CROSS-TALK BETWEEN NICOTINIC ACETYLCHOLINE (NACHR) AND SEROTONIN (5HT3R) RECEPTORS IN SYMPATHETIC NEURONS

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In Partial Fulfillment of the Requirements For the Degree of Master of Science
In the Department of Physiology
University of Saskatchewan
Saskatoon

By

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ABSTRACT

Serotoninergic type 3 receptors (5HT3Rs) are members of the Cys-loop family of ligand-gated ion channels (LGIC), which includes nicotinic ACh, glycine, GABA-A and GABA-C receptors. All members of this family are widely expressed in the central and peripheral nervous systems, where they mostly participate in fast synaptic transmission. Activation of 5HT3Rs on vagal sensory nerve endings affect respiration, circulation, emesis and nociception; and in the central nervous system they are implicated in anxiety, depression, and drug dependence. In contrast, the function of 5HT3Rs in sympathetic neurons has not been fully determined. We discovered that 5HT3Rs interact with nicotinic acetylcholine receptors (nAChRs), the main drivers of the fast cholinergic autonomic synapse, through cross-talk mechanisms. We examined cross-talk by the patch-clamp technique on cultured mouse superior cervical ganglia (SCG) neurons. Co-stimulation of 5HT3Rs and nAChRs resulted in the generation of a combined current that was smaller than arithmetically predicted if the receptors did not interact with one another. This interaction, which we quantified as mean peak amplitude and mean ionic charge, was dependent on activation of 5HT3Rs and nAChRs, and independent of metabotropic receptors, Ca^{2+} entry and Ca^{2+} second messenger pathways, and of the direct action of 5HT on nAChRs. Preliminary data using an antibody targeted to the M3-M4 linker region of the 5HT3A subunit revealed that 5HT3Rs and nAChRs possibly cross-talk through physical interactions. These results revealed a potential role of the 5HT3R in the regulation of sympathetic synaptic transmission through cross-talk inhibition of nAChRs.
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TABLE OF CONTENTS

PERMISSION TO USE .................................................................................................................. i

ABSTRACT .................................................................................................................................. ii

ACKNOWLEDGEMENTS ........................................................................................................... iii

TABLE OF CONTENTS ............................................................................................................... iv

LIST OF FIGURES AND TABLES ............................................................................................ vii

LIST OF ABBREVIATIONS ......................................................................................................... viii

1. GENERAL INTRODUCTION ................................................................................................. 1

   1.1 Sympathetic Nervous System .............................................................................................. 2

      1.1.1 Anatomy ...................................................................................................................... 2

      1.1.2 SNS Function ............................................................................................................... 4

      1.1.3 Neurotransmitters and Receptors ............................................................................... 4

      1.1.4 Modulation and Disease .............................................................................................. 7

   1.2 Two Cys-Loop Receptor Family Members: The Nicotinic Acetylcholine and Serotonin 3 Receptors .................................................................................................................. 8

      1.2.1 The 5HT3 Receptor .................................................................................................... 9

      1.2.2 The Nicotinic Acetylcholine Receptor ...................................................................... 10

2. CHARACTERIZATION OF 5HT-INDUCED MODULATION OF nAChR

   MEDIATED CURRENTS ............................................................................................................. 13

      2.1 Introduction .................................................................................................................... 13
2.2 Methods ........................................................................................................................................... 14
  2.2.1 Primary SCG Cultures .................................................................................................................. 14
  2.2.2 Electrophysiology and Agonist Application .................................................................................... 15
  2.2.3 Statistics and Analysis .................................................................................................................... 18
2.3 Results .................................................................................................................................................. 18
  2.3.1 Peak amplitudes of nAChR- and 5HT3R-mediated currents were non-additive ........................................ 20
  2.3.2 Ionic Charges conducted by nAChRs and 5HT3Rs were non-additive ........................................... 23
  2.3.3 Activation of 5HT3Rs during extended ACh application induced inhibition of ACh-evoked currents ................................................................. 26
  2.3.4 Does the inhibition of ACh-evoked current require the activation of 5HT3Rs? ....................................... 29
  2.3.5 Does 5HT act directly on nAChRs? ................................................................................................. 32
  2.3.6 Cross-talk between 5HT3Rs and nAChRs was Ca\(^{2+}\)-independent .............................................. 35
  2.3.7 Is cross-talk between the 5HT3Rs and nAChRs dependent on physical interaction? .......................... 38

3. GENERAL DISCUSSION .......................................................................................................................... 40
  3.1 Experiments and Analysis .................................................................................................................. 40
    3.1.1 Predicting the current ..................................................................................................................... 40
    3.1.2 The interaction between 5HT- and ACh-evoked currents ............................................................... 41
    3.1.3 Cross-talk was independent of Ca\(^{2+}\) second messenger pathways ........................................ 42
3.1.4 The specificity of cross-talk to 5HT3Rs and nAChRs ............................ 43
3.1.5 Does 5HT have a direct effect on nAChRs? ............................................... 44
3.1.6 The physical interaction of 5HT3Rs and nAChRs ................................. 46
3.2 Summary ........................................................................................................ 46
3.3 Significance of Findings .................................................................................. 47
3.4 Future Directions ............................................................................................. 49
3.5 Final Words ..................................................................................................... 50

4. REFERENCES .................................................................................................... 51
LIST OF FIGURES AND TABLES

Figure                                      Page

1-1  Sympathetic Efferent Pathway                                      3

2-1  Fast-Step Perfusion System                                       16

2-2  nAChR- and 5HT3R-mediated currents were non-additive             22

2-3  nAChR- and 5HT3R-mediated ionic charges were non-additive        25

2-4  Short application of 5HT or mCPBG application during a prolonged ACh application induced inhibition of ACh-evoked currents                     28

2-5  5HT inhibited ACh and nicotine-evoked currents in the presence of 5HT3R antagonists                                      31

2-6  5HT did not act directly on nAChRs                                 34

2-7  Cross-talk between 5HT3Rs and nAChRs did not require the presence of Ca^{2+}         37

2-8  The polyclonal α-5HT3A antibody against the intracellular M3-M4 linker in the α-5HT3A subunit interferes with nAChR and 5HT3R cross-talk                         36

Table                                      Page

2-1  Antagonists                                                                 17

2-2  Agonists                                                                 17
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tr>
<td>2HPβCD</td>
<td>2-hydroxypropyl-β-cyclodextrin</td>
</tr>
<tr>
<td>5HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>5HT3R</td>
<td>serotonin type 3 receptor</td>
</tr>
<tr>
<td>AC</td>
<td>adenylate cyclase</td>
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<td>acetylcholine</td>
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<td>action potential</td>
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<tr>
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<td>calcitonin gene related peptide</td>
</tr>
<tr>
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</tr>
<tr>
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<tr>
<td>EPSC</td>
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</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<tr>
<td>--------------</td>
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<tr>
<td>gLTP</td>
<td>ganglionic long-term potentiation</td>
</tr>
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<td>g-protein coupled receptor</td>
</tr>
<tr>
<td>Hist</td>
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</tr>
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<td>LGIC</td>
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<td>m5HTR</td>
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<td>noradrenaline</td>
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<td>phosphoinositide 3-kinase</td>
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</tr>
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<td>phospholipase C</td>
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<tr>
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<td>peripheral nervous system</td>
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<tr>
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<td>SCG</td>
<td>superior cervical ganglion</td>
</tr>
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<tr>
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<tr>
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CHAPTER 1

GENERAL INTRODUCTION

The function of the autonomic nervous system (ANS) is to maintain physiological homeostasis. Three autonomic divisions participate in this critical task, including the sympathetic (SNS), the parasympathetic (PSNS), and the enteric (ENS) nervous systems. In this thesis we will concentrate particularly on function and regulation of the SNS. The SNS is important for homeostasis. It modulates a wide variety of body functions, including vascular dynamics, sweating, piloerection and temperature control, bronchial dilatation, pupillomotor function, heart rate, GI motility, and reproduction (Langley, 1903; Janig and McLachlan, 1992; Boron and Boulpaep, 2009). In addition to homeostasis, the SNS plays a pivotal role in the control of the body’s immediate adaptations to acute environmental stressors. A classical physiological outcome driven by SNS is the “fight-or-flight response” (Jansen et al., 1995). This is triggered by a perceived harmful or threatening stimulus. This response causes an increase in heart rate, respiration, focus, and overall arousal and energy providing the body with increased strength and speed in anticipation of fighting or running (Boron and Boulpaep, 2009). Malfunctions of the ANS, or dysautonomia, can compromise multiple organ systems. The symptoms can include cardiovascular problems such as resting tachycardia and orthostatic hypotension, metabolic disorders such as hypoglycemia unawareness in diabetes, and gastrointestinal (GI) problems such as constipation. In patients with long standing diabetes, dysautonomia contributes to the manifestation of cardiovascular disease which accounts for up to 80% of premature deaths (Canadian Diabetes Association; http://www.diabetes.ca/Section_About/prevalence.asp). The key to understanding how the SNS functions in health and disease is to study how it
communicates within itself and with the rest of the body. Function and regulation of the SNS is mediated by communication through its neurotransmitters and receptors. How a subset of these components modulate the SNS is the focus of this thesis.

1.1 Sympathetic Nervous System

1.1.1 Anatomy

The SNS is one of the three divisions of the ANS, complemented by the parasympathetic nervous system (PSNS) and the enteric nervous system (ENS). These systems are autonomic because of their abilities to function without modulation from supraspinal centers (Boron and Boulpaep, 2009).

CNS control of the SNS lies in the nucleus tractus solitarii (NTS), but also includes the area postrema, ventrolateral medulla, medullary raphe, reticular formation, locus coeruleus, and the parabrachial nucleus (Boron and Boulpaep, 2009). The NTS receives and transmits signals with the paraventricular nucleus of the hypothalamus, as well as the central nucleus of the amygdala (Andresen and Kunze, 1994). It also has peripheral afferents from chemo- and baroreceptors from the arteries, heart, lungs, airways, GI system, liver, and tongue. Medial lesion or pharmacological blockade of the NTS will affect baroreflex responses in heart rate, blood pressure, and sympathetic nerve activity (Andresen and Kunze, 1994).

In the SNS, the most important efferent projections from the NTS travel along the intermediolateral column of the spinal cord (Andresen and Kunze, 1994; Fig. 1-1). The NTS projections within this column synapse onto preganglionic neuronal cell bodies located in the
thoracic 1 to lumbar 3 sections (T1 to L3) of the spinal cord. The preganglionic neurons exit the spinal cord at the ventral roots, via white rami communicantes (myelinated fibers), only at sections T1 to L3. There are two groups of ganglia that the preganglionic neurons synapse onto, the left and right sympathetic chains (paravertebral ganglia, shown in Fig. 1-1), flanking each side of the spinal cord, and the prevertebral plexus (not shown in Fig. 1-1), consisting of a network of interconnecting ganglia and nerve fibers that run adjacent to- and branch alongside-major arteries in the abdomen. The sympathetic chains extend beyond their vertebral origins, going from the cervical to the coccygeal levels of the spinal cord. This arrangement serves as a distribution system that enables preganglionic neurons, which are limited to the thoracic and upper lumbar segments, to activate postganglionic neurons organized in autonomic sympathetic ganglia that innervate all body segments. However, there are fewer ganglia than there are spinal segments because some of the segmental ganglia fuse during development. An example of this is the superior cervical ganglion (SCG), which is the first ganglion of the sympathetic chain, and represents the fused cervical ganglia of C1 through C4 (Boron and Boulpaep, 2009).

The SCG is particularly useful in SNS research because of its large size, ease of access, and well characterized physiology (Savastano et al., 2010; Asamoto, 2005; Tubbs et al., 2002). The SCG supplies sympathetic innervations to the head and neck, sweat glands in the face and forehead, smooth muscle of eyelid, pupillary dilator muscle in iris, gustatory glands, and heart.
Thus, cultured SCG neurons are an accepted in vitro model to study sympathetic function at the cellular level.

1.1.2 SNS Function

Langley and Anderson (Langley, 1903) first defined the major functions of the SNS (and PSNS). Working mainly on cats, they were able to describe SNS anatomy, and showed that that the SNS modulates a wide variety of body functions, including vasoconstriction, vasodilatation, sweating, piloerection, bronchial dilatation, pupillomotor function, GI motility, and reproduction (Langley, 1903; Janig and McLachlan, 1992). Within the same time period (1915), Walter Cannon coined the idea that the SNS has generalized effects, especially in comparison to the effects of the PSNS (Janig and McLachlan, 1992).

Today, many studies have confirmed their findings, and have added considerable details to how the system functions (Janig and McLachlan, 1992; Boron and Boulpaep, 2009), including blood clotting time, glucose release, insulin secretion, and blood cell mobilization by spleen contraction. In addition, we now know the SNS is a ‘diverging’ system, in that the postganglionic fibers outnumber the preganglionic fibers by an estimated 200 times, which broadens the areas controlled by the signal originating in the brainstem.

1.1.3 Neurotransmitters and Receptors

Perhaps the most significant advance in the understanding of the SNS was the identification of the neurotransmitters and receptors that form the basis of its function. The
neurotransmitter systems can be organized into (1) the neurotransmitters and receptors involved in synaptic transmission, and (2) the neurotransmitters and peptides involved in modulation of SNS functions.

One of the main characteristics of the SNS is that the synapse between the preganglionic neuron and the postganglionic neuron at the sympathetic ganglia is cholinergic, while the synapse between the postganglionic neuron and the target tissue is adrenergic (Lundberg, 1996). The effects caused on the target tissue depend on the type of receptors that that tissue expresses (Boron and Boulpaep, 2009).

The effect of noradrenaline and adrenaline on the target tissue is mediated by multiple types of adrenergic receptors (AR) (Boron and Boulpaep, 2009). There are two main types of adrenergic receptors, α and β, with different patterns of expression and function. All adrenergic receptors are G-protein coupled receptors, with α1-ARs activating phospholipase C (PLC) and α2-ARs acting through inhibitory G-proteins (Gαi). The β-ARs (β1-, β2-, and β3-ARs) all act through Gαs protein and have distinct tissue distribution. The β1-ARs are highly expressed in the heart, where their activation increases cardiac output. The β2-ARs are highly expressed in the lungs, where they relax the smooth muscle in the airways. The β3-ARs are expressed mainly in adipose tissue, where they are involved in lipolysis (Boron and Boulpaep, 2009).

As mentioned before the synapses in the sympathetic chain are cholinergic and driven by the release of acetylcholine (ACh), which activates nicotinic ACh receptors (nAChR) and muscarinic ACh receptors in the postganglionic neuron. nAChRs are ligand-gated ion channels (LGIC) that are cation permeable (Na+, K+, and Ca2+), and are responsible for the excitatory postsynaptic currents (EPSC) that drive SNS ganglionic transmission (section 1.2.2 describes this in more detail). This differs from muscarinic ACh receptors (and ARs), which are G-protein
coupled receptors (GPCR). There are three main types of muscarinic receptors expressed in sympathetic ganglia, the M1, M2, and M3 subtypes. M1 receptors are present in the postsynaptic membrane and facilitate ganglionic transmission through PLC activation. M2 receptors are Goi coupled and are present on the presynaptic membrane where they are responsible for negative feedback regulation. M3 receptors are present in the smooth muscle of sweat glands and stimulate secretion through PLC activation (Lundberg, 1996; Boron and Boulpaep, 2009).

There are a couple of exceptions to the SNS functional organization described above. In the case of the sweat glands, the postganglionic fibers are cholinergic like the preganglionic fibers. The second case is in the adrenal medulla where preganglionic neurons synapse onto chromaffin cells, which take on the role of postganglionic neurons. These cells primarily release adrenaline (Boron and Boulpaep, 2009).

There are numerous other neurotransmitters and neuromodulators which “tune” SNS function. These transmitters have classically been called the non-adrenergic non-cholinergic (NANC) transmitters and they include vasoactive intestinal peptide (VIP), substance P (SubP), calcitonin gene related peptide (CGRP), numerous tachykinins, histamine (Hist), nitric oxide (NO), ATP, and neuropeptide Y (NPY). These transmitters can be responsible for co-inducing vascular smooth muscle contraction, such as with ATP release in postganglionic terminals onto ionic purinergic P2X or G-protein coupled P2Y receptors (Lundberg, 1996). NANC transmitters can also be responsible for inducing vasodilation, such as in the case of the gaseous transmitter NO, synthesized by the enzyme nitric oxide synthase (NOS) from L-arginine (Lundberg, 1996). New research is continuing to discover how these NANC transmitters modulate the SNS and how they are relevant in SNS disorders.
1.1.4 Modulation and Disease

The SNS is commonly known as the “fight-or-flight system”, but its function extends beyond extreme circumstances. The SNS is constantly working such as during eating, exercise, or sex. The SNS actively helps to regulate body temperature, deal with visceral pain, control bladder and bowel movements, and trigger emesis during nausea. Other factors, such as stress, anxiety and fear can significantly influence SNS function. This complexity requires fine control, which is mediated by a large number of neurotransmitters and peptides, and their respective receptors. Thus, examining transmitters and their receptors is a good starting point when studying the pathologies of the SNS. The focus of this section and the rest of the thesis will be on the receptors within the sympathetic ganglia, and how they are involved in the function and dysfunction of the SNS.

Normal SNS function is maintained by fast synaptic transmission at sympathetic ganglia, which can be detected by the generation of EPSCs mediated by nAChRs on the postganglionic neuron. Thus, pathological conditions that target the function of the nAChRs can lead to serious autonomic conditions. This occurs in type I and type II diabetes, where the hyperglycemia-induced accumulation of reactive oxygen species (ROS) in the cytoplasm of postganglionic SCG neurons led to autonomic neuropathy in diabetic mice (Campanucci et al. 2010). They found that ROS targeted conserved cysteine residues located in the cytoplasmic side of nAChR pores, inactivating the channels (Campanucci et al., 2008, 2010). Inactivation of these receptors led to depression of autonomic synaptic transmission and the onset of autonomic complications associated with neuropathy in diabetes. Animal models expressing nAChRs in which the
conserved cysteine residues have been mutated to a non-oxidizing residue (e.g. alanine) were resistant to the hyperglycemia-mediated effect (Campanucci et al., 2010). Although nAChRs are driving sympathetic transmission, there are other neurotransmitters modulating the function of the postganglionic neuron.

1.2 Two Cys-Loop Receptor Family Members: The Nicotinic Acetylcholine and Serotonin 3 Receptors

The nAChRs and 5HT3Rs are members of the Cys-loop receptor super-family, which in mammals includes the GABA<sub>A</sub> and glycine ion channels (Lester et al., 2004). These are called Cys-loop receptors because of a characteristic 13 amino acid loop formed by a disulfide bond between two cysteine residues in the extracellular N-terminal of each subunit (Tsetlin, et al., 2011; Jackson and Yakel, 1995). All Cys-loop receptors are pentameric structures formed from 5 homologous or heterologous subunits (Cooper et al., 1991; Lester et al., 2004). Starting from the large N-terminal, each subunit crosses the membrane 4 times, with each domain named M1, M2, M3, and M4 (Peters et al., 2005). The M2 section of each subunit combines to create the ion selective pore of the channels, with the M1-M2 intracellular region in close proximity determining conductivity and kinetics (Tsetlin et al., 2011). The M3-M4 intracellular loop is particularly large, at 140 amino acids long in the mouse 5HT3A (Maricq et al., 1991). This large region is particularly active in channel gating, receptor assembly and trafficking, and as a phosphorylation target for kinases such as protein kinase C (PKC) (Peters et al., 2005; Coultrap and Manchu, 2002). A few amino acid differences within the M2 filter region split the Cys-loop
receptors into two categories: the anion (glycineR, GABA<sub>A</sub>) and cation (nAChR, 5HT3R) selective channels (Gunthorpe and Lummis, 2001).

1.2.1 The 5HT3 Receptor

The 5HT3R is unique amongst 5HT receptors in that it is an ion channel, while the rest are metabotropic GPCRs. Of the Cys-loop receptors, it is the most closely related to the nAChR, with 27% and 30% amino acid homology with the α2 and α7 nAChR subunits, respectively (Jackson, 1995; Noda et al., 1983). 5HT3Rs can be found in the CNS, including the hippocampus, striatum, lateral amygdala, area postrema, and NTS, but are mainly found in the PNS, such as the myenteric plexus, submucosal plexus, nodose ganglion, SCG, vagus nerve, and DRG (Jackson and Yakel, 1995). These channels mediate fast activating and fast desensitizing currents (Jackson and Yakel, 1995). In the CNS, they are known to be involved in anxiety and emesis; the latter is especially relevant in chemotherapy. Specific antagonists for 5HT3Rs such as granisetron, ondansetron, and tropisetron are widely used as anti-emetics in conjunction with chemotherapy (Mochizuki et al., 1999; Jackson and Yakel, 1995).

The pentameric structure of 5HT3Rs can be homomeric, composed of only 5HT3A subunits, or heteromeric, composed of the 5HT3A and 5HT3B subunits (Davies et al., 1999; Dubin et al., 1999; Hanna et al., 2000). The 5HT3B subunit has ~45% sequence identity with the 5HT3A subunit, but cannot form a functional homomeric structure (Park et al., 1995). Receptors containing the 5HT3A are found in the spinal cord, and peripheral ganglia, while those containing the 5HT3B subunit are found in the nodose, superior cervical, trigeminal, and dorsal
root ganglia (Morale and Wang, 2002). 5HT3Rs are activated by 5HT and are non-selective cation channels, highly permeable to \( \text{Na}^+ \), \( \text{K}^+ \) and \( \text{Ca}^{2+} \) ions (Yang et al., 1992).

The homomeric and heteromeric 5HT3Rs have marked functional differences in ion-permeabilities, current-voltage relationship, and single channel conductance (Davies et al., 1999). \( \text{Ca}^{2+} \) permeability is higher for the homomeric form, which is accompanied by inward rectification. In contrast, the heteromeric receptors have more linear current-voltage relationships and larger single channel conductances than homomeric receptors (Peters et al., 2005).

Currently, there is little information regarding the exact source and pattern of release of 5HT within the sympathetic ganglia. Small intensely fluorescent (SIF) cells within the SCG of guinea pigs and rats are known to contain 5HT, and to be regulated by preganglionic cholinergic neurons (Kanagawa et al., 1986; Hadjiconstantinou et al., 1982). Thus, the examination of SIF cells would be important for understanding the ganglionic circuit. Other sources of 5HT can come from the blood that supplies the sympathetic ganglia (Tubbs et al., 2002). Most 5HT within blood is synthesized in the enterochromaffin cells of the gut (Gershon, 1999), and absorbed and stored by blood platelets (Ohkawa et al., 2005). In fact, free 5HT levels were found to be low in serum, at 5.7 ± 3 nM (Hirowatari et al., 2004).

### 1.2.2 The Nicotinic Acetylcholine Receptor

The nAChRs have been one of the most heavily studied LGICs in history due to their critical function in mammalian physiology, diverse tissue expression, and the flexibility their subunit-diversity imparts on receptor function. Our understanding of the structure of Cys-loop receptors stems from the study of the nAChR rich electric organ of the Torpedo electric ray
(Brejc et al., 2001; Miyazawa et al., 2003; Unwin, 2005). The nAChRs have major functions in both the CNS and PNS. In the CNS, where they are mostly expressed presynaptically, they participate in the modulation of hippocampal inhibitory circuitry and dopamine transmission in the basal ganglia; while in the PNS, where they are expressed mostly postsynaptically, they are involved in receiving motor inputs at neuromuscular junctions and driving autonomic ganglionic transmission (Hurst et al., 2013). Similar to 5HT3Rs, these channels mediate fast depolarizing currents, carried by Na\(^+\), K\(^+\), and Ca\(^{2+}\) ions. The nAChRs have been implicated in Alzheimer’s disease, Parkinson’s disease, and nicotine addiction (Albuquerque et al., 2009).

Like other Cys-loop receptors, the structure of the nAChRs is pentameric (Cooper et al., 1991). In muscles, the nAChRs are mainly composed of 2 different subunit combinations: α1, β1, δ, and γ in embryonic muscle, and α1, β1, δ, and ε in postnatal muscle (Saito, 2002). Both combinations include two α1 subunits, where the ACh binding pocket is located (Albuquerque et al., 2009). In CNS and PNS neurons, there are 7 α subunits (α2-7, 9, 10), and 3 β subunits (β2-β4) (Albuquerque et al., 2009). Thus far, the α3-5, α7, β2, and β4 subtypes have been identified in SCG (Gotti and Clementi, 2004; Rust et al., 1994; Corrivea and Berg, 1993). Most nAChRs in the SCG are formed by the heteromeric α3β4 combination (55%), while α3β4α5 and α3β4β2 correspond to 24% and 21% respectively (David et al., 2010; Skok, 1999; Voitenko, 2001). All heteromeric combinations include two α subunits and the only functional homomeric receptors are formed by α7 subunits (Skok, 2002; Role, 1999).

In the SCG, nAChRs are responsible for the generation of fast EPSCs (Derkach et al., 1983). Currents carried by α3β4-containing receptors show strong inward rectification, caused by the pore blocking effect of intracellular spermine at positive potentials (Haghighi and Cooper, 2000). In addition, nAChRs are known to be highly Ca\(^{2+}\) permeable compared to other cations.
(Trouslard et al., 1993). High Ca\textsuperscript{2+} permeability allows the receptor to activate multiple Ca\textsuperscript{2+} dependent kinases, including phosphoinositol 3 kinase (PI3K), PKC, protein kinase A, calmodulin-dependent protein kinase II (CAM kinase II), and extracellular signal-regulated kinases (ERK) (Albuquerque et al., 2009). In addition to these cellular effects of activating nAChRs in central and peripheral neurons, they can also interact with other LGICs, such as P2X\textsubscript{2} receptors. The main focus of this thesis will be to study the functional interaction between the nAChRs and 5HT\textsubscript{3}Rs in sympathetic neurons, and the consequence on SNS function.
CHAPTER 2

CHARACTERIZATION OF 5HT-INDUCED MODULATION OF nAChR-MEDIATED CURRENTS

2.1 Introduction

Interaction, or cross-talk, between receptors has been particularly studied in the Cys-loop receptor super-family. Both the 5HT3Rs and nAChRs show the ability to interact with other LGICs, especially the purinergic P2X receptors. In the guinea pig intestine, and when co-expressed in *Xenopus* oocytes, cross-talk between 5HT3Rs and P2X2Rs was revealed by the co-application of 5HT and ATP, which evoked a current smaller than the addition of individually evoked currents (Barajas-Lopez et al., 2002; Boue-Grabot et al., 2003). The mechanism behind this effect was determined to be independent of Ca$^{2+}$, receptor phosphorylation, G-protein coupling, and GABA$_c$ receptors, suggesting a physical receptor-receptor interaction. Mutagenesis experiments show that these receptors were interacting at the P2X2 C-terminus and the 5HT3 M3-M4 linker (Boue-Grabot et al., 2003, 2004).

nAChRs have also been shown to cross-talk with P2XR in a similar fashion to the interaction between P2X2Rs and 5HT3Rs. In both rat SCGs and guinea pig myenteric neurons, co-application of ACh and ATP produced smaller currents than were expected if the P2X and nAChR receptors worked independently (Zhou and Galligan, 1998; Nakazawa, 1994). Experiments in which P2X2Rs and α3β4-containing nAChRs were heterologously co-expressed in *Xenopus* oocytes revealed that P2X2Rs inhibited the opening of nAChRs, and that the effect was mediated by physical interaction (Khakh et al., 2000; Decker and Galligan, 2010).
These findings emphasize the ability of the 5HT3Rs and nAChRs to interact with other LGICs, and point to the possibility that they interact with each other. nAChRs are essential for fast synaptic transmission in autonomic ganglia, and deletion of the α3 subunit, which is the highest expressed α-nicotinic subunit in SCGs, abolishes autonomic function in mice (Xu et al., 1999). Despite not being directly involved in fast synaptic transmission, 5HT3Rs are also expressed in the SCG (Tecott et al., 1993; Morales and Wang, 2002), but their contribution to autonomic function is still unclear.

In the current thesis I examined the interaction between 5HT3Rs and nAChRs in primary cultures of mice SCG neurons. My findings reveal that these receptors interact with each other, possibly through a cross-inhibitory mechanism involving physical interaction.

2.2 Methods

2.2.1 Primary SCG Cultures

All experiments were approved by the University of Saskatchewan’s Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Primary SCG from neonatal C57BL/6 mice between postnatal day 1 to 3 (P1-P3) were used for the preparation of the dissociated SCG neuron cultures. The methods used to dissociate the neurons have been described previously (McFarlane & Cooper, 1992; Campanucci et al., 2008). Briefly, mice were euthanized by cervical transection in a sterile environment. SCG were collected into a petri dish with serum-containing media (L15 supplemented with vitamins, cofactors, penicillin-streptomycin and 5mM glucose, and 10% horse serum). Forceps were used to remove the remaining efferent and afferent nerves from the ganglia. Once the
ganglia were cleaned, they were enzymatically dissociated (0.1% trypsin; Worthington, Freehold, NJ, USA) at 37°C for 30-45 min, followed by mechanical dissociation with fire-polished pipettes.

The resulting cell suspension was transferred to growth media consisting of L-15 supplemented with vitamins, cofactors, penicillin-streptomycin, 5% rat serum, 7S Nerve Growth Factor (10 ng/ml) and 5 mM of glucose. The neurons were plated on laminin-coated coverslips, which were attached to modified 35 mm tissue culture dishes. Cells were maintained at 37°C in a 95% air and 5% CO₂ environment and fed every 3-4 days with fresh growth media. To eliminate non-neuronal cells, cultures were treated with cytosine arabinoside (10 μM; Sigma, St. Louis, MO, USA) from day 2 to day 4. Cells were allowed to recover and develop for two weeks, after which they were subjected to electrophysiological experiments.

2.2.2 Electrophysiology and Agonist Application

Agonist-evoked currents were recorded using the whole-cell patch-clamp technique (Hamill et al., 1981). All cells were voltage-clamped at -60 mV. Membrane currents were recorded with a Multiclamp 700A patch-clamp amplifier (Axon Instruments, Molecular Devices LLC., Sunnyvale, CA) at room temperature and sampled at 5 kHz. Recording electrodes were filled with intracellular fluid (ICF) with the following composition (in mM): 60 KAc, 70 KF, 5 NaCl, 1 MgCl₂, 1 CaCl₂, 2 MgATP, 10 EGTA, and 10 HEPES, with pH adjusted to 7.2 using KOH. The extracellular fluid (ECF) contained the following composition (in mM): 140 NaCl, 5.4 KCl, 0.33 NaH₂PO₄, 0.44 KH₂PO₄, 1 MgCl₂, 1 CaCl₂, 10 HEPES, and 5 glucose, with pH adjusted to 7.4 using NaOH. For calcium free experiments, ECF containing 0 mM Ca²⁺/50 μM EGTA and ICF containing 5 mM BAPTA was perfused for 10-15 min before recording (Barajas-
Tetrodotoxin (TTX, 0.5µM) was added to the ECF to avoid the generation of spontaneous action potentials.

Cells were exposed to agonists using a three barrel pipette controlled by a SF-77B fast-step perfusion system (Warner Instruments LLC, Hamden, CT; Fig. 2-1).

Agonist application protocols were controlled by pClamp 10 software (Molecular Devices LLC, Sunnyvale, CA). ECF perfusion through all three barrels was turned on during recording, with each barrel fed by a set of syringe reservoirs containing control and agonist solutions. Perfusion was driven by a pressurized system (VPP-6, Warner Instruments LLC, Hamden, CT). Chamber fluids were siphoned out at a rate matching that of perfusion.

**Figure 2-1. Fast-Step Perfusion System.** A three barrel glass pipette was used to direct fluid flow over the selected cell. Drug application was achieved by stepping pipette over to a barrel which was perfusing the agonist (Adapted from Warner Instruments).
<table>
<thead>
<tr>
<th>Antagonists</th>
<th>Type</th>
<th>Receptor</th>
<th>Working Concentration</th>
<th>Dose Determination</th>
<th>Company</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asenapine</td>
<td>Anti-psychotic</td>
<td>5HT2</td>
<td>20nM</td>
<td>50-1000nM affected *5HT- and *ACh-induced current amplitude and desensitization rate</td>
<td>Sigma – A7861</td>
<td>Meltzer et al., 2009</td>
</tr>
<tr>
<td>Methiothepin</td>
<td>Anti-psychotic</td>
<td>5HT1, 5HT6, 5HT7</td>
<td>20nM</td>
<td>50-1000nM affected 5HT- and ACh-induced current amplitude and desensitization rate</td>
<td>Sigma – M149</td>
<td>Schoeffter et al., 1996</td>
</tr>
<tr>
<td>Atropine</td>
<td>Competitive</td>
<td>M1-M5</td>
<td>1µM</td>
<td>See reference</td>
<td>Sigma – A0132</td>
<td>Morishima et al., 2013</td>
</tr>
<tr>
<td>MDL 72222</td>
<td>Competitive</td>
<td>5HT3</td>
<td>500nM</td>
<td>&lt;500nM does not block 5HT3R-mediated currents and 1000nM inhibits ACh-induced current</td>
<td>Tocris - 0640</td>
<td>Fozard, 1984</td>
</tr>
<tr>
<td>Y25130</td>
<td>Competitive</td>
<td>5HT3</td>
<td>10nM</td>
<td>&lt;5nM does not block 5HT3R-mediated currents</td>
<td>Tocris - 0380</td>
<td>Sakamori, 1992</td>
</tr>
</tbody>
</table>

Table 2-1. Antagonists.
*all antagonist concentrations were determined based on agonist concentrations used in thesis

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Type</th>
<th>Receptor</th>
<th>Working Concentration</th>
<th>Dose Determination</th>
<th>Company</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5HT</td>
<td>Non-specific, endogenous</td>
<td>Multiple 5HTRs</td>
<td>100µM</td>
<td>Compared with size of current and charge of other currents (see section 3.1.1)</td>
<td>Sigma – H9523</td>
<td>Mochizuki et al., 1999</td>
</tr>
<tr>
<td>mCPBG</td>
<td>Specific</td>
<td>5HT3R</td>
<td>50µM</td>
<td>&quot;</td>
<td>Sigma – C144</td>
<td>Kilpatrick et al., 1990</td>
</tr>
<tr>
<td>ACh</td>
<td>Non-specific, endogenous</td>
<td>Multiple AChRs</td>
<td>15-100µM</td>
<td>&quot;</td>
<td>Sigma – A6625</td>
<td>Campanucci et al., 2010</td>
</tr>
<tr>
<td>Nicotine</td>
<td>specific</td>
<td>Nicotinic AChRs</td>
<td>50µM</td>
<td>&quot;</td>
<td>Sigma - 36733</td>
<td>Hu et al., 2007</td>
</tr>
</tbody>
</table>

Table 2-2. Agonists.
2.2.3 Statistics and Analysis

All currents evoked by co-application of agonists were compared to a predicted current. The predicted current (always reported in red) was calculated in three steps. First, the ACh and 5HT currents stimulated before co-application of agonists were added together within Clampfit software, and then the peak amplitude or charge of the summated current was measured. Then, the same two currents stimulated after co-application were added together, and the peak amplitude or charge of the current was measured. Finally, the two peak amplitude values were averaged to obtain the predicted current. These calculations took into consideration the typical changes in amplitude of the 5HT and ACh-evoked currents.

All current peak amplitudes were normalized to the cell capacitance and expressed as current density (pA/pF). Ionic charge was calculated as the integrated area under the current trace (from 0.2s before agonist onset to 2s after removal) and expressed in nanocoulombs (nC). All values were reported as mean ± SEM.

We used the paired t-test to compare mean current densities and mean charge, with the level of significance set at P < 0.05.

2.3 Results

To test the hypothesis that the nAChRs and 5HT3Rs expressed in SCG neurons were involved in cross-talk mechanisms, we used a modified protocol previously described to examine cross-talk between 5HT and ATP-evoked currents (Boue-Grabot et al., 2003). To do this, we first evoked whole-cell currents by applying ACh and 5HT or the 5HT3R selective agonist methyl-
chlorophenylbiguanide (mCPBG) separately (for 1s), followed next by the co-application (1s) of ACh and 5HT (or mCPBG). At the end of the stimulation protocol we re-probed with 5HT and ACh separately as a recovery test. Figure 2-2 outlines these experiments, showing currents from representative cells using 5HT (Fig. 2-2A) and mCPBG (Fig. 2-2C). Representative current examples show individually stimulated currents before (left), co-applied agonists (center), and individual currents (recovery) after co-application of agonists (right). Note the predicted current obtained by the addition of the individual evoked currents (for calculation see methods) is represented in red (Fig. 2-2A-F).

As previously described (see section 1.2.1 and 1.2.2) the kinetic profiles of ACh and 5HT-evoked currents in cultured SCGs were markedly different. While both inward currents were fast activating, the ACh-evoked currents were slightly slower than the 5HT-evoked currents (David et al., 2010; Solt et al., 2007). In addition, the desensitization phase of the individual currents was markedly different, with 5HT-evoked currents desensitizing at a faster rate and showing total desensitization by 1-2s (Fig. 2-2).

It is important to note that to obtain an accurate predicted current we chose agonist concentrations that produced currents similar in size. But because currents evoked through each receptor type varied in amplitude over the course of an experiment, with ACh-evoked currents potentiating (~10%) and 5HT-evoked currents running-down (~25%), we were required to compensate for these changes by averaging the currents before and after the co-application of agonists to calculate the predicted current.
2.3.1 Peak amplitudes of nAChR- and 5HT3R-mediated currents were non-additive

Figure 2-2A shows a representative example where ACh- and 5HT-evoked currents were non-additive. The comparison between the actual (black trace) and the predicted (red trace) current revealed that the fast desensitization characteristic of 5HT3Rs was absent in the actual current. In fact, the kinetics of the actual current resembled that of the nAChR mediated current, i.e. with slow desensitization. In addition, the amplitude of the actual current was approximately 25% smaller than that of the predicted current, suggesting there were cross-inhibitory effects between the receptors (Fig. 2-2B, P < 0.001, n = 14 neurons).

5HT can also activate multiple 5HT-metabotropic receptors (m5HTRs) in SCG neurons, which are known to express 5HT1, 5HT2, 5HT3, 5HT6, and 5HT7 receptors (Pierce et al., 1996). To test whether m5HTRs contributed to the cross-inhibitory effects between ACh- and 5HT-evoked currents, we used the 5HT3R specific agonist, mCPBG (50µM), instead of 5HT (Fig. 2-2C). Under these conditions the actual current was not only smaller than the predicted but the effect was more pronounced than when using 5HT, resulting in an actual current approximately 40% smaller than the predicted (Fig. 2-2D, P < 0.01, n = 6).

SCG neurons also express metabotropic (muscarinic) ACh receptors, including M1, M2, and M3 subtypes, which have been reported to hyperpolarize or depolarize the neuronal resting potential through K+ and Ca2+ channels (Lundberg, 1996; Boron and Boulpaep, 2009). Therefore, to test whether metabotropic receptors contribute to the cross-inhibitory effect we next repeated our experiments in the presence of antagonists to block all possible muscarinic receptors and m5HTRs naturally expressed in SCG neurons. We used the muscarinic antagonist atropine (Atr, 1µM); the 5HT1R, 5HT6R and 5HT7R antagonist methiothepin (Met, 20nM); and
the 5HT2R antagonist asenapine (Ase, 20nM). This drug cocktail allowed us to obtain currents mediated solely by nAChRs and 5HT3Rs. Consistent with our data showing that 5HT3Rs were involved in the cross-inhibition (Fig. 2-2C and 2-2D), the actual currents generated in the presence of the antagonist cocktail remained smaller than predicted, suggesting that metabotropic receptors were not involved in cross-inhibition (Fig. 2-2E and 2-2F, P < 0.001, n = 15 neurons).
Figure 2-2. nAChR and 5HT3R-mediated currents were non-additive. nAChR- and 5HT3R-mediated currents were evoked by single or combined application of agonists in cultured SCG (A, C & E) Representative example traces, show the characteristics of the individual and combined currents used in this experiments. (A & B) The co-application of ACh- and 5HT evoked non-additive currents (P < 0.001, n = 14 neurons). (C & D) The co-application of ACh and the 5HT3R specific agonist mCPBG also induced non-additive currents (P < 0.01, n = 6 neurons). (E & F) The co-application of 5HT and ACh in the presence of the metabotropic antagonists atropine (Atr, 1µM), methiothepin (Met, 20nM), and asenapine (Ase, 20nM) evoked non-additive currents as well (P < 0.001, n = 15 neurons). All current amplitudes (B, D, F) were normalized to cell capacitance (current density, pA/pF) and expressed as mean ± SEM. (* P < 0.05; ** P < 0.01; *** P < 0.001).
2.3.2 Ionic charges conducted by nAChRs and 5HT3Rs were non-additive

As explained above, the kinetic profiles of the currents mediated by 5HT3Rs and nAChRs were markedly different, particularly due to the faster activation and desensitization of the 5HT-evoked currents. These differences were not considered when calculating the predicted current using peak amplitudes. Therefore, to account for these differences, we calculated and compared the ionic charge for each current.

To obtain the ionic charge we integrated the area under the curve in a given current trace. We calculated the ionic charge from 0.2s before agonist application to 3s after agonist removal, inclusive. This time period was chosen to encompass the entirety of the currents.

As with peak amplitude analysis, the charges conducted by nAChRs and 5HT3Rs were non-additive. Figure 2-3A shows representative current traces indicating the difference between the actual and predicted currents, which was summarized by plotting the mean ionic charge (Fig. 2-3B; P < 0.01, n = 8 neurons). In addition, it can be observed that with lower ACh concentrations (15µM instead of 50µM), the actual combined current had kinetic features that resembled those of the 5HT3R-mediated current (faster activation and desensitization). This suggests that although the actual currents obtained by co-application of agonists resembled those generated by nAChRs, both receptors contribute to the kinetics of the evoked current and the extent of the contribution is determined by the concentration of the agonists.

The resemblance of the actual combined current to the 5HT3R-mediated current was more pronounced when applying mCPBG and ACh, than when using 5HT (Fig. 2-3C and D), showing faster activation and desensitization kinetics (Fig. 2-3C). Again the values of the actual
ionic charges were significantly less than predicted, with a stronger effect when using mCPBG instead of 5HT (Fig. 2-3D, P < 0.001, n = 9 neurons).

These data indicate that even after accounting for the different current kinetics, the 5HT3R- and nAChR-mediated currents were still non-additive.
Figure 2-3. nAChR and 5HT3R-mediated ionic charges were non-additive. These experiments summarize the currents evoked by single and combined applications of agonists expressed as ionic charge (area under the curve) calculated from t = 0.8 to 4s. Please note the concentration of ACh was lowered to 15µM to obtain comparable 5HT and ACh ionic charges. (A & B) The combined application of ACh (15µM) and 5HT (100µM) resulted in significantly lower actual charges than predicted (P < 0.01, n = 8). (C & D) The combined charges resulting from co-application of the 5HT3R specific agonist mCPBG (50µM) with ACh (15µM) also resulted in significantly lower charges than predicted (P < 0.001, n = 9). All data in this figure is presented as mean ± SEM, in nanocoulombs (nC). Means are tested with the paired t-test (* P < 0.05; ** P < 0.01; *** P < 0.001)
2.3.3 Activation of 5HT3Rs during extended ACh application induced inhibition of ACh-evoked currents

So far we demonstrated that the currents mediated by the two receptors were non-additive. Next we concentrated on identifying the characteristics of this interaction. In this series of experiments, we aimed to examine whether 5HT3Rs inhibit the nAChRs. Unfortunately, the possibility that nAChRs inhibit 5HT3Rs was not possible due to the fast desensitization kinetics of the latter. To test for 5HT3R inhibition of nAChRs, we designed experiments where 5HT (100µM; 1s) was applied during a prolonged (100µM; 5s) ACh-evoked current (Fig. 2-4). Charges were compared by integrating the area under the curve. We have arbitrarily selected the time from $t = 2.25s$ (immediately when 5HT application begins) to $3.75s$ (0.5s after 5HT removal). This time period allowed us to encompass the entire time course of the depression of ACh-evoked currents.

The results from these experiments supported the previous findings, i. e. 5HT and ACh-induced currents were non-additive, but also described two aspects of the interaction unseen in the 1s application protocol. The first aspect was that application of 5HT during the desensitization phase of ACh-evoked currents caused an inhibition. This was clearly seen in the combined agonist trace in figure 2-4A. When 5HT was applied during the ongoing ACh-evoked currents, not only did the currents not increase, but under this experimental protocol, they decreased. The effect was even stronger when using mCPBG to selectively activate 5HT3Rs (Fig. 2-4C). A more detailed example of the inhibitory strength of mCPBG on ACh-evoked currents is shown in figure 2-4F, where we applied mCPBG for the last 6s of a 7s long application of ACh, resulting in an inhibition of approximately 80% of the current. The ionic
charge data for 5HT and mCPBG applications are summarized in figures 2-4B and D, respectively. The combined actual currents were significantly smaller than the predicted currents for both 5HT (Fig. 2-4B, P < 0.05, n = 6) and mCPBG (Fig. 2-4D, P < 0.05, n = 4). To ensure that the inhibition induced by activation of 5HT3Rs was not an artifact due to possible disturbances in the ACh concentration during the fast-step protocol, we controlled for this by removing 5HT (or mCPBG) from the solution reservoir, leaving only ACh. Figure 2-4E shows a representative control trace where there was no disturbance of the ACh-evoked current.

The second, and puzzling, aspect of the interaction is also depicted in more detail in figure 2-4F, where we extended the application of mCPBG in the above protocol to 6s. As expected, mCPBG depressed the ACh-evoked current as shown in our previous experiment; however, this extended trace shows that the depression does not correspond with the kinetics of the mCPBG-evoked current. The mCPBG-evoked current reaches maximal amplitude at the beginning of agonist application and decays by desensitization over time (see example trace in Fig. 2-3C). Thus, this should result in a stronger inhibition at the beginning of the mCPBG application. This was clearly not the case in figure 2-4F, where an inverse effect is shown. Based on these puzzling findings, the next set of experiments aimed to further examine the relevance 5HT and 5HT3Rs to the cross-inhibitory effect between the two receptors.
Figure 2-4. Short application of 5HT or mCPBG during a prolonged ACh application induced inhibition of ACh-evoked currents. 1s of 5HT (100µM) or mCPBG (50µM) was applied during a 5s application of ACh (100µM), 1s after ACh onset. Red traces and bars indicate predicted currents. Ionic charges were measured from immediately before 5HT/mCPBG application, up to 0.5s after removal (1.5s range). (A & B) Show the depression in prolonged (5s) ACh-evoked current caused by 1s application of 5HT, which resulted in significantly less charge than predicted (P < 0.05, n = 6 neurons). (C & D) Application of mCPBG also depressed ACh-current, indicating that the effect was specific to the 5HT3R. The actual charges were significantly less than predicted (P < 0.05, n = 4). (E) Representative example showing the control for the perfusion fast-step system, where 5HT was removed from the reservoir containing 5HT and ACh, leaving only ACh. Note, the lack of depression of the ACh-evoked current in the absence of 5HT, indicating the effect was entirely due to 5HT or mCPBG application. (F) Prolonged application of mCPBG not only depressed ACh-evoked current, but also this effect was inversely related to the 5HT3R-mediated current kinetics. All data in figure is presented as mean ± SEM in nanocoulombs (nC) (* P < 0.05; ** P < 0.01; *** P < 0.001).
2.3.4 Does the inhibition of ACh-evoked current require the activation of 5HT3Rs?

Our results summarized in section 2.3.3 suggested that once nAChRs have been activated, activation of 5HT3Rs causes the inhibition of the ACh-evoked current. However, the inconsistency between the kinetic properties of 5HT3R-mediated currents and the characteristics of the inhibition made us wonder whether this effect was indeed mediated by 5HT3Rs or an alternative mechanism. To examine if the 5HT- and mCPBG-induced inhibition of ACh-evoked currents were dependent on the activation of 5HT3Rs, we next repeated our experiments in the presence of 5HT3R selective antagonists. We used two different 5HT3R specific competitive antagonists: MDL 72222 (bemisetron, 500nM) and Y25130 (10nM) (Fozard, 1984; Sakamori et al., 1992). The antagonists were applied to all perfusion solutions. First we recorded a set of control current traces for ACh and 5HT (not shown), followed by recording in the presence of the antagonists (see Fig. 2-5). Before recording in the presence of antagonist, we confirmed 5HT-evoked currents were completely inhibited by the antagonists (not shown). Figure 2-5 displays representative traces for experiments showing the effect of 1s application of 5HT on 5s application of ACh (or nicotine)-evoked currents.

If 5HT3R activation was indeed mediating the inhibition of ACh-evoked currents we would expect that the use of the antagonists would prevent this effect. However, 5HT induced a depression in the ACh-evoked currents despite being antagonized by MDL 72222 (Fig. 2-5A and B) and Y25130 (Fig. 2-5C and D). Next, to confirm that this inhibitory effect of 5HT was acting on nAChRs, we repeated the experiment using the nAChR specific agonist, nicotine (50μM, Fig. 2-5B and D). These results showed that in the presence of 5HT3R specific antagonists, 5HT induced a depression in ACh- and nicotinic-evoked currents.
In summary, our results using pharmacological blockers for 5HT3Rs revealed that 5HT still caused a marked inhibition of nAChRs. These unpredicted results were only observed when 5HT was applied during a long ACh-evoked current and contrasts with our previous experiments (Fig. 2-2C), in which 5HT3Rs were required to cause the cross-talk effects. Therefore, further experiments are required to increase our N values and to evaluate the relevance of this findings, as well as to repeat these experiments in the presence of m5HTR antagonists and mCPBG, which were conditions not tested thus far that would help to clarify these differences. In the next section we will consider an alternative mechanism for this effect, namely if 5HT has a direct effect on nAChRs.
Figure 2-5. 5HT inhibited ACh and nicotine-evoked currents in the presence of 5HT3R antagonists. Two competitive 5HT3R specific antagonists, MDL 72222 (500nM) and Y25130 (10nM) were applied immediately following control recordings (using the 5s ACh protocol discussed previously). Each antagonist was perfused for 2min prior to and during the recording of the second set of traces. Confirmation of complete 5HT current blockage was done for every cell (not shown). (A & B) In the presence of MDL 72222, which completely inhibited 5HT-evoked currents, 5HT was still able to inhibit ACh- and nicotine-evoked currents, respectively. (C & D) Similarly results were obtained in the presence of Y25130, which completely blocked 5HT-evoked currents (not shown), yet 5HT still inhibited ACh- and nicotine-evoked currents, respectively.
2.3.5 Does 5HT act directly on nAChRs?

Data presented in the previous section suggested that 5HT could directly interact with nAChRs without activation of 5HT3Rs. In fact, this possibility has been previously considered in rat trigeminal neurons, where 5HT directly inhibited nAChRs in a non-competitive manner (Hu et al., 2007). Therefore, to explore the possibility that 5HT directly inhibited nAChRs in SCG neurons, we performed a dose response curve for ACh-evoked currents with and without 5HT pre-incubation, and in the continuous presence of blockers for muscarinic receptors, m5HTRs and 5HT3Rs.

Using the experimental protocol previously described by Hu et al. (2007), we first exposed the cell to ACh and 5HT individually without any antagonists, demonstrating the expression of these receptors (see Fig. 2-6A for diagram of experiment). Next, we perfused the cell with Atr (1µM), Met (20nM), Ase (20nM), and MDL72222 (500nM), blocking all muscarinic receptors, m5HTRs and 5HT3Rs. After 2min, ACh and 5HT-evoked currents were re-probed to check for both normal ACh-evoked currents and complete blockage of 5HT3R. After this confirmation, we bathed the cell in 5HT (100µM) for 5min. Next, we stimulated the cell with ACh again to check for any current changes. Following this, we removed 5HT and allowed nAChRs to recover, after which we applied ACh one last time to test for recovery. The 3rd ACh trace was compared to the average of the 2nd and 4th ACh-evoked traces in each experiment (see Fig. 2-6A). The entire process was repeated for 10, 100, and 1000µM concentrations of ACh (Fig. 2-6B), and the results were plotted onto a dose response chart.

Remarkably, our data indicated there was no significant changes between the dose responses obtained with or without 5HT (Fig. 2-6C; P > 0.05; 10µM, n = 5; 100µM, n = 6;
Although these were preliminary results and they needed to be confirmed by increasing not only the N values but also the concentrations tested to properly fit the data to a sigmoidal dose response curve, it is important to note that the concentration range tested here corresponds to the one showing marked differences in trigeminal neurons (Hu et al., 2007). Therefore, our findings suggest that different mechanisms to those described in trigeminal neurons underlie the cross-inhibition of ACh-evoked currents in SCG neurons.
Figure 2-6. **5HT did not act directly on nAChRs.** (A) The first ACh- and 5HT-induced currents demonstrated normal currents in SCG neuron. Following this, Atr (1µM), Met (20nM), Ase (20nM), and MDL 72222 (500nM) were perfused onto the neuron to eliminate muscarinic AChRs, m5HTRs and 5HT3Rs. Next, 2min after this perfusion, the ACh and 5HT-evoked currents were tested again demonstrating that 5HT3R-mediated currents were eliminated and nAChR-mediated currents were intact. This was then followed by a 5 min application of 5HT (100µM) and next by ACh. This 3rd trace of ACh was expected to be inhibited if 5HT directly interacts with nAChRs. The final ACh current was obtained 2min after 5HT removal as recovery. For analysis, the average of the 2nd and 4th traces (red in B and C) was compared to the 3rd trace (black in B and C). (B) Example traces from 10, 100, and 1000µM ACh. (C) Dose response comparison between currents recorded with and without 5HT show no difference at all three concentrations of ACh used (P > 0.05 at all points; 10µM, n = 5; 100µM, n = 6; 1000µM, n = 4). Current densities are displayed as mean ± SEM.
2.3.6 Cross-talk between 5HT3Rs and nAChRs was Ca\(^{2+}\)-independent

There is considerable evidence supporting the involvement of Ca\(^{2+}\) in the regulation of both 5HT3Rs and nAChRs through direct and indirect mechanisms. The M3-M4 intracellular loop of both receptors is known to be targeted by the Ca\(^{2+}\)-dependent PKC. The 5HT3A receptor subunit had been shown to be potentiated by PKC (Coultrap and Manchu, 2002), while nAChRs were also regulated by PKC in addition to numerous other Ca\(^{2+}\)-dependent kinases (Albuquerque et al., 2009). Increases in extracellular Ca\(^{2+}\) also directly regulated both of these receptors, inhibiting 5HT3R currents (van Hooft and Wadman, 2002; Niemeyer and Lummis, 2001) and increasing the open probability of nAChRs (Amador and Dani, 1995). Moreover, removal of extracellular Ca\(^{2+}\) eliminated ganglionic long term potentiation (gLTP) in sympathetic ganglia (Koyano et al., 1984; Briggs et al., 1985). Finally, recent evidence suggests that cross-talk between the 5HT3Rs and nAChRs in CNS presynaptic terminals is mediated through Ca\(^{2+}\) or Ca\(^{2+}\)-dependent second messengers (Dougherty and Nichols, 2009).

Since our findings suggest that 5HT may act through other mechanisms not involving direct inhibition of nAChRs, we explored the possibility that Ca\(^{2+}\) may have contributed to the inhibitory effect. To examine the dependence of cross-talk on Ca\(^{2+}\), experiments were performed in the absence of this cation. To achieve this, neurons were bathed in extracellular solution containing 0mM Ca\(^{2+}\)/50µM EGTA and intracellular (pipette) solutions containing the Ca\(^{2+}\) chelator BAPTA (5mM; Barajas-Lopez, 2002). After achieving the whole-cell configuration, a 10min period was allowed for BAPTA to diffuse into the cell cytoplasm before recording currents. The 1s ACh protocol described in figure 2-2 was used to record ACh- and 5HT-induced currents.
Our results showed that Ca\textsuperscript{2+} was not required to induce the cross-talk of ACh-evoked current by 5HT (Fig. 2-7). The combined actual current was significantly smaller in amplitude than predicted (Fig. 2-7B; P < 0.01, n = 5). Therefore, much like the cross-talk between P2X2Rs and 5HT3Rs (Barajas-Lopez et al., 2002; Boue-Grabot et al., 2003), the interaction between nAChRs and 5HT3Rs does not seem to depend on Ca\textsuperscript{2+}. 
Figure 2-7. Cross-talk between 5HT3Rs and nAChRs did not require the presence of Ca\(^{2+}\). The same 1s ACh protocol as described in figure 2-2 was used in this experiment. However, in this experiment, Ca\(^{2+}\) was removed from the intracellular and extracellular solutions. Cells were bathed in extracellular solution containing 0mM Ca\(^{2+}\)/50uM EGTA and patched with intracellular solution containing the Ca\(^{2+}\) chelator BAPTA (5mM) (Barajas-Lopez, 2002). After achieving whole-cell, 10min was allowed for BAPTA to diffuse into the cytoplasm before recording currents. (A) A representative cell recorded without Ca\(^{2+}\). The combined actual current remained smaller in amplitude than predicted. (B) Current densities of actual and predicted currents were analyzed and found to be significantly different (P < 0.01, n = 5). (* P < 0.05; ** P < 0.01; *** P < 0.001).
2.3.7 Is cross-talk between the 5HT3Rs and nAChRs dependent on physical interaction?

The data presented so far indicate that the interaction between 5HT3Rs and nAChRs does not depend on Ca\(^{2+}\), the direct effect of 5HT on nAChRs, or metabotropic receptors. Since it was shown that P2X2/5HT3 receptors, and nACh/P2X receptors interact in a physical manner, we next examined the possibility that 5HT3Rs inhibit nAChRs through physical interactions (particularly with their intracellular domains).

Previous reports have shown that the 5HT3Rs and P2X2Rs interact physically at the cytosolic M3-M4 linker of 5HT3Rs (Boue-Grabot et al., 2003, 2004). In order to test this, an antibody targeted to the M3-M4 linker of the 5HT3A subunit (i.e. amino acids 342-355; Alomone Labs.) was used in an attempt to prevent the interaction with nAChRs at that site. The antibody was applied through the patch pipette and allowed to diffuse into the cytosol before recording. Figure 2-8 includes representative traces of these experiments. The antibody dose used here was 1:200, which was the recommended dose for immunocytochemistry and Western blotting (Alomone Labs.; Fig. 2-8A), and a more concentrated 1:20 dilution (Fig. 2-8B).

These data showed that while the combined actual currents were still smaller than predicted at 1:200 dilutions (Fig. 2-8A), this effect was less pronounced at 1:20 dilutions (Fig. 2-8B). More importantly, allowing the antibody more time to diffuse intracellularly (10min) decreased this difference making the actual currents similar to the predicted current (with the exception of kinetics). Unfortunately, since these were preliminary experiments, there was not enough data (n = 3) to statistically analyze actual versus predicted currents. However, this data supports for the first time that a physical interaction between 5HT3Rs and nAChRs may be responsible for the cross-talk mechanisms described in this thesis.
Figure 2-8. The polyclonal antibody against the intracellular M3-M4 linker in the α-5HT3A subunit interferes with nAChR and 5HT3R cross-talk. The antibody was diluted in the intracellular solution and applied through the patch pipette and allowed 5-10 min to diffuse to the cytosol before recording. Two antibody dilutions were used: (A) 1:200 and (B) 1:20. A 5 min diffusion time did not totally prevent the cross-talk between the receptors at either dose, but the 10 min diffusion time minimized the difference between the actual and predicted currents. Note, the 1:20 dilution had a stronger effect preventing the interaction between the receptors even at 5 min diffusion time. There was not enough data collected to compare the means of the combined actual and predicted currents.
CHAPTER 3

GENERAL DISCUSSION

In this thesis we revealed a novel cross-inhibitory mechanism between nAChRs and 5HT3Rs. Using a specific agonist and multiple antagonists, we showed that the effect was specific to nAChRs and 5HT3Rs, and that no metabotropic receptors were required for the cross-inhibition. In addition, we found that the effect was Ca$^{2+}$ independent and did not involve the previously described direct effect of 5HT on nAChRs (Hu et al., 2007). Rather, here we describe a cross-talk mechanism that is possibly mediated by receptor-receptor interaction.

3.1 Experiments and Analysis

3.1.1 Predicting the current

The key measure of our effect laid in the difference between the combined actual 5HT and ACh evoked currents and the one predicted. Thus, the calculation of the predicted current was a crucial factor in determining cross-talk between 5HT3Rs and nAChRs.

Two factors influenced how we calculated the predicted current. First, both 5HT and ACh-evoked currents changed in amplitude over time. ACh-evoked currents slightly potentiated at the beginning of the experiment, while 5HT-evoked currents ran down continuously over time. To account for these changes, the predicted current was calculated by averaging the currents occurring before and after the combined application of the agonists. Second, since both of the currents had different kinetics, with 5HT having a fast activation and desensitization and ACh
having a slower desensitization phase, the peak amplitudes of each current did not occur at the same point in time. Thus, the summation of the peak amplitude values would result in an inaccurate predicted peak. To address this issue, we used Clampfit software to arithmetically combine the obtained single currents to generate a predicted current, resulting in a single larger current with different peak amplitude and kinetics. Thus, our method provides a more accurate predicted current; however, it is worth noting that this calculation assumes that the ACh and 5HT-evoked currents changed linearly with time.

To calculate the predicted current we also considered the contribution of 5HT3Rs and nAChRs to the predicted current. For example, if the ACh-evoked current was five times larger than the 5HT-evoked current, as was observed in many cells, the predicted current would not be statistically different than the individual ACh-evoked current. Therefore, in order to make the calculation of the predicted current more reliable, we emphasized the difference between the actual and predicted currents by adjusting the concentrations of ACh and 5HT/mCPBG to produce currents similar in amplitude or charge.

3.1.2 The interaction between 5HT- and ACh-evoked currents

It initially seemed by examining figure 2-2A and B that there was cross-talk occurring between ACh- and 5HT-evoked currents. We originally used the peak amplitudes of the evoked currents to calculate and compare the actual vs. predicted currents generated by co-application of agonist. However, the accuracy of this method was questionable mainly due to the marked difference in current kinetics for these two receptors. Due to the fast activation and desensitization of 5HT3R-mediated currents vs. the slower activation and desensitization of
nAChR-mediated currents, the use of peak amplitude, which measures one single time point, will not take into consideration current changes occurring before and after the time of the peak. Therefore, to compare the actual vs. predicted currents in a way that would more closely represent the current experienced by the cell, we measured the ionic charge conducted through each receptor by integrating the area under the current traces (Fig. 2-3). Our results measuring ionic charge confirmed our original findings using peak amplitude.

In addition, these experiments also revealed the effect of lowering the concentration of one of the agonists on the desensitization of the actual combined current. As explained before, to calculate the ionic charge we reduced the concentration of ACh to 15µM (instead of 50µM), which resulted in an increase in the desensitization rate of the actual combined current (Fig. 2-3A). These experiments suggest that 5HT3Rs are still functional in the actual combined currents but at 50µM ACh, the nAChRs are mostly responsible for the slower current kinetics.

3.1.3 Cross-talk was Ca\textsuperscript{2+}-independent

To investigate whether Ca\textsuperscript{2+} entry through both 5HT3Rs and nAChRs could participate in cross-talk mechanisms we designed experiments in Ca\textsuperscript{2+} free conditions, as previously described for synaptosomes (Dougherty and Nichols, 2009). In these experiments, 5HT3Rs and nAChRs inhibited each other’s function through Ca\textsuperscript{2+} ions. However, our results showed that Ca\textsuperscript{2+} and Ca\textsuperscript{2+}-dependent second messenger pathways were not involved in the mechanisms behind cross-talk in the SCG (see Fig. 2-7). It is important to note that the type of receptors and their anatomical location are markedly different between the synaptosomes and the SCG, which may account for the difference in the cross-talk mechanisms. In the CNS, nAChRs mostly contain α4
subunits and they are located pre-synaptically where they are mainly involved in the regulation of neurotransmitter release. On the other hand, nAChRs in the PNS (particularly in the SCG) mostly contain α3 subunits and they are located post-synaptically where they drive autonomic synaptic transmission (reviewed by Hurst et al., 2013). Thus, these differences between the CNS and PNS may be essential for the type of regulation the nAChRs are subjected to. While Ca^{2+} is a key player during neurotransmitter release, and directly regulates the interaction between 5HT3Rs and nAChRs at synaptosomes, other mechanisms could be more relevant for the interaction at sympathetic ganglia, including a physical interaction.

3.1.4 The specificity of cross-talk to 5HT3Rs and nAChRs

Since SCG neurons express multiple receptors that respond to both ACh and 5HT, to study the nature of the cross-talk mechanisms between these currents we used specific agonist and antagonists to isolate the currents mediated by solely nAChRs and 5HT3Rs.

We used multiple methods to isolate currents through 5HT3Rs and nAChRs because pharmacological specificity among members of the Cys-loop family has been shown to be insufficient (Papke et al., 2004), something we have also observed in our own experiments (1µM MDL 72222 inhibits nAChRs, not shown). First we used the nAChR specific agonist nicotine and the 5HT3R specific agonist mCPBG (see Fig. 2-2C, 2-3C, 2-4C). Under these conditions we demonstrated that the actual current generated by the co-application of agonists was still significantly smaller than the calculated predicted current. These results suggested that the cross-talk required nAChRs and 5HT3Rs.
Second, to isolate currents mediated through 5HT3Rs and nAChRs, we created a cocktail of antagonists that blocked metabotropic receptors sensitive to ACh and 5HT (Fig. 2-2E). These antagonists permitted us to use the endogenous agonists. These conditions also resulted in combined currents that were smaller than predicted, thus also suggesting that cross-talk required nAChRs and 5HT3Rs.

Here, the use of agonists specific to 5HT3Rs and nAChRs, and antagonists that isolate currents by 5HT3Rs and nAChRs were able to show that cross-talk was specific to these two receptors. In fact, our findings indicated that cross-talk was even stronger when mCPBG was used (Fig. 2-2D, Fig. 2-3D, Fig. 2-4D). Thus, these data strongly suggest that the cross-talk mechanisms observed specifically involved 5HT3Rs and nAChRs.

3.1.5 Does 5HT have a direct effect on nAChRs?

To study whether 5HT3Rs were cross-talking with nAChRs, or vice versa, we designed experiments in which 5HT or mCPBG was applied in the middle of a prolonged ACh pulse (see Fig. 2-4). Unfortunately we could not do the opposite experiment, in which ACh was applied during a long 5HT pulse, because of the strong desensitization of the 5HT3R-mediated current. Our experiments confirmed that activation of 5HT3Rs during prolonged ACh-evoked currents induced not only a non-additive current, but also an inhibition of the ACh-evoked current. This effect was stronger in the case of mCPBG application (Fig. 2-4B). An interesting aspect of this inhibition is that it is inversely proportional to the amount of current that should be passing through the 5HT3R due to the activation by mCPBG. This aspect made us wonder if this effect was mediated by an alternative mechanism contributing to the interaction of the receptors but
independent of 5HT3Rs. To confirm this, we repeated the experiments in the presence of the 5HT3R specific antagonists, MDL 72222 and Y25130 (Fig. 2-5). Surprisingly, under these conditions, 5HT still caused an inhibition of the ACh-evoked currents. These puzzling results raised the question whether in these conditions, where 5HT is applied during a prolonged ACh application, 5HT maybe acting directly on nAChRs without involving the function of 5HT3Rs.

To address this possibility that 5HT directly acts on nAChRs, we repeated the experiments performed by Hu et al. (2007), who previously demonstrated that 5HT had a direct effect on nicotinic-evoked currents in sensory neurons. Our results were in stark contrast to what was published before (Fig. 2-6C). In SCG neurons, 5HT did not produce any changes in the dose response relationship of the ACh-evoked current suggesting that it did not have any direct effect on nAChRs, either competitive or non-competitive. A few reasons may explain this difference. First, our data comes from primary cultures from autonomic neurons, which may respond differently to 5HT than sensory neurons. Second, we used antagonists for m5HTRs and muscarinic ACh receptors in the generation of our ACh dose-response curve in the presence and absence of 5HT. It is worrying that these antagonists were omitted by Hu et al., particularly since some of the effects reported could be explained by metabotropic receptor function. Thus, we concluded from our own data that the 5HT3R did not directly interact with the nAChR.

Note that the experiments presented in figure 2-6 do not support our findings with the prolonged ACh application experiments described above (Fig. 2-4). Here, 5HT may have been acting differently, perhaps directly, on nAChRs during the desensitization phase. This possibility needs to be addressed in future experiments where the potential direct effect of 5HT is tested using the prolonged ACh application protocol.
3.1.6 The physical interaction of 5HT3Rs and nAChRs

So far our findings revealed that the cross-talk between 5HT and ACh-evoked currents do not require metabotropic receptors, Ca\(^{2+}\) ions or Ca\(^{2+}\)-mediated 2\(^{nd}\) messenger pathways, or, at least in part, the direct effect of 5HT on nAChRs. Therefore, we next examined the possibility of physical interaction between the two receptors. This was a mechanism that had been previously described between P2X/nAChRs and P2X2/5HT3Rs (see section 2.1).

To address this possibility of physical interaction, we performed experiments with an antibody against the M3-M4 linker of the 5HT3A subunit (Fig. 2-8). To increase the quantity of antibodies bound to the 5HT3A M3-M4 linker, we used a polyclonal antibody. Our preliminary results suggested that cross-talk inhibition between the 5HT3R and nAChRs depended on the availability of the M3-M4 linker of 5HT3A. These data suggested for the first time a modulation by 5HT3Rs on nAChRs in autonomic neurons mediated receptor-receptor interaction. However, further experiments need to be performed, increasing time and concentration of antibody incubation to ensure complete interference of the M3-M4 linker. In addition, these experiments should be done at 37°C instead of room temperature to ensure optimal antibody binding.

3.2 Summary

The data summarized in this thesis showed for the first time a cross-talk inhibition of nAChRs driven by 5HT3Rs. This effect was not dependent on Ca\(^{2+}\), metabotropic receptors, or, at least in part, the direct inhibition of nAChRs by 5HT. In addition, this effect was specific to 5HT3Rs and nAChRs. This novel inhibitory effect is relevant to understand autonomic
physiology, particularly since autonomic synaptic transmission is strictly driven by nAChRs, and therefore modulation of their function maybe important for both physiological and pathological conditions.

3.3 Significance of Findings

We have discussed earlier that understanding the neurotransmitters and receptors of the SNS was critical to knowing how it behaves in normal and pathological states. Yet, in the literature there was extremely little information on the role of 5HT and the 5HT3R in the SNS (1.1.3). This may be because the role of 5HT in the PNS was being overshadowed by its role in the CNS (Villalon and Centurion, 2007). Our work aimed to significantly advance knowledge on the role of 5HT and the 5HT3R in the modulation of nAChR, and therefore SNS function.

The purpose of ACh release in the SCG is to induce fast autonomic synaptic transmission and generation of action potentials that could propagate to the target tissues. Thus, the modulatory effect of 5HT3Rs on the sympathetic tone, by cross-talk with nAChRs, suggests a potential role of 5HT on SNS physiology, for example reducing heart rate, blood pressure, and bronchodilation.

In fact, evidence for 5HT3R regulation of the SNS had been found in vivo. In a model of orthostatic intolerance in rats, in which the heart responds pathologically to inversion by lowering heart rate instead of raising it, the administration of two 5HT3R specific antagonists, MDL 72222 and ondansetron recovered the rats’ abilities to increase heart rate (Martel et al., 1998). Why or how blocking 5HT3Rs recovered the heart rate in this rat model was unknown,
and perhaps inhibition of nAChRs by 5HT3R by cross-talk in sympathetic ganglia may play a role in the decreased heart rate seen in orthostatic intolerance.

In humans, 5HT3R antagonists are used frequently as an antiemetic during chemotherapy (see section 1.2.1). This pattern of use has produced documented cases of bradycardia associated with granisetron use on children (Buyukavci et al., 2005). Although, it is unclear if cross-talk between nAChRs and 5HT3Rs is involved, these findings further support a role for 5HT3Rs in the SNS. In addition, hypertension has also been linked to 5HT3R function through gLTP (Johnston, 1992; Brown and McAfee, 1982; Koyano et al., 1985), which can be prevented by the blockage of 5HT3Rs with specific antagonists (Alkadhi et al., 2001, 2005; Gerges et al., 2002). These reports demonstrate the complexity of 5HT3R function within the body, and the need for whole animal experiments to understand how 5HT3Rs modulate physiological function.

The 5HT3R and its effects on the SNS may also be critical for pathological conditions that target the autonomic nervous system, such as diabetic autonomic neuropathy (see section 1.1.4). Recently it was reported that the nAChR have a cysteine residue sensitive to oxidation (Campanucci et al., 2008; 2010), which is particularly targeted in diabetes by the glucose-induced accumulation of ROS. Oxidation of nAChRs in diabetic mice leads to depression of autonomic function and serious dysautonomia. 5HT3Rs also contained an analogous region with a conserved cysteine residue, located in the intracellular M1-M2 in the same position as the nAChRs. Our own unpublished observations reveal that this receptor is also sensitive to ROS, which causes a fast and irreversible run-down that can be prevented by previous exposure to antioxidants. The latter might be significant for understanding the function of the SNS under pathological conditions. Thus, further experiments on this effect, and how it affects cross-talk with
3.4 Future Directions

The key question left unanswered within this work was the mechanism behind the cross-talk of these receptors. We have thus far only been able to eliminate a few possibilities including Ca\(^{2+}\), direct ligand-receptor interaction, and metabotropic receptors. At this stage, the most likely mechanism is receptor-receptor interaction between nAChRs and 5HT3Rs. We have begun preliminary experiments probing the 5HT3A M3-M4 linker, but there are numerous other directions we can progress in. The most anticipated experiments we plan to do is the mutation of the M3-M4 linker of 5HT3A and expression of the nAChR and 5HT3R within *Xenopus laevis* oocytes. These experiments would effectively isolate the two LGICs from the many confounding factors found in native neurons, and would also allow identification of the molecular determinants responsible for cross-talk.

Another issue we have considered, but have been unable to test was the possibility that the subunits of 5HT3R and nAChR were combining together to form a chimeric channel. This has been shown as a possibility between 5HT3A and α4 receptor subunits; at least when injected into *X. laevis* oocytes (van Hooft et al., 1998). The resulting chimeric channel was biphasic, responsive to 5HT, but not ACh. As described in section 1.2.2, the primary nAChR type expressed in SCG neurons was α3β4. Note that the same work by van Hooft et al. (1998) also tested the α3 subunit, which did not co-assemble with the 5HT3A subunit to form a functional channel. Therefore, a 5HT3A/α4 chimeric channel may form only a small portion of the total functional channels in SCG neurons. Nevertheless, given this possibility, it may be worth examining, especially when it becomes possible in our laboratory to use a system capable of expressing the neuronal nAChR.
3.5 Final Words

Here we show a cross-talk inhibition between the nAChRs and 5HT3Rs in mice SCG neurons. 5HT and ACh-induced currents were non-additive when the agonists were applied together. This interaction was also specific to the nAChRs and 5HT3Rs, as shown by the use of specific agonists, mCPBG (5HT3R) and nicotine (nAChR), and antagonists, MDL 72222 and Y25130 (5HT3R), methiothepin and asenapine (metabotropic 5HT receptors), and atropine (muscarinic receptors). It was also apparent that cross-talk inhibition did not depend on 5HT directly acting on the nAChR, although it could not be entirely discarded as an explanation in some experimental conditions. In addition to non-additive currents, it was also shown that 5HT induced depression in ACh-evoked current, which is physiological relevant for understanding the function of the sympathetic synapse. These results have many potential implications on SNS function in health and disease. Unfortunately, we have yet to discover the mechanism behind the cross talk. Further work will be necessary to complete the examination of the 5HT3R and nAChR interaction.


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