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Nicotinic Signaling: Alpha3 Beta4 Heteromers, Alpha5 Subunits, And The Prototoxin Lypd6b

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NICOTINIC SIGNALING: ALPHA3 BETA4 HETERO HETEROMERS, ALPHA5 SUBUNITS, AND THE PROTOTOXIN LYPD6B

A Dissertation Presented

by

Vanessa Ochoa

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ABSTRACT

Prototoxin proteins have been identified as members of the Ly6/uPAR super family whose three-finger motif resembles that of αbungarotoxin. Though they are known to modify the function of nAChRs, their specificity is still unclear. Our lab identified three prototoxin proteins in the chicken ciliary ganglion: Ch3ly, Ch5ly, and Ch6ly. Ch6ly was later identified as prostate stem cell antigen (PSCA), and specifically decreased the amount of calcium influx through the homomeric α7 nAChR subtype. I then identified Ch3ly and Ch5ly as LY6E and LYPD6B, respectively. I focused my attention on LYPD6B because of its expression in the brain. This dissertation tests whether LYPD6B is a prototoxin protein that specifically co-localizes with and modifies the function of the heteromeric α3β4* nAChRs (the other nAChR subtype expressed in the chicken ciliary ganglia). In the first part of my dissertation I performed intracellular two-electrode voltage clamp on Xenopus oocytes co-expressing human LYPD6B and different stoichiometries of the α3β4* nAChR, these included two (α3)2(β4)3 with β4–α3–β4–β4–α3 and β4–α3–β4–α3–β4 stoichiometries, two (α3)3(β4)2 with stoichiometries β4–α3–α3–β4–α3 and β4–α3–β4–α3–α3, two (α3β4)2(α5D) β4–α3–α5D–β4–α3 and β4–α3–η–α3–α5D, and (α3β4)2(α5N) with stoichiometries β4–α3–α5N–β4–α3 and β4–α3–β4–α3–α5N. Concatemeric constructs are designed to link nAChR subunits, thus when translated it is done so as a single polypeptide. LYPD6B increased the acetylcholine (ACh) potency and desensitization rate, but decreased the maximum current response (Imax) for the (α3)3(β4)2 nAChR subtype. Yet, LYPD6B only decreased the Imax for the (α3β4)α5 D-variant and not the N-variant (associated with increase nicotine consumption). For the second part of my dissertation, I determined if the expression of LYPD6B correlated with nAChRs in an activity dependent manner. Though LYPD6B mRNA expression correlates with nAChR subunit mRNA expression levels, it seemed to be independent of nAChR activity. To determine if fluorescent co-localization occurs between LYPD6B and a specific nAChR subtype, I genetically engineered LYPD6B to express a human influenza hemagglutinin (HA) epitope tag and cloned into a chicken retrovirus. LYPD6B was shown to co-localize only with the α3β4* heteromeric and not the homomeric α7 nAChRs, in a nAChR activity dependent manner. This study adds to the complexity of a prototoxin’s function by suggesting that the specificity is dependent on nAChR type and stoichiometry. It is the first in identifying a prototoxin protein, LYPD6B, which specifically modulates the function of the (α3)(β4), and (α3β4)2(α5 D-variant) heteromeric nAChR subtypes. For the (α3β4)2(α5 D-variant) nAChR subtype LYPD6B decreased the Imax. Such observation may be telling of a novel mechanism involved with nicotine dependence. For the (α3)(β4) nAChR subtype LYPD6B increases its ACh sensitivity, desensitization rate, while decreasing Imax. Additionally, the co-localization of LYPD6B and α3β4* nAChRs in the lack of nAChR activity highlights the relevance of the functional effects α3β4* nAChRs exhibit due to LYPD6B. Such relevance may be the utilization of limiting ACh amounts.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF TABLES</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>vi</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LIST OF FIGURES</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>vii</td>
</tr>
</tbody>
</table>

## CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Nicotinic acetylcholine receptors (nAChRs)</td>
<td>2</td>
</tr>
<tr>
<td>α5 subunit containing nAChRs</td>
<td>10</td>
</tr>
<tr>
<td>The Ly6 Superfamily</td>
<td>12</td>
</tr>
<tr>
<td>Prototoxin proteins are part of the Ly6 Superfamily</td>
<td>14</td>
</tr>
<tr>
<td>The avian ciliary ganglion as a model for studying nAChRs</td>
<td>18</td>
</tr>
<tr>
<td>Purpose of this research</td>
<td>21</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>22</td>
</tr>
</tbody>
</table>

## CHAPTER 2: THE PROTOTOXIN LYPD6B MODULATES HETEROMERIC ALPHA3 BETA4 CONTAINING NICOTINIC ACETYLCHOLINE RECEPTORS (NACHRS) BUT NOT ALPHA7 HOMOMERS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>36</td>
</tr>
<tr>
<td>Introduction</td>
<td>37</td>
</tr>
<tr>
<td>Material and Methods</td>
<td>40</td>
</tr>
</tbody>
</table>
CHAPTER 3: EXPRESSION OF LYPD6B AND COLOCALIZATION WITH ALPHA3 CONTAINING NICOTINIC ACETYLCHOLINE IN PARASYMPATHETIC NEURONS

Abstract .............................................................................................................. 82

Introduction ......................................................................................................... 83

Material and Methods .......................................................................................... 87

Results ................................................................................................................... 94

Transcript expression and immunofluorescence of LYPD6B in the presence and absence of nAChR activity .................................................................................. 94
LYPD6B specifically co-localizes with a3* heteromeric nAChRs ......................... 95

Discussion ............................................................................................................ 97

Literature Cited .................................................................................................... 102

Tables ................................................................................................................... 108
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPTER 4: SUMMARY AND CONCLUSIONS</td>
<td>114</td>
</tr>
<tr>
<td>Significance of the results</td>
<td>118</td>
</tr>
<tr>
<td>Future Directions</td>
<td>126</td>
</tr>
<tr>
<td>Comprehensive Bibliography</td>
<td>128</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>153</td>
</tr>
<tr>
<td>Abstract</td>
<td>154</td>
</tr>
<tr>
<td>Introduction</td>
<td>155</td>
</tr>
<tr>
<td>Material and Methods</td>
<td>157</td>
</tr>
<tr>
<td>Results</td>
<td>159</td>
</tr>
<tr>
<td>Discussion</td>
<td>160</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>163</td>
</tr>
<tr>
<td>Tables</td>
<td>165</td>
</tr>
<tr>
<td>Figures</td>
<td>166</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

### CHAPTER 2

| Table 1 | The effects of LYPD6B on functional characteristics of nAChR concatemers | 69 |

### CHAPTER 3

| Table 1 | α3* nAChRs but not α7 colocalize with LYPD6B | 108 |

### APPENDIX

| Table 1 | All α7β2α5 nAChR subtypes respond to MG 624 similarly | 165 |
### LIST OF FIGURES

**CHAPTER 2**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Alignment of amino acid sequences for LY6E and LYPD6B from Gallus gallus and Homosapiens</td>
<td>71</td>
</tr>
<tr>
<td>Figure 2</td>
<td>A LYPD6B decreases ACh Log EC50 for ((\alpha 3)_3(\beta 4)_2) heteromeric nAChRs</td>
<td>72</td>
</tr>
<tr>
<td>Figure 3</td>
<td>LYPD6B does not affect the ACh Log EC50 for (\alpha 3\beta 4\alpha 5) heteromeric nAChRs</td>
<td>73</td>
</tr>
<tr>
<td>Figure 4</td>
<td>LYPD6B does not affect the ACh Log EC50 for (\alpha 7) homomeric nAChRs</td>
<td>74</td>
</tr>
<tr>
<td>Figure 5</td>
<td>LYPD6B decreases the ACh induced maximum current response for ((\alpha 3)_3(\beta 4)_2)</td>
<td>75</td>
</tr>
<tr>
<td>Figure 6</td>
<td>LYPD6B affects the ACh induced maximum current response for (\alpha 3\beta 4\alpha 5)D-vanriant nAChRs</td>
<td>77</td>
</tr>
<tr>
<td>Figure 7</td>
<td>LYPD6B decreased the time constant ((\tau)) from steady-state desensitization for (\alpha 3\beta 4) (*) heteromeric nAChR concatemers</td>
<td>78</td>
</tr>
<tr>
<td>Figure 8</td>
<td>LYPD6B did not affect the time constant ((\tau)) from steady-state desensitization for (\alpha 3\beta 4\alpha 5) heteromeric nAChR concatemers</td>
<td>79</td>
</tr>
<tr>
<td>Supplemental figure 1</td>
<td>There is no difference in relative fluorescence values in oocytes expressing concatemers that exhibited a decrease in ACh maximum induced current response due to LYPD6B</td>
<td>80</td>
</tr>
</tbody>
</table>
CHAPTER 3

Figure 1. Lack of nAChR activity increases nAChR subunit mRNA levels, but does not increase LYPD6B........................................109

Figure 2. Genetically engineered LYPD6B expresses HA epitope tag.................................................................110

Figure 3. LYPD6B colocalizes with α3* nAChRs in the ciliary ganglia of avian embryos kept in constant darkness...............111

Figure 4. LYPD6B colocalizes with α3* nAChRs but not α7 in the ciliary ganglia of avian embryos kept in constant darkness and light.................................................................112

APPENDIX

Figure 1. Determining CHRNA5 levels in neuroblastoma cell lines and testing MG 624 efficacy.................................166

Figure 2. Quantitative PCR on shRNA transfected SH-EP cells.................................................................167

Figure 3. CHRNA5 knockdown in SH-EP cells did not affect proliferation rate, MG 624 or MLA.................................168

Figure 4. The LogIC50 did not shift for the different α7β2α5 nAChR stoichiometries.........................................................169
CHAPTER 1

COMPREHENSIVE LITERATURE REVIEW

I. Introduction

In the simplest terms, the cholinergic system is defined by the cholinergic neurons that transmit and respond to action potentials, and the proteins involved in mediating such signals. The neurotransmitter within the brain that triggers a cholinergic synaptic response is acetylcholine (ACh; Albuquerque et al., 2009). The enzyme choline acetyltransferase synthesizes acetylcholine by transferring an acetyl group from acetyl-CoA to choline (Albuquerque et al., 2009). The termination of a cholinergic response occurs with the breakdown of ACh into its two components by the enzyme acetylcholinesterase (Albuquerque et al., 2009). There are two types of receptors that are activated by ACh and mediate cholinergic signaling, muscarinic and nicotinic acetylcholine receptors (Albuquerque et al. 2009). The muscarinic acetylcholine receptors (mAChRs) are coupled to heterotrimeric guanine nucleotide-binding proteins (G proteins), which allow them to regulate second messenger activity (Eglen, 2006). Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels (Albuquerque et al., 2009). Both receptor types are located pre- and postsynaptically with a discrete expression pattern both in the central and peripheral nervous system. Thus, the cholinergic system is known to be involved in an array of human behaviors. In our lab we
study the cholinergic system at the level of the nicotinic acetylcholine receptors (nAChRs).

II. Nicotinic acetylcholine receptors (nAChRs)

Nicotinic acetylcholine receptors (nAChRs) are a diverse group of ligand-gated ion channels. Based on evolutionary and physiological properties, nAChRs have been divided into three different categories (Le Novere and Changeux, 1995): 1) Skeletal muscle nAChRs, made up of α, β, γ, or ε subunits, 2) neuronal heteromeric nAChRs, made up of a combination of α2–α6 and β2–β4 subunits, and 3) neuronal homomeric nAChRs, made up of all α7, α8, α9, or α10 subunits. However, the α7 subunit has also been shown to form a receptor with the β2 subunit (Thomsen et al., 2015). All nAChRs are pentameric, composed of five subunits (Cooper et al., 1991). Each subunit has an extracellular NH$_2$-terminal domain of about 200 amino acids, four transmembrane domains (TM), two intracellular loops (the second intracellular loop is located between the third and fourth TM and varies in size), and a relatively short extracellular COOH-terminal (Albuquerque et al., 2009). Though certain nAChRs are more permeable to particular cations, in general the TM2 forms a hydrophilic non-selective cation pore (Albuquerque et al., 2009). The binding of the endogenous ligand ACh will activate the receptor, opening the pore, and allowing the flow of cations (Le Novere et al., 2002).
The ligand-binding site is a hydrophobic pocket formed between two neighboring subunits, the positive and negative face, which are defined by specific characteristics. A conserved property within the extracellular NH$_2$-terminal towards TM1 is a cysteine-cysteine pair separated by 13 amino acids, the pair forms a Cys-loop due to disulfide bonding (Karlin et al., 1986). This characteristic defines an $\alpha$ subunit, determines ligand-binding site, and is termed as the positive face of a nAChR subunit (Lukas et al., 1999).

Additionally, $\alpha$ subunits (with the exception of $\alpha5$) express hydrophobic aromatic amino acids required for ligand binding (Karlin et al., 1986; Sine and Engel, 2006). The $\beta$ subunit expresses three essential amino acids, leucine 112, methionine 114, and tryptophan in position 53, which are required for ligand recognition and is known as the negative face of a nAChR subunit (Sine and Engel, 2006). The $\alpha$ subunits that form homomeric nAChRs contain both positive and negative face characteristics (Albuquerque et al., 2009).

Although neuronal heteromeric and homomeric nAChRs share common physiological features, the specific properties of each receptor state differ between the two nAChR subtypes. Both receptor subtypes may physiologically exist and spontaneously interchange among three different conformational states: resting (closed), active (open), and desensitized (closed) (Le Novere et al., 2002). A nAChR exists in the closed state in the absence of ligand. In a closed state there are two factors that do not allow the passage of cations. 1) The diameter of the pore and 2) The hydrophobic amino acids of all five TM2 domains, which face the pore of the receptor, thus acting as a barrier (Albuquerque
et al., 2009). A nAChR may enter the open state when a ligand such as ACh is bound. However, one characteristic that affects the ease of ligand binding is the receptor’s affinity towards that particular ligand. Studies have revealed that both the α and β subunits affect the potency of multiple agonists (Luetje and Patrick, 1991). Two-electrode voltage clamp recordings, done on Xenopus oocytes injected with a 1:1 cRNA concentration ratio of human α2, α3, α4, β2, and β4 nAChR subunits, compared agonist potency by comparing the normalized partial dose-response curves of four agonists (ACh, nicotine, cytisine, and DMPP) for the α2β2, α2β4, α3β2, α3β4, α4β2, α4β4 nAChRs (Luetje and Patrick, 1991). For example, the nAChRs expressing a β4 subunit (α2β4, α3β4, α4β4) have a greater affinity to cytisine than the other agonists tested (Luetje and Patrick, 1991). Additionally, nAChRs have different sensitivities to antagonists, including α-bungarotoxin (Loring and Zigmond, 1988). Also, the ease of ligand binding is affected by the cooperative binding property. If the binding of a ligand to a receptor further increases the receptor’s affinity to the ligand, then this is termed as cooperative ligand binding and evident through the hill coefficient. A greater hill coefficient indicates positive cooperative binding and a smaller hill coefficient indicates less cooperative binding. The nAChRs that exhibit the greatest cooperative binding to ACh are as follows: α3β4, α2β4, homomeric α7, α4β4, α2β2, α3β2, and α4β2 (Elliott et al., 1996). When nAChRs enter the open state, cations flow across the membrane, as previously mentioned. When considering the open state of a nAChR, there are two channel properties that affect the cation flow: 1) the duration of open time and
2) conductance of the channel. In general nAChRs with varying $\alpha$ and $\beta$ subunits exhibit a <0.1-8 ms open time duration with a conductance’s ranging from 5-45 ps (Role, 1992). In the presence of prolonged ligand exposure, nAChRs may transition from an open state to a desensitization state. The order of desensitization rates from fastest to slowest is as follows: homomeric $\alpha_7$, heteromeric $\alpha_3\beta_2$, $\alpha_4\beta_2$, $\alpha_3\beta_4$, $\alpha_2\beta_2$, $\alpha_4\beta_4$, and $\alpha_2\beta_4$ (Elliott et al., 1996).

Nicotinic AChRs also express allosteric effector binding sites, which have the ability to modulate the properties of each physiological state. The binding of an effector to an allosteric binding site modifies the energy barriers between any two given physiological states (Bertrand and Gopalakrishnan, 2007). Positive allosteric modulators (PAM) lower the energy barrier between the closed and open state, and increase an open state response in the presence of agonist (Bertrand and Gopalakrishnan, 2007). Thus, there are two types of PAMs. Type I will increase the peak current response either by transiting the receptor into an open state at lower agonist concentrations (increasing agonist potency) or stabilizing the open state of the receptor in response to agonist (Bertrand and Gopalakrishnan, 2007). Type II will increase the peak current response and change the desensitization profile in response to agonist (Bertrand and Gopalakrishnan, 2007). Negative allosteric modulators (NAM) increase the energy barrier between the closed and open state, and decrease an open state response in the presence of agonist (Bertrand and Gopalakrishnan, 2007).
The expression of the different nAChR subtypes is regulated. The first step in regulating nAChR expression begins at the DNA level. Although there is not much evidence specifically identifying transcription factors that regulate the expression of nAChRs, there is some research investigating the α3/β4/α5 gene cluster and their coordinated expression. The CHRNA3/CHRNB4/CHRNA5 gene organization is located on chromosome 15q24 and will express α3/β4/α5 nAChR subunits respectively (Duga et al., 2001). The CHRNA3 and CHRNB4 transcripts are transcribed in the same direction, whereas CHRNA5 is transcribed in the opposing direction (Duga et al., 2001). Furthermore, the 3’ tail end of the CHRNA5 transcript overlaps the 3’ tail end of the CHRNA3 transcript (Duga et al., 2001). The coordinated expression of the gene cluster is regulated by upstream promoter elements. Studying β43’ enhancer’s involvement in regulating the CHRNA3/CHRNB4/CHRNA5 gene cluster transcription followed its identification upstream of the 3’ end of the CHRNB4 transcript (McDonough and Deneris, 1997). By producing transgeneic mice that express a plasmid with a mutated β43’ enhancer, Xu et al. determined a decrease in the expression of CHRNA3, CHRNB4, and CHRNA5 transcripts (Xu et al., 2006). The group also identified conserved noncoding region 4 (CNR4) to regulate the expression of CHRNA3 and CHRNB4, but not CHRNA5 (Xu et al., 2006). Other evidence has also determined that the expression of CHRNA3 and CHRNB4 transcripts are regulated through the Ras-dependent MAPK pathway via NGF activation, and that the expression may be increased or decreased depending on the cell type (Nakayama et al., 2000). The second step in regulating the expression of nAChRs is the proper folding and assembly of the subunits, which occurs
in the endoplasmic reticulum. There is evidence identifying essential amino acids within the NH₂ extracellular terminal, of the α7 and α3 nAChR subunits, in regulating proper subunit interactions for the formation of the functional homomeric α7 and heteromeric α3 nAChRs (Dunckley et al., 2003; Wanamaker et al., 2003). The third step in regulating the expression of nAChRs is at the level of embedding functional receptors into the plasma membrane. One way, cholinergic neurons regulate the appropriate folding and monitor glycosylation for proper nAChR plasma membrane insertion is through chaperone proteins. A well document endogenous nAChR chaperone protein is RIC3. By recording α7 nAChR currents, performing a co-immunoprecipitation, and quantifying radiolabeled alpha-bungarotoxin from α7 and RIC3 co-transfected human kidney tsA201 cells, Lansdell et al. determined RIC3 to increase α7 nAChR expression and function, thus identifying RIC3 as an α7 nAChR chaperone protein (Lansdell et al., 2005). Yet, exogenous molecules also behave as chaperone proteins and upregulate the expression of nAChRs. An example of this is nicotine. Nicotine is not only known to upregulate the expression of the α4β2 nAChRs it is also believed to favor a higher nicotine affinity subtype, possibly by facilitating the formation of a specific α4β2 nAChR stoichiometry (Buisson and Bertrand, 2002; Moroni et al., 2006; Wonnacott, 1990). Though chaperone proteins have the ability to facilitate proper nAChR plasma membrane insertion, large amounts of evidence identify the majority of nAChRs to be kept in intracellular pools as opposed to being sent to lysosomes, and inserted into the plasma membrane, if conditions
permit (Fenster et al., 1999b; Jacob et al., 1986; Pakkanen et al., 2006; Whiteaker et al., 1998).

Where in the nervous system and at the neuronal cellular level the different nAChR subtypes are expressed has an impact on biological functions. The most abundantly expressed nAChRs, in the CNS, are the homomeric \( \alpha_7 \) and the heteromeric \( \alpha_4\beta_2 \) receptors (Couturier et al., 1990; Schoepfer et al., 1988). There are a few nuclei (e.g. medial habenula) within the brain that express distinct nAChRs; these include the \( \alpha_3\beta_4 \) and \( \alpha_3\beta_4\alpha_5 \) (Scarr et al., 2013). The ventral tegmental area is known to have high levels of the \( \alpha_6 \) and \( \beta_3 \) nAChR subunits (Scarr et al., 2013). In the CNS, nAChRs are expressed at presynaptic terminals, and modulate synaptic transmission by regulating the release of neurotransmitters. By radiolabeling the alpha-bungarotoxin and nicotine-binding sites, nAChRs at presynaptic terminals were identified within the interpeduncular nucleus and dopaminergic projections to striatum and accumbens (Clarke et al., 1986; Clarke et al., 1987; Clarke and Pert, 1985). Additionally, studying synaptosome preparations revealed that presynaptic nAChRs regulate the release of norepinephrine, dopamine, GABA, serotonin, and ACh (el-Bizri and Clarke, 1994; King, 1990; Lapchak et al., 1989; Lena et al., 1993). Nicotinic AChRs at the postsynaptic level involved in mediating fast EPSPs in the CNS have been difficult to identify (Role and Berg, 1996). A reason for this might be the location of cholinergic projections, which are known to be deep within the limbic system (Role and Berg, 1996). Another, reason may be their implications in other functions besides fast EPSPs. In contrast to the CNS, the autonomic nervous system
abundantly expresses the α3 containing nAChRs; these include a combination of α3β4, α3β4α5, and sometimes α3β2 (Conroy and Berg, 1995; Vernallis et al., 1993). They are located postsynaptically and involved in mediating nicotinic transmission (Langley and Anderson, 1892).

Non-traditional functions of nAChRs such as gene transcription, neuron survival, dendritic branching, and facilitating the reversal of the Cl⁻ gradients, may be explained through their high permeability to Ca²⁺. Although nAChRs are considered non-selective cations, they can be more permeable to Ca²⁺ than Na⁺ and K⁺. This is particularly true for the α7 nAChR (Castro and Albuquerque, 1995). The α7 nAChR has the highest permeability to Ca²⁺ permeability of all nAChRs (Role and Berg, 1996). Additionally, the Ca²⁺ permeability is independent of membrane potential unlike the N-methyl-D-aspartic acid (NMDA) receptors, which are blocked by Mg²⁺ at resting membrane potentials (Role and Berg, 1996). The Ca²⁺ influx through nAChRs is known to switch on multiple cellular pathways. In differentiated PC12 cells, a 100μM nicotine exposure increased the mRNA levels of the c-fos transcription factor (Greenberg et al., 1986). Furthermore, when those cells were exposed to ethylene glycol tetraacetic acid (EGTA), a Ca²⁺ chelating agent, no increase in c-fos transcript was observed, thus the increase in c-fos via activation of nAChRs is Ca²⁺ dependent (Greenberg et al., 1986). Additionally, the influx of Ca²⁺ through activated nAChRs is known to activate protein kinase such as calmodulin-dependent protein kinase, phosphoinositide 3-kinase, protein kinase C, and protein kinase A (Albuquerque et al., 2009). In turn these kinases activate the
transcription factor, cAMP response element binding protein (CREB) (Albuquerque et al., 2009).

III. The $\alpha_5$ nAChR subunit

Structurally the $\alpha_5$ subunit resembles the other $\alpha$ nAChR subunits, but functionally it lacks a ligand-binding site. It is considered an $\alpha$ subunit because it contains the cysteine-cysteine pair at the NH$_2$- extracellular terminal (Albuquerque et al., 2009). However, a key polar ligand-binding amino acid, tyrosine, is replaced by the charged amino acid, aspartic acid (Albuquerque et al., 2009) so ACh cannot bind. Thus, in order for the $\alpha_5$ subunit to form a functional nAChR, it must be incorporated into the pentamer in combination with other alpha subunits. There is evidence that $\alpha_5$ has the ability to form a nAChR with the $\alpha_3$, $\beta_4$, and $\beta_2$ subunits (Girod et al., 1999; Yu and Role, 1998). There is also evidence that $\alpha_5$ forms a receptor with the $\alpha_7$ subunit (Anand et al., 1993; Girod et al., 1999; Listerud et al., 1991).

The $\alpha_5$ subunit is known as a modulator because channel properties are modified when it is incorporated into an nAChR pentamer. A larger conductance and low ACh affinity occurs in the heteromeric $\alpha_3\beta_4$ and $\alpha_3\beta_2$ nAChRs when combined with the $\alpha_5$ subunit (Yu and Role, 1998). Furthermore, the addition of the $\alpha_5$ subunit to nAChRs containing
the α3 subunit causes a significant increase in calcium influx compared to the homomeric α7 nAChR (Yu and Role, 1998).

The α5 nAChR subunit has an impact on human behavior. The α5 nAChR subunit was first identified to be associated with nicotine dependence through a genome wide association study (GWAS). The GWAS was performed on 1929 subjects, and a single nucleotide polymorphism (SNP) located within the α5 nAChR subunit gene (CHRNA5) was identified (Bierut et al., 2007; Bierut et al., 2008; Saccone et al., 2007). The SNP replaces the 398th amino acid from an aspartic amino acid (D-variant) to asparagine (N-variant) (Bierut et al., 2008), and it is located within the second major intracellular loop of the nAChR subunit (Hung et al., 2008). The SNP located within CHRNA5 is noted as α5(D398N) (Bierut et al., 2008).

Although the significance of the SNP located in the second intracellular loop of the human α5 nAChR subunit is unclear, animal models have elucidated some functional and behavioral consequences. In order to determine the functional role of the α5 N-variant subunit included in the heteromeric α3β4 nAChR, intracellular two-electrode voltage clamp was conducted on Xenopus oocytes expressing the human receptor (George et al., 2012). The inclusion of the α5 N-variant did not change the acetylcholine dose response curve when compared to α3β4 nAChRs including the α5 D-variant (George et al., 2012). However, it reduces the maximum acetylcholine induced current response (George et al.,
2012). The behavioral consequences of the α5 N-variant can be inferred through the CHRNA5 knockout mouse. The CHRNA5 knockout mouse continues to consume high concentrations of nicotine in comparison to the wild type (Fowler et al., 2011), this behavior was also reproduced by knocking down the CHRNA5 in the medial habenula (MHb) of rats (Fowler et al., 2011). It was concluded that the α5 nAChR subunit is required and necessary in the inclusion of nAChRs involved in the inhibitory pathway of high nicotine consumption, but not in the rewarding pathway (Fowler et al., 2011; Fowler et al., 2013). This data is indicative of nAChR networks that include the α5 subunit to be involved in smoking behavior.

IV. The Ly6 Superfamily

The superfamily Ly6 is a large family made up of proteins with specific characteristics. Molecules from the Ly6 superfamily have a small molecular weight ranging from 12-20 KD and conserved 8-10 cysteine residues that form specific disulfide bonds. Commonly, they are phosphatidyl inositol anchored to cell surface glycoprotein’s, although they may also be secreted, and are encoded by murine chromosome 15 (Gumley et al., 1995). Initial isolation of Ly6 like proteins occurred in the immune system of the mouse (Gumley et al., 1995). In general, their functions were determined as T-cell activation, lymphocyte homing, and leukocyte migration (Gumley et al., 1995; Hanninen et al., 1997). Since then, the superfamily of Ly6 like molecules has expanded and now includes proteins from invertebrates such as Drosophila and Caenorhabditis elegans to vertebrates.
such as rat and human (urokinase plasminogen activator receptor uPAR) (Gumley et al., 1995).

Interestingly, neurotoxins such as α-bungarotoxin and cobratoxin have the Ly6 superfamily characteristics. Neurotoxins have been isolated from the venom of multiple snake families (Endo and Tamiya, 1987). They were first identified as competitive antagonists of the nAChRs found at the motor end-plate of the neuromuscular junction (Endo and Tamiya, 1987). There are two groups of neurotoxins, short chain and long chain (Endo and Tamiya, 1987). The short chain neurotoxins are made up of 60-62 amino acids and have 4 conserved disulfide bonds formed between cysteine residues (Tsetlin, 2015). The long chain neurotoxins are made up of 66-75 amino acids with 5 conserved disulfides bonds and a longer C-terminal tail (Tsetlin, 2015). These characteristics allow both the short and long chain neurotoxins to fold into a three-fingered beta structural loop motif (Tsetlin, 2015). This secondary protein structure is formed from their 4 conserved disulfide bonds (Tsetlin, 2015). The short and long chain neurotoxins are known to bind to the α subunit of the muscle nAChRs with high affinity (Endo and Tamiya, 1987). However, only the long chain neurotoxins such as alpha-bungarotoxin and cobratoxin are known to bind to the neuronal homomeric α7 nAChRs (Endo and Tamiya, 1987). X-ray and NMR structures have identified the second fingered loop of the long chain neurotoxins to interact with the extracellular NH₂-terminal agonist binding site of the α nAChR subunit (Tsetlin, 2015).
V. Prototoxin proteins are part of the Ly6 Superfamily

Proteins identified as part of the Ly6 superfamily and expressed in the nervous system are termed prototoxins. Because they share protein characteristics with neurotoxins such as α-bungarotoxin, it is hypothesized that many prototoxins modulate nAChRs. The GPI-linked prototoxins studied intensively are LYNX1, LYNX2, LYPD6, and prostate stem cell antigen (PSCA) (Darvas et al., 2009; Dessaud et al., 2006; Hruska et al., 2009; Miwa et al., 1999).

LYNX1 was the first Ly-6-like molecule to be identified as a prototoxin. The idea that LYNX1 interacts with nAChRs was first supported by its localization. Through in-situ hybridization performed on mice brain slices, LYNX1 mRNA was identified in the purkinje cell body layer of the cerebellum, deep cortex layers such as layers V and VI, CA3 pyramidal neurons of the hippocampal formation, and mitral cells of the olfactory bulb (Miwa et al., 1999). The expression pattern of LYNX1 was similar to the expression pattern of the α4β2 and α7 nAChRs. Thus, immunofluorescently stained HEK293 cells transfected with the α4β2 nAChRs and LYNX1 showed co-localization (Ibanez-Tallon et al., 2002). Additionally, hippocampal CA3, pyramidal, and amygdala neurons stained positive for α7 and LYNX1 (Ibanez-Tallon et al., 2002). Following its localization, LYNX1 was established it as a nAChR allosteric modulator. Macroscopic currents from *Xenopus* oocytes injected with α4 nAChR subunit, β2 nAChR subunit, and LYNX1
cRNA exhibited a decrease in the fast time constant (tau) of desensitization (Ibanez-Tallon et al., 2002). Also, the ACh EC50 was increased (Ibanez-Tallon et al., 2002). Collectively, this data was interpreted as LYNX1 increasing the desensitization rate of α4β2 while decreasing its sensitivity to ACh. This conclusion is further supported by whole cell recordings from neurons located in the medial habenula of the LYNX1 knockout mouse. When 20μM nicotine was perfused onto the medial habenula neurons of the LYNX1 knockout mouse, an increase in maximum current response was observed compared to wild type (Miwa et al., 2006).

LYNX2 was the second Ly-6 like molecule to be identified as a prototoxin, and was also shown to affect behavior. An interaction between LYNX2 and nAChRs was first established through co-immunoprecipitation experiments performed on transfected HEK293 cells, which confirmed the interaction between LYNX2 with α7 and α4β2 nAChRs (Ibanez-Tallon et al., 2002; Miwa et al., 1999; Tekinay et al., 2009). Similarly to LYNX1, LYNX2 also decreases the fast time constant of desensitization (tau) and increases the ACh EC50, as measured from Xneopus oocyte whole cell recordings (Tekinay et al., 2009). LYNX2, on the other hand, is linked to anxiety and fear behavior. The LYNX2 knockout mouse spends less time in the open arms from an elevated plus maze and less time in a well-lighted area (Tekinay et al., 2009). Both behavioral characteristics are trademarks of a more anxious and fearful mouse.
PSCA was the third Ly-6 like molecule to be identified as a prototoxin (Hruska et al., 2009). Again, PSCA transcript levels correlated with CHRNA7 (α7) nAChR subunit transcript levels. The detection was done through qPCR on multiple chicken neural tissue derived from telecephalon, cerebellum, ciliary ganglia, sympathetic ganglia, and dorsal root ganglia (Hruska et al., 2009). PSCA was also shown to modulate the function of the α7 nAChR. Neurons from dissociated embryonic day 14 chicken ciliary ganglia, which over expressed PSCA, exhibited a decrease in intracellular Ca^{2+} levels in response to 10µM nicotine (Hruska et al., 2009). Also, the decreased Ca^{2+} influx was not further diminished when 50nM α-bungarotoxin was applied (Hruska et al., 2009). These results indicate that the expression of PSCA blocks Ca^{2+} influx through the α7 nAChRs (Hruska et al., 2009). The biological relevance of PSCA was demonstrated when it was expressed in the chicken ciliary ganglia at embryonic day 8 (E8) with a chicken retrovirus. Under these conditions PSCA rescued choroid neurons from programmed cell death (Hruska et al., 2009).

In contrast to the other prototoxins, LYPD6 seems to behave as a positive modulator of nAChRs. The expression of LYPD6 was established through qPCR and in-situ hybridization. Both methods illustrated LYPD6 to be highly expressed in the brain, sympathetic ganglia, dorsal root ganglia, and the trigeminal ganglione of the mouse (Darvas et al., 2009). Whole cell recordings were obtained from trigeminal neurons of genetically engineered transgenic mice over expressing LYPD6 in order to identify
LYPD6 as a nAChR modulator. No change in the nicotine EC50 was observed in the transgeneic mice compared to wild type (Darvas et al., 2009). However, at 1 and 2 mM nicotine an increase in inward Ca\(^{2+}\) currents was observed (Darvas et al., 2009).

Secreted prototoxins from the Ly6 superfamily have also been identified; some examples of these include SLURP-1 and SLURP-2. Both of these were initially isolated from human blood and urine (Tsetlin 2015). Soon after, their discovery, they were shown to modulate nAChRs. Macroscopic ACh currents recorded from Xenopus oocytes expressing the α7 nAChR were increased when perfused with 20nM SLURP-1 (Chimienti et al., 2003). In a similar fashion, SLURP-2 affected the α3 heteromeric nAChRs (Tsetlin 2014).

In summary, the number of genes encoding Ly6 family members likely to be prototoxins is very large, and a number of questions on how these family members may regulate nicotinic signaling still remains. Although, prototoxins have a similar protein structure as α-bungarotoxin it is still unclear as to where these proteins are binding on nAChRs. If prototoxin proteins do bind to nAChRs at a competitive antagonist site, then it would be expected to inhibit nAChR function. However, inhibition of nAChR function has only been observed with LYNX1 (Miwa et al., 1999), suggesting that prototoxins bind to an allosteric binding site. Additionally, α-bungarotoxin is known to specifically bind and inhibit the function of the homomeric α7 nAChR, implying prototoxins to only inhibit
the function of the α7 receptor subtype. Yet, this is not the case since LYNX1 and LYNX2 are known to regulate the α7 as well as the α4β2 nAChRs (Miwa et al., 1999; Tekinay et al., 2009). All of this evidence suggests prototoxins to bind to an allosteric binding site on nAChRs, but it is still unclear as to how these prototoxins may be modulating the function of nAChRs.

VI. The avian ciliary ganglion as a model for studying nAChRs

The well-established characteristics of the avian ciliary ganglion make it an excellent model for studying nAChRs. The ganglion sits behind the eye, which makes it easy to access and isolate for further manipulation. It is composed of two types of neurons, choroid and ciliary, both having specific characteristics (Marwitt et al., 1971). The ganglion is innervated by neurons from the accessory ocular nucleus, which activates the nAChRs of the choroid and ciliary neurons in response to light. Both neuron types express two nAChR subtypes: the homomeric α7 and heteromeric α3* containing nAChRs, the latter include combinations of α3β4, α3β4α5, and sometimes α3β2 (Corriveau and Berg, 1993; Wevers et al., 1999). Though both neurons express both nAChR subtypes, their localization vary according to neuron type. At avian embryonic day 10, the ciliary neurons form pseudospines from small somatic dendrites that over-fold on themselves. These pseudospines are highly enriched in clustered α7 nAChRs that are activated by ACh spill over (Shoop et al., 2002). In contrast, the choroid neurons
express $\alpha_7$ nAChRs over the surface of the cell body. Both neuron types express the $\alpha_3^*$ nAChRs at postsynaptic densities, and are vital for autonomic transmission (Blumenthal et al., 1999; Chiappinelli and Giacobini, 1978; Corriveau and Berg, 1993; Kirchgessner and Liu, 1998; Mandelzys et al., 1994; Obaid et al., 1999). There are other differences between the neurons, besides their nAChR expression patterns. The ciliary neurons are larger in size, regulate pupil diameter in response to light by innervating the ciliary muscle and iris, and synapses onto ciliary neurons are specialized calyx structures (Shoop et al., 2002). The choroid neurons are smaller in size, regulate nutrient delivery to the retina in response to light by innervating the choroid smooth muscle, and stain positive for somatostatin (Epstein et al., 1988; Meriney and Pilar, 1987).

The developmental stages of the ganglion are well known, documented, and characterized, which has facilitated investigation on the functions of nAChRs during neurodevelopment. Between embryonic days 8-14 (E8-14), major steps in neurodevelopment are occurring. During this critical period there is a distinct nAChR expression pattern. A ten-fold increase in protein expression of the $\alpha_3^*$ heteromeric containing, as well as a six-fold increase in the $\alpha_7$ homomeric containing nAChRs, have been observed by using radiolabeled antibodies (Chiappinelli and Giacobini, 1978; Corriveau and Berg, 1994; Smith et al., 1985). Additionally, whole cell patch clamp recordings on dissociated ciliary ganglion neurons demonstrate an increase in the amount of inward current, capacitance, and current density for both the homomeric $\alpha_7$ and
heteromeric $\alpha 3^*$ containing nAChRs (Blumenthal et al., 1999). Thus, these observations implicate the $\alpha 7$ and $\alpha 3^*$ nAChRs in regulation of developmental processes. One major step in this critical developmental period is neural programmed cell death, were 50% of newly born neurons die (Landmesser and Pilar, 1974b). When the homomeric $\alpha 7$ nAChR selective drugs MLA and $\alpha$-bungarotoxin are applied to dissociated avian ciliary ganglia about 90% of the neurons are rescued from the programmed cell death (Bunker and Nishi, 2002; Hruska and Nishi, 2007). However, when a non-selective nAChR drug is applied, cell death is exacerbated (Maderdrut et al., 1988; Meriney et al., 1987). These data suggest programmed neuron death is dependent only on current going through the homomeric $\alpha 7$ nAChR. Another critical event that occurs is neuron maturation. A phenotype of an immature neuron is the expression of the chloride transporter NKCC1. This transporter pumps Na$^+$, K$^+$, and larger amounts of Cl$^-$ ions into the cell; thus channel opening will cause an outward flow of Cl$^-$ ions and depolarize the cell membrane.

Blocking $\alpha 7$ homomeric and $\alpha 3^*$ heteromeric nAChRs in the avian ciliary ganglia and mouse hippocampus with methyllycaconitine (MLA) and dihydro-\(\beta\)-erythroidine (DH\(\beta\)E), respectively, inhibited the transition of GABA from an excitatory to inhibitory transmission. This was attributed to an increase in NKCC1 expression and an increase in GABA response (Liu et al., 2006). Thus $\alpha 3^*$ and $\alpha 7$ nAChRs are implicated in the maturation of neurons within the dentate gyrus of the hippocampus as well as the avian ciliary ganglion (Campbell et al., 2010; Liu et al., 2006).
VII. Purpose of this research:

The main focus of my dissertation was to determine if the prototoxin protein, LYPD6B, interacted with a specific type of nAChR. This question was addressed by two different approaches. The first approach determined whether heteromeric $\alpha_3\beta_4*$ (any combination of $\alpha_3$, $\beta_4$, and $\alpha_5$ subunits) nAChRs were affected by the presence of LYPD6B. As part of this project, I injected cRNA into four different *Xenopus* oocyte groups: 1) oocytes expressing a variety of human $\alpha_3\beta_4*$ pentamers 2) oocytes co-expressing a variety of human $\alpha_3\beta_4*$ pentamers plus LYPD6B 3) oocytes expressing a variety of human $\alpha_3\beta_4(\alpha_5)$ pentamers and 4) oocytes co-expressing a variety of human $\alpha_3\beta_4(\alpha_5)$ pentamers plus LYPD6B. A concatemer approach was taken to restrict the $\alpha_3\beta_4*$ and $\alpha_3\beta_4(\alpha_5)$ stoichiometries. nAChR concatemers are designed so that every nAChR subunit is linked to each other; thus, the cRNA is translated as a single polypeptide as opposed to individual subunits. The ACh potency, desensitization rate, and maximum current were established using two electrode voltage clamp recordings. The second approach was to determine the expression pattern of LYPD6B in the avian ciliary ganglion and its ability to co-localize with $\alpha_3\beta_4*$ or $\alpha_7$ nAChRs. For this project, I genetically engineered LYPD6B to express the human influenza hemagglutinin (HA) epitope tag, since there are no commercial antibodies against avian proteins. The modified LYPD6B/HA was cloned into a chicken retrovirus in order to express LYPD6B/HA in the avian ganglion and perform immunocytochemistry against HA, allowing visualization of LYPD6B and different nAChR subtypes.
Literature Cited:


The prototoxin LYPD6B modulates heteromeric alpha3 beta4 containing nicotinic acetylcholine receptors but not alpha7 homomers.

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### Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>ACh</td>
<td>acetylcholine</td>
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<tr>
<td>EC50</td>
<td>concentration of a compound where 50% of the maximum response is observed</td>
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<tr>
<td>ELISA</td>
<td>enzyme linked immunoassay</td>
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<tr>
<td>EST</td>
<td>expressed sequence tag</td>
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<tr>
<td>Imax</td>
<td>maximum current</td>
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<tr>
<td>Ly6/uPAR</td>
<td>Ly-6/urokinase plasminogen activator receptor</td>
</tr>
<tr>
<td>LYNX</td>
<td>Ly-6/neurotoxin</td>
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<tr>
<td>nAChR</td>
<td>nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>PSCA</td>
<td>prostate stem cell antigen</td>
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<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
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Abstract

Prototoxins are a diverse family of membrane-tethered molecules expressed in the nervous system that modulate nicotinic cholinergic signaling, but their functions and specificity have yet to be completely explored. We tested the selectivity and efficacy of LYPD6B on α3β4, α3α5β4, and α7-containing nicotinic acetylcholine receptors (nAChRs). To constrain stoichiometry, fusion proteins encoding concatamers of human α3, β4, and α5 (D and N variants) subunits were expressed in Xenopus oocytes and tested with or without LYPD6B. We used two-electrode voltage-clamp to quantify responses to acetylcholine (ACh): agonist sensitivity (EC50), maximum agonist induced current (Imax), and time constant of desensitization (τ). For β4–α3–α3–β4–α3 and β4–α3–β4–α3–α3, LYPD6B decreases EC50 from >631 µM to approximately 100 µM, reduces Imax by at least 59%, and decreases τ. For β4–α3–α5D–β4–α3 and β4–α3–β4–α3–α5D, LYPD6B decreases Imax by 63% and 32% respectively. Thus, LYPD6B acts only on (α3)3(β4)2 and (α3)2(α5D)(β4)2 and does not affect the properties of (α3)2(β4)3, α7, and (α3)2(α5N)(β4)2 nAChRs. Therefore, LYPD6B acts as a mixed modulator that enhances the sensitivity of (α3)3(β4)2 nAChRs to ACh while also reducing ACh induced whole cell currents. LYPD6B also negatively modulates α3β4 nAChRs that include the α5 D common human variant, but not the N variant associated with nicotine dependence.

LYNX1, LYNX2, Ly6/uPAR, CHRNA5, PSCA
Introduction

Because nicotinic cholinergic signaling is known to be involved in an array of human behaviors such as learning, memory, addiction, and attention (Picciotto et al., 2000; Rezvani and Levin, 2001), the discovery of endogenous modulators of nicotinic acetylcholine receptors (nAChRs) is significant. These modulators are proteins known as prototoxins, so named because they share structural characteristics with 3-fingered snake venom proteins such as α-bungarotoxin and cobra toxin (Miwa et al., 2011; Miwa et al., 2012). Prototoxins belong to a larger family known as Ly-6/urokinase plasminogen activator receptor (Ly6/uPAR). At the primary structural level, prototoxin proteins have 8-10 conserved cysteine residues that allow for the formation of disulfide bonds, constraining the protein to the three-fingered motif secondary structure (Holford et al., 2009). A number of prototoxins that are expressed in the nervous system and act as allosteric modulators of nAChRs have been identified. These include LY-6/neurotoxin 1 (LYNX1), LYNX2 (also known as LYPD1), LY6H, LYPD6, and PSCA (Dessaud et al., 2006; Horie et al., 1998; Hruska et al., 2009; Miwa et al., 1999; Zhang et al., 2010).

When considering the interactions of prototoxins with nAChRs, the complexity of nAChR structure must be taken into consideration. Functional nAChRs are either homomeric or heteromeric pentamers. Functional homomeric nAChRs can be assembled exclusively from α7, α8, or α9 subunits (Albuquerque et al., 2009; Elgoyhen et al., 1994).
while the heteromeric nAChRs are composed of combinations of $\alpha_2 - \alpha_6$ and $\beta_2 - \beta_4$ subunits (Albuquerque et al., 2009; Lindstrom et al., 1996). Additionally, the $\alpha_7$ nAChR subunit has the ability to form heteromeric nAChRs with $\alpha_8$, $\alpha_5$, or $\beta_2$ subunits, and the $\alpha_9$ has the ability to form a heteromeric nAChR with the $\alpha_{10}$ subunit (Elgoyhen et al., 2001; Girod et al., 1999; Keyser et al., 1993; Moretti et al., 2014; Morley et al., 1998; Murray et al., 2012; Rothlin et al., 2003; Thomsen et al., 2015). The varying subtypes, and even alternate stoichiometries within a given subtype contribute to different receptor properties, including channel kinetics and conductance, ligand potency, and desensitization rate (Albuquerque et al., 2009; Moroni and Bermudez, 2006; Moroni et al., 2006; Murray et al., 2012; Nelson et al., 2003; Tapia et al., 2007). The availability of varying combinations of $\alpha$ and $\beta$ subunits can lead to the expression of multiple nAChR subtypes within the same cell (Conroy and Berg, 1995; Halvorsen and Berg, 1986; Vernallis et al., 1993).

The modulatory effects of prototoxins on nAChR signaling have been studied in a limited number of nAChR subtypes. The first prototoxin to be identified was LYNX1. An increase in induced acetylcholine macroscopic currents is observed when soluble LYNX1 is perfused onto *Xenopus* oocytes expressing $\alpha_4\beta_2$ or $\alpha_7$ (Miwa et al., 1999); however, when co-expressed with $\alpha_4\beta_2$, LYNX1 increases the rate of desensitization by acetylcholine (Ibanez-Tallon et al., 2002; Ibanez-Tallon et al., 2004). LYNX1 knockout
mice also have greatly enhanced responses to ACh in the habenula (Miwa et al.), and similar effects were observed for LYNX2 (Tekinay et al., 2009).

Because autonomic neurons express a limited number of nAChR subtypes (homomeric α7 and heteromeric α3β4*-nAChRs: any combination of α3 with β4 as well as α3, β4 with α5 (Conroy and Berg, 1995; Halvorsen and Berg, 1990)), they are a useful system for studying the subtype selectivity of prototoxins. We previously discovered three prototoxins that are expressed in parasympathetic neurons of the Gallus ciliary ganglion. One of the prototoxins, prostate stem cell antigen (PSCA), blocks calcium influx through the α7 nAChRs but not α3-containing nAChR heteromers in ciliary ganglion neurons (Hruska et al., 2009). In addition, expressing PSCA prior to the period of developmental cell loss, rescues neurons from dying (Hruska et al., 2009).

Here, we report that the other two prototoxins in the Gallus ciliary ganglion are LY6E and LYPD6B. Of these two, LYPD6B is expressed in the nervous system (http://mouse.brain-map.org) (Miwa et al., 2012). We therefore hypothesized that, in contrast to PSCA, LYPD6B selectively modulates the function of heteromeric α3β4* nAChRs. Since heteromeric receptors can be composed of pentamers containing two or three alpha subunits, we restricted the stoichiometry by expressing pentameric concatemers in Xenopus oocytes (George et al., 2012). These concatemers are composed of all five nAChR subunits expressed as a single fusion protein with linkers between each subunit that allow the polypeptide to fold into a functional pentamer (Carbone et al.,
2009; George et al., 2012; Kuryatov and Lindstrom, 2011). The linker allows complete control of the subunit stoichiometry and associations to be exercised. Expression studies of nAChR concatemers have faithfully mimicked pharmacological properties of multiple natively expressed nAChR subtypes, including $\alpha_3\beta_4^*$ nAChRs (Carbone et al., 2009; George et al., 2012; Kuryatov and Lindstrom, 2011). Our results show that LYPD6B reduces the EC50 value for ACh while also reducing the net whole cell current induced by ACh on $\alpha_3\beta_4$ nAChRs containing three $\alpha_3$ subunits as opposed to those containing only two. In addition, LYPD6B reduces the whole cell current induced by ACh through $\alpha_3\beta_4^*$ nAChRs containing the $\alpha_5(D)$ subtype, while having no effect on the $\alpha_5(N)$ subtype associated with nicotine dependence.

**Material and Methods**

*Chemicals*- All buffer components and pharmacological reagents (acetylcholine and atropine) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fresh stock drug solutions were made daily and diluted as required.

*nAChR concatemers and monomeric $\alpha_7$ plasmids*- Pentameric nAChR concatemers were designed and constructed from human nAChR subunits with subunit sequences $\beta_4-\alpha_3-\beta_4-\alpha_3-X$ (George et al., 2012) or $\beta_4-\alpha_3-X-\beta_4-\alpha_3$ where $X$ is $\beta_4$, $\alpha_3$, $\alpha_5(D$-variant), or $\alpha_5(N$-variant). These series generated pairs of identical pentamers that varied in the location of the inserted subunit $X$ within the length of the fusion protein. Thus,
these served as controls for whether isoform selective effects of LYPD6B were consistently observed between the two series (eg, between (α3)3(β4)2 generated by β4–α3–β4–α3-α3 or β4–α3–α3–β4–α3). In order to express the concatemer as a single polypeptide the Kozac and signal peptide sequences were removed from all subunits with the exception of the first subunit. Additionally, a 40-amino acid sequence that includes the C-terminal tail of the preceding subunit and alanine-glycine-serine repeats arranged in order to encode enzyme restriction sites are inserted in the linker region between each subunit. In either series, the first agonist-binding site occurs between the negative face of the first β4 subunit and the positive face of the neighboring α3 subunit (George et al., 2012). Concatemeric receptors and the monomeric α7 subunit were expressed from the pSGEM oocyte high expression vector (George et al., 2012).

RNA synthesis- Concatemeric plasmids were linearized with NheI for two hours at 37°C and treated with proteinase K for 30 min at 50°C. The DNA was purified with the Qiagen Qiaquick PCR purification kit protocol (Qiagen, Venlo, Limburg, Netherlands). The synthesis and clean up of cRNA was done by following the protocol from the mMessage mM Machine T7 kit (Applied Biosystems/Ambion, Waltham, MA, USA). Reactions were treated with TURBO DNase (1 unit for 15 min at 37°C), and cRNAs were purified by lithium chloride precipitation.
Oocyte preparation and RNA injection- Ready to inject *Xenopus* oocytes were purchased from Ecocyte Bioscience (Austin, TX, USA). Oocytes were stored at 13°C in incubation buffer (82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂·6H₂O, 1 mM CaCl₂·2H₂O, 1 mM Na₂HPO₄, 5 mM HEPES, 600 µM theophylline, 2.5 mM Na pyruvate, 50 U/ml penicillin, 50 µg/ml streptomycin, 50 µg/ml gentamycin sulfate; pH to 7.5). Injection electrodes were pulled glass micropipettes that were broken to achieve an outer diameter of 40 µm (resistance of 2-6 mΩ), and pipettes were used to inject 60 nl containing 20 ng of α3β4 or α3β4α5 heteromeric nAChRs cRNA/oocyte, or 10 ng of the monomeric α7 subunit cRNA/oocyte. The monomeric α7 subunit was co-injected with the chaperone protein RIC3 at a 1:50 mass ratio to enhance functional expression (Halevi et al., 2002). Oocyte coinjections with the receptor as well as the prototoxin LYPD6B were performed at a 1:1 cRNA concentration by weight per oocyte.

Dose and maximum current response recordings of *Xenopus* oocytes expressing α3β4*-nAChR concatemers and monomeric α7-nAChRs- Recordings were performed five days post injection. *Xenopus* oocytes were voltage-clamped at -70 mV with an Axoclamp 900A amplifier (Molecular Devices, Sunnyvale, CA, USA). Recordings were sampled at 10kHz (low-pass Bessel filter, 40 Hz; high pass filter, DC), and the resulting traces were saved to disk (Molecular Devices Clampex v10.2). Data from oocytes with leak currents (I_leak)> 50 nA were excluded from recordings. Agonist (ACh) was applied using a 16 channel, gravity-fed, perfusion system with automated valve control (AutoMate...
Scientific, Inc., Berkeley, CA). All solutions contained atropine sulfate (1.5 µM) to block muscarinic responses. Oocytes expressing the α7 homomeric or α3β4*-nAChR concatemers were perfused with receptor agonist (ACh) for 5 s with 60 s washout times between each subsequent application.

Data Analysis for LogEC50 and maximum current response- LogEC50 and maximum current values (Imax) was determined from individual oocytes. For the LogEC50, responses were normalized to the maximum current response. Normalized current responses were plotted against ACh concentration and the LogEC50 was determined through non-linear least squares curve-fitting (GraphPad Prism 6.0, GraphPad Software, Inc., La Jolla CA, USA) using unconstrained, monophasic logistic equations to fit all parameters. LogEC50 values are presented as the mean ± 95% confidence interval (CI).

For determination of LYPD6B effects on Imax, responses from oocytes coexpressing the nAChRs and LYPD6B were normalized to control oocytes (oocytes only expressing the nAChRs under question). Normalized LogEC50 and Imax responses for oocytes expressing a defined nAChR concatemer averaged with or without co-expression of LYPD6B were averaged, and then tested for significant differences between groups using Student’s t test (GraphPad Prism 6.0, GraphPad Software, Inc., La Jolla, CA, USA).

Desensitization rate of α3β4*-nAChR concatemers

Recordings were performed seven days post injection. *Xenopus* oocytes were voltage-clamped and desensitization kinetics was measured as previously described (Eaton et al.,
1.0 mM ACh (corresponding to the EC100 value) was applied by using 1 channel from a 16 channel, gravity-fed, perfusion system (AutoMate Scientific, Inc.; Berkeley, CA, USA). Because perfusion rates can influence channel desensitization or recovery from activation, the flow rate of the system (6 mL/min) was continuously monitored and measured between groups. Oocytes expressing concatemers were perfused with receptor agonist ACh for 20-45 minutes; all α5 containing nAChRs were perfused for approximately 20 min, (α3)3(β4)2 nAChRs were perfused for approximately 25 min, and (α3)2(β4)3 nAChRs were perfused for approximately 45 min. Perfusion times were determined according to the amount of time required for the entire population of receptors to reach 10% of the maximum response; this allows for an accurate measurement of the desensitization time constant (τ).

**Data Analysis for current recovery at steady-state desensitization recordings**

The rate of desensitization was determined from individual oocytes. Each oocyte’s current responses were observed during prolonged agonist application, and normalized to the maximum response in each individual case. Normalized current responses were plotted as a function of time, and desensitization was fitted with a one phase or two phase exponential decay (GraphPad Prism 6.0, GraphPad Software, Inc., La Jolla, CA, USA). However, there was no significant increase in the quality of fit between the one phase and two phase model in any of the measurements taken, either from individual oocytes or for averaged data across multiple oocytes. Therefore, the single-site fit model was chosen to
determine $\tau$. The statistical significance of any difference between $\tau$ values in the presence or absence of LYPD6B was determined by using a Student’s t test (GraphPad Prism 6.0).

**Oocyte ELISA**

Following recordings, oocytes expressing the concatemer versus concatemer plus LYPD6B were incubated on ice for one hour with a 1:50 dilution of primary antibody (concentrated mAb35 rat anti AChR; (Developmental Studies Hybridoma Bank, Iowa City, IA, USA,) in blocking solution (incubation buffer, 10% horse serum, and 0.1% sodium azide). Oocytes were then washed, fixed on ice for an hour with Zamboni’s fixative (0.2 M Na$_2$HPO$_4$, 4% paraformaldehyde, 0.2 M NaH$_2$PO$_4$, and 10% picric acid, pH 7.2), blocked, and incubated for at least one hour at room temperature in a 1:250 dilution of biotinylated anti-mAb35 rat, (Vector laboratories, Burlingame, CA, USA) in modified blocking solution (block solution minus sodium azide), washed, then incubated at room temperature for an hour with 1:500 dilution beta-galactosidase avidin D (Vector laboratories, Burlingame, CA, USA). Control oocytes were incubated in modified block solution. Oocytes were then washed and incubated at room temperature for 24 hours with 100µM 4-methylumbelliferyl β-D-galactopyranoside (Molecular Probes, Eugene, OR, USA). The reaction was stopped by adding 15µl of 10M NaOH. Fluorescence was measured at excitation and emission of 450 and 350 nm respectively with a Biotek synergy H4 hybrid reader (Biotek, Winooski, VT, USA).
Data Analysis for oocyte ELISA- The average relative fluorescence values per group were determined from each oocyte fluorescence value normalized to the average fluorescence of control oocytes (oocytes incubated without beta-galactosidase avidin D). Student’s t test (GraphPad Prism 6.0) was used to test for statistically significant differences between the average of relative fluorescence values of oocytes expressing the concatemer alone versus concatemer plus LYPD6B.

Results

Identifying prototoxins in Gallus parasympathetic neurons

In our previously reported study, six EST sequences similar to the structure of Mus lynx1 were identified and three (Ch6ly, Ch3ly, and Ch5ly) were found to be expressed in the embryonic ciliary ganglion (Hruska et al., 2009). Ch6ly was identified as prostate stem cell antigen (PSCA) (Hruska et al., 2009). Prior to undertaking the present study, we determined by BLAST that Ch3ly is LY6E and Ch5ly is LYPD6B. Gallus LYPD6B and LY6E share a 72% and 44% amino acid identity respectively with the Homo sapiens LYPD6B and LY6E (Fig. 1A and 1B). When translated, both sequences have the canonical characteristics of a prototoxin protein: conserved 8-10 cysteine residues with defined disulfide bonding pattern that forms the three finger tertiary structure similar to the toxin alpha-bungarotoxin (Fleming et al., 1993). Since LYPD6B is expressed in the brain, we focused our attention on LYPD6B (Miwa et al., 2012). LYPD6B is located on
chromosome 2 and is also known as LYPD7 (Fig 1C). Though LYPD6B and LYPD6 are similar in name they are encoded by different genes and are not splice variants of each other (Fig.1C).

*Preferential modulation of ACh sensitivity by LYPD6B of specific concatamers*

We tested the effects of LYPD6B on four different $\alpha_3\beta_4$ nAChR concatamers expressed in *Xenopus* oocytes using two-electrode voltage clamp to measure responses to ACh: two nAChRs with a composition of $(\alpha 3)_2(\beta 4)_3$ having concatemeric sequences of $\beta 4-\alpha 3-\beta 4-\alpha 3$ and $\beta 4-\alpha 3-\beta 4-\alpha 3-\beta 4$ and two nAChRs with a composition of $(\alpha 3)_3(\beta 4)_2$ having concatemeric sequences of $\beta 4-\alpha 3-\alpha 3-\beta 4-\alpha 3$ and $\beta 4-\alpha 3-\beta 4-\alpha 3-\alpha 3$. The current responses of *Xenopus* oocytes expressing each $\alpha 3\beta 4$ nAChR concatemer were compared to those expressing the same concatemer in the presence of LYPD6B. The responses to different concentrations of ACh for the $\alpha 3\beta 4$ heteromers are illustrated in Figure 2. The EC50 value of ACh was not affected by LYPD6B in $(\alpha 3)_2(\beta 4)_3$ concatamers: $\beta 4-\alpha 3-\beta 4-\alpha 3$ and $\beta 4-\alpha 3-\beta 4-\alpha 3-\beta 4$ (Fig. 2A,B). In contrast, LYPD6B decreases the EC50 value of ACh for $(\alpha 3)_3(\beta 4)_2$ concatamers: $\beta 4-\alpha 3-\beta 4-\alpha 3$ and $\beta 4-\alpha 3-\beta 4-\alpha 3-\alpha 3$ (Fig. 2C, D) from 104 $\mu$M (CI 90-120 $\mu$M) to 22 $\mu$M (CI 20-23 $\mu$M) and 670$\mu$M (CI 638-703 $\mu$M) to 95$\mu$M (CI 85-105 $\mu$M) for $\beta 4-\alpha 3-\alpha 3-\beta 4-\alpha 3$ and $\beta 4-\alpha 3-\beta 4-\alpha 3-\alpha 3$ respectively.
In addition to α3β4, autonomic neurons express α3β4α5 nAChRs (Vernallis et al., 1993). Genome-wide association studies on humans have identified a rare allele of the gene encoding the α5 subunit CHRNA5 that is associated with nicotine dependence in cigarette smokers (Bierut et al., 2007; Bierut et al., 2008). The common allele of CHRNA5 encodes the α5 D-variant, and the allele associated with nicotine dependence encodes the α5 N-variant. Therefore, we tested the effects of LYPD6B on four different concatemers encoding α3β4α5 nAChRs. Two of the α3β4α5 nAChRs have a composition of (α3)2(β4)3(α5D) with sequences of β4–α3–α5D–β4–α3 and β4–α3–β4–α3–α5D, and the other two are (α3)2(β4)3(α5 N) with sequences of β4–α3–α5N–β4–α3 and β4–α3–β4–α3–α5N. The responses to ACh of Xenopus oocytes expressing α3β4α5 nAChR concatemers were compared to those co-expressed with LYPD6B (Fig. 3). Regardless of the presence of the α5 D-variant or the α5 N-variant, the co-expression of LYPD6B does not alter the EC50 value to ACh of the four different stoichiometries tested (Fig. 3; Table 1).

Since autonomic neurons prominently express homomeric α7 nAChRs in addition to heteromeric α3β4* nAChRs (Blumenthal et al., 1999; Vernallis et al., 1993), we tested the effects of LYPD6B on α7 nAChRs (Fig.4). The homomeric α7 nAChR requires the chaperone protein, RIC3, in order to traffic to the cell surface (Halevi et al., 2002; Williams et al., 2005); therefore, Xenopus oocytes were either co-injected with mRNAs encoding the chaperone protein RIC3 and the α7 nAChR subunit monomer at a 50:1
ratio, or with RIC3, α7, and LYPD6B at a 50:1:1 concentration ratio. The ACh EC50 value for α7 nAChR homomers is not affected by the presence of LYPD6B (Fig 4; Table 1).

*Preferential modulation by LYPD6B of maximum current induced by ACh in specific concatemers*

The second functional component tested was the maximum current response to ACh (Imax). LYPD6B significantly decreases the Imax of both (α3)_3(β4)_2 nAChR concatemers (β4–α3–α3–β4–α3 and β4–α3–β4–α3–α3) by approximately 30% and 60% respectively (Fig. 5 and 6). LYPD6B does not alter the Imax for either of the two (α3)_2(β4)_3 nAChR concatemers or that of the homomeric α7 nAChRs. These differences in Imax cannot be attributed to differences between the number of pentamers expressed on the surface of the oocytes because we cannot detect any differences in cell surface mAb35 binding to oocytes expressing these constructs in the presence or absence of LYPD6B (Suppl. Fig. 1).

LYPD6B decreased the Imax for the β4–α3–α5D–β4–α3 and β4–α3–β4–α3–α5D nAChRs by approximately 30% and 60% respectively (Fig. 5 and 6). There is no change in the Imax for either format of α3β4α5 concatemers tested (Fig. 6). Thus, LYPD6B selectively reduces the Imax of α5 D-variant containing α3β4 nAChRs compared to those containing the α5 N-variant associated with heavy smoking.
Preferential modulation of desensitization rate to ACh in specific concatemers by LYPD6B

The third functional component tested was the desensitization rate of the nAChRs. For each nAChR, the desensitization time constant ($\tau$) was measured during an acetylcholine perfusion time period long enough for the inward current to reach ten percent of its initial response. LYPD6B decreased the time constant for desensitization ($\tau$) of only the $(\alpha_3)_2(\beta_4)_2$ concatemers $\beta_4-\alpha_3-\alpha_3-\beta_4-\alpha_3$ and $\beta_4-\alpha_3-\beta_4-\alpha_3-\alpha_3$ (Fig. 7; Table 1), indicating an increase in the desensitization rate of the receptors. LYPD6B did not change the desensitization rates to ACh to $(\alpha_3)_2(\beta_4)_3$ or any of the $\alpha_{5D}$ or $\alpha_{5N}$ containing $\alpha_3\beta_4$ heteromers (Fig. 8). Note that our perfusion system is capable of exchanging solutions on a sub-second timescale (38), at least an order of magnitude faster than the measured $\tau$ values. This ensures that $\alpha_3\beta_4$-nAChR desensitization effects can be measured accurately since they (and not solution exchange effects) are by far the predominant contributor to the observed $\tau$ values. For the same reason, it was not possible to measure accurately the desensitization rate for the homomeric $\alpha_7$ nAChR in our system ($\alpha_7$-nAChR desensitization occurs much more rapidly than solution exchange) (Papke and Porter Papke, 2002).
Discussion

This study identifies LYPD6B as a prototoxin that modulates the function of $\alpha 3\beta 4^*$ heteromeric, but not $\alpha 7$ homomeric nAChRs. Further, the effects of LYPD6B are dependent on the particular subunit composition and stoichiometry of $\alpha 3\beta 4$ containing nAChRs. For $(\alpha 3)_3(\beta 4)_2$ nAChRs, LYPD6B enhances the sensitivity to ACh, yet decreases the $I_{\text{max}}$ and increases the rate of desensitization to ACh, while these effects are not seen with $(\alpha 3)_2(\beta 4)_3$ nAChRs. When the $\alpha 5$ subunit is introduced, LYPD6B decreases the $I_{\text{max}}$ induced by ACh only when the $\alpha 5$ D-variant is present, but has no effect when the $\alpha 5$ N-variant associated with heavy smoking is included. Importantly, LYPD6B had no apparent effect on ACh responsiveness of $\alpha 7$ subunit containing homomers. This demonstrates that the effects of LYPD6B on $\alpha 3\beta 4^*$ nAChR function are highly subtype- and stoichiometry selective and illustrates a previously unappreciated complexity in prototoxin modulation of nAChRs.

In comparison to the two best-studied prototoxins to date, LYNX1 and LYNX2 (LYPD1), LYPD6B exhibits a much higher selectivity and complexity in modulating nicotinic signaling. LYNX1 and LYNX2 decrease the sensitivity to ACh as well as enhance the rate of desensitization of both $\alpha 7$ homomeric and $\alpha 4\beta 2$ heteromeric nAChRs in *Xenopus* oocytes (Miwa et al., 2006; Tekinay et al., 2009), thereby acting as a “brake” on nicotinic signaling. This is supported by studies of the LYNX1 knockout mouse where nicotinic responses are enhanced in the habenula (Miwa et al., 2006). LYNX1 also co-
localizes and co-immunoprecipitates with homomeric $\alpha 7$ nAChRs in the amygdala, CA3/CA1 hippocampal neurons, and thalamic reticular nuclei (Ibanez-Tallon et al., 2002). In contrast, LYPD6B enhances ACh sensitivity yet also decreases the Imax to ACh on only “low sensitivity” $\alpha 3\beta 4$ pentamers containing three $\alpha 3$ subunits and has no detectable effect on $\alpha 7$ containing homomers. LYNX1 facilitates the assembly and trafficking of the low sensitivity $(\alpha 4)_3(\beta 2)_2$ nAChRs (Nichols et al., 2014); such trafficking might explain the observed increase in ACh EC50 (ACh decreased sensitivity) of $\alpha 4\beta 2$ nAChRs when expressed from subunit monomers and coexpressed with LYNX1 (Ibanez-Tallon et al., 2002). However, in our study, differences in nAChR subunit assembly cannot explain the enhanced ACh sensitivity of $(\alpha 3)_3(\beta 4)_2$ because of the fixed stoichiometry of our concatemeric constructs. As a result, changes in EC50 values in our system must be due to a direct allosteric effect of LYPD6B on the function of $(\alpha 3)_3(\beta 4)_2$ nAChR.

The differential responses of $(\alpha 3)_3(\beta 4)_2$ and $(\alpha 3)_2(\beta 4)_3$ nAChR concatemers to LYPD6B suggest that the additional $\alpha 3/\alpha 3$ interface provides a structural site for LYPD6B allosterism. These two types of stoichiometries have been identified for both $\alpha 3\beta 4$ and $\alpha 4\beta 2$ heteromeric nAChRs (Moroni et al., 2006; Nelson et al., 2003) with distinct pharmacological properties including a higher sensitivity to ACh for the heteromers that contain 2 alpha nAChR subunits (Eaton et al., 2014; Harpsoe et al., 2011; Moroni et al., 2008). This concept of $\alpha/\alpha$ interface sites for allosteric modulation is supported by the
observation of modulation by divalent cations at an α4/α4 interface (Moroni et al., 2008). When LYPD6B is coexpressed with the less sensitive (α3)3(β4)2, the EC50 to ACh becomes comparable to that of the more sensitive (α3)2(β4)3. With α4β2 heteromeric nAChRs, the additional α4/α4 interface is thought to act equivalently to a co-agonist site by producing a second phase of low affinity function when it is engaged by high agonist concentration (Eaton et al., 2014; Harpsøe et al., 2011). Thus, if the α3/α3 interface parallels the behavior of the α4/α4 interface, then LYPD6B may be influencing the interaction of agonist at the additional alpha-alpha interface. Similar studies such as ours that constrain stoichiometry of α4β2 nAChRs while examining interaction with LYNX1 and LYNX2 have yet to be performed. Prototoxin proteins have a similar protein structure to alpha-bungarotoxin, thus it is assumed that they interact with nAChRs near the agonist binding site (Fleming et al., 1993; Gumley et al., 1995); however, this may differ among prototoxins based upon structural characteristics such as the length of each of the three fingers and according to the nAChR subtypes with which they interact. It is also worth noting that many features of conventionally-recognized agonist binding interfaces are conserved at non agonist binding interfaces (Eaton et al., 2014; Harpsøe et al., 2011; Moroni et al., 2008), so prototoxin interaction sites on nAChRs could conceivably be located at any subunit pair interface.

Our results showing that LYPD6B decreased the maximum induced ACh current response only in concatemers containing an α5 D-variant and not the α5 N-variant
suggests a possible importance in nicotine dependence. Of the many single nucleotide polymorphisms (SNPs) identified in CHRNA5 through a genome-wide association study of nicotine dependence, only one was a variant in the coding region (Bierut et al., 2007; Bierut et al., 2008; Saccone et al., 2007). This SNP replaces the 398th amino acid, aspartic acid (D), with an asparagine (N) amino acid in the intracellular loop of the nAChR subunit (Bierut et al., 2008; Saccone et al., 2007). The amino acid sequences surrounding the SNP are conserved across eight species, highlighting the importance of this region (Hung et al., 2008). The α5 subunit is known to modulate the properties of nAChRs in which it is incorporated (George et al., 2012; Kuryatov et al., 2011), and α5 knockout mice as well as α5 knockdown rats exhibit reduced aversion to nicotine (Fowler et al., 2011; Fowler et al., 2013). Our studies confirm our previous finding that nAChRs containing the α5 N-variant exhibit a decreased response to ACh when compared to those with the α5 D-variant. However, LYPD6B decreases the Imax to ACh only in α3β4 heteromers containing the normal α5D variant as opposed to the α5N variant associated with nicotine dependence. The fact that such a dramatic difference was seen in the effects of LYPD6B on α3β4 [α5D vs. α5N] suggests that individuals expressing the α5N-variant may lose an important mechanism for modulation of α3β4α5-nAChR function. It is clear that considerably more studies of nAChRs and their associated prototoxins in pathways regulating nicotine reward and aversion must be pursued.
Because nicotinic signaling has important functions in guiding behavior, nAChRs have adapted a variety of means to fine-tune their signaling. First, cells can regulate the subtypes of nAChRs that are expressed, and where they are expressed. Second, neurons can express varying nAChR subunit stoichiometries within the same overall nAChR subtype(s), which further affect receptor properties. Third, prototoxin proteins belonging to the Ly6-uPAR family can modulate nAChR responsiveness. The data presented in this paper identifies LYPD6B as a prototoxin that modulates a specific stoichiometry of α3β4 nAChR subtype as well as modulating the responsiveness of nAChRs only containing a specific variant of α5, serving as another means of fine-tuning nicotinic signaling. The exquisite selectivity for subtype, and even stoichiometry, of LYPD6B modulation of nAChR activity, together with the diversity of effects observed in this study, indicates that the nAChR-regulatory roles of at least some prototoxins are likely to be complex and highly specialized.
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Appendix

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Table 1. The effects of LYPD6B on functional characteristics of nAChR concatemers

The indicated parameters were measured in Xenopus oocytes expressing the indicated nAChR concatemers with and without coexpression of LYPD6B using two electrode voltage clamp after applying ACh by perfusion. Data are presented as the mean ± S.E., with numbers of individual oocytes tested (n). The α7 nAChRs were formed from mRNA encoding the CHRNA7 monomer rather than produced as a concatemer. Student’s t-test was performed to determine statistical significant differences between concatemeric nAChR function in the presence or absence of LYPD6B, * p-value ≤ 0.05, ** p-value ≤ 0.01, and *** p-value ≤ 0.001.
<table>
<thead>
<tr>
<th>nAChR concatemer</th>
<th>ACh LogEC50</th>
<th>Imax % Normalized</th>
<th>( \tau ) (secs) Normalized</th>
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<tr>
<td></td>
<td>-</td>
<td>+ LYPD6B</td>
<td>-</td>
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<tr>
<td>( \beta 4\alpha 3\alpha 3\beta 4\alpha 3 )</td>
<td>-3.2±0.03 (10)</td>
<td>-4.1±0.01 (14)***</td>
<td>100</td>
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<td>( \beta 4\alpha 3\beta 4\beta 4\alpha 3 )</td>
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<td>-4.3±0.01 (10)</td>
<td>100</td>
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<td>( \beta 4\alpha 3\alpha 5D\beta 4\alpha 3 )</td>
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<td>-4.0±0.01 (9)</td>
<td>100</td>
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<td>( \beta 4\alpha 3\alpha 5N\beta 4\alpha 3 )</td>
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<td>( \alpha 7 )</td>
<td>-3.6±0.04 (6)</td>
<td>-3.6±0.03 (6)</td>
<td>100</td>
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Figure 1. Alignment of amino acid sequences for LY6E and LYPD6B from *Gallus gallus* and *Homo sapiens*. A. Mature protein alignment of the *Gallus gallus* LYPD6B, Genbank accession number XM_422156, and *Homo sapien* prototoxin LYPD6B, Genbank accession number XM_006712283. B. Mature protein alignment of the *Gallus gallus* LY6E, Genbank accession number NM_204775, and *Homo sapien* prototoxin LY6E, Genbank accession number NM_002346. For both A and B, asterisk identifies the consensus between amino acids and the shaded cysteine residues are required for a three-finger motif secondary structure. C. A cartoon representation of human
Figure 2. LYPD6B decreases ACh Log EC50 for (α3)3(β4)2 heteromeric nAChRs. Oocytes injected, with a 1:1 RNA concentration ratio of a nAChR to LYPD6B, were perfused with different concentrations of ACh. Closed circles are recordings collected from oocytes co-expressing the receptor and prototoxin. Open circles are recordings collected from oocytes expressing the receptor alone. A. β4α3β4α3 compared to β4α3β4α3 with LYPD6B; n=11, 10, ACh concentration (10^{-6} to 10^{-2.5}). B. β4α3β4α3β4 compared to β4α3β4α3β4 with LYPD6B; n=12, 13, ACh concentration (10^{-6} to 10^{-2.5}). C. β4α3α3β4α3 compared to β4α3α3β4α3 with LYPD6B; n=5, ACh concentration (10^{-5.5} to 10^{-2}). D. β4α3β4α3α3 compared to β4α3β4α3α3 with LYPD6B; n=17, 18, ACh concentration (10^{-5.5} to 10^{-2}). Data points represent averages (±S.E), and the drug potency differences between the receptor versus the receptor and LYPD6B groups were analyzed using a student t-test (see “Materials and Methods”).
Figure 3. LYPD6B does not affect the ACh Log EC50 for $\alpha_3\beta_4\alpha_5$ heteromeric nAChRs. Oocytes injected, with a 1:1 RNA concentration ratio of a nAChR and LYPD6B, were perfused with ACh. Closed circles are recordings collected from oocytes co-expressing the receptor and prototoxin. Open circles are recordings collected from oocytes expressing the receptor alone.

A. $\beta_4\alpha_3\alpha_5D\beta_4\alpha_3$ compared to $\beta_4\alpha_3\alpha_5D\beta_4\alpha_3$ with LYPD6B; n=9, ACh concentration (10$^{-5.5}$ to 10$^{-2}$).

B. $\beta_4\alpha_3\alpha_5N\beta_4\alpha_3$ compared to $\beta_4\alpha_3\alpha_5N\beta_4\alpha_3$ with LYPD6B; n=9, 8, ACh concentration (10$^{-5.5}$ to 10$^{-2}$).

C. $\beta_4\alpha_3\alpha_5D$ compared to $\beta_4\alpha_3\beta_4\alpha_3\alpha_5D$ with LYPD6B; n=9, 9, ACh concentration (10$^{-5.5}$ to 10$^{-2}$).

D. $\beta_4\alpha_3\beta_4\alpha_3\alpha_5N$ compared to $\beta_4\alpha_3\beta_4\alpha_3\alpha_5N$ with LYPD6B; n=7, 9, ACh concentration (10$^{-5.5}$ to 10$^{-2}$). Data points represent averages (+S.E), and the drug potency differences between the receptor versus the receptor and LYPD6B groups were analyzed using a student t-test (see “Materials and Methods”).
Figure 4. LYPD6B does not affect the ACh Log EC50 for α7 homomeric nAChRs.

Oocytes injected with the monomeric α7 nAChR subunit, chaperon protein RIC3, and prototoxin LYPD6B RNA at a 1:50:1 volume amounts respectively. They were perfused with ACh. Closed circles are recordings collected from oocytes co-expressing the receptor and prototoxin. Open circles are recordings collected from oocytes expressing the receptor alone. A. homomeric α7 nAChR expressed as monomers compared to homomeric α7 nAChR with LYPD6B; n=6, 6, acetylcholine concentration (10^{-5.5} to 10^{-2}). Data points represent averages (±S.E), and the drug potency differences between the receptor versus the receptor and LYPD6B groups were analyzed using a student t-test (see “Materials and Methods”).
Figure 5. LYPD6B decreases the ACh induced maximum current response for (α3)3(β4)2.
An average of individual oocyte recorded traces of nAChR concatemers compared to concatemers co-expressed with LYPD6B. As well as a bar graph of normalized induced ACh response expressed as a percent normalized to the control for all of the α3β4β4α3 heteromeric concatemers with LYPD6B. A. β4α3β4α3β4α3 receptor at ACh doses (10^-6 to 10^-2.25M), n=6. A1. β4α3β4β4α3 and plus LYPD6B, n=10. B. β4α3β4α3β4 receptor at ACh doses (10^-6 to 10^-2.25M), n=6. B1. β4α3β4α3β4 and plus LYPD6B. C. β4α3β4α3β4α3 receptor at ACh doses (10^-5.5 to 10^-2M), n=6. C1. β4α3β4α3β4α3 and plus LYPD6B. The oocytes co-expressing the LYPD6B and β4α3β4α3β4α3 exhibit about a 30% decrease induced ACh maximum response p value 0.0001. D. β4α3β4α3β4α3 receptor at ACh doses (10^-5.5 to 10^-2M), n=6. D1. β4α3β4α3β4α3 and plus LYPD6B. The oocytes co-expressing the LYPD6B and β4α3β4α3β4α3 exhibit about a 60% decrease induced ACh maximum response p value 0.0001. and E. α7α7α7α7α7α7 receptor at ACh doses (10^-5.5 to 10^-2M), n=6. E1. α7α7α7α7α7α7 and plus LYPD6B, n=6.
Figure 6. LYPD6B affects the ACh induced maximum current response for α3β4α5D-variant nAChRs. An average of individual oocyte recorded traces of nAChR concatemers compared to concatemers co-expressed with LYPD6B. As well as a bar graph of normalized induced ACh response expressed as a percent normalized to the control for all of the α3β4α5D heteromeric concatemers with LYPD6B. A. β4α3α5Dβ4α3 receptor at ACh doses (10^{-5.5} to 10^{-2}M), n=6. A1. β4α3α5Dβ4α3 and plus LYPD6B. The oocytes co-expressing the LYPD6B and β4α3α5Dβ4α3 exhibit about a 30% decrease induced ACh maximum response p value 0.0001. B. β4α3α5Nβ4α3 receptor at ACh doses (10^{-5.5} to 10^{-2}M), n=6. B1. β4α3α5Nβ4α3 and plus LYPD6B. C. β4α3β4α3α5D receptor at ACh doses (10^{-5.5} to 10^{-2}M), n=6. C1. β4α3β4α3α5D and plus LYPD6B. The oocytes co-expressing the LYPD6B and β4α3β4α3α5D exhibit about a 60% decrease induced ACh maximum response p value 0.0001. D. β4α3β4α3α5N receptor at ACh doses (10^{-5.5} to 10^{-2}M), n=6. D1. β4α3β4α3α5N and (+) LYPD6B.
Figure 7. LYPD6B decreased the time constant (τ) from steady-state desensitization for α3β4* heteromeric nAChR concatemers. A. Graphs of the averaged normalized time in seconds versus the averaged normalized ACh current response per α3β4* nAChR subtype with and without LYPD6B. Expression of concatemer subtype alone is represented by the black trace and concatemer plus LYPD6B is represented by the gray trace. From left to right and top to bottom: β4α3α3β4α3, β4α3β4β4α3, β4α3β4α3α3, and β4α3β4α3β4. B. A quantitative bar graph of the time constant (τ) for each group. Concatemer alone is represented by the black bar and concatemer plus LYPD6B is represented by the gray bar. The β4α3α3β4α3 and β4α3β4α3α3 nAChR concatemers when expressed with LYPD6B showed a statistical significant decrease in the time constant. p value 0.02 and 0.03 respectively.
Figure 8. LYPD6B did not affect the time constant (τ) from steady-state desensitization for α3β4α5 heteromeric nAChR concatemers. A. Graphs of the averaged normalized time in seconds versus the averaged normalized ACh current response per α3β4α5 nAChR subtype with and without LYPD6B. Concatemer alone is represented by the black trace and concatemer plus LYPD6B is represented by the gray trace. From left to right and top to bottom: β4α3α5Dβ4α3, β4α3α5Nβ4α3, β4α3β4α3α5D, and β4α3β4α3α5N. B. A quantitative bar graph of the time constant for each group. The black bar represents concatemer alone and the gray bar represents concatemer plus LYPD6B. There was no statistically significant difference among the time constants.
Supplemental Figure 1. There is no difference in relative fluorescence values in oocytes expressing concatamers that exhibited a decrease in ACh maximum induced current response due to LYPD6B. A. A bar graph of relative fluorescence values. Concatamer alone is illustrated in the black bar and concatemer with LYPD6B is illustrated in the gray bar (n= oocyte). Relative fluorescence values were normalized to an average fluorescence value from oocytes incubated without the avidin-beta-galactodise. From left to right: β4α3α3β4α3 (n= 11), β4α3α3β4α3 plus LYPD6B (n= 11), β4α3β4α3α3 (n= 11), β4α3β4α3α3 plus LYPD6B (n= 13), and control oocytes injected with β4α3β4α3α3 (n= 6). β4α3α5Dβ4α3 (n= 9), β4α3α5Dβ4α3 plus LYPD6B (n= 9), β4α3β4α3α5D (n= 13), β4α3β4α3α5D plus LYPD6B (n= 12), and control oocytes injected with β4α3α5Dβ4α3 plus LYPD6B (n= 4).
Expression of LYPD6B and colocalization with alpha3 containing nicotinic acetylcholine receptors in parasympathetic neurons

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Abstract

Prototoxins belong to the Ly6 superfamily and are known to modulate the function of nicotinic acetylcholine receptors (nAChRs). Yet, the regulation of mRNA expression and specificity for nAChR subtypes are still unclear. We have previously shown that PSCA reduces nicotine-induced increases in α7 nAChRs and that LYPD6B acts as a positive modulator of α3 containing heteromeric nAChRs. In this study we used the avian ciliary ganglion as a model to determine if nAChR activity regulates mRNA expression and co-localization of LYPD6B with α7 versus heteromeric α3* nAChRs. Because light is the stimulus driving nicotinic transmission in ciliary ganglia, we measured our two variables (mRNA expression and co-localization) from avian embryos kept in constant darkness versus constant light during embryonic day 7 (E7)-E14. The transcript levels of LYPD6B, CHRNA3, CHRNA5, CHRN4, and CHRNA7 increased in ciliary ganglia from embryos kept in constant dark compared to embryos kept in constant light. Daily application of nAChR antagonist drugs, α-methyllycaconitine (MLA) and Dihydro-β-erythroidine hydrobromide (DHβE) in embryos kept in constant light also caused an increase in CHRNA3, CHRNA5, CHRN4, and CHRNA7. However, LYPD6B expression levels were not restored in the drug treated group. When embryos were reared in constant dark, LYPD6B co-localized with mAb35 binding, which recognizes α3, α5 and β4 subunits. LYPD6B did not colocalize with alpha bungarotoxin binding, a marker for α7 containing nAChRs. These results provide further evidence that LYPD6B is a modulator of α3* nAChRs rather than α7 containing nAChRs.
Introduction

Signaling mediated by nicotinic acetylcholine receptors (nAChRs) is essential for controlling neural activity in the CNS and PNS. In the CNS, behaviors affected by nicotinic signaling are nicotine reinforcement, locomotion, working memory, anxiety, and depression (Picciotto et al., 2000). In the PNS nicotinic signaling is responsible for fast synaptic transmission at peripheral autonomic ganglia and at the neuromuscular junction (Campbell et al., 2010; Gray et al., 1996; MacDermott et al., 1999; McGehee et al., 1995; Wonnacott et al., 1989; Xu et al., 1999). Because of this extensive list of functions the cholinergic system has adapted a variety of mechanisms that modulate the nicotinic signaling.

Recent studies have suggested that one means of modulating nicotinic signaling is through the expression of endogenous prototoxin proteins. Prototoxin proteins are part of the Ly6 urokinase plasminogen activator receptor (Ly-6/uPAR) superfamily (Gumley et al., 1995). The proteins are small (12-20 kDa), express a glycosylphosphatidylinositol anchor (GPI anchor) (Gumley et al., 1995), and possess conserved 8-10 cysteine residues that form specific disulfide bonds, and allow for a tertiary three-finger motif, homologous to snake venom neurotoxins (α-bungarotoxin and α-cobratoxin) (Fleming et al., 1993; Gumley et al., 1995).
Because prototoxins share a similar protein structure to neurotoxin proteins (nAChR antagonists), it is hypothesized that prototoxins interact and modulate the response of nAChRs. Indeed, the prototoxin LYNX1 co-localizes with and increases the acetylcholine (ACh) induced macroscopic current generated by α7 and α4β2 nAChRs (Ibanez-Tallon et al., 2002; Miwa et al., 1999). An increase in the desensitization rate and ACh EC50 for both the α7 and α4β2 nAChRs was observed in Xenopus oocytes co-expressing LYNX2 and α7 or LYNX2 and α4β2 nAChRs (Tekinay et al., 2009). Another example is seen in transgenic mice over expressing LYPD6. The over expressing LYPD6 transgeneic mice exhibited an increase in Ca^{2+} influx specifically through the heteromeric α3* nAChRs of the trigeminal ganglia neurons (Darvas et al., 2009). All of these studies identify prototoxin proteins as modulators of nAChR signaling, however, they fail to demonstrate their specificity towards a specific nAChR subtype.

The avian ciliary ganglion is a convenient model system to study the function, expression, and biological relevance of prototoxins. The development of the ciliary ganglion has been well documented, and many critical events occur between embryonic day 8 (E8) and E14 (Landmesser and Pilar, 1974a, b), including programmed cell death, peripheral synaptogenesis, and morphological differentiation of two subtypes, choroid and ciliary neurons (Marwitt et al., 1971). Both ciliary and choroid neurons express two
subtypes of nAChRs: the homomeric α7 and the heteromeric α3 containing nAChRs (α3*) (Chiappinelli and Giacobini, 1978; Conroy and Berg, 1995; Vernallis et al., 1993). The latter group is made up of α3β4, α3β4α5, and sometimes α3β2 (Conroy and Berg, 1995).

We initially identified six prototoxin genes through a search of chicken EST databases. Of these, only three were expressed in the avian ciliary ganglion: prostate stem cell antigen (PSCA; (Hruska et al., 2009)), LYPD6B, and LY6E (Ochoa et al, submitted manuscript). PSCA was not expressed at E8 but was present at E14, indicating a possible role in programmed cell death (Hruska et al., 2009). When PSCA was misexpressed at E8 of the avian ciliary ganglion, choroid neurons were rescued from natural programmed cell death and a decrease in Ca$^{2+}$ influx specifically through homomeric α7 nAChRs was observed (Hruska et al., 2009). Additionally, mRNA PSCA expression and co-localization specifically with the homomeric α7 nAChR subtype in the avian ciliary ganglia are nAChR activity dependent (Otto. S. and Nishi R, unpublished data).

In contrast to PSCA, LYPD6B selectively modulates the function of α3* containing nAChRs but not α7. This was determined by systematically expressing concatemeric α3β4(α5) nAChRs in Xenopus oocytes with or without LYPD6B and using 2 electrode voltage clamp to measure responses to acetylcholine (ACh). LYPD6B decreased ACh EC50, increased the rate of desensitization to ACh, but decreased the maximum ACh
current response specifically for the $\alpha_3^*$ containing nAChRs (Ochoa et al, submitted manuscript).

In the present study we determined if the expression of LYPD6B and its co-localization with $\alpha_3^*$ nAChR subtypes are regulated by the activity of the avian ciliary ganglion nAChRs. The function of neurons in the ciliary ganglion is to cause pupillary constriction and enhance blood flow in response to light entering the eye. This system is active by E10 and fully developed by E14 (Landmesser and Pilar, 1972). Because of this well-established circuit, we utilized light as the stimulus in determining if nAChR activity regulated the expression of LYPD6B and its co-localization with $\alpha_3^*$ nAChRs. We also quantified the expression of CHRNA3, CHRNA5, CHRN4, and CHRNA7 transcripts ($\alpha_3$, $\alpha_5$, $\beta_4$, and $\alpha_7$ nAChR subunits) to determine if LYPD6B expression and the nAChR subunits coincided.
Material and Methods

Tissue- Premium SPF fertile chicken eggs from Sunrise Farms were incubated at 100°F. Cilary ganglia were collected by dissecting avian embryos at embryonic day 14 (E14).

RNA extraction and cDNA synthesis- Cilary ganglia from E14 avian embryos was collected and immediately flash frozen with dry ice. The RNA extraction was performed with the TRI reagent (Molecular Research Center, Cincinnati, OH)/ chloroform method. The cDNA was reverse transcribed from 1µg of total RNA with the Superscript III enzyme, oligo-dT, and random hexamers from the Superscript III reverse transcriptase kit (Invitrogen, Carlsbad, CA).

Real-time quantitative PCR (qPCR)- qPCR for LYPD6B was performed on the 7500 standard Real-Time PCR system (Applied Biosystems, Waltham, MA) using TaqMan probe and primers. Probe was labeled with 6 FAM reporter dye at its 5’ end and black hole quencher (BHQ) at their 3’ end. Primer set for LYPD6B was designed against LYPD6B nucleic acid sequence, Genbank accession number XM_422156. LYPD6B forward primer 5’ GGAGAACCTCGGGTGGCAGC3’ and reverse primer 5’ CACACCTTCCCCACTGTCTCCACA 3’. qPCR for the CHRNA3, CHRNA5, CHRN4, and CHRNA7 transcripts were performed by using TaqMan Gene expression assays (Applied Biosystems/Ambion, Waltham, MA). The constitutive gene control used
to normalize gene expression was chicken S17 ribosomal binding protein (Chrps) (Darland et al., 1995). The forward primer: 5’ AACGACTTCCACACCAACAA 3’ and the reverse primer: 5’ CTTCATCAGGTGGGTGACAT 3’. Relative transcript expression and number was determined using Sequence Detection Software (SDS) version 1.4.

*Designing and cloning LYPD6B/HA-RCASBP(A):* The insertion of the human influenza hemagglutinin (HA), amino acid sequence YPYDVPDYA was done with site-directed mutagenesis through PCR using the QuikChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). The forward primer is as follows 5’ CCCCACTCCATTCCAAACAGTTTTTATCCTTTATGATGTCCTGATTATGCTAA GTGCTTTACCTGTG 3’. The reverse primer is as follows: 5’ CACAGGTAAGCACCTTAGCATAATCAGGAACATCATAAGGATAAAAAACTG TTTGGAAATGGAGTGGG 3’. The HA epitope tag was inserted in frame into the full length of LYPD6B after the signal sequence but before the three finger-motif. The modified LYPD6B expressing HA was then cloned into the Slax13Nco1 plasmid. For in frame amino acid reading of LYPD6B/HA cloning must be done into the 5’ Nco1 and 3’ BamH1 restriction sites of slax13Nco1 (Morgan and Fekete, 1996). To clone into the restriction sites stated above an internal Nco1 enzyme restriction site was removed, 5’ Nco1 site was add, and a 3’ BamH1 site was added with site-directed mutagenesis as previously stated. The LYPD6B/HA-slax13Nco1, was cut with Cla1, and the LYPD6B-HA insert was cloned into the avian retroviral vector RCASBP(A) (Federspiel and Hughes, 1997).
Concentrating LYPD6B/HA-RCASBP(A) virus: Infective LYPD6B/HA-RCASBP(A) viral particles were generated by transfecting DF-1 chicken fibroblast cells with 1µg of LYPD6B/HA-RCASBP(A) plasmid DNA using Lipofectamine 3000 Transfection Kit (Invitrogen, Carlsbad, CA). Conditioned media containing viral stocks collected from DF-1 cells were concentrated ~ 20-fold by ultra-centrifugation at 90,000 x g at 4°C for 3 hours (Morgan and Fekete, 1996). Concentrated stocks were titered by limiting dilution and infectivity of cells as measured by staining with Charles River rabbit antiserum p27gag antibody. Stocks with concentration of > 10^7 infectious particles/ml were used for in vivo infection. Viral particles (60-120 nl) were injected into the mesencephalic enlargement of the neural tube of Hamburger/Hamilton stage (St.) 8-9 or St. 10-13 embryos using Drummond Nanoject microinjector. The shells were sealed with a glass coverslip and sterile vacuum grease and incubated at 37°C to the desired stage.

Exposure of avian embryos to constant light or dark: Avian embryos were windowed at Hamburger/Hamilton stage 9 and incubated in Model “1502” circulated Air Incubator (G.Q.F Manufacturing Co, Savannah, GA) set to 100°F. Once avian embryos reached E8 the three groups (constant dark addition of saline, constant light addition of saline, and constant light addition of MLA+DhβE) were incubated in two side-by-side sister clear plastic lidded Marsh Automatic incubators (Lyon Electronic Co, San Diego, CA). Both incubators were maintained at a constant temperature of 100°F and water was added as needed to maintain a constant humidity. The group kept in constant darkness had its
plastic lid entirely covered in aluminum foil. The group kept in constant light was kept in an incubator with a fluorescent light (Helical 20Watt CFL mercury bulb, GE, Fairfield, CT) suspended above it and left on 24 hours a day until embryos were harvested. The addition of 100 µl of saline and drugs onto the embryos was done daily. Saline (0.9% NaCl) or an MLA+DhβE drug cocktail in saline was added to yield a final concentration of 20 nM MLA (Sigma Aldrich St. Louis, MO) and 5 µM DhβE (Sigma Aldrich St. Louis, MO) dripped onto the chorioallantoic membrane. Concentrations were calculated assuming an average 60ml volume per egg (Kaiser et al., 2002; Liu et al., 2006).

Dissociating and staining E14 avian ciliary ganglia neurons- Ciliary ganglia collected from E14 chicken embryos were dissociated in 1mg/ml collagenase (catalog #4176; Worthington, Lakewood, NJ), 1mg/ml hyaluronidase (catalog # 2592; Worthington) and 10 mg/ml bovine serum albumin (BSA; Sigma Aldrich St. Louis, MO) dissolved in Earle’s balanced salt solution (EBSS) at 37°C for 15 min. Reaction was terminated by diluting in EBSS; cells were further dissociated by incubating ganglia for 5 min in 0.3% trypsin (catalog # 3703; Worthington) at 37°C followed by trituration. Reaction was terminated with EBSS and 10% (v/v) heat inactivated horse serum, 2% (v/v) fetal bovine serum (Invitrogen, Carlsbad, CA), 52U/ml penicillin, 50mg/ml streptomycin and 2 mM glutamine, and 20 ng/ml GDNF (PeproTech, London, UK).

Cells were plated on poly-d-lysine (0.5 mg/mL of Borate Buffer) and laminin (1 mg/mL of PBS) coated glass cover slips at a 1.5-2 ciliary ganglia/per coverslip concentration and
incubated at 37°C for 3 hours (acute culture) to allow neurons to settle before being labeled. After 3 hours of incubation neurons were stained and processed. Cover slips containing neurons were incubated at room temperature for 15 minutes in zamboni’s fixative (0.2 M Na₂HPO₄, 4% paraformaldehyde, 0.2 M NaH₂PO₄, and 10% picric acid pH to 7.2). The cells were then washed three times with EBSS and incubated on ice for an hour with the called for primary antibody. Primary antibodies diluted in EBSS: concentrated rat anti mAb35 (Developmental Studies Hybridoma Bank, Iowa City, IA, 1:50 dilution); mouse anti HA (Covance, Princeton, NJ, 1:1000); and Alexa-488 conjugated α-bungarotoxin (Invitrogen, Carlsbad, CA, 1:500 dilution at a final concentration of 2nM). The cells were then rinsed three times with EBSS and placed in blocking solution (1X PBS and 10% horse serum) for at least an hour at room temperature. Followed by an hour at room temperature incubation with secondary antibodies diluted in EBSS: Alexa fluor 488-conjugated affinity purified donkey anti-rat (Jackson ImmunoResearch Laboratores, West Grove, PA, 1:800, 1.6 nM) and Cy3-conjugated affinity purified donkey anti-mouse (Jackson ImmunoResearch Laboratores, West Grove, PA, 1:800, 1.6 nM), then rinsed three times with EBSS and attached to a colorfrost slide (Fisher Scientific, Waltham, MA) using Mowiol (Calbiochem/EMD Biosciences, San Diego, CA).

*Imaging ciliary ganglia cells -* Cells were imaged in the UVM Neuroscience COBRE Imaging Core Facility with a 60X/1.4 Plan Apochromatic objective using 1.512 refractive oil, on an inverted Olympus IX70 scope using a DeltaVision Deconvolution Restoration
Microscopy system (Applied Precision, Issaquah, WA) attached to a CoolSNAP HQ CCD camera using interline Sony ICX285 CCD (Photometrics, Tucson, AZ). Data acquisition was set at 0.1 μm/pixel. Thresholds were established using no primary controls. Serial optical sections were acquired in 200 nm steps inclusive of the entire volume of the cell. The entire coverslip was panned for cells and imaged. Images were deconvolved using SoftWoRx image analysis software version 3.5.1 (Applied Precision Inc., Issaquah, WA). Deconvolution uses iterative algorithms to de-blur the 3-D image stack, increase the contrast, reduce background, and capacitate resolution to exceed the Raleigh limit (Landmann and Marbet, 2004; McNally et al., 1999). Deconvolution used 10 iterations per stack. Three-dimensional (3D) reconstructions were created from deconvolved images.

*Pearson Coefficient analysis* - 3D images were converted into 2D projections using SoftWoRx Vision software. Using SoftWoRx, a Pearson's coefficient was measured in order to quantify the amount of colocalization between two channels, (480-500nm FITC and 541-569nm Cy3). The Pearson’s coefficient provides a value describing the linear relationship between two channels in all the voxels of a 2D image. This can range from -1 to 1. However, 0 and negative numbers indicate a lack of colocalization. The higher the number is, above 0, the greater the degree of colocalization. The Pearson’s coefficient obtained is for the entire cell body of the deconvolved 2D image. The mean Pearson coefficient ± standard error (SEM) was calculated. These Pearson’s coefficients were analyzed for significance using Graphpad Prism software (GraphPad Prism 6.0, 92
GraphPad Software, Inc., La Jolla CA). Unless otherwise noted two-tailed student t-test with an alpha level of $p < 0.05$ were considered significant.
Results

*Transcript expression and immunofluorescence of LYPD6B in the presence and absence of nAChR activity*

In order to determine if the expression of LYPD6B is regulated through nAChR activity, E7 embryos were incubated for 7 days under three conditions: constant light exposure with a daily application of saline, constant dark exposure with a daily application of saline, and constant light exposure with a daily application of a final concentration of 20 nM of MLA and 5 µM of DhβE, which completely blocks nicotinic transmission through the ganglion. LYPD6B mRNA levels were higher in the ciliary ganglia of avian embryos kept in constant darkness compared to the ciliary ganglia of avian embryos kept in constant light (fig. 1A). However, the LYPD6B mRNA levels were not restored in the third group of embryos kept in constant light with a daily application of nicotinic antagonists MLA and DhβE (fig. 1A). The expression of CHRNA3, CHRNA5, CHRNA7 and CHRNB4 transcripts were also higher from ciliary ganglia of avian embryos kept in constant dark versus constant light (fig 1B). Furthermore, application of the nicotinic antagonists, MLA and DhβE, also exhibited an increase in CHRNA3, CHRNA5, CHRNA7 and CHRNB4 mRNA levels comparable to that of embryos kept in the dark.

Because it is known that LYPD6B increases the ACh sensitivity of the α3* heteromeric nAChRs, we hypothesized that LYPD6B would specifically co-localize with the α3*
nAChRs in the avian ciliary ganglia and that this co-localization is regulated by nAChR activity. Co-localization between LYPD6B and α3* was determined by staining neurons from dissociated ciliary ganglia through immunocytochemistry. However, there are currently no commercial available antibodies against avian prototoxin proteins. Thus, we genetically engineered LYPD6B to express a human influenza hemagglutinin (HA) epitope tag. The HA epitope tag was inserted after the signal sequence but before the three finger motif (fig 2A). We then inserted LYPD6B/HA into an avian retrovirus plasmid. Avian df-1 fibroblasts cells were transfected with the LYPD6B/HA retrovirus plasmid, so that the active virus could be collected and concentrated from the cell media. Proper processing of LYPD6B/HA was determined with a Western blot against the HA epitope tag of LYPD6B from transfected versus non-transfected avian df-1 fibroblasts cells. The Western blot shows three bands: 14kDa, 17kDa, and 20kDa (fig 2B). The entire protein length, signal sequence, and GPI cleavage site for LYPD6B are identified and documented on ensembl (www.ensembl.org). Thus, it was determined that each band represents the expected processed LYPD6B proteins. The 20kDa band represents the full length LYPD6B with the addition of HA, the 17kDa represents LYPD6B/HA minus the signal sequence, and the 14kDa represents LYPD6B/HA minus the signal sequence and GPI linkage (fig 2B).

**LYPD6B specifically co-localizes with α3* heteromeric nAChRs**

In order to characterize the co-localization of LYPD6B and nAChRs within the avian ciliary ganglia, we performed immunocytochemistry on neurons from dissociated ciliary
ganglia and calculated a Pearson’s coefficient of co-localization. The Pearson’s coefficient is statistically significant for the co-localization of LYPD6B and $\alpha_3^* \text{nAChRs}$ from the ciliary ganglion of avian embryos kept in constant darkness versus avian embryos kept in constant light with the addition of saline (from a Pearson’s coefficient of $0.4530 \pm 0.026$; n=13 to $0.3653 \pm 0.0308$; n=13; p value < 0.05) (Table 1, fig 3A-C).

Since LYPD6B is known to modify the function of $\alpha_3^* \text{nAChRs}$ and not $\alpha_7$ nAChRs, we wanted to determine if LYPD6B specifically co-localized with $\alpha_3^*$ and not $\alpha_7$ nAChRs. This was done by comparing the measured Pearson’s coefficient for the co-localization between LYPD6B and $\alpha_3^*$ versus LYPD6B and $\alpha_7$ nAChRs, from previously stained cells. The Pearson’s coefficient is significantly higher for the co-localization of LYPD6B and $\alpha_3^* \text{nAChRs}$ in the ciliary ganglion of avian embryos kept in constant darkness compared to the co-localization of LYPD6B and the homomeric $\alpha_7$ nAChRs (from $0.4530 \pm 0.026$; n=13 to $0.139 \pm 0.022$; n=4; p value 0.0001) (fig. 4A, B, and E). The Pearson’s coefficient is also significantly higher for the co-localization of LYPD6B and $\alpha_3^* \text{nAChRs}$ in the ciliary ganglia of avian embryos kept in constant light compared to the co-localization of LYPD6B and the homomeric $\alpha_7$ nAChRs (from $0.3653 \pm 0.0308$; n=13 to $0.228 \pm 0.029$; n=6; p value 0.01) (fig. 4C, D, and E).
Discussion

This study aimed to 1) determine the regulation of LYPD6B mRNA expression 2) to determine the condition/s necessary for specific co-localization between LYPD6B and α3* nAChRs and 3) to further support LYPD6B specificity towards α3* nAChRs. Although there is a correlation of gene expression between LYPD6B and CHRNA3, CHRNA5, CHRNB4 and CHRNA7 transcripts, their gene expressions seem to be regulated by two different mechanisms. Interestingly CHRNA3, CHRNA5, CHRNB4 and CHRNA7 transcripts seem to be regulated by a nAChR activity mechanism, whereas LYPD6B seems to be regulated independent of nAChR activity. Additionally, a mechanism dependent on nAChR activity also seemed to drive the co-localization of LYPD6B and α3* nAChRs. Lastly LYPD6B failed to co-localize with the α7 nAChRs, which further supports the specificity LYPD6B exhibit towards the α3* nAChRs.

Because LYPD6B modulates the function of α3* nAChRs, we hypothesized that nAChR activity would regulate the expression of LYPD6B. The LYPD6B expression levels were quantified through qPCR, which gives insight to transcript levels and does not necessarily translate to the amount of protein. However, LYPD6B is known to regulate the channel properties of the α3* nAChRs (Ochoa and Nishi, submitted manuscript), thus its biological function suggest the relevance of translating LYPD6B transcript levels. Therefore, we conclude that the transcript expression levels of LYPD6B are equivalent to
the amount of protein levels. The mRNA levels of LYPD6B increased from E14 embryos lacking an active nAChR activity (embryos kept in the dark), implying LYPD6B expression to be regulated by nAChR activity. Yet, blocking nAChR activity with nAChR antagonist drugs failed to restore LYPD6B expression levels, comparable to the levels found from embryos kept in the dark. Thus, a mechanism independent of nAChR activity must be involved in the regulation of LYPD6B gene expression. We then went on to investigate the expression of CHRNA3, CHRNA5, CHRNB4, and CHRNA7 transcripts. CHRNA3, CHRNA5, CHRNB4, and CHRNA7 transcripts increased from avian embryos lacking nAChR activity (embryos kept in the dark), but in contrast to LYPD6B, embryos having nAChR activity blocked exhibited an increase in CHRNA3, CHRNA5, CHRNB4, and CHRNA7 transcripts comparable to the levels observed in embryos lacking nAChR activity. In this case CHRNA3, CHRNA5, CHRNB4, and CHRNA7 transcripts seem to be regulated by nAChR activity, at least in the developing ciliary ganlion. Such observation is similar to what is observed in the extensor digitorum longus muscle of adult rats, in which stimulation of the muscle decreased the amount of acetylcholine receptors expressed on the surface of the muscle (Fambrough, 1979). In conclusion, the expression of LYPD6B and CHRNA3, CHRNA5, CHRNB4, and CHRNA7 transcripts coincide with each other but seem to be regulated by two different mechanisms: one independent of nAChR activity and the other dependent on nAChR activity.
Though there is an extensive amount of research identifying neural innervation as a regulator of nAChR gene expression, it is controversial as to whether this is due to nerve activity or a released growth factor. All of the experiments severed the pre- and postganglionic inputs, nerves coming from the accessory oculomotor nucleus and exiting the ciliary ganglion respectively from post-hatched chickens (Boyd et al., 1988; Jacob and Berg, 1987, 1988; McEachern et al., 1989). Using quantitative PCR, microscopic images, radiiodinated antibodies, and Northern blot, researchers determined that the mRNA expression of nAChR subunits of the ciliary ganglion of newly hatched chickens are regulated through innervation and target tissues (Brumwell et al., 2002; Jacob and Berg, 1987; Levey et al., 1995; McEachern et al., 1989). It is clear that the innervating nerve is regulating nAChR expression, however, none of these studies aimed to identify the factor/s released from the innervating nerve. Furthermore, innervation to the avian ciliary ganglion and synaptogenesis of the ganglion to its targets occurs between E4-E14 (Landmesser and Pilar, 1974), thus, these studies identify neural innervation as a component of nAChR gene expression after these events have occurred. In contrast, this study identifies a mechanism of nAChR subunit mRNA expression during the development of innervation, synaptogenesis, and programmed cell death.

The idea of a prototoxin protein interacting with all nAChRs subtypes is supported by the LYNX1 studies. Through in-situ hybridization and immunocytochemistry, LYNX1 mRNA and protein was demonstrated to be present in the deep layers of the cortex, hippocampus, and cerebellum a pattern similar to the α4β2 and α7 nAChRs in the Mus
Additionally, the use of a monoclonal antibody against purified LYNX1 protein demonstrated co-localization of LYNX1 and α4 as well as α7 nAChRs within the neurons of the cortex, amygdala, habenular complex, and substantia nigra (Ibanez-Tallon et al., 2002). In that same study LYNX1 was shown to increase the ACh induced macroscopic current in both α4 and α7 nAChRs (Ibanez-Tallon et al., 2002). These data identify LYNX1 as a promiscuous prototoxin, modulating different nAChR subtypes. In contrast, this study determines LYPD6B to co-localize exclusively with the α3* nAChR subtypes. Such observation is further supported by the macroscopic current recordings collected on Xenopus oocytes co-expressing LYPD6B and α3* nAChRs (Ochoa, V. and Nishi R, manuscript in preparation). LYPD6B decreased the ACh EC50, time constant desensitization rate (tau), and maximum induced current response for the low sensitivity (α3)(β4) nAChRs but did not affect the α7 homomeric nAChRs (Ochoa and Nishi, submitted manuscript).

Lastly, the most interesting data collected in this study was the observed co-localization between LYPD6B and α3* nAChRs to occur in the absence of nAChR activity (embryos kept in constant darkness). The co-localization of LYPD6B and α3* nAChRs implies an important function in increasing ACh potency for the α3* in the absence of nAChR activity. The biological importance of such co-localization, in the avian ciliary ganglion, was not investigated in this study. However, an intriguing idea is maintaining...
homeostatic activity within a circuit. Two concepts identified as regulators of
homeostatic activity are: 1. Balance between excitatory and inhibitory inputs and 2. An
excitatory threshold set point within each neuron (John and Berg, 2015). Modifying these
two processes can affect the homeostatic activity of an intact neural circuit, and have
aberrant effects. Thus, neurons must express mechanisms that allow the restoration of
their homeostatic activity.
Literature Cited


**Table 1. α3* nAChRs but not α7 colocalize with LYPD6B.** A table summarizing the Pearson’s coefficient for the co-localization of nAChRs and HA (the presence of α3* nAChRs and LYPD6B). As well as the Pearson’s coefficient for the co-localization of α-bungarotoxin (α-btx) with HA (the presence of α7 nAChRs and LYPD6B).

<table>
<thead>
<tr>
<th>Primary antibody staining</th>
<th>Pearson’s coefficient Dark incubation</th>
<th>Pearson’s coefficient Light incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA/ mAb35</td>
<td>0.4530 ± 0.026; n=13</td>
<td>0.3653 ± 0.0308; n=13</td>
</tr>
<tr>
<td>HA/α-btx</td>
<td>0.139 ± 0.022; n=4</td>
<td>0.228 ± 0.029; n=6</td>
</tr>
</tbody>
</table>
Figure 1. Lack of nAChR activity increases nAChR subunit mRNA levels, but does not increase LYPD6B. A. Quantitative PCR on LYPD6B from avian ciliary ganglia kept in constant light, dark, and light plus an addition of MLA/DhβE. mRNA levels for LYPD6B increased in the group kept in constant darkness compared to the group kept in constant light and constant light with the addition of MLA/DhβE; n=4; p value ~0.01. B. Quantitative PCR on α3, β4, α5, and α7 nAChR subunits from avian ciliary ganglia kept in constant light, dark, and light plus an addition of MLA/DhβE. mRNA levels for all nAChR subunits increased in the group kept in constant darkness as well as the group kept in constant light with the addition of MLA/DhβE compared to the group kept in constant light; n= 2-4.
Figure 2. Genetically engineered LYPD6B expresses HA epitope tag. A. Western blot identifies LYPD6B to be a glycosylphosphatidylinositol anchored protein. A. A cartoon image of LYPD6B protein with the insertion of the HA epitope tag. B. A Western blot of avian df-1 fibroblast cells not infected versus infected with the avian retrovirus expressing LYPD6B/HA.
Figure 3. LYPD6B colocalizes with α3* nAChRs in the ciliary ganglia of avian embryos kept in constant darkness. A. An image of a neuron stained for HA in red and α3* nAChRs in green from dissociated ciliary ganglia of avian embryos kept in constant darkness. The arrows indicate colocalization. B. An image of a neuron stained for HA in red and α3* nAChRs in green from dissociated ciliary ganglia of avian embryos kept in constant light. C. A graph of each neuron’s Pearson’s coefficient from the two groups: avian embryos kept in constant darkness versus constant light. There is a higher Pearson’s coefficient for LYPD6B and α3* nAChRs from avian embryos kept in constant darkness (n = 13) versus constant light (n = 13); p value < 0.05.
Figure 4. LYPD6B colocalizes with α3* nAChRs but not α7 in the ciliary ganglia of avian embryos kept in constant darkness and light. A. An image of a neuron stained for HA in red and α3* nAChRs in green from dissociated ciliary ganglia of avian embryos kept in constant darkness. B. An image of a neuron stained for HA in red and α7 nAChRs in green from dissociated ciliary ganglia of avian embryos kept in constant darkness. C. An image of a neuron stained for HA in red and α3* nAChRs in green from dissociated ciliary ganglia of avian embryos kept in constant light. D. An image of a neuron stained for HA in red and α7 nAChRs in green from dissociated ciliary ganglia of avian embryos kept in constant light. E. A graph of each neuron’s Pearson’s coefficient from the four groups: avian embryos kept in constant darkness stained for LYPD6B and α3* versus neurons stained for LYPD6B and α7 nAChRs. Avian embryos kept in constant light stained for LYPD6B and α3* versus neurons stained for LYPD6B and α7 nAChRs. There is a higher Pearson’s coefficient for LYPD6B and α3* nAChRs (n = 13) versus LYPD6B and α7 (n = 4) from the avian embryos kept in constant darkness as well as embryos kept in constant light (n = 13, 6, respectively), p value 0.0001 and 0.01 respectively.
CHAPTER 4
SUMMARY AND CONCLUSIONS

The principal finding in this dissertation is the identification of another prototoxin member of the Ly6 superfamily, LYPD6B, which specifically modifies function and co-localizes with $\alpha_3^*$ containing heteromeric nAChRs. *Xenopus* oocytes co-expressing human LYPD6B and $\alpha_3^*$ nAChRs exhibited an increase in ACh sensitivity, desensitization rate, and a decrease in the ACh induced maximum current response for the low sensitivity $(\alpha_3)_2(\beta_4)_2$ nAChR subtype, but no functional effects were observed for the homomeric $\alpha_7$ nAChR. Additionally, LYPD6B only decreased the maximum ACh-induced maximum current for the $\alpha_3^*$ heteromeric nAChRs containing an $\alpha_5$ variant and not the $\alpha_5$ N-variant associated with nicotine dependence. Furthermore, in the avian ciliary ganglion of embryos kept in constant darkness (reduced nAChR activity), LYPD6B co-localizes with $\alpha_3^*$ heteromeric, but not with $\alpha_7$ homomeric nAChRs. These results identify a mechanism in modifying the amount of nicotinic signaling a cholinergic neuron receives. The biological relevance of such modulation, within the context of the avian ciliary ganglia, will be explored in this discussion.

Programmed cell death during neurodevelopment is well studied in the avian ciliary ganglion. Programmed cell death is known to occur between stage 34 and 40, which is
equivalent to embryonic day 8-14 (E8-E14) (Landmesser and Pilar, 1974b). During this period about 50% of the ciliary ganglion neurons are eliminated, and because peripheral synaptogenesis is also occurring, neuron survival seems to be supported by target released neurotrophic factors. Indeed, such conclusion is supported by the rescuing of ciliary ganglion neurons due to the over-expression of chCNTF in vivo (Finn et al., 1998; Finn and Nishi, 1996). However, a significant number of neurons die at E6 well before synaptogenesis has occurred, implying that there is another mechanism involved in supporting neuron survival.

The activity of nAChRs is involved in programmed cell death during neurodevelopment of the avian ciliary ganglion. Blocking activity through the nAChRs exacerbated cell death during this programmed cell death period. More specifically avian embryos treated with chlorisondamine, pempidine, or d-tubocurare reduced the survival of the neurons (Maderdrut et al., 1988; Meriney et al., 1987). Yet, application of specific α7 nAChR antagonist, such as α-bungarotoxin (αbtx) and methyllycaconitine (MLA), rescued ciliary ganglion neurons (Bunker and Nishi, 2002; Hruska and Nishi, 2007), an indication that a signal specifically through the α7 nAChRs induces cell death during the programmed cell death period. Thus, neurons must express a mechanism that regulates α7 signaling.
Because the current identified prototoxins reduce the amount of inward current going through the nAChRs, we hypothesized PSCA to interact with α7 nAChRs and reduce the amount of inward current, and this reduction in inward current is a mechanism in supporting neuron survival during programmed cell death. This hypothesis was further supported by two sets of evidence 1. in-situ hybridization and qPCR methods both illustrate a correlation in expression between PSCA and α7, suggestive of an interaction between the two proteins and 2. PSCA expression pattern, absent at E8 present at E14, suggest PSCA to have a function in programmed cell death. The hypothesis was then tested by misexpressing PSCA in the avian ciliary ganglion at E8 and determining if ciliary ganglion neurons were rescued from their programmed cell death phenomena (Hruska et al., 2009). It was concluded that PSCA misexpression rescued only the choroid neurons (Hruska et al., 2009). In order to demonstrate that the choroid neuron survival was due to a decrease in α7 activity calcium imaging was performed on dissociated E8 ciliary ganglia, that over expressed PSCA, in the presence of TTX and cobalt (Hruska et al., 2009). The ciliary ganglia neurons exhibited a $[Ca^{2+}]_i$ decrease in response to 10µM nicotine, and the current response could not be further reduced with the application of αbtx, suggestive that the residual Ca$^{2+}$ response is due to the α3* nAChRs (Hruska et al., 2009). In conclusion PSCA is acting as a negative modulator (brake) and reducing the function specifically for the α7 nAChR and the interaction of PSCA and α7 rescues choroid neurons from their programmed cell death process.
Since, PSCA regulates the activity of α7 nAChRs we hypothesized that its mRNA expression and co-localization to be nAChR activity dependent. When avian embryos were kept in constant light exposure PSCA expression levels increased (Otto and Nishi, unpublished), presumably because of an increase in nAChR activity. Furthermore, the constant light exposure drove a higher Pearson’s coefficient for the co-localization between PSCA and α7 in ciliary and choroid neurons, whereas a smaller Pearson’s coefficient was observed for the co-localization of PSCA and α3* nAChRs (Otto and Nishi, unpublished data). The major conclusion drawn from these experiments is the specificity of PSCA towards the α7 nAChRs seems to be driven by nAChR activity.

In contrast to PSCA, LYPD6B is a complex allosteric modulator specifically for the α3* heteromeric nAChRs. In the presence of LYPD6B low ACh concentrations are able to activate the (α3)(β4)2 nAChRs and allow current to flow through (reference to Chapter 2). However, as ACh concentrations increase LYPD6B decreases the amount of current that may flow through the (α3)(β4)2 receptors as seen in the reduced Imax (reference to Chapter 2). Additionally, LYPD6B does not affect ACh potency for (α3β4)2(α5D-variant) nAChRs, but does decrease the amount of current flowing through the receptor (reference to Chapter 2). In this aspect LYPD6B behaves similarly to PSCA, where a decrease in nAChR function is observed due to a prototoxin.
Because the expression of PSCA was increased by nAChR activity, we questioned if LYPD6B was also regulated by the same mechanism. Again avian embryos were kept in constant light, but this time LYPD6B mRNA levels failed to increase (reference to Chapter 3). They did however increase from embryos kept in constant darkness. These data show LYPD6B does not increase with nAChR activity. Furthermore, the constant darkness drove a higher Pearson’s coefficient for the co-localization between LYPD6B and $\alpha_3^*$ in ciliary ganglia neurons, whereas the opposite was observed for the co-localization of LYPD6B and $\alpha_7$ nAChRs (reference to Chapter 3). In conclusion PSCA and LYPD6B act through opposing mechanisms, possibly to promote cell survival. PSCA protects neurons from programmed cell death by reducing excessive influx through $\alpha_7$ nAChRs. In contrast, LYPD6B enhances ACh sensitivity of the $\alpha_3^*$ nAChRs, which may promote trophic effects and support neuron survival.

**Significance of the results**

Since blocking the $\alpha_7$ signal rescues neurons and blocking all nAChR activity exacerbated neuron death, signaling coming through the other nAChR subtype ($\alpha_3^*$ nAChRs) must be involved in neuron survival. Such hypothesis could be used to explain the increase in co-localization between the LYPD6B and $\alpha_3^*$ nAChRs from avian embryos kept in constant darkness. The circuitry stimulated by light will release ACh and activate the nAChRs located on the ciliary and choroid neurons of the ciliary ganglia.
However, in the dark this circuitry remains off, leaving very minimal amounts of ACh due to spontaneous activity. If the signaling from $\alpha_3^*$ nAChRs is required for the support of neuron survival then the neuron would have to adapt a mechanism which would allow the $\alpha_3^*$ receptors to be activated even at low ACh concentrations. Based upon the functional data presented in this dissertation, the interaction of LYPD6B and $\alpha_3^*$ nAChRs increases the ACh sensitivity, thus the co-localization of LYPD6B and $\alpha_3^*$ in the dark would be a way to increase the probability of receiving a constant signal through the $\alpha_3^*$ nAChRs.

Though the implications LYPD6B has on programmed cell death were not investigated in this dissertation, I am proposing an experiment that will further support such hypothesis. A straightforward experiment would be knocking down LYPD6B and through a stereology based method count ciliary and choroid neurons from ciliary ganglia of avian embryos kept in constant darkness. From this experiment one would expect a decrease in cell number for the LYPD6B knockdown group versus the group that still expressed LYPD6B.

In order to explore other possible functions of LYPD6B it is pertinent to discuss the mechanisms cholinergic neurons use in order to regulate the amount of received nicotinic signaling. One mechanism is modifying nAChR response properties by regulating the stoichiometry of heteromeric nAChRs. Both the $\alpha_3\beta_4$ and $\alpha_4\beta_2$ heteromeric nAChRs
exist in two isoforms: those containing a ratio of 2α:3β or 3α:2β (Moroni and Bermudez, 2006). For the α4β2 heteromeric nAChRs the two isoforms differ in their channel properties, cation permeability, and response to chronic nicotine. The (α4)2(β2)3 is known as the high sensitivity form because it is more sensitive to ACh, whereas the (α4)3(β2)2 is known as the low sensitivity form because it is less sensitive to ACh (Mazzaferro et al., 2011). By measuring the Ca\textsuperscript{2+} reversal potential it was determined that the low sensitivity (α4)3(β2)2 receptor had a higher permeability to Ca\textsuperscript{2+} then the high sensitivity (α4)2(β2)3 nAChRs (Tapia et al., 2007). Chronic nicotine exposure to α4β2 nAChR transfected HEK 293 cells portrayed channel properties similar to the low sensitivity (α4)2(β2)3 receptor form (Kuryatov et al., 2005; Nelson et al., 2003), suggesting that nicotine behaves as an assembly chaperone only for the low sensitivity isoform.

Another mechanism in regulating the amount of nicotinic signaling received is increasing or decreasing expression of a particular nAChR type. Though increasing or decreasing expression of a nAChR may occur at the transcription level a post-translational approach is through exocytosis and endocytosis. Exocytosis is the fusion of small vesicles to the plasma membrane allowing a variety of proteins to be located in the plasma membrane. Endocytosis is the removing of small vesicles from the plasma membrane, thus removing where proteins are located (Tojima and Kamiguchi, 2015). The last method is through post-translational modifications. Examples of these include palmitylation, myristoylation,
glycosylation, and phosphorylation all of which have been known to occur in the α4β2 and α7 nAChRs (Alexander et al., 2010; Fenster et al., 1999a; Hoffman et al., 1994; Kawai and Berg, 2001; Ramarao and Cohen, 1998).

Though data presented in this dissertation demonstrates that LYPD6B modulates the response properties of the α3* heteromeric nAChR, it does not exclude the possibility of having chaperone protein-like functions. An example of this would be the assembly of a particular stoichiometry for heteromeric nAChRs; such example has been seen with LYNX1. By conducting a donor-recovery after photobleaching (DRAP) FRET experiment on mouse N2a cells transfected with a particular nAChR subunit plus or minus LYNX1 it was determined that LYNX1 stabilized the α4/α4 dimer. More specifically, cells co-transfected with α4-GFP and α4-mCherry showed an increased in FRET efficiency in the presence of LYNX1 (Nichols et al., 2014). However, these observations were not observed with cells co-transfected with β2-GFP and β2-mCherry (Nichols et al., 2014). These data imply LYNX1 to stabilize the low sensitivity α4β2 nAChRs that is those that have an α4/α4 interface. Additionally, the ACh EC50 from N2a cells transfected with α4-GFP, β2-wt nAChR subunits, and LYNX1-mCherry resembled the ACh EC50 for the low sensitivity (α4)3(β2)2 nAChRs (Nichols et al., 2014).
Another chaperone protein-like function is increasing or decreasing the expression of nAChRs, in other words the idea that LYPD6B has the ability to traffic nAChRs. Again an example of this has been observed for a different prototoxin protein, Ly6h. The amount of $\alpha_7$ expressed on the surface of HEKtsa cells, previously transfected with YFP-$\alpha_7$ cDNA alone or in combination with Ly6h, was quantified via a biotinylation labeling method. The cells were labeled with biotin, lysed, biotin was then immunoprecipitated with streptavidin, and Western blotted to determine the levels of internal versus surface $\alpha_7$ (Puddifoot et al., 2015). The cells that were co-transfected with Ly6h exhibited a decrease in surface of $\alpha_7$ expression (Puddifoot et al., 2015), an indication that Ly6h is retaining the $\alpha_7$ nAChR.

A provocative idea is the possible function/s prototoxins may have on diseases characterized by a nicotinic signaling imbalance, such as Alzheimer’s disease (AD). AD is one of the most common causes of dementia (Kumar et al., 2015). Though there are two types of AD the more common type is known as sporadic (SAD) (Kumar et al., 2015). The characteristics of AD include the accumulation of amyloid $\beta$ (A$\beta$) plaques around neurons and the hyperphosphorylation of microtubules due to the tau protein, which leads to the formation of neurofibrillary tangles inside neurons (Kumar et al., 2015). These events are thought to cause an increase in cholinergic neuron death. As a consequence of cholinergic neuron death is a decrease in nicotinic signaling, this decrease in nicotinic signaling is thought to be a contributing factor in the cognitive
deficits observed in AD patients (Kumar et al., 2015). However, the identification of cholinergic neuron death has been done on post-mortem tissue and fails to explain what may contribute to cognitive deficits at earlier time points.

I propose that an imbalance in nicotinic signaling, due to deficiencies in prototoxin protein expression, may be a cause of the cognitive deficits observed in AD patients. Such idea has been explored for the prototoxin LYNX1. An H&E staining on coronal sections through the dorsal striatum of the LYNX1 knockout mouse portrayed a vacuolating phenotype (Miwa et al., 2006). It was concluded that such vacuolating phenotype was due to an increase in neuron death. This conclusion was also supported by the mixed cortical cultured neurons, from the LYNX1 knockout mouse, which exhibited a decrease in percent neuron survival when exposed to 100µM of glutamate (Miwa et al., 2006). Additionally, the cortical cultured neurons were not protected by a10µM nicotine pre-treatment (Miwa et al., 2006). It was concluded that the absence of LYNX1 increased a neurons susceptibility to glutamate excitotoxicity, because of increased nicotinic signaling (Miwa et al., 2006). It would be interesting to determine if LYPD6B behaves similarly to LYNX1. Based upon the functional data presented in this dissertation, LYPD6B decrease the amount of signal coming through the α3* nAChRs, AD patients that fail to express or lose the expression of LYPD6B would have a higher signal coming through the α3* nAChRs making neurons more susceptible to excitotoxicity.
Prototoxins may also have function/s in nicotine dependence, which is attributed by the imbalance nicotinic signaling of the reward and aversion pathways. The nicotine reward pathway has been identified as dopaminergic neurons located in the ventral tegmentum area of the midbrain and project to the nucleus accumbens (Corrigall et al., 1994; Corrigall et al., 1992), these dopaminergic neurons are known to express the α4, α6, β2, and β3 nAChR subunits (Klink et al., 2001; Zoli et al., 2002). In contrast the aversion pathway highly expresses the α3, α5, and β4 nAChR subunits in the medial habenula of the diencephalon, and have glutamatergic projects to the interpuduncular nucleus (De Biasi and Salas, 2008; Girod et al., 2000; Mata et al., 1977; Ren et al., 2011; Sheffield et al., 2000; Staines et al., 1980; Vincent et al., 1980).

Because of a single nucleotide polymorphism (SNPs) identified in the CHRNA3, CHRNA5, and CHRNB4 genes (Wen et al., 2014), there has been a large interest in determining the relationship α3, α5, and β4 nAChR subunits have in regards to nicotine dependence. The α5 knockout mouse via intracranial self-stimulation continuously administered nicotine compared to wild type; this was attributed by the absence of a nicotine aversion effect (Fowler et al., 2011). Furthermore, the α5 knockout mouse had less of a cFos immunoreactivity staining compared to wild type mice brains, an indication of less neuronal activity (Fowler et al., 2011). These data suggest that the incorporation of the α5 nAChR subunit leads to the activation of the nicotine aversion pathway. When determining how this information relates to the α5 D versus α5 N-variant, comparisons
of the physiological properties between the $\alpha_5$ N-variant versus D must be made. The incorporation of the $\alpha_5$ D-variant into the ($\alpha_3\beta_4$) nAChRs exhibits an increase in receptor function (George et al., 2012). Meaning there is a greater amount of current flowing through the receptor when activated by ACh (George et al., 2012). The incorporation of the $\alpha_5$ N-variant into the ($\alpha_3\beta_4$) nAChRs also increases receptor function but is statistically less than what is observed for ($\alpha_3\beta_4$)$_2$$\alpha_5$ D-variant nAChRs (George et al., 2012). This decrease in the amount of current flowing through the ($\alpha_3\beta_4$)$_2$$\alpha_5$ N-variant nAChRs may fail to activate the nicotine aversion pathway. Similarly, LYPD6B is seen to decrease the ACh induced maximum current response of the $\alpha_3\beta_4$ heteromeric nAChRs containing an $\alpha_5$ D-variant subunit, such observation may be another mechanism in nicotine dependence. The genome wide association study (GWAS) identified a SNP in the CHRNA5 gene to be associated with nicotine dependence and this may be due to the absence of the aversion pathway. However, the SNP fails to explain why adolescents display a blunted withdrawal symptom. It would be interesting to investigate the expression of LYPD6B in wild type adolescent mice, and determine if the expression of LYPD6B within the medial habenula of wild type adolescent mice fails to activate the nicotine aversion pathway similarly to the $\alpha_5$ knockout mouse.
Future directions

The first list of future experiments will further test the conclusion that LYPD6B specifically modulates the heteromic $\alpha_3^*$ nAChRs. A study to be performed in the future would be testing LYPD6B against other heteromic nAChRs. A common heteromic nAChR highly expressed in the brain and known to exist in two stoichiometry subtypes is the $\alpha_4\beta_2$ nAChR (Luetje et al., 1990; Moroni and Bermudez, 2006). Additionally, because my experiments were intracellular macroscopic recordings of Xenopus oocytes co-expressing LYPD6B and $\alpha_3^*$ heteromic or LYPD6B and $\alpha_7$ homomic nAChRs, it would be pertinent to perform single channel recordings to further support the functional characteristics observed by LYPD6B against the $\alpha_3^*$ heteromic and $\alpha_7$ homeric nAChR subtypes.

The second set of experiments aims to understand the regulation of LYPD6B expression in the avian ciliary ganglion. The data in this dissertation demonstrates LYPD6B mRNA levels to increase from avian ciliary ganglion of embryos kept in constant darkness, suggesting that LYPD6B expression is dependent on the lack of nAChR activity. However, when nAChR activity was blocked with the application of nAChR antagonist drugs, LYPD6B expression was not restored. Thus, LYPD6B expression is regulated independently of any nAChR activity. Future experiments would aim to determine the factor/s that regulate the mRNA LYPD6B expression. Additionally, a pixel intensity
comparison from immunoflourescence experiments staining for HA/LYPD6B on neurons from dissociated avian ciliary ganglia kept in constant dark versus constant light would further support the data demonstrating the increase in LYPD6B expression from avian embryos kept in constant darkness.
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APPENDIX

Targeting the α5 nicotinic acetylcholine receptor (nAChR) subunit as a treatment for neuroblastoma.

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Abstract

Neuroblastoma is a pediatric extra-cranial solid tumor that accounts for only 7% of malignancies in children less than 15 years of age, but makes up 15% of pediatric oncology deaths. Thus it is an aggressive cancer for which new treatments are badly needed. In an effort to identify a drug that targets neuroblastoma, we identified, MG 624, a nicotinic acetylcholine receptor (nAChR) antagonist, by testing its efficacy in killing that tumor initiating cells (TICs) isolated from the bone marrow of patients with stage IV neuroblastoma (unpublished data). We discovered that several different TIC cell lines, several neuroblastoma cell lines (SKN-SY5Y; SH-KCN, SH-KCNR, and SH-EP), and many primary neuroblastoma tumors showed highly elevated levels of transcripts encoding CHRNA5 (α5 nAChR subunit) when compared to normal human sympathetic ganglia. There was no detectable expression of the CHRNA3 (α3) and CHRN4 (β4) and no relative difference in CHRNA7 (α7) and CHRN2 (β2) nAChR transcripts. In contrast, skin-derived neural precursors did not express detectable levels of any nAChR subunit transcript. We hypothesize that the α5 subunit contributes to the neuroblastoma phenotype, and forms a novel functional nAChR with subunits α7 and β2, and that this receptor subtype is the target of the drug MG 624. We have confirmed MG624 to kill SH-EP cells with the same concentration dependence as on neuroblastoma TICs. However, an MG 624 IC50 shift was not observed from intracellular recordings of *Xenopus* oocytes expressing a variety of (α7β2)2α5 nAChR concatemers. The data
presented in this paper suggest $\alpha_5$ nAChR subunit to correlate with neuroblastoma, thus a novel biomarker for neuroblastoma.

**Introduction**

Neuroblastoma is one of the most common childhood extra-cranial solid tumors. It accounts for more than 7% of malignancies in patients less than fifteen years of age and makes up 15% of oncology deaths (Maris et al., 2007). Neuroblastoma occurs in the sympathoadrenal progenitor cells, and tumors are formed in the adrenal medulla, sympathetic ganglia, and 65% occur in the abdomen (Maris et al., 2007). Current treatments for neuroblastoma include surgery, chemotherapy, radiotherapy, and biotherapy, all of which are inefficient, with a less than 50% long term survival in high risk cases (Maris et al., 2007). This inefficiency could be due to neuroblastoma stem cells that are dormant and are capable of being activated and differentiated into tumor like cells. Thus, it is clear that a more specific treatment for neuroblastoma is needed.

One way to study neuroblastoma is through the culturing of tumor-initiating cells (TICs). Our collaborators, Drs. David Kaplan and Giselle Sholler, have isolated TICs from bone marrow aspirates of stage 4 neuroblastoma patients. TICs have features of neuroblastoma stem cells, such as self-renewal and the ability to form metastatic tumors in mice (with injecting as little as ten cells) (Hansford et al., 2007). In an effort to identify a more
specific drug treatment, Dr. Kaplan has screened multiple drugs on TICs. One drug, MG 624, which is a nicotinic acetylcholine receptor (nAChR) antagonist, was shown to be the most effective in killing TICs (unpublished data). This led to the characterization of nAChR expression in TICs. TICs showed an increase in α5 expression levels compared to cells from normal non-cancerous pediatric sympathetic ganglia following drug administration. Also, there was no detectable expression of the α3 or β4 subunits, and no relative difference in α7 and β2 expression levels.

The α5 subunit is known as an accessory subunit. Because the α5 subunit does not have a ligand binding site, it cannot form a functional nAChR on its own; It needs to be combined with other α subunits and/or with other α and β subunits. We hypothesize that the α5 subunit can form a novel functional nAChR with subunits α7 and β2 to contribute to the neuroblastoma phenotype, and that this receptor subtype is the target of the drug MG 624.

The sensitivity of α7β2α5 nAChRs against MG 624 was tested by intracellular two electrode voltage clamp recordings on Xenopus oocytes expressing a variety of α7β2α5 nAChR stoichiometries. To ensure the expression of α7β2α5, Xenopus oocytes were injected with an α7β2α5 mRNA concatemer. A nAChR concatemer is designed so that each subunit is linked together and is translated as a single polypeptide, thus restricting the stoichiometry. Cell growth rate was characterized in a stable SH-EP α5 (CHRNA5)
knockdown cell line in order to determine if the α5 nAChR subunit contributes to the Neuroblastoma tumor phenotype. The α7β2α5 nAChRs did not respond differently to MG 624 and the SH-EP CHRNA5 knockdown cell line failed to change in proliferation rate. Thus, the α5 nAChR subunit does not seem to directly be involved in the neuroblastoma phenotype. However, elevated CHRNA5 mRNA expression in the neuroblastoma tumors and cell lines suggest a potential neuroblastoma biomarker.

**Material and Methods**

*Cloning*- Native human subunit protein sequences for α7, β2, and α5 nAChR subunits were encoded by nucleotide sequences optimized for expression in vertebrate expression systems. In order to express the concatemer as a single polypeptide the Kozac and signal peptide sequences were removed from all subunits with the exception of the first subunit. Additionally a 40- amino acid sequence that includes the C-terminal tail of the preceding subunit and alanine-glycine-serine repeats arranged in order to encode enzyme restriction sites are paired in between each subunit. Subunits were interchanged by the digesting with the appropriate restriction enzyme. Concatemeric receptors were expressed from the pSGEM oocyte high expression vector.

*Cell culture*- Neuroblastoma cell lines were grown at 37°C in 5% CO₂. They were maintained in 10% (v/v) fetal bovine serum, 20 U/ml penicillin, 20 mg/ml streptomycin, 2 mM L-glutamine, and 6 mg/ml glucose in modified L15CO₂.
RNA extraction and qPCR- RNA was extracted from confluent cells using the TRI reagent/ chloroform method. qPCR primers were designed using MacVector against the CHRNA5 sequence obtained from http://www.ensembl.org/index.html. CHRNA5 expression levels were determined using the syber green based method.

Transfections- SH-EP cells were transfected using JetPEI (polyplus transfection, Illkirch, France). Transfected SH-EP cells were selected with G418 (Sigma, St. Louis, MO, USA) at 500 mg/ml.

In-vitro transcription- 2 µg of nAChR concatemers DNA was linearized with the enzyme EcoR1. In-vitro transcription was performed with the enzyme T7 RNA polymerase using the kit MegaScript from Ambion. RNA was purified through the MegaClear kit from Ambion.

Electrophysiological Recordings and analysis- Xenopus oocytes were purchased and harvested through EcoCyte Bioscience (ecocyte-us.com) and two-electrode voltage-clamp recording was performed as described in (Boorman et al., 2000). 70 ng of cRNA was injected into oocytes with a 50:1 molar ratio RIC3:concatemer. Recording was done at -70 mV in OR2 solution (115 mM NaCl, 2 mM CaCl2, and 2 mM KCl) in the presence of 1.5 mM atropine. Drug application was perfused for 5 secs then washed for 30 secs. 15 ACh concentrations were used starting at 10 mM and diluted by quarter log units.
Results

To confirm if CHRNA5 mRNA levels are elevated in neuroblastoma cell lines, I performed quantitative PCR on a sample of tumor initiating cells (TICs), sy5y neuroblastoma cell line, SH-EP neuroblastoma cell line, and a skin progenitors (SKPs) sample as a control. The CHRNA5 mRNA levels were elevated in the TIC sample, sy5y, and SH-EP neuroblastoma cell line compared to SKPs (Fig. 1 A). Though the SH-EP neuroblastoma cell line showed the smallest increase in CHRNA5 mRNA expression it still was elevated when compared to the control group (SKPs). For this reason an MG 624 growth assay against SH-EP cells was performed. First a MG 624 dose response curve against SH-EP cells was performed via a calcein AM assay. The EC50 for MG 624 against SH-EP cells was determined to be 5 μM (Fig. 1 B). SH-EP cells were then incubated for four days with a daily application of 5 μM MG 624. About 50% of plated SH-EP cells were viable at two days post MG 624 exposure (Fig. 1 C).

Because SH-EP cells were affected by MG 624 exposure I performed a knockdown by transfecting cultured SH-EP cells with four different shRNAs against CHRNA5. The four shRNA plasmids tested were noted as 1253, 904, 873, and a negative control (CHRNA5 scramble). An initial quantitative PCR on transfected SH-EP cells was done to determine if there was a knockdown (Fig. 2 A). The most effective shRNA in knocking down CHRNA5 was 1253 (Fig. 2 B). The knockdown was about 70% compared to SH-EP cells not transfected (Fig. 2 B).
A stable CHRNA5 knockdown SH-EP cell line was established by the 1253 shRNA. A calcein AM assay was performed against the stable CHRNA5 knockdown SH-EP cell line (1253), transfected SH-EP cells with negative shRNA control (NC), and SH-EP cells not transfected. The growth of all three-cell lines was measured over three days. There was no significant difference in relative fluorescence, thus no difference in viable cells among the three cell lines tested (Fig. 3 A). We then went on to determine if the stable CHRNA5 knockdown SH-EP cell line was insensitive to MG 624. The 1253, NC, and untransfected SH-EP cell lines were comparably sensitive to MG 624 (Fig. 3 B). No change in MG 624 sensitivity suggests MG 624 to act on another source besides a nAChR. Therefore, we performed another calcein AM assay against all three cell lines exposed to methyllycaconitine (MLA). All three-cell lines (1253, NC, and untransfected SH-EP cells) are not affected by MLA (Fig. 3 C). Additionally, the sensitivity SH-EP cells exhibit against MG 624 further supported the idea that a nAChR is the target of MG 624. Therefore, I performed intracellular voltage clamp recordings on Xenopus oocytes expressing a variety of \( \alpha 7\beta 2\alpha 5 \) nAChRs to determine if there is a shift in the MG 624 IC50. All \( \alpha 7\beta 2\alpha 5 \) nAChRs tested responded to MG 624, comparably (Fig. 4 A).

**Discussion**

The mRNA expression of CHRNA5 in neuroblastoma TICs and cell lines resemble the expression identified in other neuron types. Neural crest cells are destined to be sympathetic ganglia, the neuroblastoma progenitors, and it was determined that cultured
quail neural crest cells express CHRNA5, CHRNA3, and CHRNB4 for 12 days (Howard et al., 1995). Additionally, the developing chicken ciliary ganglion has a 400 fold mRNA increase in CHRNA5 from embryonic day 8 to 18, a greater increase than the CHRNA3, CHRNB4, and CHRNA7 transcripts (Corriveau and Berg, 1993). The sympathetic ganglion also expresses the CHRNA5, CHRNA3, CHRNB4, and CHRNA7 transcripts (Gardette et al., 1991). However, in these studies the expression of CHRNA5 is accompanied by the expression of CHRNA3, CHRNB4, CHRNB2, and CHRNA7. An interesting observation in this study is the absence of CHRNA3 and CHRNB4 mRNA expression.

Because the \( \alpha_5 \) nAChR subunit is known as the “accessory subunit”, its functions are related to the modulation of nAChR signaling. The autonomic ganglion expresses the CHRNA5, CHRN3, and CHRNB4 nAChR subunit transcripts and are known to be involved in fast EPSPs (Rassadi et al., 2005; Ullian et al., 1997). The CHRNA5 Knockout mouse exhibits increase nicotine consumption, due to the decrease in cholinergic neuron activity within the medial habenula (Fowler et al., 2011). Though these studies identify the \( \alpha_5 \) nAChR subunit to directly affect the cholinergic signaling, there are no studies that investigate the developmental function of the \( \alpha_5 \) nAChR subunit.

Though this paper determined CHRNA5 to not have a direct role in the neuroblastoma phenotype it does not discard the importance the \( \alpha_5 \) nAChR subunit may have in neuroblastoma. All of the TICs, neuroblastoma cell lines, and primary tumors express
elevated CHRNA5 mRNA levels. However, knocking down CHRNA5 did not alter the SH-EP phenotype. Thus, CHRNA5 is not directly involved in the neuroblastoma phenotype, but may be identified as a biomarker for neuroblastoma.
Literature Cited


Table 1. All $\alpha 7\beta 2\alpha 5$ nAChR subtypes respond to MG 624 similarly. There is no significant change in the MG 624 LogIC50 and Hill slope for all of the different for $\alpha 7\beta 2\alpha 5$ nAChR stoichiomtries.

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<th>nAChR type</th>
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<th>Hill Slope</th>
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<td>$\alpha 7\alpha 7\alpha 7\alpha 7$</td>
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Figure 1. Determining CHRNA5 levels in neuroblastoma cell lines and testing MG 624 efficacy. A. CHRNA5 transcript levels are elevated in neuroblastoma TICs and cell lines. B. A calcein AM assay determined viable SH-EP cells based off of relative fluorescence. The EC50 for MG 624 against SH-EP cells is 5 µM. C. Four day 5 mM MG 624 exposure on SH-EP cells versus DMSO exposure. At two days of MG 624 exposure 50% of the cells were viable. MG 624 treated cells are closed circles and DMSO treated cells are open circles.
Figure 2. Quantitative PCR on shRNA transfected SH-EP cells. A. All shRNA transfections knocked down the CHRNA5 transcript. B. The 1253/shRNA was the most effective in knocking down the CHRNA5 transcript, with a 70% knockdown.
Figure 3. CHRNA5 knockdown in SH-EP cells did not affect proliferation rate, MG 624 or MLA. A. 1253/shRNA SH-EP cell line to proliferate at a comparable rate to the negative control and untransfected SH-EP cells. B. 1253/shRNA SH-EP cell line MG 624 sensitivity is comparable to the negative control and untransfected SH-EP cells. C. MLA exposure to 1253/shRNA SH-EP cell line did not affect viability and is comparable to controls.
Figure 4. The LogIC50 did not shift for the different $\alpha_7\beta_2\alpha_5$ nAChR stoichiometries. A. MG 624 dose response curves for $\alpha 7\beta 2\alpha 5$ nAChRs.