manner to
ALLO at the GABA\textsubscript{A} receptor, and thus has been utilized as a synthetic analog of ALLO, the capacity for ganaxolone to act at the nAChR has not been characterized. Another explanation for these findings may be that the decrease in nicotinic signalling that we previously observed may be derived from the conversion of ALLO into its related metabolites within the brain slice, rather than by ALLO itself.
Fig. 5.2. Ganaxolone does not influence α4β2* nAChR responses in medial prefrontal cortex layer VI pyramidal neurons. A: In males, inward current responses to 1 mM ACh (30 s) were significantly affected by 5 min ganaxolone treatment (one-way ANOVA, p = 0.03), although no single post-ALLO change in current response was different from DMSO (Tukey’s post hoc test, p > 0.2 for each comparison). B: In males, inward current responses to 1 mM ACh (30 s) were not significantly affected by 20 min ganaxolone treatment (one-way ANOVA, p = 0.4). C: In females, inward current responses to 1 mM ACh (30 s) were not significantly affected by 5 min ganaxolone treatment (one-way ANOVA, p = 0.4). D: In females, inward current responses to 1 mM ACh (30 s) were not significantly affected by 20 min ganaxolone treatment (one-way ANOVA, p = 0.6). All recordings were made in the continuous presence of 200 nM atropine and 10 nM MLA. All values are displayed as mean ± SEM.
5.4.3 Isolating the effect of allopregnanolone on α4β2* nAChR function using a 3α-HSD inhibitor

In the following set of experiments, we sought to isolate the effects of ALLO by inhibiting its potential conversion to 5α-DHP or allopregnanediol within brain slices. Indomethacin has previously been demonstrated to inhibit the actions of the 3α-HSD (Fukabori et al. 1992; Smithgall and Penning 1985), thereby allowing for the study of the primary effects of ALLO. We conducted this experiment by testing the effect of 20 min exposure to 100 nM ALLO to decrease α4β2* nAChR function in female mPFC layer VI principal neurons because this physiologically-relevant concentration of ALLO was shown in Fig. 5.1D to significantly affect nicotinic inward currents in this population of neurons. This experiment was performed by measuring inward current response to 1 mM ACh, allowing for 5 min of washout, and then applying either 10 μM indomethacin by itself (INDO) or 100 nM ALLO in the presence of 10 μM INDO (ALLO + INDO) in bath for 20 min. After a 20-min application, the 1 mM ACh (30s) response was measured again and the percent change from baseline was calculated. We compared changes in nicotinic responses in female mPFC layer VI principal neurons following 20 min exposure to DMSO control, ALLO (100 nM) by itself, INDO (10 μM) by itself, and ALLO + INDO (100 nM and 10 μM, respectively). One-way ANOVA for the change in peak inward current response from baseline reveals a significant effect of treatment (Fig. 5.3A, F (3,33) = 9.5, p = 0.0001). Application of indomethacin by itself changed the nicotinic inward current response by +3.6 ± 2.8% from baseline (n = 9 cells from 3 mice), which was not significantly different from DMSO control (Tukey’s post hoc test, p < 1.0; n = 9 cells from 6 mice) but was significantly different from the application of 100 nM ALLO (-17.4 ± 3.0% from baseline; Tukey’s post hoc test, p = 0.01; n = 10 cells from 4 mice). As reported earlier, 100 nM ALLO significantly changed the nicotinic response by -17.4 ± 3.0% from baseline (Tukey’s post hoc test, p = 0.01). The change to nicotinic inward current response after the
application of INDO was significantly different from change in response after the application of 100 nM ALLO (Tukey’s post hoc test, p = 0.01), and significantly different from the change in response after the application of ALLO+INDO (+16.0 ± 4.8% from baseline; Tukey’s post hoc test; n =10 cells from 3 mice).

In the experiment above, indomethacin should have acted to isolate for the effects of exogenous ALLO application. Since the effect of ALLO differed from that of ALLO plus indomethacin, we hypothesized that the observed inhibition of nicotinic signalling following ALLO administration may have resulted from the conversion of ALLO by endogenous 3α-HSD into 5α-DHP or allopregnanediol, which then acted directly to decrease nAChR function. The next set of experiments was performed to test this postulate by measuring changes in nicotinic responses in female mPFC layer VI principal neurons following 20 min exposure to DMSO control, 5α-DHP (100 nM), ALLO (100 nM) or allopregnanediol (100 nM). These experiments were conducted in the presence of CNQX (20 μM) and BCC (10 μM) to ensure that responses are mediated by postsynaptic α4β2* nAChRs. The change to nicotinic responses was significantly affected by treatment (Fig. 5.3B, one-way ANOVA, F(3, 32) = 9.0, p = 0.0002). Following exposure to 100 nM 5α-DHP, the nicotinic inward current elicited by application of 1 mM ACh (30s) was changed by +21.5 ± 5.5% from baseline (n = 9 cells from 4 mice). This change was not significantly different from the change in the nicotinic current response after exposure to DMSO control (Tukey’s post hoc test; p = 0.1; n = 9 cells from 6 mice), but was significantly different from the change in the nicotinic current response after exposure to ALLO (-17.1±2.5% of baseline; Tukey’s post hoc test, p < 0.0001; n =9 cells from 3 mice). As reported earlier, 100 nM ALLO significantly changed the nicotinic response by -17.1 ± 2.5% from baseline (Tukey’s post hoc test; p < 0.05).
Following exposure to 100 nM allopregnanediol, the current elicited by application of 1 mM ACh (30s) was changed by +9.3 ± 6.1% from control (n = 9 cells from 4 mice). This change was again, not significantly different from the change in the nicotinic current response after exposure to DMSO control (Tukey’s post hoc test; p = 0.9), but was significantly different from the change in the nicotinic current response after exposure to ALLO (Tukey’s post hoc test, p = 0.008). These findings demonstrate that the progesterone metabolites 5α-DHP, ALLO and allopregnanediol differ in their capacity to modulate nicotinic signalling. However, these findings also suggest that the decrease in nicotinic response after ALLO is applied in the absence of indomethacin is not due to its conversion to 5α-DHP or allopregnanediol.
Fig. 5.3. Allopregnanolone and its metabolites 5α-DHP and allopregnanediol differ in their capacity to alter the function of α4β2* nicotinic receptors. A: The change to inward current responses following application of 1 mM ACh (30 s) was significantly affected by treatment (one-way ANOVA, p = 0.0001). Percent change to nicotinic responses was significantly different between ALLO and DMSO (*p = 0.01), indomethacin (INDO; *p = 0.01), and ALLO in the presence of indomethacin (ALLO + INDO; ****p < 0.0001). Typical traces are shown below the histogram plot. B: The change to inward current responses following application of 1 mM ACh (30 s) in the continuous presence of CNQX and bicuculline was significantly affected by treatment (one-way ANOVA, p = 0.0002). Percent change to nicotinic responses was significant different between ALLO and DMSO (*p < 0.05), 5α-DHP (****p < 0.0001) and allopregnanediol (**p = 0.008). Typical traces are shown below the histogram plot. All recordings were made in the continuous presence of 200 nM atropine and 10 nM MLA. Application of ACh (30s) indicated by gray bar. All values are displayed as mean ± SEM.
5.4.4 Interaction between allopregnanolone at the mPR complex and α4β2* nAChR function

ALLO has been demonstrated to mediate rapid effects through agonist action at the membrane progesterone receptor (mPR) (Kelder et al. 2010), which has more-recently been proposed to act in conjunction with the progesterone membrane receptor component 1 (PGRMC1) to form the mPR complex (Thomas et al. 2014). The binding of ALLO to the mPR complex can elicit numerous changes through downstream second messenger cascades. Particularly, it has been suggested that the mPR activates an inhibitory G protein (G_i) resulting in downregulation of cAMP, or, through the alteration of intracellular Ca^{2+} to produce rapid changes in neuron function by regulating channel protein transportation, function, and expression (Charles et al. 2010; Petersen et al. 2013; Rekawiecki et al. 2008; Thomas et al. 2014). To test for the involvement of mPR complex in mediating ALLO effects on nAChR function, we compared alterations to nAChR function following 20 min exposure to ALLO, the mPR agonist ORG OD 02-0, and ALLO plus the PGRMC1 antagonist AG-205. Experiments were performed in mPFC layer VI neurons of female mice because nAChR function in this neuronal population is decreased by 20 min exposure to 100 nM ALLO (Fig. 5.1D). The change to measured peak inward current responses to 1 mM ACh (30 s) was similar after exposure to 100 nM ALLO (-17.4 ± 3.0% from baseline; n = 10 cells from 4 mice) and 100 nM ORG OD 02-0 (-15.9 ± 6.0% from baseline; n = 10 cells from 4 mice) (Fig. 5.4; Dunnett’s post hoc test, p = 1.0). To further investigate the potential role of the mPR complex in mediating ALLO effects at the nAChR, we also compared the change to nicotinic inward currents following exposure to 100 nM ALLO versus 100 nM ALLO plus 5 nM AG-205. Results from this experiment demonstrated a significant difference between changes in nicotinic currents between ALLO (-17.4 ± 3.0% from baseline; n = 10 cells from 4 mice) and ALLO plus AG-205 (+16.6 ± 3.8% from baseline, n = 9 cells from 4 mice) (Dunnett’s post hoc test, p = 0.0001).
It has been suggested that the mPR complex couples to intracellular signalling cascades via heteromeric G proteins (but see (Kasubuchi et al. 2017; Smith et al. 2008)). One potential pathway involves mPR activation of phospholipase C, followed by inositol phospholipid breakdown and intracellular Ca\(^{2+}\) mobilization, and/or diacylglyceride (DAG) and protein kinase C (PKC) activation (Charles et al. 2010; Petersen et al. 2013; Rekawiecki et al. 2008; Thomas et al. 2014). Accumulating evidence demonstrates that phosphorylation of the nAChR acts to ensure proper receptor expression, distribution, and signalling that is consistent with cellular demands (Fenster et al. 1999; Lee et al. 2015; Nakayama et al. 1993; Nishizaki and Sumikawa 1998). Phosphorylation by PKC is a known modulator of nAChR signalling (Kamerbeek et al. 2016; Lee et al. 2015; Nuutinen et al. 2006; Pollock et al. 2009). We therefore investigated the possible role for PKC activation in mediating ALLO inhibition of \(\alpha4\beta2^*\) nAChR function. This was performed by comparing changes to ACh-induced nicotinic currents following exposure to 100 nM ALLO versus 100 nM ALLO following cellular exposure to the PKC inhibitor peptide PKC-I (19-36) (10 \(\mu\)M). Specifically, PKC-I (19-36) was diluted into the internal solution with 0.1% DMSO vehicle. One sample \(t\) test demonstrates that inward current response was not affected by 20 min exposure to 0.1% DMSO vehicle in the internal solution (\(p > 0.3\)). After the successful patch of a mPFC layer VI principal neuron, internal solution was allowed to dialyze into the cell for 5 min before conducting any recordings. Similar to previous experiments, we compared alterations to nAChR function following 20 min exposure to ALLO, but in the continuous presence of PKC-I (19-36). Under these conditions, there was a significant difference between the change to nicotinic currents after exposure to ALLO (-17.4 ± 3.0% from baseline; \(n = 10\) cells from 4 mice) and ALLO plus PKC-I (19-36) (-2.3 ± 3.0% from baseline, \(n = 9\) cells from 3 mice) (Dunnett’s \textit{post hoc} test, \(p < 0.05\)).
Fig. 5.4. Interaction between ALLO and the mPR complex in modulating \( \alpha 4\beta 2^* \) nAChR function. The change to inward current responses to 1 mM ACh (30 s) was significantly affected by treatment (one-way ANOVA, \( p = 0.00001 \)). Percent change to the nicotinic response was not significantly different between ALLO versus ORG OD 02-0 treatment (Dunnett’s post hoc test, \( p = 1.0 \)). There was a significant difference between changes to the nicotinic response following exposure to ALLO versus ALLO plus AG-205 (Dunnett’s post hoc test, \( ****p = 0.0001 \)). There was a significant difference between changes to the nicotinic response following exposure to ALLO versus ALLO plus PKC-I (19-36) (Dunnett’s post hoc test, \( *p < 0.05 \)). Typical traces shown below the histogram plot. All recordings were made in the continuous presence of 200 nM atropine and 10 nM MLA. Application of ACh (30s) indicated by grey bar. All values are displayed as mean ± SEM.
5.5 Discussion

Results from this study provide evidence that ALLO negatively modulates α4β2*-nAChR function in mPFC layer VI pyramidal neurons. By characterizing the response in both males and females, we present in situ evidence of the modulatory effects of ALLO at two different concentrations, one of which is physiologically relevant. At 100 nM, ALLO decreases nicotinic signalling in the female mouse. However, ALLO and its metabolites, 5α-DHP and allopregnanediol differ in their capacity to mediate nicotinic signalling, where 5α-DHP and allopregnanediol do not inhibit α4β2*-nAChR signalling. We have also shown using ORG OD-02 and AG-205, an agonist and antagonist of the mPR complex, respectively, that the inhibitory actions of ALLO on the nAChR may be mediated via this mPR complex. Furthermore, the downstream modulatory effect of ALLO at α4β2*-nAChRs in mPFC layer VI pyramidal neurons is likely to be PKC dependent, as inactivation of PKC phosphorylation prevented ALLO from inhibiting nAChR response. These findings suggest that ALLO modulates α4β2* nAChR function through the activation of the mPR complex, and through secondary signalling cascades requiring PKC. As nAChRs play an important role in influencing excitatory output of pyramidal neurons, our findings suggest that ALLO can play an important role in influencing the proper function of neuronal networks by mediating nAChR activity.

5.5.1 Inhibition of nicotinic response in the mPFC

The mPFC receives dense cholinergic innervation from the nucleus basalis of the basal forebrain (Bloem et al. 2014; Mechawar and Descarries 2001; Mesulam 1995; Zaborszky et al. 1999). Cholinergic input is layer-specific in the mPFC and each layer has a different pattern of nAChR expression (Dajas-Bailador and Wonnacott 2004; Gulledge et al. 2007; Yakel 2013), suggesting
that nAChRs modulate mPFC output in a layer-specific manner to influence specific cognitive functions. nAChRs are expressed on both pyramidal and interneurons, where they are thought to be responsible for regulating excitability, synaptic communication and cognitive function. Consistent with previous findings by the Bullock et al. (1997), our current findings demonstrate that ALLO (100 nM and 10 μM) significantly inhibits α4β2*-nAChR function in mPFC neurons. The activation of α4β2* nAChRs in mPFC layer VI pyramidal neurons has been of particular interest, as mice lacking the β2 nAChR subunit exhibit decreased cognitive function and re-expressing β2-containing nAChRs in mPFC layer VI pyramidal neurons specifically restores this deficit (Guillem et al. 2011). In cultured neurons, stimulation of nAChR limits neurite outgrowth (Lipton et al. 1988; Nordman and Kabbani 2012), a process which is mediated in a Ca2+-dependent manner (Pugh and Berg 1994). Consistent with these effects, stimulation of heteromeric nAChRs containing the α5 subunit, which have the highest Ca2+ permeability of the α4β2* subtypes, appear to mediate the retraction of apical dendrites in the mPFC layer VI pyramidal neurons right after the third week of postnatal maturation (Bailey et al. 2012). Therefore, the modulation of nicotinic receptor signalling in layer VI of the mPFC by ALLO may play an important role in influencing the changes in neuron morphology which contribute to layer-specific outputs that are important to the proper function and development of the mPFC circuity.

We demonstrated that while the higher concentration of 10 µM ALLO inhibited α4β2* nAChR-mediated signalling in both male and female mPFC layer VI pyramidal neurons, 100 nM ALLO was able to inhibit nicotinic signalling in pyramidal neurons of female mice. There are known sex differences in nicotinic excitation in the brain, but mechanisms behind these differences have not been elucidated. For example, sex differences have been reported in the developmental regulation
of nicotinic receptors in midbrain dopamine neurons, where there were changes in both nicotine potency and efficacy during the transition between adolescent to adulthood in males, but not in females (Azam et al. 2007). Additionally, there are known developmental sex differences in nicotinic excitation in the mPFC. Specifically, in mPFC layer VI pyramidal neurons, nicotinic currents are larger and observed in a greater proportion of cells in males than in female mice. Additionally, mean nicotinic current response is greatest in males during the third week of postnatal development whereas in females, response is greatest during the fourth week (Alves et al. 2010). One explanation of these differences may be due to sex differences in the ability of the brain to metabolize acetylcholine. It was reported that there are 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, males have a reliably greater amount of acetylcholinesterase activity than females (Akmaev and Fidelina 1996). Thus, brain cholinergic structures may be differently controlled between the sexes due to differing levels of acetylcholinesterase activity. However, the work conducted by Alves et al, 2010 was also conducted using carbachol which cannot be metabolised by acetylcholinesterase, therefore suggesting that sex differences in the mPFC may be facilitated by other mechanisms. Our current study proposes that differences in the capacity of ALLO to modulate nAChR signalling may explain the observed sex differences in nicotinic excitation in the brain. This is consistent with our findings as ALLO decreases nAChR currents in female mice to a greater extent than in male mice during this period of development. It has long been recognized that the presence or absence of steroid hormones during development can produce long term neurodevelopmental consequences, and that steroid hormones can modulate the development of sex differences in cognitive functions (Goldman et al. 1974; MacLusky et al. 1979). Our study suggests that, although mice were
investigated at P14-21 prior to the gonadal surge of sex steroids during adolescence, the *de novo* production of neurosteroids during early postnatal development may exert sex-specific effects on nicotinic signalling, where female mice appear to be more susceptible to the effects of ALLO. Therefore, studies conducted in both the male and female brain will be important, as this may help improve our understanding of how sex differences in nicotinic signalling in the brain may influence cognition and the risk for neuropsychiatric disorders.

### 5.5.2 Rapid effects of allopregnanolone to modulate nicotinic receptor function

The classic actions of progesterone metabolites involve the nuclear progesterone receptor (PR). While ALLO does not directly activate PRs, back-conversion to 5α–DHP by 3α-HSD allows for ALLO to mediate its effects through PRs (Belelli and Lambert 2005; Rupprecht et al. 1993) and thus regulate gene expression, a process which typically occurs over a time scale of hours (DeMarzo et al. 1991; Edwards et al. 1991; Evans 1988). However, it has also been reported that ALLO can have rapid non-genomic effects, and that these actions can occur within minutes by activating membrane receptors and their intracellular transduction pathways (Frye 2001; Hwang et al. 2009; Meyerson 1972). In our current study, effects of ALLO were present by 5 min, but significantly more pronounced after 20 min of exposure, suggesting a rapid non-genomic action of ALLO in modulating α4β2* nAChR function. The most-extensively investigated rapid non-genomic actions of neurosteroids are those at synaptic and extrasynaptic GABA_A receptors (Purdy et al. 1991), which are part of the same cys-loop superfamily of ion channels as nAChRs. Previous studies have demonstrated that at a low nanomolar concentration, ALLO acts allosterically to enhance the action of the natural ligand GABA, while at a high micromolar concentration, ALLO directly gates the GABA_A receptor channel complex (Callachan et al. 1987; Puia et al.
Therefore, understanding how ALLO directly acts at nAChRs at different concentrations will be important in determining how it may alter nicotinic signalling within the brain. The inhibitory effects observed in our current slice preparation following 20 min application of ALLO do not appear to result from the conversion of ALLO to 5α-DHP or allopregnanediol, as neither of these metabolites exert an inhibitory effect on α4β2* nAChR when bath-applied for 20 minutes. This finding suggests that the ALLO metabolite may be unique in its capacity to rapidly exert non-genomic actions leading to downstream signalling cascades and changes in cellular function. The study of the rapid effects of ALLO at GABA<sub>A</sub> receptors led to the development of the synthetic compound ganaxolone, which is also a positive allosteric modulator of the GABA<sub>A</sub> receptor. However, to the best of our knowledge, the effect of ganaxolone at nAChRs has not been investigated. Our findings demonstrate that in slices from the mouse brain, ganaxolone and ALLO applied at the same concentrations do not exert similar effects on α4β2* nAChR function. Understanding the mechanism behind the rapid non-genomic actions of ALLO at nAChRs, and comparing this with ALLO action at GABA<sub>A</sub> receptors will be important. This may provide us with further understanding of how ALLO may affect diverse aspects of neural cell physiology (Stromberg et al. 2006), play an important role in modulating stress (Gunn et al. 2015; Purdy et al. 1991), and be involved in neurological disorders such as epilepsy (Smith et al. 2007) and Alzheimer’s disease (Irwin et al. 2011), all of which are also recognized diseases related to nAChR dysfunction.

5.5.3 Involvement of the mPR complex in allopregnanolone modulation of α4β2* nAChR function. The findings from this study suggest that ALLO exerts its action at nAChRs in a rapid, non-genomic manner. This may be mediated through the activation of the mPR and PGRMC1, which
recently has been considered to be components of a single mPR complex (Thomas et al. 2014). mPR sequences suggest that these receptors contain seven trans-membrane domains and therefore have led to their classification as a novel group of G protein-coupled receptors (GPCRs) which mediate rapid actions through intracellular signalling pathways (Tang et al. 2005; Thomas and Pang 2012). When bound to neurosteroids, the mPRα, mPRβ, and mPRγ subtypes are thought to act through the inhibitory G_{αi/o} signalling pathway to decrease membrane-bound adenylyl cyclase, and subsequently decrease cAMP and PKA levels (Thomas and Pang 2012). However, as the G_{βγ} dimer is also released during this signalling process, neurosteroid binding to the mPR complex can activate PLC to increase the release of Ca^{2+} from intracellular stores (Ashley et al. 2006; Kowalik et al. 2013). Additionally, DAG which acts downstream of PLC may also increase PKC activity. Therefore, activation of the mPR may initiate multiple signalling cascades and thus generate multiple effects on cell function. It is important to note that some literature suggests that mPRs do not act as a classical GPCR, and therefore, the various mPR downstream signalling activities have not been fully understood or characterized (Kasubuchi et al. 2017; Smith et al. 2008). In our present work, use of the mPR agonist ORG OD-02 and the PGRMC1 antagonist AG-205 demonstrated that the mPR complex plays a role in mediating ALLO effects on nAChR function, whereby activation of the mPR complex leads to a decrease in α4β2* nAChR function and the inactivation of the complex overrides ALLO inhibition, leading to an overall increase in α4β2* nAChR function. Additionally, our data suggest that activation at the mPR complex may initiate a signalling cascade that is PKC-dependent (such as through PLC), as inactivation of PKC phosphorylation prevented ALLO from modulating nAChR signalling in principal neurons.

Several studies have demonstrated that PKC modulates nicotinic receptor function, and suggest
that phosphorylation of nAChRs may impact nicotinic function by altering their desensitization kinetics or by regulating their expression on the cell surface membrane (Kamerbeek et al. 2016; Lee et al. 2015; Nuutinen et al. 2006; Pollock et al. 2009). Of interest to our findings, it has also been demonstrated that the M_1 isoform of mACHRs, which is also a GPCR, mediates an inhibitory action at the α7 nAChR in hippocampus interneurons (Shen et al. 2009). These actions were mediated through the PKC-dependent signal transduction cascade, where the inhibition of PLC, Ca^{2+}, and PKC significantly reduced the capacity for M_1 receptors to inhibit α7 nAChR function. In a study using neurosecretory cells from insects, the activation of M_1 mAChRs via two different PKC isozymes, both up- and down-regulated nAChR function (Courjaret et al. 2003). These actions are very similar to what we have demonstrated in this study, where ALLO may be acting at another membrane bound protein, in this case, the mPR complex, to influence nAChR function. Specifically, our findings suggest that this process may be mediated by PKC, as the addition of a PKC inhibitor to our internal patch solution prevented the inhibition of nAChR response after exposure to ALLO. It is possible that when ALLO binds to the mPR complex, downstream signalling cascades activate PLC to increase the release of Ca^{2+} from intracellular stores (Ashley et al. 2006; Kowalik et al. 2013), as well as DAG to increase PKC activity. This is consistent with the findings by Coujaret et al. (2003) and Shen et al. (2009), in that this allows for Ca^{2+} to be situated in a prime position to select which PKC isozyme will regulate nAChR function by phosphorylation. Based these findings, we propose that ALLO exerts a similar inhibitory effect on nAChR function by activating the mPR complex, which functions as a GPCR that initiates PKC-dependent signalling to reduce α4β2* nAChR function (Fig. 5.5). Consistent with this model, the mPRα subtype and PGRMC1 exhibit overlapping distributions in specific brain regions including the cerebral cortex (Intlekofer and Petersen 2011; Meffre et al. 2013). Therefore, the mPR complex
is appropriately situated to mediate the observed ALLO-induced changes to nAChR function in mPFC layer VI neurons. Future studies investigating the presence of these components on specific neuron subtypes of the mPFC, including both pyramidal and interneurons, will be of importance because this will aid in determining how progesterone metabolites such as ALLO can modulate specific classes of neurons. Additionally, investigating the role of specific PKC isozymes at the nAChR will help to determine precisely how the $\alpha4\beta^2*$ nAChRs are inhibited in our model, as different isozymes have been associated with mediating distinct cellular effects (Kamerbeek et al. 2016; Nuutinen et al. 2006; Pollock et al. 2009). While much is still unknown about the process by which phosphorylation influences desensitization of nAChRs, this process functions as a mechanism that limits both amplitude and duration of nAChR activation by cholinergic input. Determining specific PKC isozyme(s) affecting the nAChR will be important in our understanding of whether exposure to ALLO results in desensitization, or whether ALLO alters the time which nAChRs require to recover from the closed state (Miyazawa et al. 2003; Quick and Lester 2002; Taly et al. 2009). Similarly, determining if specific isozymes selectively target nAChR isoforms will also be important as nAChRs of the $(\alpha4)_2(\beta2)_3$ stoichiometry are more sensitive to nicotine-induced up-regulation and more sensitive to desensitization induced by low concentration of agonists, whereas nAChRs of the $(\alpha4)_3(\beta2)_2$ stoichiometry have a higher permeability to Ca$^{2+}$ and more rapid desensitization kinetics than the $(\alpha4)_2(\beta2)_3$ stoichiometry (Albuquerque et al. 2009; Gotti et al. 2009; Kuryatov et al. 2008; Lopez-Hernandez et al. 2004; Tapia et al. 2007). These findings may help to elucidate the mechanism by which ALLO alters nAChR function under different physiological conditions.
Understanding the mechanism by which ALLO modulates nAChR function within the mPFC will be important as ALLO is involved in a number of processes involving mPFC function. These processes include the neuroprotection and prevention of cell death (Djebaili et al. 2004), regulation of higher-order cognition, such as preventing memory impairment (Escudero et al. 2012) and the regulation of emotions, such as by acting as an anxiolytic (Pibiri et al. 2008). This study represents the first demonstration of crosstalk between the mPR complex and nAChR in any system. It may therefore improve our understanding of how these two systems work together to regulate neuronal excitability, synaptic transmission, and synaptic plasticity to regulate higher-order cognitive functions.
Fig. 5.5. Working model of the mechanism by which allopregnanolone exerts inhibitory effects on the nicotinic acetylcholine receptor in principal neurons of the medial prefrontal cortex. We propose that ALLO activates the membrane progesterone receptor (mPR) complex, which consists of the mPR and progesterone mediated complex 1 (PGRMCI). The activated mPR complex induces downstream signalling cascades including protein kinase C (PKC), which phosphorylates the nicotinic acetylcholine receptor (nAChR) to decrease its activity when bound with acetylcholine (ACh). In this model, we have not excluded the possibility that ALLO may also have direct effects at the nAChR.
5.5.4 Conclusion

Nicotinic acetylcholine receptor (nAChR)-mediated signalling plays an important role in the normal development and function of mPFC circuitry that facilitates higher-order cognitive functions. ALLO is an important neurosteroid that exerts rapid non-genomic actions in neurons and is involved in mediating numerous brain processes. Our results demonstrate that ALLO inhibits α4β2* nAChR function in mPFC layer VI pyramidal neurons. Based on our findings, we suggest that ALLO exerts this inhibitory effect by activating the mPR complex, which acts as a GPCR to ultimately initiate a PKC-dependent signal to reduce α4β2* nAChR activation by ACh. Our findings present a potentially important avenue for understanding how ALLO can influence the excitatory output of pyramidal neurons, and thus influence the proper function of neuronal networks.
CHAPTER 6

Summary and Future Directions
The proper development and function of the HF and PFC depend on the interaction between the neurotransmitter ACh and nAChRs. Within the brain, the α4β2* isoform is one of the primary mediators of such ACh signalling. While the location and functional role of α4β2* nAChRs have been well characterized in the mPFC, the location, function and developmental regulation of these receptors within the HF are not well known, leaving a gap in our understanding as to how and where α4β2* nAChRs can mediate proper neural circuitry during critical periods of development. Additionally, there is growing evidence to suggest that the progesterone metabolite, ALLO, plays a role in regulating the proper function of the HF and the PFC (Djebaili et al. 2004; Pibiri et al. 2008; Wang et al. 2005), whereby it acts to influence neural physiology, higher-order cognitive functions, as well as stress responses. However, while it has been demonstrated that ALLO can significantly alter α4β2* nAChR-mediated signalling in cellular expression and ex vivo studies, the effect of ALLO on α4β2* nAChR function has not been investigated in situ.

Given the importance of nicotinic signalling in the HF and mPFC toward normal cognitive functions, the goal of this work was to develop a foundation towards understanding the location, function and developmental regulation of α4β2* in HF signalling in principal neurons during development, and the role of ALLO in modulating α4β2* nicotinic receptor function during this period.

This work was divided into three objectives:

- **Objective 1**: I determined whether functional heteromeric α4β2* nAChRs are present in CA1 principal neurons of the developing mouse HF.
- Objective 2: I characterized α4β2* nAChRs function across the primary regions of the HF in young postnatal mice.

- Chapter 3: I determined the effect of ALLO on α4β2* nAChR function within living neurons of the mPFC of young postnatal mice.

6.1 Objective 1 Review

The objective of this study was to determine whether functional heteromeric nAChRs are present in developing mouse hippocampus CA1 pyramidal neurons. Based on *in situ* expression analysis of the α5 subunit, which can only exert functional influence when incorporated into heteromeric α4β2* nAChRs, we hypothesized that the expression of functional α4β2* nAChRs within hippocampal CA1 pyramidal neurons is developmentally regulated and in rodents peaks during the first two weeks of postnatal life. I used whole-cell electrophysiology to show that heteromeric nicotinic receptors mediate direct inward currents, depolarization from rest and enhanced excitability in hippocampus CA1 pyramidal neurons of male mice. Measurements were made throughout postnatal development to provide a thorough developmental profile for these heteromeric nicotinic responses, which were greatest during the first two weeks of postnatal life and decreased to low adult levels shortly thereafter. All measurements were conducted in the presence of muscarinic ACh receptors and α7 subunit-containing nAChRs, demonstrating that nicotinic responses were mediated by heteromeric nAChRs. Pharmacological experiments found that responses are blocked by a competitive antagonist of α4β2* nicotinic receptors and augmented by a positive allosteric modulator of α5 subunit-containing receptors, which is consistent with expression studies suggesting the presence of α4β2* and (α4β2)α5 nicotinic receptors within the developing CA1 pyramidal cell layer. These findings demonstrate that functional heteromeric
nicotinic receptors, likely of the $\alpha4\beta2^*$ isoform, are present on CA1 pyramidal neurons during a period of major hippocampal development.

The presence of functional heteromeric nAChRs on CA1 pyramidal neurons during early postnatal development may have important implications for our understanding of normal and aberrant hippocampus development. The development of CA1 pyramidal neurons in mice is characterized by increased dendrite complexity and length over the first two to four weeks of postnatal life for basal dendrites (Nishimura et al. 2011) and over at least the first five weeks for apical dendrites (Jacobson et al. 1988). Nicotinic signalling plays an important role in neuron morphological development. Experiments in genetically modified mice demonstrate that nAChRs containing the $\beta2$ subunit contribute to the production of dendritic spines \textit{in vivo} for pyramidal neurons of the cerebral cortex and CA1 area of the hippocampus (Ballesteros-Yanez et al. 2010; Lozada et al. 2012b). Moreover, for cultured rat olfactory bulb neurons and for hippocampal adult-born neurons, nAChR activation has been found to promote neurite/dendrite elongation (Campbell et al. 2010; Coronas et al. 2000). The presence of $\alpha4\beta2^*$ nAChRs during early development places them in a prime position to play an important role in the establishment of hippocampal cognitive networks via these developmental effects. Additionally, in this study, I demonstrated that nicotine at concentrations relevant to those seen in the brain of smokers desensitizes heteromeric nAChRs in young postnatal CA1 pyramidal neurons. Both nicotine and $\alpha4\beta2^*$ nAChR agonists can improve attention and working memory in ADHD (Plessen et al. 2006; Wilens and Decker 2007; Wilens et al. 2006) and the augmentation of nicotinic neurotransmission is a proposed therapeutic strategy for ASD (Deutsch et al. 2010; Ghaleiha et al. 2013). Therefore, this work may also be used to
inform efforts for pharmaceutical development aimed to mitigate or treat multiple neurodevelopmental disorders involving the hippocampal nicotinic system.

6.2 Objective 2 Review

The objective of this study was to characterize α4β2* nAChR function across the primary regions of the HF in young postnatal mice. As early postnatal expression of heteromeric nAChR subunits varies in magnitude across regions of the HF (Didier et al. 1995a; Machaalani et al. 2010; Winzer-Serhan and Leslie 2005), we hypothesized that α4β2* nAChRs mediate direct inward current and depolarization responses in principal neurons across the HF of the young postnatal mouse, and that α4β2* mediated responses vary in magnitude across regions. However, as these expression studies were either focused on the male mouse or, did not differentiate between the two sexes, we also investigated whether there were sex differences in α4β2* mediated responses in the developing HF. Chapter 3 of this thesis describes my whole-cell electrophysiological investigation of visually-identified principal neurons of the DG, CA1, CA3, SUB and ECVI principal neurons from young postnatal male mice. Chapter 4 of this thesis describes my whole-cell electrophysiological investigation of visually-identified principal neurons of the DG, CA1, CA3, SUB and ECVI principal neurons from young postnatal female mice.

In Chapter 3, I found that heteromeric nAChR stimulation elicits direct passive inward current and depolarization responses in principal neurons of all HF regions investigated in young postnatal male mice, and that the magnitude of these passive responses is greater in the SUB and ECVI than in the hippocampus proper regions CA1, CA3 and DG. Interestingly, despite this regional variation in the magnitude of passive responses, heteromeric nicotinic receptor stimulation increased the
excitability of active principal neurons by a similar amount in all regions in the male HF. Pharmacological experiments found this similar excitability response to be regulated by small conductance calcium-activated potassium (SK) channels, which exhibit regional differences in their influence on neuron activity that offset the observed regional differences in passive nicotinic responses. These findings demonstrate that SK channels play a role in coordinating the magnitude of heteromeric nicotinic excitability responses across the HF at a time when nicotinic signalling drives the development of this cognitive brain region.

In Chapter 4, I first conducted whole-cell electrophysiological recordings in female mice at the equivalent ages of the young postnatal, juvenile, and adult period. I found that glutamatergic principal neurons in the CA1 of female mice are transiently excited by postsynaptic heteromeric nAChRs during early postnatal development, with the greatest function occurring during the first two weeks of postnatal life. These findings are similar to those which were reported in male mice. Then, I found that heteromeric nAChR stimulation elicits direct passive inward current and depolarization responses in principal neurons of all HF regions investigated in young postnatal female mice as well. However, in contrast with previous observations of nicotinic response in young postnatal male mice, where the active response is generally equal across HF regions because of an mAHP generated by SK channels, in female mice, the active response is not equal across HF regions as there was no mAHP observed in the ECVI. This is interesting, as without an mAHP, ECVI principal neurons in female mice demonstrate a hyper-excitable nature and are not restrained from firing. These findings suggest that differences in the presence or absence of the mAHP component in male and female mice, respectively, can alter nicotinic excitability in the ECVI
principal layers and influence the active output of these neurons during the early development of the HF circuitry.

Previous studies have demonstrated that spontaneous coordinated activity of immature neuronal networks appears in the rodent hippocampus during the first postnatal week (Ben-Ari et al. 1989; Crepel et al. 2007; Garaschuk et al. 1998). While these oscillations are mediated by excitatory GABAergic neurotransmission to form giant depolarizing potentials (GDPs) in the HF (Nardou et al. 2009; Sipila et al. 2005), both α7 and β2 subunit-containing nAChRs also exert regulatory actions on network-driven GDPs (Le Magueresse et al. 2006; Maggi et al. 2001). In male mice, the finding that functional heteromeric nAChRs are present on all principal neuron types of the young postnatal HF, and that their activation leads to similar-magnitude active excitability responses in active neurons across all regions of the HF, suggests that postsynaptic α4β2* nAChRs may be involved in the formation of these coordinated and organized rhythms within the developing HF during the same time that GABAergic neurotransmission is excitatory. My finding that ECVI neurons have greater nAChR excitability in females than in males during early this developmental period is interesting, as there is also evidence of sex differences in the duration of GABA_A receptor-mediated excitation during normal brain development (Galanopoulou 2008). Particularly, the time course for the switch of GABA_A receptor-mediated excitation to inhibition occurs earlier in females than in males. This suggests that while ECVI principal neurons in female mice demonstrate a hyper-excitable nature and generate a larger active nicotinic response than in males during this developmental period, there is also less excitatory GABAergic neurotransmission in females. Potentially then, while there are sex differences in nAChR and GABAR function during early development, the two systems may be coordinating in a sexually
dimorphic manner to facilitate the development of more organized activity in the HF circuitry, such as theta or gamma oscillations (Bragin et al. 1995; Fellous and Sejnowski 2000; Mohajerani and Cherubini 2006). Importantly, the presence of sex differences in heteromeric nAChR responses in ECVI principal neurons during early postnatal development has direct implications for the understanding of neurodevelopmental disorders. Not only has the dysfunction of hippocampal nAChRs been linked with specific neurodevelopmental and neurological disorders, such as attention-deficit hyperactivity disorder (Levin 2002), epilepsy (Dani 2000; Labate et al. 2013; Roshan-Milani et al. 2003), schizophrenia (Tizabi 2007; Tregellas et al. 2010), and depression (Mineur et al. 2017), but being male or female also contributes to the risk of developing neurodevelopmental disorders, where there are known sex differences in their incidence, clinical manifestations, and therapeutic response (Savic and Engel 2014). It will be important to determine whether these sex differences in nAChR-mediated signaling contribute to differential regulation of HF network activity, so that we can better inform our efforts towards understanding the functional basis for neurological disorders.

6.3 Objective 3 Review

The objective of this study was to investigate whether ALLO modulates α4β2* nAChR function in mPFC layer VI principal neurons. As previous studies using mouse striatal and thalamic synaptosomes demonstrated that ALLO acts as a non-competitive inhibitor of nAChRs, we hypothesized that ALLO negatively modulates α4β2* nAChRs within living principal neurons. Using whole-cell electrophysiological recording of visually-identified layer VI pyramidal neurons located within acute mPFC slices of male and female mice aged P14-21, I demonstrated that ALLO exposure does indeed decrease α4β2* nAChR function in these neurons. By characterizing the
effect of ALLO in both males and females, I present in situ evidence of the modulatory effects of ALLO at three different concentrations, two of which are of physiological relevance. At 100 nM, ALLO decreases nicotinic signalling in the female mouse. I also demonstrated that the activation of the mPR complex mediates the inhibitory effects of ALLO at α4β2* nAChRs, and that this is facilitated by PKC. These results present the first demonstration of crosstalk between the mPR complex and nAChR systems in any system, and may therefore assist our understanding of how these two systems work together to regulate neuronal excitability, synaptic transmission, and synaptic plasticity to support medial prefrontal cognitive networks.

The finding that ALLO can inhibit α4β2* nAChR function in mPFC layer VI principal neurons has important implications. Various studies have demonstrated that the proper development of mPFC circuitry is dependent on nAChR-mediated signalling. The expression of α4β2* nAChRs in layer VI pyramidal neurons has been of particular interest as mice lacking the β2 nAChR subunit exhibit decreased attention performance and re-expressing β2-containing nAChRs in mPFC layer VI pyramidal neurons specifically restores attention performance (Guillem et al. 2011). Additionally, layer VI pyramidal neurons are also directly mediated by postsynaptic (α4β2)α5 nAChRs (Bailey et al. 2010; Kassam et al. 2008; Poorthuis et al. 2013b; Tian et al. 2011), and the stimulation of these (α4β2)α5 receptors appears to mediate the retraction of apical dendrites in the mPFC layer VI pyramidal neurons during postnatal maturation (Bailey et al. 2012). As the proper development of the mPFC circuitry is dependent on α4β2* nAChR–mediated signalling, altered levels of ALLO during development may have negative consequences on the development of the mPFC. For example, during periods of stress, there is an increase in the de novo production of ALLO, which may therefore influence the proper function of α4β2* nAChR–mediated signalling.
In this study, the finding that ALLO modulates nicotinic signalling through secondary messenger cascades provides us with an additional avenue towards understanding how ALLO can rapidly affect diverse aspects of neural physiology (Stromberg et al. 2006) and modulate stress (Gunn et al. 2015; Purdy et al. 1991), as well as how ALLO may be dysregulated in neurological disorders such as epilepsy (Smith et al. 2007) and Alzheimer’s disease (Irwin et al. 2011), which are also recognized diseases related to nAChR dysfunction.

6.4 General Discussion and Future Directions

Nicotinic signalling plays an important role in coordinating the response of neuronal networks in many brain regions. nAChRs are particularly important during pre- and postnatal circuit formation, whereby signalling by nAChRs influences neuronal survival (Bunker and Nishi 2002; Dajas-Bailador and Wonnacott 2004; Meriney et al. 1987), promotes neurite elongation (Coronas et al. 2000), determines dendritic shaft and spine synapses (Lozada et al. 2012a; b), and coordinates synchronized neuronal activity (Myers et al. 2005). Numerous findings suggest that nicotinic signalling is necessary for the proper function of both the HF and mPFC. Several studies also provide evidence of anatomical connections between the HF and the mPFC, which may serve to facilitate higher-order cognitive functions (Ferino et al. 1987; Hoover and Vertes 2007; Swanson 1981). Specifically, one pathway originates in the CA1 and SUB, and terminates at the infralimbic, prelimbic, and anterior cingulate mPFC (Cenquizca and Swanson 2007; Hoover and Vertes 2007; Swanson et al. 1981), while the other pathway originates in the mPFC, and can project to EC, and then converge at the dorsal HF (Cenquizca and Swanson 2007; Eichenbaum et al. 2007; Navawongse and Eichenbaum 2013; Vertes 2004). The circuitry between the HF and mPFC has been suggested to act as a functional loop that facilitates a bidirectional flow of information.
between the two regions in order to facilitate higher-order cognitive processes such as learning and memory, while damage to the loop has been linked to aberrant cognitive functions.

The work presented in this thesis serves to improve our understanding of how α4β2* nAChR-mediated signalling can facilitate the proper development and function of both the HF and mPFC in two fundamental ways.

First, the work conducted in this thesis demonstrates that functional heteromeric α4β2* nAChRs are present in CA1, CA3, DG, SUB, ECVI principal neurons during early postnatal development in both male and female mice. While the first week of early postnatal development in rodents corresponds to approximately the third trimester in humans, and the second to third postnatal week in rodents corresponds to early childhood in humans, key developmental processes during the first two weeks of young postnatal development include peak brain growth, increasing axonal and dendritic density, as well as synaptogenesis (Clancy et al. 2001; Clancy et al. 2007; Pressler and Auvin 2013; Semple et al. 2013; Workman et al. 2013). Based on the work presented in this thesis, I have proposed that the presence of α4β2* nAChR on young postnatal HF principal neurons during early development may serve to modulate network-driven GDPs, and therefore promote the proper development of synchronized activities in the mature brain. Previous studies have demonstrated that while excitatory GABAergic neurotransmission forms GDPs in the HF (Nardou et al. 2009; Sipila et al. 2005), both α7 and β2 subunit-containing nAChRs also exert regulatory actions on network-driven GDPs (Le Magueresse et al. 2006; Maggi et al. 2001). These findings complement the established functional role of α4β2* nAChRs in mPFC principal neurons, which is also to facilitate excitatory actions in the mammalian cognitive network (Bailey et al. 2012;
Guillem et al. 2011). Therefore, one implication of this work is that the functional presence of α4β2* nAChRs in excitatory principal neurons of both the HF and mPFC during early development can assist in the creation of a synchronized excitatory network between the HF and mPFC, which may be important in developing the synchronized networks of the mature brain. It could be assumed then, that the aberrant development of this α4β2* nAChR-facilitated system may lead to certain forms of cognitive deficits or in severe cases, neurological disorders and diseases. For example, during the second week of postnatal development where GABAergic and glutamatergic signalling exert excitatory responses, the HF is highly susceptible to seizures (Ben-Ari and Holmes 2006). Therefore, aberrant development of α4β2* nAChR-mediated signalling during early development may interfere with the development of neuronal circuits and increase the risk for seizures or related epileptic disorders. To delineate the cellular mechanisms underlying the function of HF and mPFC networks, it is critical to understand how synaptic inputs from various afferents are integrated and drive neuronal activity. Generally, using the rodent model, we can understand the connectivity of neurons by either simultaneously recording synaptically connected neurons, or measuring the response of a neuron that is connected to a presynaptic neuron that is activated by a stimulation electrode. Our understanding of neuronal function is likely to be aided by recent advances in methodology such as those that allow for the recording of simultaneous activity of a large number of neurons in distributed neuronal networks, or those that help to eliminate the technical limitation that do not allow for the activation of specific inputs in a slice preparation because of spatial segregation (Chen et al. 2017; Fiath et al. 2016; Parent et al. 2010). Together, these advances may serve to assist our understanding of how the functional circuitry between the HF and the mPFC is developmentally regulated or how they coordinate to mediate higher order cognitive processes.
As previously discussed, dysfunction in nicotinic signalling is implicated in an array of disorders involving learning and memory, and has been linked with a variety of developmental neurological disorders such as ASD, ADHD, epilepsy, schizophrenia and addiction (Changeux 2010; Drenan and Lester 2012; Gotti and Clementi 2004; Hurst et al. 2013; Loveland et al. 2008; Picciotto et al. 2012; Picciotto and Zoli 2008; Schumann et al. 2004). Furthering our understanding of the basic physiology of nAChRs during early development is critical in that it will aid in the development of therapeutics to treat or reduce the symptoms of nAChR dysfunction at an early stage. Particularly, knowing how, when and where these receptors are located will help us to understand how nicotine and/or other therapies targeting nAChRs may influence the function of cognitive circuits involving the HF and mPFC. For example, several studies have demonstrated the ability of nicotine to increase cognitive performance in ADHD patients (Plessen et al. 2006; Wilens and Decker 2007; Wilens et al. 2006) and clinical trials are being performed to test the efficacy of nAChR subtype specific agonists to increase cognitive performance in ADHD patients (Deutsch et al. 2010; Ghaleiha et al. 2013). Furthermore, schizophrenia patients exhibit deficits in cognition, alterations in the microcircuitry of the PFC, and multiple nAChRs have been linked to the disease. It is known that individuals with schizophrenia persistently seek out nicotine, potentially to compensate for the lower expression of nAChR, in order to improve cognitive functioning (Tizabi 2007; Wallace and Bertrand 2013). However, the precise deficits in nAChR signalling in these patients are unknown, and the mechanisms by which nicotine can exert relief for these individuals has yet to be discovered. Future research may be directed towards understanding how changes to α4β2* nAChR signalling in both the HF and mPFC in principal neurons during early development
may underlie the development of such neurological disorders and diseases, and assist in finding suitable avenues to treat and mitigate them.

Second, the work conducted in this thesis also demonstrates that ALLO negatively modulates α4β2* nAChR function in living neurons. By characterizing the response in both males and females, I present in situ evidence that physiological levels of ALLO modulate nAChR function. Particularly, at 100 nM, ALLO significantly decreases nicotinic currents in mPFC layer VI pyramidal neurons of the female mouse. ALLO levels not only increase in the CNS during different phases of the estrous cycle, but also increase during times of stress (Barbaccia et al. 1997; Lovick 2012; Purdy et al. 1991), and therefore alterations to its expression levels may have an important functional impact on nAChR-mediated signalling. As the HF and mPFC depend on the normal function of α4β2* nAChRs on its neurons, altered production of endogenous ALLO within these brain regions may have a significant negative impact on the development and function of these two regions.

The work in this thesis also demonstrates that the mPR complex may be involved in mediating the inhibitory effects of ALLO at α4β2* nAChRs, and that this is likely facilitated by PKC-dependent signalling. This is the first demonstration of crosstalk between the mPR complex and nAChR, and may therefore assist our understanding of how these two systems work together during early development to regulate neuronal excitability, synaptic transmission, and synaptic plasticity. Future work will be directed towards investigating whether this inhibition of nAChRs by ALLO is also present across other regions of the brain, such as in the HF, in order to fully understand its impact on normal brain function. As well, it will be important to conduct immunohistochemistry
or rt-PCR studies in order to determine the presence of the mPR complex on specific neuron subtypes in the HF and mPFC, including both pyramidal and interneurons, as well as in glia. This will help us to understand how ALLO precisely influences neuronal networks through the modulation of nicotinic receptor function. Currently, there are conflicting reports regarding the mechanisms by which the mPR complex mediates its downstream effects (Kasubuchi et al. 2017; Petersen et al. 2013; Smith et al. 2008; Thomas et al. 2014). It will therefore be important to determine whether the mPR complex mediates changes through GPCR mechanisms, or others, such as the adiponectin receptors, which are part of the PAQR family as it also contains 7 transmembrane domains but does not associate with G proteins, or through PGRMC1 affiliated mechanisms such as by association with P450 proteins. Pharmacological control studies or proteomic analysis can also serve to assist our understanding of which PKC isozymes facilitate this inhibition of α4β2* nAChR function. For example, the use of a pan-inhibitor of GPCR can first help to elucidate whether the observed effects are mediated by GPCR. This can be followed with the use of selective G protein inhibitors, such as those of the Gi pathway to narrow down the pathway towards PKC isozymes. The use of the PLC inhibitor U73122, or DAG inhibitor R59022 can also further elucidate the downstream pathway before utilizing specific inhibitors of PKC isozymes, such as the classical PKC isozymes which require Ca^{2+}, DAG, and a phospholipid for activation, novel PKC isozymes which require DAG but not Ca^{2+} for activation, and atypical PKC isozymes which do not require DAG or Ca^{2+} for activation. The complementary use of proteomics to measure phosphorylation of the nAChR subunits, or mPR subunits, can also be useful in clearly elucidating the role that ALLO plays modulating cellular functions. This will also be important as the actions of different isozymes have demonstrated unique functional outcomes (Lee et al. 2015; Nishizaki and Sumikawa 1998; Pollock et al. 2009). Finally, while this thesis presents one working
model for how ALLO can modulate α4β2* nAChR function, we have not eliminated the possibility that ALLO can also act directly at nAChRs, and therefore conducting binding assays to measure the allosteric modulation by ALLO of ACh binding to the nAChR will assist in our development of a more comprehensive picture of how ALLO inhibits α4β2* nAChR function in living neurons.

Together, the work presented in this thesis serves to improve our understanding of how α4β2* nAChR-mediated signalling may facilitate the proper development and function of the both the HF and mPFC, as well as support the development of strategies to improve early cognitive development or therapeutic treatments to reduce and/or mitigate neurological disorders that involve cognitive deficits.

6.5 Conclusions

The work presented in this thesis demonstrates that functional heteromeric α4β2* nAChRs are present in the developing mouse HF during a time when nicotinic signalling drives the development of this cognitive brain region. It also provides a characterization of α4β2* nAChR function in CA1, CA3, DG, SUB, and ECVI principal neurons during early development in both male and female mice. Finally, the work presented here provides evidence that α4β2* nAChR function in living neurons is negatively modulated by the neurosteroid ALLO during early development. Future work will explore in further detail the mechanisms by which ALLO negatively modulates α4β2* nAChR signalling and how this modulation influences the proper development and function of the HF and PFC. It is the overall aim of this thesis to add novel fundamental knowledge to improve our understanding of how α4β2* nAChR-mediated signalling
can facilitate the proper development and function of both the HF and mPFC, as well as to support the development of strategies to improve early cognitive development or identify therapeutic treatments to mitigate the impact of neurological disorders that involve cognitive functions.
References


Agster KL, and Burwell RD. Hippocampal and subicular efferents and afferents of the perirhinal, postrhinal, and entorhinal cortices of the rat. *Behavioural brain research* 254: 50-64, 2013.


Bell KA, Shim H, Chen CK, and McQuiston AR. Nicotinic excitatory postsynaptic potentials in hippocampal CA1 interneurons are predominantly mediated by nicotinic receptors that contain alpha4 and beta2 subunits. *Neuropharmacology* 61: 1379-1388, 2011.


Cheng Q, and Yakel JL. Activation of alpha7 nicotinic acetylcholine receptors increases intracellular cAMP levels via activation of AC1 in hippocampal neurons. *Neuropharmacology* 95: 405-414, 2015a.


Fanselow MS, and Dong HW. Are the dorsal and ventral hippocampus functionally distinct structures? Neuron 65: 7-19, 2010.


Fellous JM, and Sejnowski TJ. Cholinergic induction of oscillations in the hippocampal slice in the slow (0.5-2 Hz), theta (5-12 Hz), and gamma (35-70 Hz) bands. Hippocampus 10: 187-197, 2000.


Ghaleiha A, Ghayasvand M, Mohammadi MR, Farokhnia M, Yadegari N, Tabrizi M, Hajiaghaee R, Yekehtaz H, and Akhondzadeh S. Galantamine efficacy and tolerability as an


Grady SR, Wageman CR, Patzlaff NE, and Marks MJ. Low concentrations of nicotine differentially desensitize nicotinic acetylcholine receptors that include alpha5 or alpha6 subunits


**Gu N, Hu H, Vervaeke K, and Storm JF.** SK (KCa2) channels do not control somatic excitability in CA1 pyramidal neurons but can be activated by dendritic excitatory synapses and regulate their impact. *J Neurophysiol* 100: 2589-2604, 2008.


Gustafsson B, and Wigstrom H. Hyperpolarization following long-lasting tetanic activation of hippocampal pyramidal cells. *Brain research* 275: 159-163, 1983.


Huang GZ, and Woolley CS. Estradiol acutely suppresses inhibition in the hippocampus through a sex-specific endocannabinoid and mGluR-dependent mechanism. *Neuron* 74: 801-808, 2012.


Intlekofer KA, and Petersen SL. Distribution of mRNAs encoding classical progestin receptor, progesterone membrane components 1 and 2, serpine mRNA binding protein 1, and progestin and ADIPOQ receptor family members 7 and 8 in rat forebrain. *Neuroscience* 172: 55-65, 2011.


Kalappa BI, Gusev AG, and Uteshev VV. Activation of functional alpha7-containing nAChRs in hippocampal CA1 pyramidal neurons by physiological levels of choline in the presence of PNU-120596. *PloS one* 5: e13964, 2010.


Kenney JW, Poole RL, Adoff MD, Logue SF, and Gould TJ. Learning and nicotine interact to increase CREB phosphorylation at the jnk1 promoter in the hippocampus. PloS one 7: e39939, 2012a.


Mineur YS, Mose TN, Blakeman S, and Picciotto MR. Hippocampal alpha7 nicotinic ACh receptors contribute to modulation of depression-like behaviour in C57BL/6J mice. *British journal of pharmacology* 2017.


Muir JL, Everitt BJ, and Robbins TW. The cerebral cortex of the rat and visual attentional function: dissociable effects of mediofrontal, cingulate, anterior dorsolateral, and parietal cortex


Naber PA, Lopes da Silva FH, and Witter MP. Reciprocal connections between the entorhinal cortex and hippocampal fields CA1 and the subiculum are in register with the projections from CA1 to the subiculum. *Hippocampus* 11: 99-104, 2001a.


Picciotto MR, and Zoli M. Neuroprotection via nAChRs: the role of nAChRs in neurodegenerative disorders such as Alzheimer's and Parkinson's disease. Front Biosci 13: 492-504, 2008.


Reynolds GP, Czudek C, and Andrews HB. Deficit and hemispheric asymmetry of GABA uptake sites in the hippocampus in schizophrenia. Biological psychiatry 27: 1038-1044, 1990.


Semenova S, and Markou A. The effects of the mGluR5 antagonist MPEP and the mGluR2/3 antagonist LY341495 on rats' performance in the 5-choice serial reaction time task. *Neuropsychopharmacology* 52: 863-872, 2007.


Shen JX, Tu B, and Yakel JL. Inhibition of alpha 7-containing nicotinic ACh receptors by muscarinic M1 ACh receptors in rat hippocampal CA1 interneurons in slices. *The Journal of physiology* 587: 1033-1042, 2009.


Sun HY, and Goodkin HP. The pervasive reduction of GABA-mediated synaptic inhibition of principal neurons in the hippocampus during status epilepticus. *Epilepsy research* 119: 30-33, 2016.

Sun Y, Yang Y, Galvin VC, Yang S, Arnsten AF, and Wang M. Nicotinic alpha4beta2 Cholinergic Receptor Influences on Dorsolateral Prefrontal Cortical Neuronal Firing during a


**Sutherland RJ, McDonald RJ, and Savage DD.** Prenatal exposure to moderate levels of ethanol can have long-lasting effects on hippocampal synaptic plasticity in adult offspring. *Hippocampus* 7: 232-238, 1997.


Thomas P. Characteristics of membrane progestin receptor alpha (mPRalpha) and progesterone membrane receptor component 1 (PGMRC1) and their roles in mediating rapid progestin actions. *Front Neuroendocrinol* 29: 292-312, 2008.


Wallace TL, and Bertrand D. Alpha7 neuronal nicotinic receptors as a drug target in schizophrenia. Expert Opin Ther Targets 17: 139-155, 2013.


