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Nicotine has a Direct Effect on Brainstem Startle-Mediating Neurons

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entitled:

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Abstract

Individuals with schizophrenia have impairments in prepulse inhibition of startle (PPI) which correlates with cognitive deficits. Nicotine improves the performance of patients and healthy individuals with PPI deficits on PPI tests. We hypothesized that nicotine directly affect startle-mediating neurons of the caudal pontine recticular nucleus (PnC) of the brainstem using electrophysiological recordings. The data revealed that nicotine (10 μM) increases leak current amplitude, reduces membrane resistance, and depolarizes the resting membrane potential. Nicotine had no significant effect on the EPSC amplitude for neither the trigeminal nor auditory stimulations. All effects were reversed only by a high dose (10 μM) of the α_7 nAChR antagonist MLA and a low dose of TMPH (100nM; antagonizes all but the α_7 nAChR). The effect of nicotine persisted in the presence of cadmium (100 μM), which blocks synaptic transmission. These results confirm the functional expression of nAChRs in the PnC and suggest a role of nAChRs in modulating startle responses directly in the brainstem.

Keywords:

Prepulse inhibition of startle, sensorimotor gating, cognitive deficits, mental health disorders, caudal pontine recticular nucleus, brainstem, electrophysiology, patch-clamp recordings

Co-Authorship

This work was carried out by Farena Pinnock whom conducted all experiments, performed the data analyses, interpreted the data and wrote the thesis document.

Dr. Susanne Schmid aided in planning the design and methodology as well as editing the thesis.

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Lab Members

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List of Abbreviations

ACh: acetylcholine

ACSF: artificial cerebral spinal fluid

AMPA: α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

ANOVA: analysis of variance

ASR: acoustic startle reflex

Ca^{2+}: calcium ions

Cd^{2+}: cadmium ions

CN: cochlear nucleus

CNS: central nervous system

CRNs: cochlear root neurons

DC: direct current

EPSC: excitatory postsynaptic current

EPSP: excitatory postsynaptic potential

GABA: gamma-aminobutyric acid

IC: inferior colliculus

ICS: intracellular solution

K^+: potassium ions

LDT: laterodorsal tegmental nucleus

LSO: lateral superior olive

mAChR: muscarinic acetylcholine receptor

MLA: methyllycaconitine

mPRF: medial pontine recticular formation

Na^+: sodium ions

nAChR: nicotinic acetylcholine receptor

NMDA: N-methyl-D-aspartic acid

PB: peribrachial nucleus

PHA 543-613: *N*-[(3*R*)-1-Azabicyclo[2.2.2]oct-3-yl]furo[2,3-*c*]pyridine-5-carboxamide

PnC: caudal pontine recticular nucleus

PNS: peripheral nervous system

PPI: prepulse inhibition of startle

PPT: pedunculopontine tegmental nucleus

Pr5: principal sensory nucleus 5

REM: rapid eye movement

SC: superior colliculus

SNR: substantia nigra, pars reticulate

TMPH: 2,2,6,6-Tetramethylpiperidin-4-yl heptanoate

TTX: tetrodotoxin

VN: vestibular nucleus

Chapter 1

Introduction

The brain continuously receives and processes sensory information to allow for the appropriate behaviour. To be able to focus one's attention and respond appropriately to external stimulation, it is crucial to select salient sensory information and suppress unimportant stimuli (Fendt 1999; Koch 1999; Martin, Kem et al. 2004; Nusbaum and Contreras 2004). In the absence of this automatic filtering mechanism, known as sensorimotor gating, individuals would have difficulty thinking coherently given the abundance of sensory stimuli throughout the environment (Geyer and Markou 1995; Gogos and Van den Buuse 2004). In fact, it is believed that the cognitive dysfunctions (e.g., memory loss, disorganized thought and non-discriminatory attention) experienced by patients with schizophrenia, for example, are in part due to sensory gating deficits (Adler, Pachtman et al. 1982; Braff and Geyer 1990; Paz, Ortiz et al. 2007). Moreover, some researchers have suggested that it is a lack of sensory filtering that may cause the hallucinations and delusions characteristic of schizophrenia (Braff and Geyer 1990; Adler, Olincy et al. 1998). Thus, it becomes apparent that some sensory input must be inhibited or 'gated out' so that individuals can pay attention to relevant sensory stimuli. More specifically, there is some evidence to suggest that presynaptic inhibition of activated sensory neurons allow for sensorimotor gating (Rudomin 2002; Nusbaum and Contreras 2004).

Chapter 2

2.1 Modulation of the startle reflex (prepulse inhibition of startle) as a measure of sensorimotor gating

The mammalian startle reflex is a highly conserved protective response to a sudden intense (acoustic, tactile or vestibular) stimulus that causes an accelerated heart rate and facial, cranial and skeletal muscles to contract (Landis and Hunt 1939; Davis 1984; Koch 1999). It is commonly measured as an eyeblink response in humans and as a whole body flinch in rodents. Startle responses can be modulated by emotions, learning, and sensorimotor gating and thus makes startle an attractive model to utilize in the investigation of underlying mechanisms (Davis 1984; Lang, Bradley et al. 1990; Schanbacher, Koch et al. 1996; Koch 1999; Bradley, Codispoti et al. 2006; Herbert and Kissler 2010). Furthermore, the anatomical structures that mediate startle share some similarities across mammals which allows for cross-species comparisons (e.g., between humans and rats) to better understand basic cognition such as sensory filtering and disease states (Koch 1999).

Prepulse inhibition (PPI) of startle is often used as an operational measure of sensorimotor gating whereby the processing of an initial non-startling stimulus (a prepulse) is protected from interference by suppressing the processing of subsequent incoming stimuli (e.g., the startle pulse; Ison and Hammond 1971; Graham 1975; Braff, Geyer et al. 2001; Swerdlow, Geyer et al. 2001; Frost, Tian et al. 2003). Thus, presentation of a brief weak sensory stimulus before an intense startle-eliciting pulse

causes a reduction in startle as the startle pulse is 'gated out' (Figure 1; Hoffman and Searle 1965; Buckland, Buckland et al. 1969; Graham 1975; Koch 1999; Giakoumaki 2006). Prepulse stimuli can include acoustic, tactile, visual and vestibular stimuli, and PPI of startle can occur when the prepulse and startle pulse are in same or different modalities (Hoffman and Fleshler 1963; Buckland, Buckland et al. 1969; Graham 1975; Pickney 1976; Hoffman and Ison 1980; Blumenthal and Gescheider 1987; Gruner 1989; Bisdorff, Bronstein et al. 1995; Li, Steidl et al. 2001; Yeomans, Li et al. 2002). Throughout this thesis, we will focus on mechanisms underlying PPI of the acoustic and tactile startle reflex.

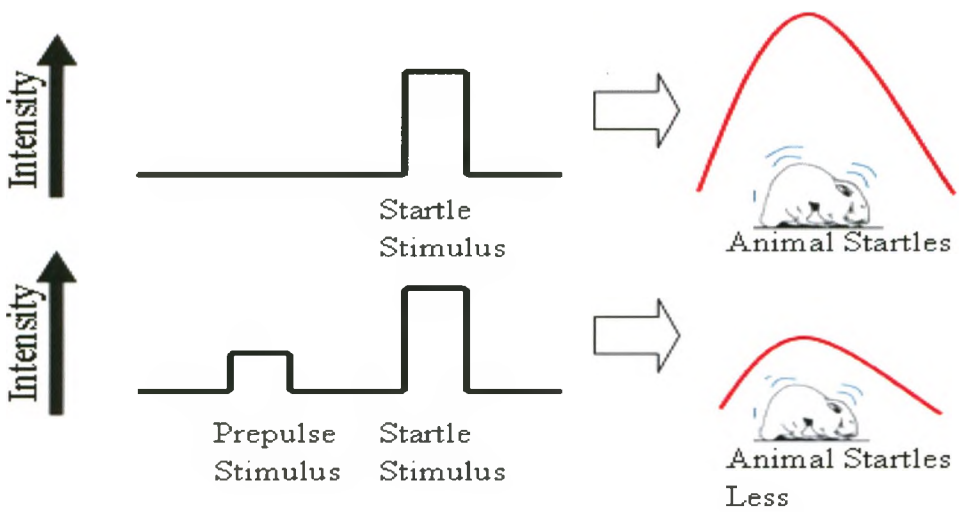


Figure 1: *Startle and prepulse inhibition*. The startle response (top): an intense stimulus causes muscles to contract. Prepulse inhibition of startle (bottom): a brief weak sub-threshold sensory prepulse presented before the startle stimulus reduces the intensity of the startle response.

2.2 Neural circuitry of startle in rats

Although there is evidence to suggest that several clinical populations (e.g., patients with Schizophrenia, Tourette's syndrome, obsessive-compulsive disorder, Huntington's disease, Parkinson's disease and attention-deficit hyperactivity disorder) are afflicted by

sensorimotor gating deficits measured as PPI disruption (Swerdlow and Geyer 1993; Swerdlow, Paulsen et al. 1995; Castellanos, Fine et al. 1996; Braff, Geyer et al. 2001; Swerdlow, Karban et al. 2001; Valls-Sole, Munoz et al. 2004; Kumari, Antonova et al. 2007; Kunugi, Tanaka et al. 2007) the neural mechanisms of PPI of startle are not yet fully understood. However, a hypothetical neural circuit has been proposed for both the startle and PPI pathway (see Figure 2 for an overview of the startle pathway).

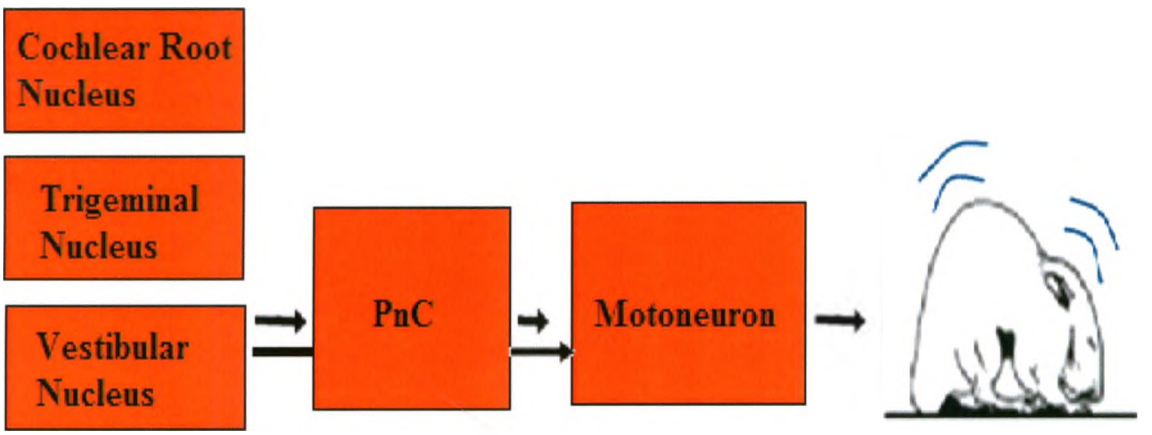


Figure 2: *Hypothetical startle pathway.* In rats, acoustic startle stimuli activate neurons in the cochlear root and tactile startle stimuli activate neurons in the trigeminal nucleus. Both nuclei project to giant neurons in the PnC which in turn excite motoneurons to induce startle. Free fall can be used as a stimulus which will excite neurons in the vestibular nucleus which then excites PnC giant neurons and/or directly elicit a startle response.

An acoustic stimulus activates the ascending auditory pathway (i.e., the dorsal and ventral cochlear nucleus (CN), the lateral superior olive (LSO) and neurons in the cochlear root nucleus). The auditory pathway, in particular the cochlear root neurons (CRNs) in rodents, send excitatory projections to the caudal pontine recticular nucleus (PnC) of the brainstem (Lingenhöhl and Friauf 1992; Lingenhohl and Friauf 1994; Koch 1999; Leumann, Sterchi et al. 2001). Excitation of giant (soma diameter greater than 35 μM) reticulospinal neurons in the PnC ultimately leads to activation of spinal

motoneurons which then elicit a startle response (Hoffman and Searle 1968; Davis 1984; Koch, Kungel et al. 1993; Lee, López et al. 1996; Koch 1999). In tactile startle, activated primary sensory neurons send excitatory projections to the principal sensory nucleus (Pr5) which in turn innervate PnC giant neurons (Scott, Frankland et al. 1999; Yeomans, Li et al. 2002; Schmid, Simons et al. 2003).

The PnC is a crucial brain structure for eliciting acoustic and tactile startle since lesions of the PnC impairs startle in both modalities (as reviewed in Yeomans, Li et al. 2002). Furthermore, the PnC is a multimodal structure that not only receives input from cochlear and trigeminal nucleus, but also from neurons in the vestibular nucleus (VN). The PnC startle-mediating neurons project directly onto spinal cord motor neurons; thus, the PnC is the sensorimotor interface of the startle response (Davis 1984; Lingenhohl and Friauf 1994; Li and Yeomans 1999; Yeomans, Li et al. 2002; Li, Du et al. 2009). Neurons from VN, however, also send direct axonal projections to the spinal cord via the vestibulospinal tract to elicit a faster motor response; therefore, vestibular startle does not rely on the PnC (Li and Yeomans 1999; Yeomans, Li et al. 2002). There are several properties of giant reticulospinal neurons in the PnC that allow for summation of input from different modalities. These neurons exhibit a low membrane resistance, long membrane time constant and low firing threshold which allows for both cross-modal temporal integration and a fast startle response (Wagner and Mack 1998; Koch 1999).

2.3 Neural circuitry of prepulse inhibition

The hypothetical primary PPI pathway is comprised of the cochlear nuclei, inferior and superior colliculi and the pedunculopontine tegmental nucleus (PPT; Figure 3; Leitner and Cohen 1985; Koch, Kungel et al. 1993; Fendt, Koch et al. 1994; Lee, López et al. 1996; Fendt 1999; Fendt, Li et al. 2001; Yeomans, Lee et al. 2006; Li, Du et al. 2009). This pathway is activated when a sensory stimulus (e.g., auditory, tactile, free fall or visual) excites its respective primary sensory area (i.e., CRN, Pr5, VN or retina) which sends axonal projections to the superior colliculus (a multimodal structure that receives input from acoustic, somatosensory and visual stimuli; Meredith and Stein 1986; Meredith, Wallace et al. 1992). Unlike other sensory stimuli, acoustic prepulses first activate the inferior colliculus (IC) which acts as a relay station between the auditory system and the SC. Once the SC is activated, it will excite PPT neurons that then send inhibitory projections to the PnC, ultimately inhibiting startle (Diederich and Koch 2005; Yeomans, Lee et al. 2006).

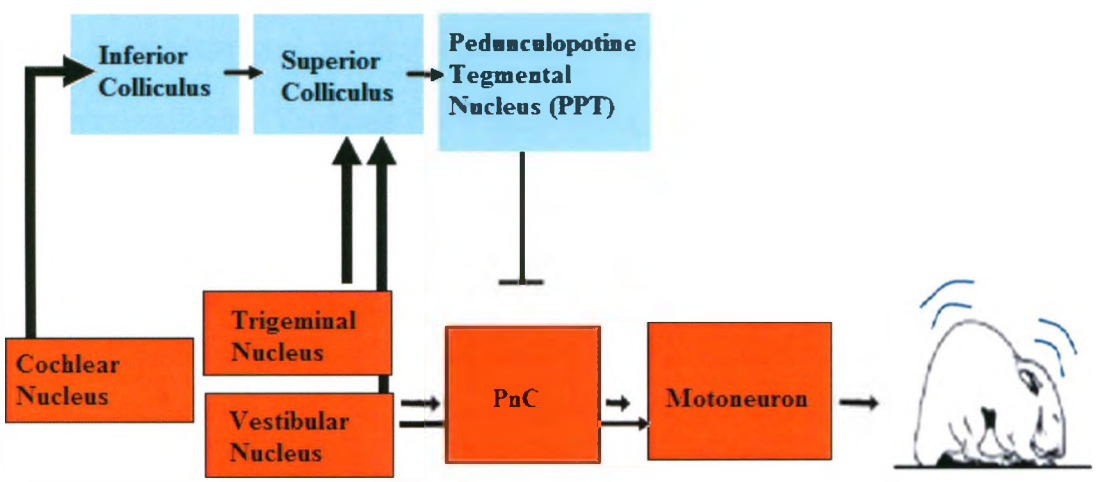


Figure 3: *Hypothetical primary PPI pathway.* Sensory stimuli excite the superior colliculus which then activate the pedunculopontine tegmental nucleus. Once the PPT is stimulated it subsequently inhibits PnC giant neuron activity. Take note that acoustic stimuli will first excite the inferior colliculus before reaching the superior colliculus.

There is significant evidence to suggest that the PPT is critical for PPI of startle. Saitoh and colleagues conducted the first experiments where the lateral tegmental area (which includes the PPT) was stimulated and they noted that startle amplitude was reduced (Saitoh, Tilson et al. 1987). Direct stimulation of the PPT elicits the same reduction in startle as the presentation of a prepulse prior to the startle stimulus (Li and Yeomans 2000). Furthermore, lesioning the PPT causes significant reduction or elimination of PPI demonstrating the necessity of the PPT for prepulse inhibition (Swerdlow and Geyer 1993; Kodsi and Swerdlow 1997; Fendt, Li et al. 2001).

This primary PPI pathway introduced so far is modulated by many different higher brain structures (see Fendt, Li et al. 2001 for review). Modulations in the limbic cortex (which includes the hippocampus, amygdala, nucleus accumbens and medial prefrontal cortex; Lewis and Shute 1967; Swerdlow, Caine et al. 1992; Bubser and Koch 1994; Wan, Caine et al. 1996; Meloni and Davis 2000; Daenen, Wolterink et al. 2003; Miller, Saint Marie et al. 2010), the substantia nigra, ventral tegmental area (VTA) and the ventral pallidum (Yeomans 1995; Blaha, Allen et al. 1996; Fendt 1999; Li and Yeomans 1999) all directly or indirectly regulate the activity of PPT, which inevitably impacts PPI of startle. Direct projections from the nucleus accumbens, substantia nigra, and ventral pallidum in particular inhibit PPT neurons (Figure 4).

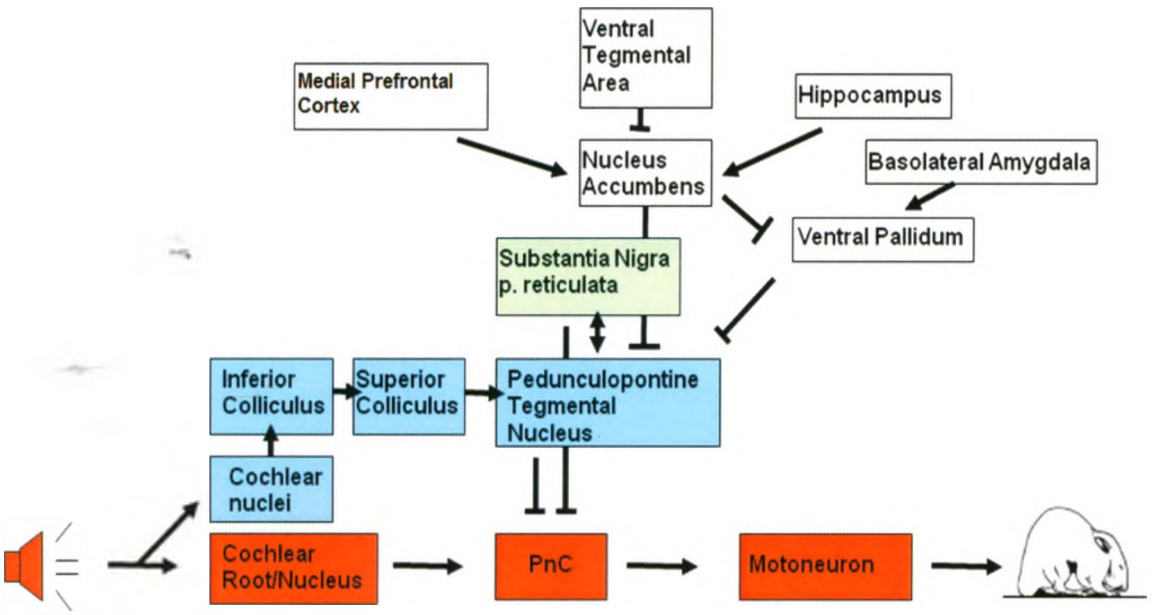


Figure 4: *Modulation of PPI from higher brain structures*. There are several midbrain and cortical brain regions that directly or indirectly suppress the activity of the PPT. As depicted in this figure, the limbic cortex, substantia nigra, VTA and ventral pallidum inhibit PPT activity. Inhibition of the PPT, a crucial structure to elicit PPI of startle, would allow for a startle response (modified, from Koch et al., 1999).

Furthermore, besides its descending inhibition to the PnC, the PPT primarily projects to these higher brain structures, especially to the thalamus, and elicits significant cortical activation (Steriade, Datta et al. 1990; as summarized in Fendt, Li et al. 2001). Cholinergic neurons within the PPT provide the strongest excitatory inputs to substantia nigra and VTA dopaminergic neurons particularly during presentation of rewards (Yeomans 1995; Blaha, Allen et al. 1996; see Fendt, Li et al. 2001). Blocking the cholinergic receptors near dopaminergic neurons block the effects of rewarding stimuli such as nicotine, for example, and cause avoidance behaviours (Yeomans and Baptista 1997). It was suggested that the PPT may be responsible for cortical activation and eliciting approach behaviours important for survival while inhibiting avoidance

behaviours by inhibiting the PnC, for example (Fendt, Li et al. 2001). As for the inhibition of the startle-mediating neurons in the PnC during PPI, studies have suggested that there are direct projections not only from cholinergic neurons of the PPT, but also from the substantia nigra and the laterodorsal tegmentum (LDT) to the PnC (Yeomans 1995; Laviolette, Priebe et al. 2000).

2.4 Pharmacology of startle and prepulse inhibition

Within the auditory system acoustic information is primarily relayed by fast glutamatergic neurotransmission (Potashner, Morest et al. 1985). Given that there is evidence to suggest that glutamate acts on reticulospinal neurons in the PnC (Greene and Carpenter 1985), Ebert and Koch examined whether or not the auditory system utilizes glutamate for synaptic transmission to the PnC (Ebert and Koch 1992). Local application of AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) and NMDA (N-methyl-D-aspartic acid) receptor antagonists into the PnC revealed that both receptors played a role in ASR, although AMPA receptors had a more significant role than that of NMDA (Ebert and Koch 1992; Krase, Koch et al. 1993).

Both GABA (gamma-aminobutyric acid) and acetylcholine (ACh; reviewed in section 1.7) have been recognized as the neurotransmitters recruited to suppress startle during PPI (Kungel, Ebert et al. 1994; Fendt 1999; Koch 1999; Bosch and Schmid 2006; Bosch and Schmid 2008; Yeomans, Bosch et al. 2010). For instance, when GABA was perfused onto rat PnC giant neurons it inhibited their spontaneous and tone-evoked activity (Kungel, Ebert et al. 1994). Together $GABA_A$ and $GABA_B$ receptor subtypes

contribute to PPI of startle through inhibition of PnC giant neurons (Fendt 1999; Yeomans, Bosch et al. 2010). Evidently, inhibiting GABA receptors on PnC giant neurons can facilitate startle (Kungel, Ebert et al. 1994 and reviewed in ; Koch 1999).

It was proposed that GABA-induced reduction of startle and PnC giant neuron activity was caused by excitation of neurons within the substantia nigra, pars reticulata (SNR) which does in fact send GABAergic projections to the PnC (Yasui, Nakano et al. 1992; Koch, Fendt et al. 2000). However, the PPT may also give rise to GABAergic projections to the PnC given that cholinergic antagonists could not completely eliminate the inhibitory effect on PnC giant neurons caused by PPT stimulation (Bosch and Schmid 2006; Bosch and Schmid 2008; Yeomans, Bosch et al. 2010). Furthermore, GABAergic neurons have been found in the PPT (Wang and Morales 2009).

2.5 Cholinergic involvement in prepulse inhibition

Semba and colleagues conducted anatomical tracing and immunofluorescence experiments and revealed that there are cholinergic projections from the PPT to the PnC, a finding later confirmed by other researchers (Semba, Reiner et al. 1990; Koch, Kungel et al. 1993; Swerdlow and Geyer 1993; Kungel, Ebert et al. 1994). Furthermore, specifically destroying the cholinergic neurons within the PPT significantly reduced PPI of startle in rats (Koch, Kungel et al. 1993; Swerdlow and Geyer 1993). Thus far, the data suggest that PPT cholinergic neurons mediate prepulse inhibition of startle via inhibition of the startle-mediating giant neurons of the PnC (as reviewed in Fendt, Li et al. 2001; Homma, Skinner et al. 2002; Yeomans, Lee et al. 2006).

Several studies have attempted to elucidate which cholinergic receptors mediate PPI. To date, both muscarinic and nicotinic cholinergic receptors (mAChR and nAChR) have been shown to be involved. Systemic or local microinjections of scopolamine (a muscarinic antagonist) into the PnC, for example, increased the ASR and/or reduced PPI (Wu, Jenden et al. 1993; Fendt and Koch 1999; Jones and Shannon 2000). Bosch and Schmid further demonstrated that it is the M_2 and M_4 mAChR subtypes that specifically mediate presynaptic PnC inhibition (Bosch and Schmid 2006; Bosch and Schmid 2008). They also noted that there was an additional postsynaptic inhibition of PnC giant neurons that could not be attributed to muscarinic receptors and suggested a possible role of nicotinic receptors. Indeed, systemic nicotine administrations enhanced PPI in rats whereas a high dose of the nonspecific nicotine antagonist (mecamylamine) decreased PPI (Acri, Morse et al. 1994; Curzon, Kim et al. 1994). Nicotine has also been shown to enhance PPI in humans (e.g., individuals with schizophrenia as well as their relatives; Adler, Hoffer et al. 1992; Hong, Wonodi et al. 2008). Evidently, both cholinergic receptors seem to be implicated in prepulse inhibition of startle.

2.6 Nicotine and its role in prepulse inhibition

Approximately 90% of patients with schizophrenia smoke, which is two to four times more than the general population (Hughes, Hatsukami et al. 1986; Ucok, Polat et al. 2004). Individuals with schizophrenia not only consume more cigarettes, but they also tend to smoke cigarettes with higher nicotine content (Olincy, Young et al. 1997) and are able to obtain more nicotine from each cigarette (Olincy, Young et al. 1997; Strand and Nyback 2005). Self-reports from those with schizophrenia suggest that alleviation of the

negative psychiatric symptoms of the disease (such as apathy) is among the reasons that they smoke (reviewed in Kumari 2005). Interestingly, studies show that individuals with schizophrenia who smoke have significantly better PPI than their non-smoker counterparts (Kumari, Soni et al. 2001; George, Termine et al. 2006; Swerdlow, Light et al. 2006). Hong and collaborators examined PPI in schizophrenia populations instructed to abstain from smoking two hours before PPI testing. Participants who received nicotine administration from a nasal spray immediately before testing had improved PPI. Furthermore, the greater their clinical symptoms, the more profound the nicotine-induced enhancement of PPI (Hong, Wonodi et al. 2008). Therefore, among schizophrenia populations nicotine has a positive effect on sensorimotor gating as measured by PPI of startle and may serve as a form of self –medication to improve maladaptive behaviour (see Kumari 2005 for a review).

The effect of nicotine on PPI, however, can also be seen in nonclinical populations. For example, nicotine enhanced PPI of startle in otherwise healthy male smokers who abstained from smoking the night prior to PPI testing, but were given one cigarette smoke immediately before the test (Kumari, Checkley et al. 1996). Given that the nicotine effect found here could be attributed to nicotine withdrawal, Kumari and colleagues conducted further testing employing non-smoker volunteers. The data collected illustrated that nicotine administered to healthy non-smokers could in fact enhance PPI of startle compared to the saline-treated group (Kumari, Cotter et al. 1997; Della Casa, Hofer et al. 1998).

Nicotine has also proven to be beneficial in animal models with deficient PPI by enhancing prepulse inhibition (Acri, Grunberg et al. 1991; Acri, Morse et al. 1994; Curzon, Kim et al. 1994; Semenova, Bespalov et al. 2003; Suemaru, Yasuda et al. 2004) without affecting baseline startle amplitude (Curzon, Kim et al. 1994; Della Casa, Hofer et al. 1998). Curzon and colleagues demonstrated that a high dose (10 mg/kg) of mecamylamine (a nonspecific nAChR antagonist) can dampen the PPI caused by both weak and strong prepulses (Curzon, Kim et al. 1994). Furthermore, DBA/2J mice undergoing nicotine withdrawal experience disrupted PPI that can be ameliorated following nicotine self-administration (Semenova, Bespalov et al. 2003). Lastly, nicotine can also improve PCP-induced PPI disruption in this mice strain and that of C3H/HEJ (Kumari 2005). The majority of research evidence suggests that nicotine plays an important role in PPI of startle in both humans and animals.

2.7 Pharmacology of nicotine receptors

Homopentameric and heteropentameric nAChRs are ligand-gated cation channels found throughout the peripheral and central nervous systems (PNS and CNS; for detailed review see Paterson and Nordberg 2000; Laviolette and van der Kooy 2004; Kumari 2005; Taly, Corringer et al. 2009; Changeux 2010). Within the PNS, nAChRs located at neuromuscular junctions include α1, β1, δ, and γ subunits, whereas neural nAChRs consist only of α and β subunits. There are nine α ($\alpha_2 - \alpha_{10}$) and three β ($\beta_2 - \beta_4$) neuronal nAChR subunits identified with several possible combinations (e.g., $\alpha_4\beta_2$ and $\alpha_3\beta_4$) that ultimately determine receptor structure, affinity to agonists and location in the brain (Elgoyhen, Johnson et al. 1994; Taly, Corringer et al. 2009). Thus far, three classes of

homomeric neuronal nAChRs have been identified (α_7, α_8 and α_9; Elgoyhen, Johnson et al. 1994; Johnson, Martinez et al. 1995; Yu and Role 1998; summarized in Paterson and Nordberg 2000).

Five nicotinic subunit assemble to form a pore and each subunit is composed of an extracellular loop, 4 transmembrane domain and an intracellular loop (see Taly, Corringer et al. 2009 for review). An agonist (i.e., nicotine or ACh) must bind to the extracellular loop of an α subunit in order to elicit its effect on nAChRs. Heteromeric nAChRs (e.g., $\alpha_4\beta_2$) have two binding sites located between the α and β subunits while the homomeric receptor subtype (e.g., α_7) can have up to 5 binding sites (Figure 5; Paterson and Nordberg 2000; Taly, Corringer et al. 2009).

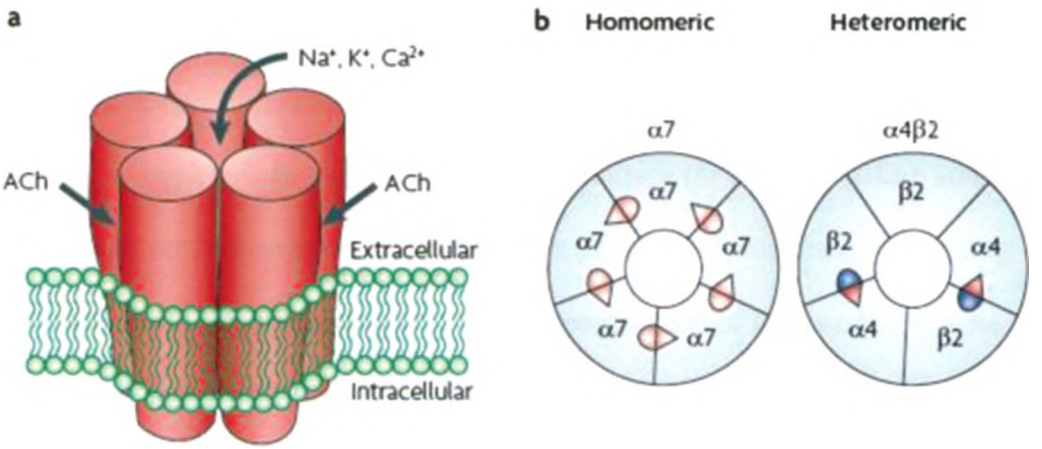


Figure 5: *Structure of nicotine receptors and binding sites*. (a) Five nicotinic subunits are arranged such that the centre forms a pore highly permeable to Na^+. The receptor is embedded into the cell membrane such that there is an extracellular and intracellular loop. (b) Semi-circles indicate binding sites of nicotine to receptors for both the α_7 (homomeric) and $\alpha_4\beta_2$ (heteromeric) subtypes. (Figure adopted from Changeux 2010)

Let us now consider the mechanism of action of cholinergic agonists on neuronal nAChRs. When an individual smokes a cigarette, nicotine moves through the bloodstream into the brain where it spreads quickly and binds to ionotropic nAChRs causing channel opening (within μs and ms; Taly, Corringer et al. 2009). Once opened, the receptors allow influx of sodium (Na^+) and calcium (Ca^{2+}) ions, as well as efflux of potassium ions (K^+). This cascade of events causes even more Ca^{2+} to permeate the cell by opening of voltage-gated Ca^{2+} channels. Activation of nAChRs often elicits increased transmitter release from dopaminergic, serotonergic, glutamatergic and GABAergic neurons which may be implicated in both basic cognition and maladaptive behaviours (e.g., sensory gating, learning and memory, and addiction; Paterson and Nordberg 2000; Laviolette and van der Kooy 2004).

Several studies have indicated that both $\alpha_4\beta_2$ and α_7 nAChRs are involved in sensory gating (Schreiber, Dalmus et al. 2002; Rudnick, Koehler et al. 2009). The α_7 nAChR subtype was found to be crucial for auditory (or sensory) gating amongst relatives of schizophrenics (Adler, Hoffer et al. 1992). Further research among schizophrenia populations has lead to the identification of the α_7 nAChR gene (CHRNA-7) located at 15q14 (Chini, Raimond et al. 1994; Freedman, Coon et al. 1997). Both receptor subtypes are ubiquitously distributed throughout the brain, particularly $\alpha_4\beta_2$-containing nAChRs which make up 90% of nicotinic receptor expression (McGehee and Role 1995; as reviewed in Kumari 2005). These two nAChRs are located in brain areas known to mediate sensorimotor gating (e.g., substantia nigra, hippocampus, VTA and PPT; see Changeux 2010 for recent review).

Nonetheless, it is currently unknown which brain areas nicotine exerts its effect on PPI. Electrophysiological evidence suggests that nicotine may be able to influence PPI by directly acting on the startle-mediating giant neurons in the PnC. The aim of this study is to further elucidate the physiological role of nicotine in prepulse inhibition of startle.

2.8 Hypothesis and research objectives

As indicated above, various behavioural and electrophysiological studies reveal that cholinergic inhibition of the PnC through muscarinic receptors plays an important role in PPI. There is also substantial behavioural evidence for the involvement of nAChRs in PPI, but it is not yet clear if nicotine affects the PnC directly or whether it exerts its action through modulation in higher brain areas. Based on the fact that muscarinic antagonists did not completely block the cholinergic inhibition in the PnC (Bosch & Schmid 2006), we hypothesize that nAChRs are directly involved in PnC inhibition. We propose that nAChRs may activate GABAergic interneurons in the PnC or may directly inhibit PnC giant neurons (Figure 6).

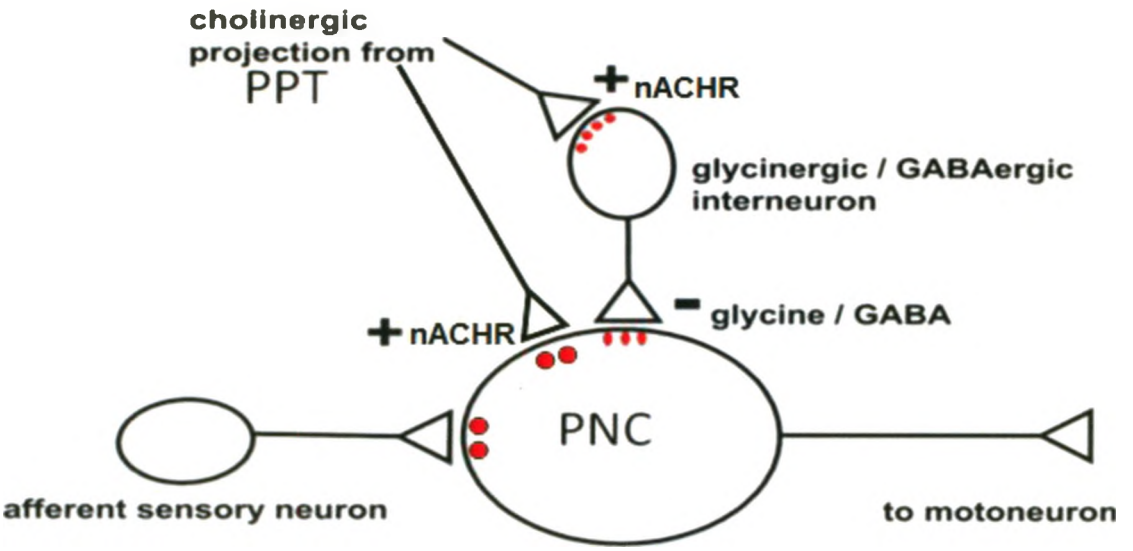


Figure 6: *Hypothesis*. Nicotinic receptors may be located on glycinergic or GABAergic interneurons. Once stimulated by the PPT, the inhibitory interneuron(s) inhibit PnC giant neuron activity. However, nicotine receptors may be directly located on PnC giant neurons and elicit postsynaptic inhibition once activated.

To test this hypothesis, we conducted electrophysiological recordings in rat brainstem slices. Our first objective was to identify whether or not nicotine has an effect on PnC giant neuron activity. We then attempted to identify if the α_7 and/or non- α_7 nAChR subtype was involved in PnC modulation by applying the α_7 specific antagonist (MLA), the specific agonist PHA 543-613 or non-α_7 antagonist (TMPH). Subsequently, we examined if the nicotine effect could be eliminated by blocking synaptic transmission which would suggest that nAChRs are not expressed by startle-mediating PnC neurons, but by modulatory neurons that synapse onto PnC neurons.

Chapter 3

Materials and Methods

3.1 *Animals*

Sprague-Dawley rat pups (Charles River Laboratories, Saint Constant, QC, Canada) were housed with their mothers on a 12-hour light/dark cycle, and mothers were given free access to food and water. Male and female pups between postnatal day 9 and 17 (where postnatal day 1 refers to the day of birth) were used to obtain acute brain slices. All experimental procedures were approved by the Institutional Animal Care Committee and performed in accordance with the Canadian and National Institute of Health Guides for Care and Use of Laboratory Animals.

3.2 *Brain Slice Preparation*

Sprague-Dawley rats were anaesthetized with isoflurane and decapitated using a guillotine. Brains were removed from the skull within approximately 1 - 3 minutes. First, the skin on the head was lifted away from the skull and was cut caudal to rostral along the midline with sharp scissors. Subsequently, the skull was cut along the midline from caudal to rostral followed by short cross incisions along Bregma and Lambda. Tweezers were used to carefully remove the upper skull and any remaining Dura Mater from the brain. The brain was rapidly removed by severing the temporal bone on either side of the brain with a pair of bone pliers and carefully cutting all the nerves on the base of the skull with a sharp scalpel. A long incision was made with a scalpel half way between Bregma and Lambda such that only the caudal portion of the brain (containing the cerebellum and

brainstem) was transferred to an ice-cold preparation solution for further slicing. The preparation solution (see Table 1) was bubbled with carbogen (95% O_2 and 5% CO_2) to limit injury and provide sufficient oxygen to the tissue.

Solutions and Chemicals

Table 1: Composition of all solutions and chemicals.

Solution	Substance	Molarity [mM]	
Preparation Solution:	KCl	2	with Carbogen:
	$MgCl_2$	2	pH 7.36
	KH_2PO4	1.2	Osmolarity:
	MgSO4	1.3	310-330 mosmol
	NaHCO3	26	
	Glucose	10	
	Sucrose	210	
	$CaCl_2$	2	
	Myoinositol	3	
	Na-Pyruvate	2	
	Ascorbic Acid	0.4	
Artificial Cerebral Spinal Fluid (ACSF):	KCl	2	with Carbogen:
	KH_2PO4	1.2	pH 7.36
	MgSO4	1.3	Osmolarity:
	NaHCO3	26	310-330 mosmol
	NaCl	124	
	Glucose	10	
	$CaCl_2$	2	
Intracellular solution (ICS):	K-Gluconat	130	with KOH:
	EGTA	0.6	pH 7.2
	$MgCl_2$	2	Osmolarity:
	KCl	5	270-280 mosmol
	HEPES	10	
Drugs	Nicotine:	10 µM	
	MLA:	10 µM	
	PHA 543-613:	30 µM	
	TMPH:	100 nM	
	Cadmium:	100 µM	

Brains were glued onto a stage with Loctite 411 instant glue. They were glued on the rostral plane with the ventral side facing towards the blade (WPI, Valet Auto Strop Blades) attached to the vibratome and the dorsal side against an agar block for support (see Table 1 for ingredients). Coronal sections (350 μm to 400 μm thick) were prepared using a vibratome (Microm HM 650V, Germany). Depending on the thickness of the slices, approximately 1 to 3 slices were collected from each brain. Slices of interest contained the caudal pontine reticular nucleus (PnC) of the brainstem as well as afferent fibres from the trigeminal and auditory pathways (see Figure 7 for a representative slice).

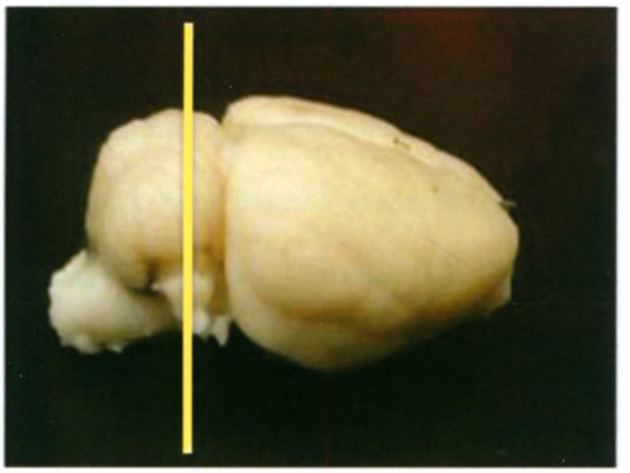
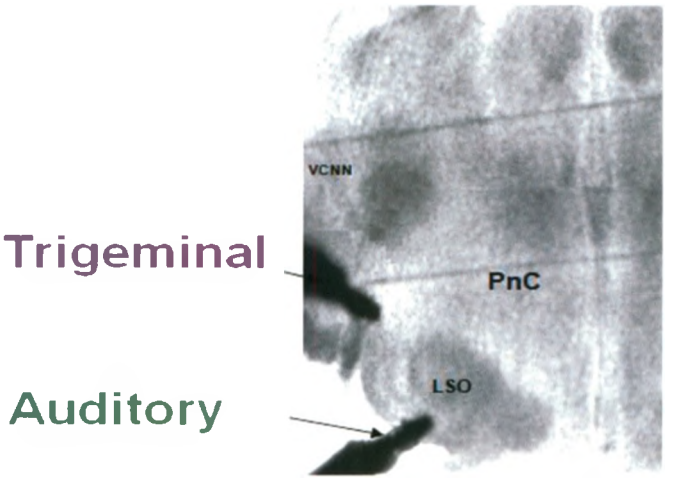
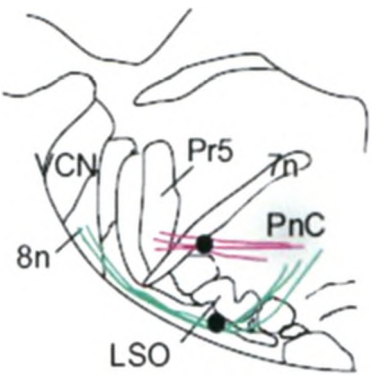


Figure 7:

(Left) Rat pup brain, yellow line indicates area where coronal slices are taken. (Below) Brain slice of interest contains PnC structure as well as afferent fibres from both the trigeminal and auditory pathways (bottom left). Right schematic illustrates that afferent fibres from the trigeminal and auditory pathways project to the PnC (adopted from Simons-Weidenmaier, Weber et al. 2006).





Immediately after sectioning, slices were transferred into calcium-free artificial cerebral spinal fluid (ACSF) bubbled with carbogen. Once all slices were collected, Ca^{2+} (2 mM) was added to the ACSF and the solution was either heated to 32°- 35° C to improve patch success. The slices were maintained at this temperature for 30 min, and subsequently brought to room temperature (20° C – 25° C) for a minimum of one hour. Slices were then transferred to a holding chamber for electrophysiological recordings (see below).

3.3 *Experimental Setup*

The experimental setup contained a superfusion holding chamber under an upright fixed stage microscope (Axioskop, Zeiss, Germany) with an infrared 40x magnification water-immersion objective (IR-Achroplan, Zeiss, Germany). The microscope was mounted onto a vibration -free table (Science Products Ltd, Hofheim, Germany) and surrounded by a Faraday cage. Within the holding chamber, slices were constrained using a custom made platinum grid. The holding chamber was constantly perfused with bubbled ACSF (200 ml/hr). The chamber outlet was connected to a suction pump to maintain optimal solution level within the bath. Experiments were conducted at room temperature or between 32° C and 34° C if the bath solution was heated (with a TC-324B Automatic Temperature Controller; Warmer Instrument Corporation).

Patch-clamp recordings were controlled visually using a digital infrared-sensitive camera (Kappa CF 8 / 4 NIR, Gleichen, Germany) with a black/ white monitor (Monacor,

CDM-1702) attached to the microscope to visualize cells. Borosilicate glass patch pipettes (GB150F-10, Science Products Ltd, Germany) were first cleaned with 70% ethanol in an ultrasonic bath to remove any dirt. Subsequently, pipettes were pulled daily prior to experiments using a micropipette puller (Model P-57, Sutter Instruments Co., Novato, USA) with a resistance of approximately 3.5 MΩ (minimum of 2 MΩ and maximum of 7.5 MΩ) and filled with sterile intracellular solution (ICS; see Table 1) .

During an experiment, the glass pipette was slipped over a chlorinated silver recording electrode (AG-15T, Science Products LTD, Hofheim, Germany) attached to a preamplifier (CV 203BU Headstage, Axon Instruments, Sunnyvale U.S.A.) which was in turn attached to a piezo-driven micromanipulator (TS-5000-150, Burleigh Instruments, Oakville, Canada). Signals from the recording electrode were sent from the preamplifier to a patch-clamp amplifier (Axopatch 200B, Axon Instruments, Sunnyvale, USA), to a digitizer (DIGIDATA 1320 A, Axon Instruments, Sunnyvale, USA) and then to a PC. A silver chloride pellet in the bath solution was used as a reference electrode. The computer program Clampex 8.2 (Axon Instruments, Sunnyvale, USA) was used to generate commands and measure cell signals. Lastly, to stimulate the trigeminal and auditory fibres within the slice, Clampex 8.2 was used to send TTL pulses via a digitizer to a stimulus unit (Master-8, A.M.P.I., Haifa, Israel) which in turn triggered the stimulation isolators. Each stimulating electrode was connected to its own isolator (ISO-Flex, A.M.P.I., Haifa, Israel) which allowed for independent adjustment of stimulation intensity.

3.4 *Electrophysiology*

Patch-clamp whole cell recordings were performed on visually identified PnC giant neurons. Only cells with a soma diameter of 35 microns or greater and within the boundaries of the PnC (after Paxinos and Watson 1998) were used for recording. Both voltage- and current-clamp experiments were carried out. During voltage-clamp experiments, cells were held at -70 mV, unless otherwise noted. In the current clamp mode, cells were brought to a holding potential of -70 mV by injecting a negative direct current (DC) if the resting membrane potential was more positive. Only cells that had a resting membrane potential more negative than -40 mV and had an access resistance no greater than 50 MΩ were used. Cells were excluded if the access resistance changed during drug perfusions by more than 30 percent of the control or if the holding current was larger than 300 pA.

3.5 *Extracellular Stimulation*

Auditory and trigeminal pathways were stimulated separately (representing acoustic and tactile stimuli) while recording from the startle mediating giant neurons in the PnC. For trigeminal stimulation, afferent fibres that extend from the principal sensory trigeminal nucleus 5 (Pr5) to PnC were stimulated with bipolar tungsten electrodes (SNEX, Science Products, Hofheim, Germany) along the mediolateral 7^{th} cranial nerves. Auditory afferent fibres were stimulated by placing the electrode ventral to the lateral superior olive.

Prior to recording from PnC giant neurons' excitatory postsynaptic currents (EPSC) in response to stimulation of trigeminal and auditory afferent fibres, the stimulation intensity was adjusted for both stimuli. Stimulus intensity was increased until maximum EPSC amplitudes were measured and then adjusted to elicit half maximum amplitudes. For PnC giant neurons, the half maximal EPSC amplitude is around -300 pA, stimulus intensity was adjusted to give EPSCs between -150 pA and -300 pA. Each stimulus pulse was 150 μs in duration.

3.6 *Experimental Protocols*

Several protocols were created and applied to each cell during recordings to ensure the viability of cells as well as to test the effects of various drugs on different parameters. Tightly controlled recordings allowed for the measurement of leak currents, membrane resistance, resting membrane potentials and synaptic currents and potentials. The protocols used are described in detail below. During all voltage-clamp experiments a -5 mV test pulse was applied to the neurons for 25 ms at the beginning of each trial to measure series and input resistance to control for stable recordings.

3.7 *Current Voltage Relation*

To ensure neuron viability and characterize the cells, cells were depolarized for 250 ms from -70 mV to potentials between -70 mV and +40 mV in steps increasing by 10 mV (VC_IV, Figure 8). Neurons that exhibited large transient inward currents (greater than -1000 pA) followed by non- or partly inactivating outward currents upon depolarization

were considered mature and viable neurons and could be included in data analysis. A similar protocol was applied in the current clamp mode: cells were brought to -70 mV by the injection of a tonic negative current. Positive currents between -70 pA and -25 pA in 5 pA steps were injected for 250 ms, while recording the membrane voltage (CC_IV).

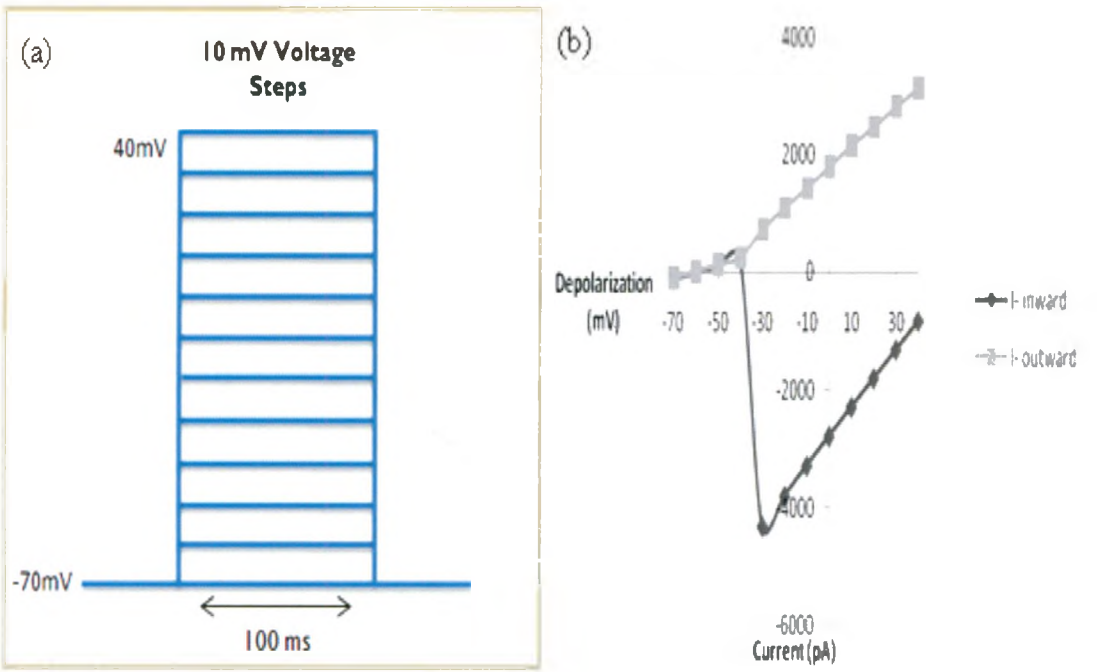


Figure 8: *Current voltage relation.* (a) Depolarizing voltage step from -70 mV to 40 mV in 10 mV increments. (b) Inward and outward currents in response to depolarization.

3.8 *Paired-pulse stimulation*

Auditory and trigeminal afferent fibres were each stimulated rapidly twice in succession [interstimulus interval (ISI): 100 ms, Figure 9], known as paired-pulse paradigm. Each stimulus pulse was 150 μs in duration and had the same intensity (modality specific). The interval between auditory and trigeminal stimulations was 100 ms. A trial consisted

of twelve paired-pulse traces delivered every five seconds. Paired-pulse stimulations were carried out in the voltage- clamp mode at a holding potential of -70 mV and in the current-clamp mode (while neurons were brought to -70 mV by a DC current injection).

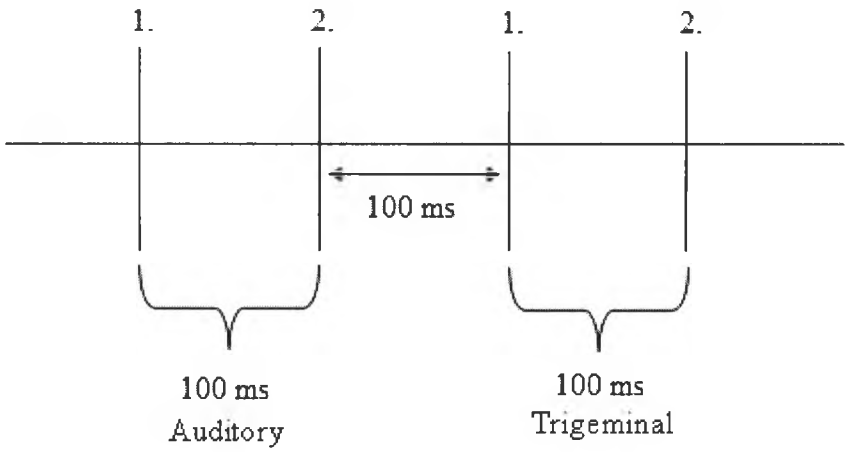


Figure 9: *Paired-pulse stimulations.* Auditory and trigeminal afferent fibres were stimulated twice. The interval between paired-pulses within and between modalities was 100 ms.

3.9 *Hyperpolarizing voltage step*

In voltage-clamp mode, a 20 mV hyperpolarizing voltage step was applied to neurons for 75 ms so that cells were taken from -70 mV to -90 mV. Each condition consisted of 100 traces applied at a frequency of 1 Hz. This hyperpolarizing voltage step allowed for the analysis of the leak current and membrane resistance (using Ohm's law; Figure 10) before and during drug perfusion. Leak current refers to the amount of current injected into to the cell in order to clamp it at -70 mV. Given that leak current is dependent on membrane resistance and resting membrane potential, the latter two parameters were also examined separately (see below).

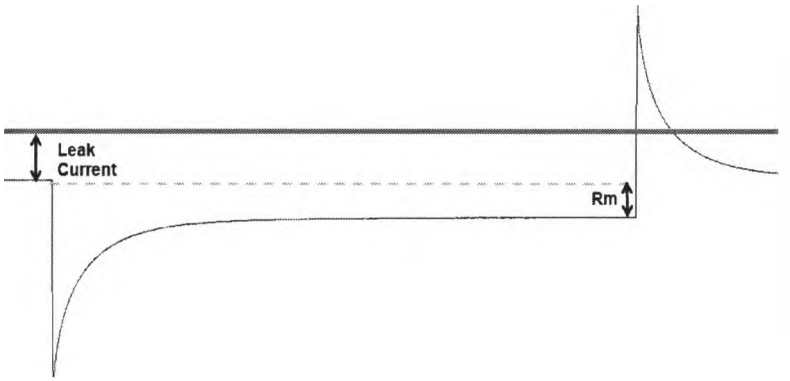


Figure10: *Hyperpolarizing voltage step*. Current in response to a -20 mV voltage pulse used to calculate leak current and membrane resistance (using Ohm's law).

3.10 *Pharmacology*

Data were collected to assess whether or not nAChRs can modulate PnC giant neuron activity. A 10 μM concentration of (+) - nicotine ditartrate (Sigma-Aldrich, Canada) was added to the ACSF and perfused onto brain slices. Upon demonstration of an effect of nicotine on PnC giant neurons, a specific agonist and antagonists were used to elucidate which nAChR subtype was mediating the nicotine effect. The α_7 specific agonist N-[(3R)-1-azabicyclo[2.2.2]oct-3-yl]furo[2,3-c]pyridine-5-carboxamide hydrochloride (PHA 543 613; Tocris Bioscience, U.S.A.) and the α_7 specific antagonist methyllycaconitine (MLA; Tocris Bioscience, USA) were applied at concentrations of 10 μM and 30 μM respectively to assess the involvement of the α_7 nAChR subtype. The non-α_7 preferring nAChR antagonist, 2,2,6,6-tetramethylpiperidin-4-yl heptanoate (TMPH; Tocris Bioscience, U.S.A.) was added to the bath superfusion at a concentration of 100 nM. Furthermore, cadmium (100 μM; Sigma-Aldrich, Canada) was perfused onto brain slices to block synaptic transmission by blocking voltage-gated calcium channels. Drugs were diluted in double distilled water or saline as a 1000 times stock solution and kept frozen until added to the bath solution immediately before perfusion. Control data were collected in the presence of ACSF alone prior to drug perfusion.

3.11 *Data Analysis*

The Clampfit 8.2 program (Axon Instruments) was used to conduct preliminary data analysis and derive the raw data for statistical analysis using a custom-built add-in for Microsoft Excel (StatEl, Ad Science, France).

Leak current was analyzed by examining current flow across cell membrane during a 20 mV hyperpolarizing voltage step. In Clampfit, the average of the first 50 traces (out of 100) during the perfusion of ACSF was used as the control condition. Two cursors were placed at a point on the averaged trace before the -20 mV step and the mean between the cursors was used as the raw score for the control leak current. Following drug perfusion, 50 traces were averaged during the maximal drug effect on leak. Again, the mean between the two cursors was used as the leak value for the drug effect.

Given that leak current is dependent on both membrane resistance and resting membrane potential, both parameters were also assessed independently. The hyperpolarizing voltage step allowed for the analysis of membrane resistance by using Ohm's law ($R = \Delta V/\Delta I$). The mean between two cursors placed at the steady-state position after the application of the -20 mV step was calculated and used as the current value in the calculation of the membrane resistance (where $\Delta V = -20mV$ and ΔI is the mean difference calculated between the two cursors). For both leak current and membrane resistance drug data were normalized to the control levels. Membrane

potential was measured by switching from voltage- to current-clamp mode and reading the potential without injecting current.

Excitatory postsynaptic current (EPSC) amplitude was measured in response to presynaptic stimulation of both trigeminal and afferent fibres. The current in response to the first stimulus pulse was analyzed in order to assess drug effects on EPSCs. All twelve traces of synaptic currents were averaged and adjusted to baseline. Subsequently, the peak amplitude was measured.

Paired-pulse ratios (PPR) were analyzed by taking the average of the twelve traces adjusted to baseline and dividing the maximum amplitudes for EPSC 2 by EPSC 1. Paired-pulse stimulation allows for the calculation of a ratio (PPR) whereby changes in the ratio suggest a possible change in presynaptic efficacy (reviewed in Xu-Friedman and Regehr 2004). Statistical analyses were carried out on the raw data for each measure parameter. A one-way ANOVA or t-test was used to assess significant differences between drug conditions for all analyses and only differences with a p value less than 0.05 were considered statistically significant. Unless otherwise noted, Fisher's LSD test was used for Post-hoc comparisons. Friedman analysis was used when normality of data was not assumed.

Chapter 4

Results: Effects of nicotine and an α_7 nAChR antagonist on PnC giant neurons

4.1 Effects of nicotine on PnC giant neurons nicotine had an effect on startle-mediating neurons in the caudal pontine recticular nucleus

To examine whether or not nicotine has an effect on PnC giant neurons, nicotine was added to the bath superfusion after control measurements. The effect of nicotine on leak current, membrane resistance, resting membrane potential and EPSC amplitudes was examined. Friedman analysis revealed that the perfusion of nicotine on PnC giant neurons significantly increased leak current from -159 pA (SEM = 20.70) to -337 pA [(SEM = 51.26); t = 4.61; n = 18, $p < 0.001$]. Nicotine simultaneously caused a drop in membrane resistance from -155 MΩ (SEM = 18.88) under control conditions to -111 MΩ (SEM = 8.72) during nicotine [t = -3.25, p = 0.005). Nicotine also significantly depolarized the resting membrane potential of PnC giant neurons from -48 mV (SEM = 2.09) to -40 mV [(SEM = 2.15); t = -7.51, $p < 0.001$]. See Figures 11 – 13 for the nicotine effect on leak, membrane resistance and potential.

T-test analyses further indicated that nicotine had no significant effect on EPSC amplitudes for cells held at -70 mV for neither the trigeminal [control: -112 pA (SEM = 9.95)c and nicotine: -96 pA (SEM = 14.24); n = 4, t = -1.29, p = 0.29] nor auditory stimulations [control: -98 pA (SEM = 30.83) and nicotine: -70 pA (SEM = 25.25); n = 3, t = -1.42, p = 0.29]. Figure 14 shows EPSC amplitudes for both trigeminal and auditory

stimulations. However, the effect of nicotine on EPSC amplitude may have failed to reach statistical significance due to receptor desensitization during continuous nicotine perfusion (see Figure 15 for receptor desensitization during nicotine). Paired-pulse ratios were also found to be unchanged. Before nicotine the ratios were 1.52 [(SEM = 0.14); t = -1.25, n = 4) and 1.51 (SEM = 0.08, t = -1.43, n = 3) for trigeminal and auditory stimulation, respectively, and during nicotine 1.67 (SEM = 0.10; p = 0.30) and 1.97 (SEM = 0.32; p = 0.28). Thus, there is no evidence for a presynaptic effect of nicotine.

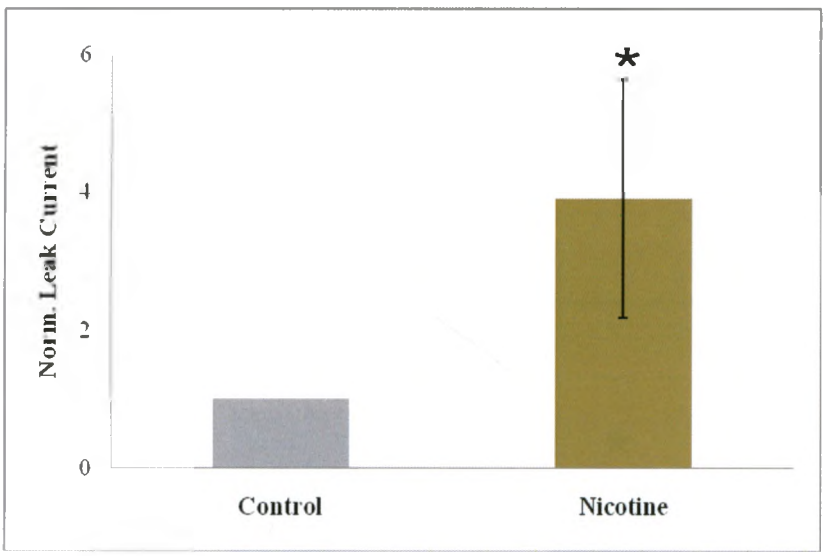


Figure 11: *Nicotine effect on leak current*. Nicotine was perfused onto brain slices after control recordings of ACSF. The addition of nicotine to the bath perfusion caused an increase in leak current (n = 18; p < 0.001).

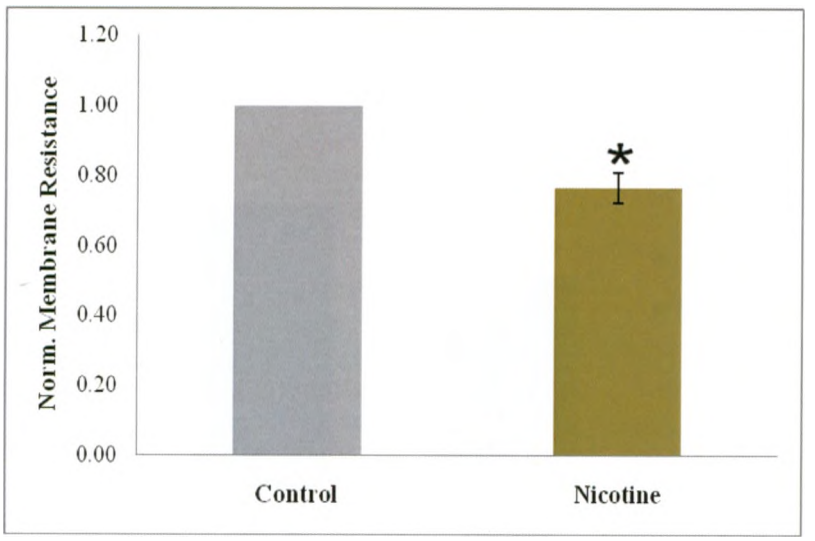


Figure 12: *Nicotine effect on membrane resistance*. Nicotine decreased membrane resistance (n = 18, p = 0.005).

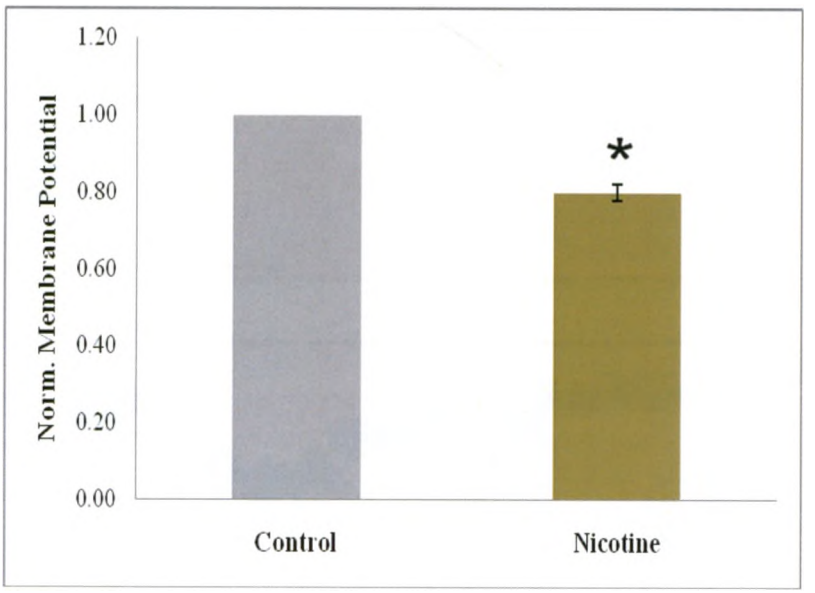


Figure 13: *Nicotine effect on resting membrane potential*. Nicotine depolarized cells' resting membrane potential (n = 18, p < 0.001).

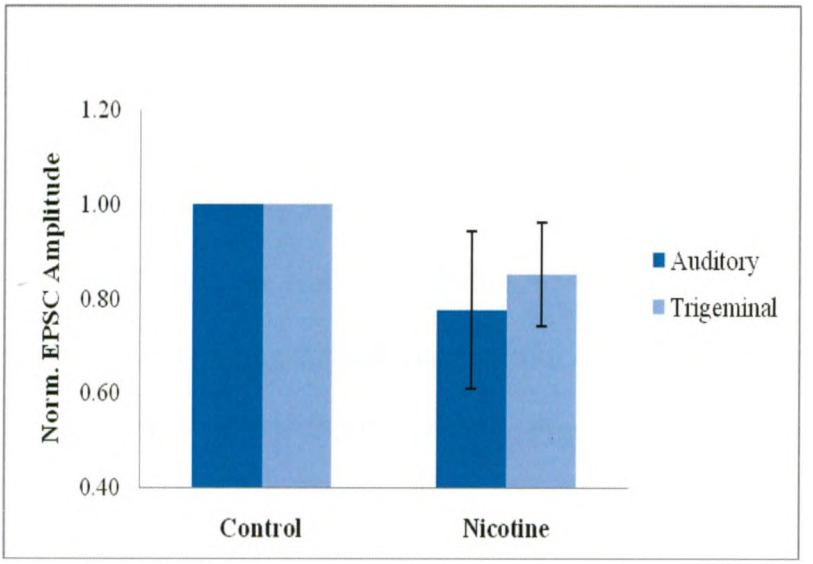


Figure 14: *Nicotine effect on EPSC amplitude*. Auditory (n = 3) and trigeminal (n = 4) afferent fibres were stimulated before and during nicotine perfusion. Nicotine did not have a significant effect on EPSC amplitude (p = 0.29 for both data sets). However, the effect of nicotine on EPSC may have been occluded due to receptor desensitization (see figure 15 as well as discussion for further explanation).

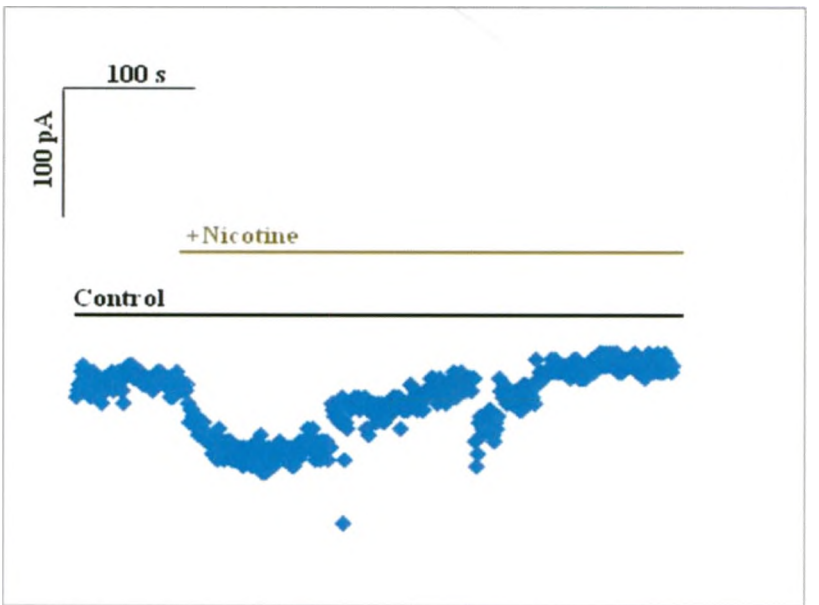


Figure 15: *Effect of prolonged nicotine perfusion on nAChRs*. Bath superfusion of nicotine elicited receptor desensitization within minutes.

4.2 Effects of a high dose of the α7-specific antagonist MLA

The high dose of MLA reversed all nicotine effects

We examined the effect the α_7 antagonist MLA (10 μM) on the nicotine effect. All nicotine effects were replicated and MLA reversed the effect of 10 μM nicotine on the leak current, bringing the leak current from -297 pA to -210 pA (SEM = 54.23 and 46.03 respectively; $F(2,5) = 25.98$, $p = 0.001$; Figure 16 and 17). However, the nicotine effect was not fully reversed since currents under MLA+ nicotine conditions also differed significantly from the control condition [ASCF; -158 pA (SEM = 95.34), $p = 0.02$]. The perfusion of MLA also recovered the membrane resistance of the neurons from -102 MΩ (SEM = 8.83) during nicotine to -111 MΩ (SEM = 10.48; $F(2, 5) = 7.08$, $p = 0.02$; Figure 18). Under control conditions, the membrane resistance was -115 MΩ (SEM = 11.24). The nicotine effect on the resting membrane potential was also reversed by 10 μM MLA from -42 mV (SEM = 2.70) to -47 mV [SEM = 3.27; $F(2, 5) = 40.57$, $p = 0.002$; Figure 19). Again, MLA did not completely reverse the effect of nicotine and thus significantly differed from the control potential which was -52 mV (SEM = 3.06, $p < 0.001$).

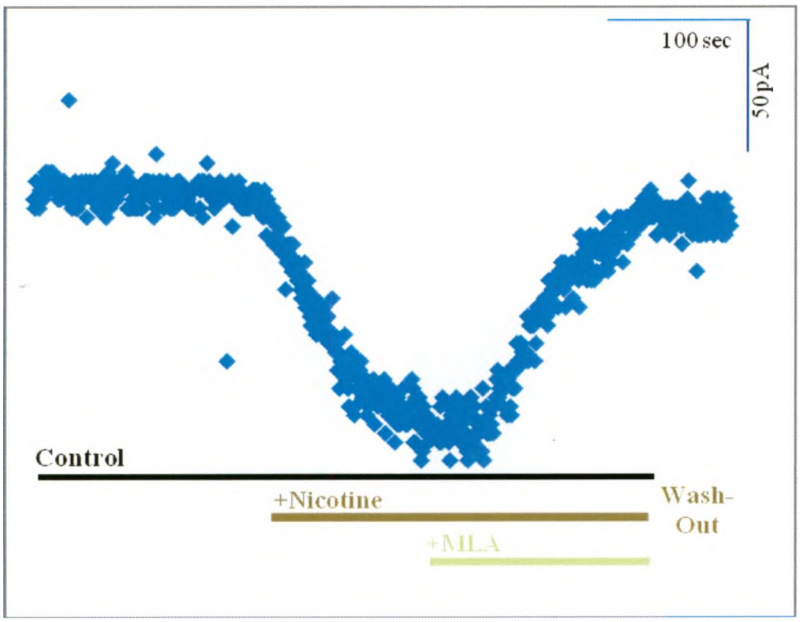


Figure 16: *Exemplary trace of MLA reversal of nicotine effect*. Nicotine (10 µM) was added to the bath perfusion followed by a high dose of MLA (10 µM). Nicotine increased leak current from -158 to -287 pA ($n = 6$; $p < 0.001$). MLA reversed this effect ($p = 0.001$).

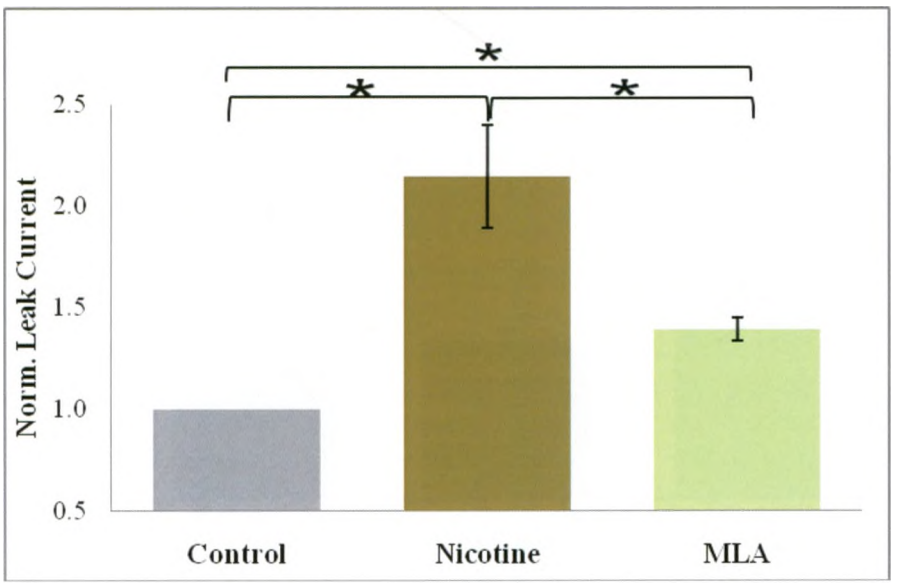


Figure 17: *High dose of MLA effect on leak current*. Nicotine (10 µM) increased leak current ($n = 6$; $p < 0.001$) and MLA (10 µM) reversed this effect ($p = 0.001$). However, MLA also significantly differed from the control ($p = 0.001$) as the drug did not completely reverse the effect of nicotine. Asterisks indicate significant differences.

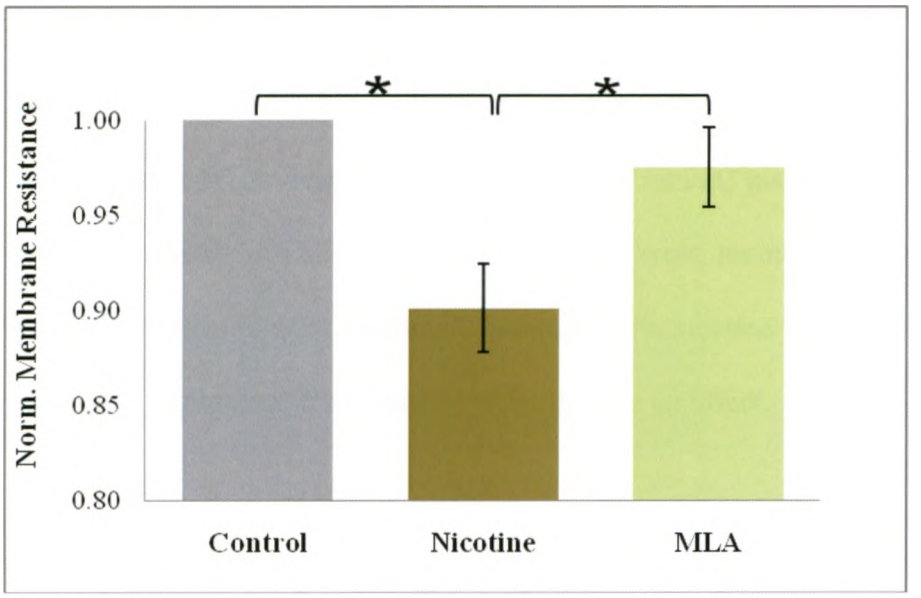


Figure 18: *High dose of MLA effect on membrane resistance.* Nicotine decreased membrane resistance (n = 6, p = 0.005) and MLA (10 μM) reversed this effect ($p < 0.02$).

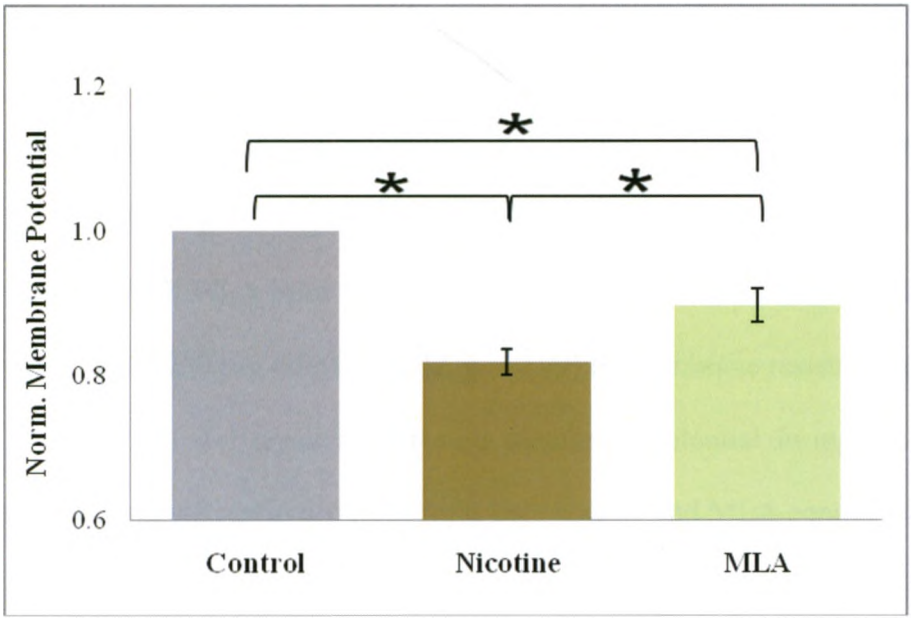


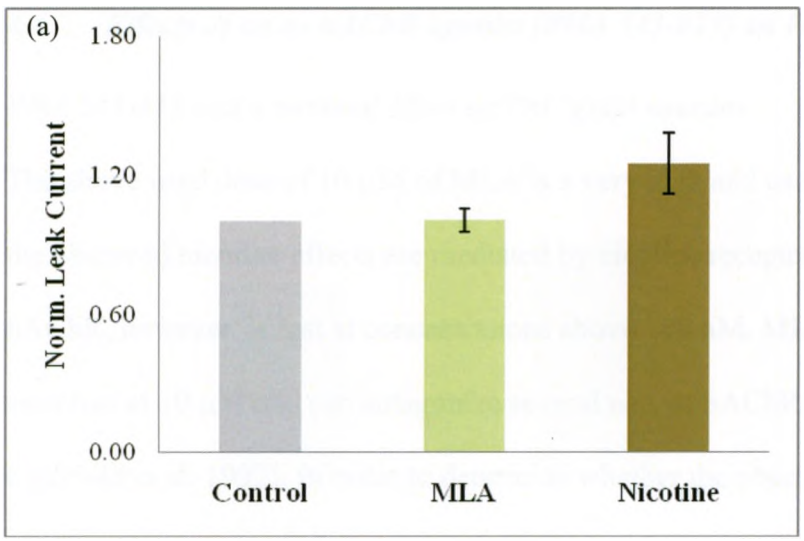
Figure 19: *High dose of MLA effect on resting membrane potential*. Nicotine depolarized cells' membrane potential (n = 6; $p < 0.001$). When MLA was added to the perfusion, the resting membrane potential of the cells was brought close to the control resting potential ($p < 0.001$); but the reversal was not complete since MLA was also significantly different than the control

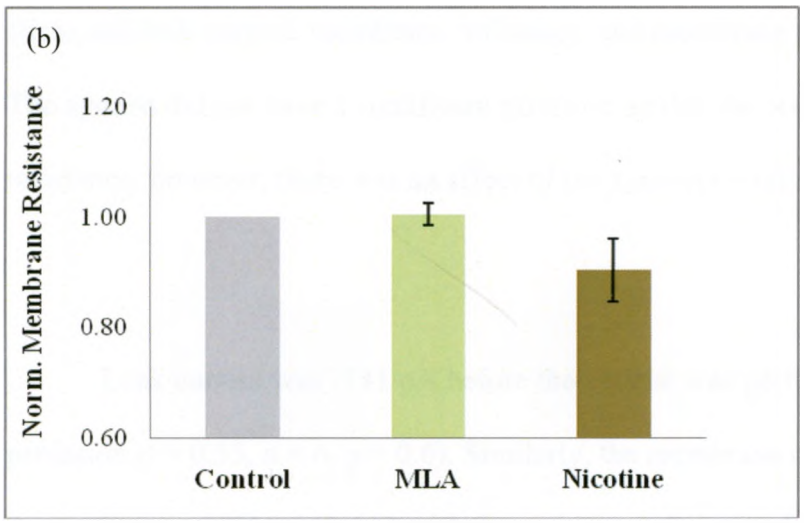
4.3 Effects of MLA perfusion prior to nicotine

Perfusion of 10 μM MLA before nicotine occluded the nicotine effect

To ensure that MLA alone did not have an effect on PnC giant neurons, slices were initially perfused with MLA (10 μM) and leak current, membrane resistance, and resting membrane potential were examined. Subsequently, nicotine was added to the perfusion with MLA to measure if nicotine would still have an effect.

MLA alone had no significant effect on any parameters measured in PnC giant neurons. In fact, when MLA was perfused on its own it did not significantly differ from the control condition when the leak current [control: -199 pA (SEM = 24.05) and MLA: -200 pA (SEM = 25.54); $F(2, 4) = 3.29$, $p = 0.09$], membrane resistance [control: -138 MΩ (SEM = 37.02) and MLA: -139 MΩ (SEM = 39.50); $F(2, 4) = 2.71$, $p = 0.13$], and resting membrane potential [control: -45 (SEM = 2.1) and MLA: -44 mV(SEM = 2.66); $F(2, 4) = 20.31$, $p = 0.13$] were examined (Figures 20a – c). When brain slices were perfused with MLA prior to addition of nicotine, there was no effect on the leak current amplitude (-250 pA, SEM = 42.42, $p = 0.09$) or membrane resistance (-124 MΩ, SEM = 35.39, $p > 0.13$). However, the resting membrane potential during nicotine addition (-41 mV) was significantly different from both control and MLA conditions ($p < 0.001$ and $p = 0.002$ respectively).





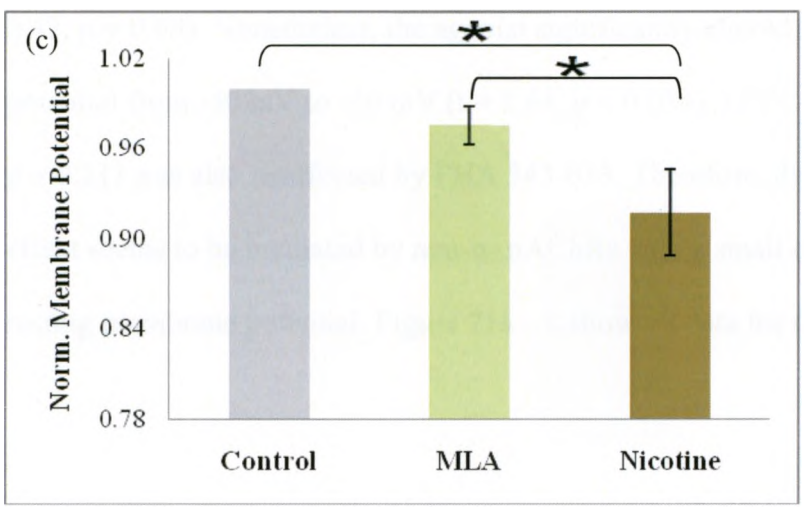


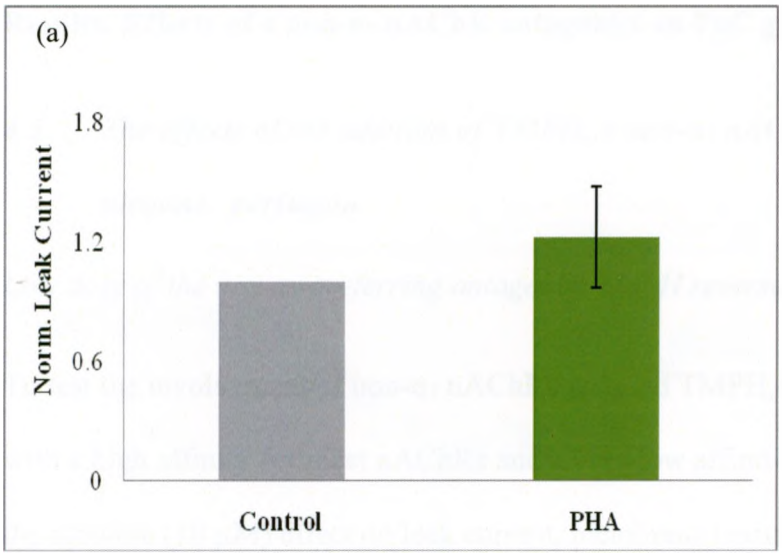
Figure 20a - c: *MLA perfusion before addition of nicotine.* The addition of MLA to the bath perfusion did not have a significant effect on PnC giant neurons. (a – b) Furthermore, in cells (n = 5) first perfused with MLA nicotine had no significant effect on leak current or membrane resistance, $p > 0.09$ and $p > 0.13$, respectively. Nicotine did significantly depolarize the resting membrane potential (n = 5; $p < 0.001$).

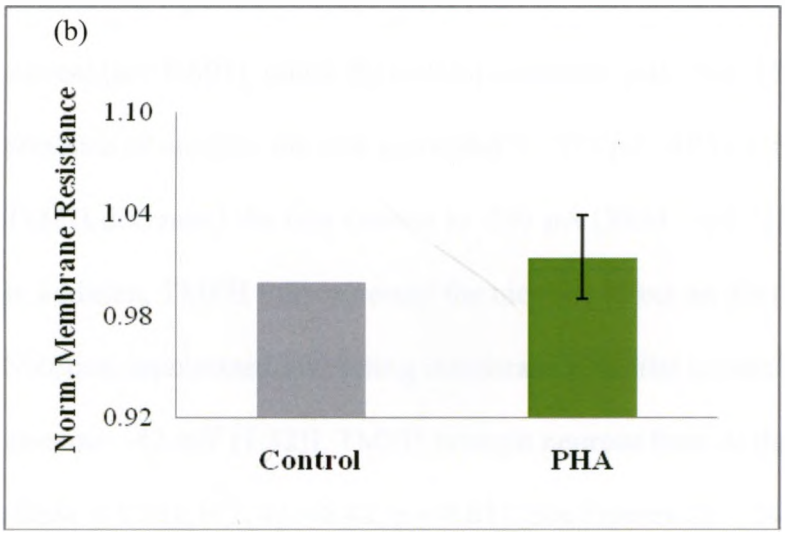
4.4 Effects of an α_7 nAChR agonist (PHA 543-613) on PnC giant neurons

PHA 543-613 had a minimal effect on PnC giant neurons

The above used dose of 10 μM of MLA is a very high and useful for demonstrating that the observed nicotine effects are mediated by nicotine receptors. The specificity for the α_7 nAChR, however, is lost at concentrations above 100 nM. MLA was found to be non-selective at 10 μM and can antagonize several non-α_7 nAChR subtypes (Drasdo, Caulfield et al. 1992). In order to determine whether the observed nicotine effects are mediated by α_7 receptors, the α_7 agonist, PHA 543-613 (30 μM), was perfused onto brain slices and leak current, membrane resistance, and membrane potential were measured. The agonist did not have a significant effect on neither the leak current nor membrane resistance; however, there was an effect of the agonist on resting membrane potential.

Leak current was -141 pA before the agonist was perfused and -139 pA after the perfusion ($t = 0.55$, $n = 6$, $p = 0.6$). Similarly, the membrane resistance was unaffected by the perfusion of PHA 543-613 with the resistance changing from -192 to -189 MΩ ($t = 0.42$, $p = 0.69$). Nonetheless, the agonist significantly altered the resting membrane potential from -50 mV to -46 mV ($t = 2.64$, $p = 0.034$). EPSC amplitude ($t = 1.44$, $n = 6$, $p = 0.21$) was also unaffected by PHA 543-613. Therefore, the majority of the nicotine effect seems to be mediated by non-α_7 nAChRs with a small effect of α_7 nAChRs on the resting membrane potential. Figure 21a – c show all data for the α_7 agonist.





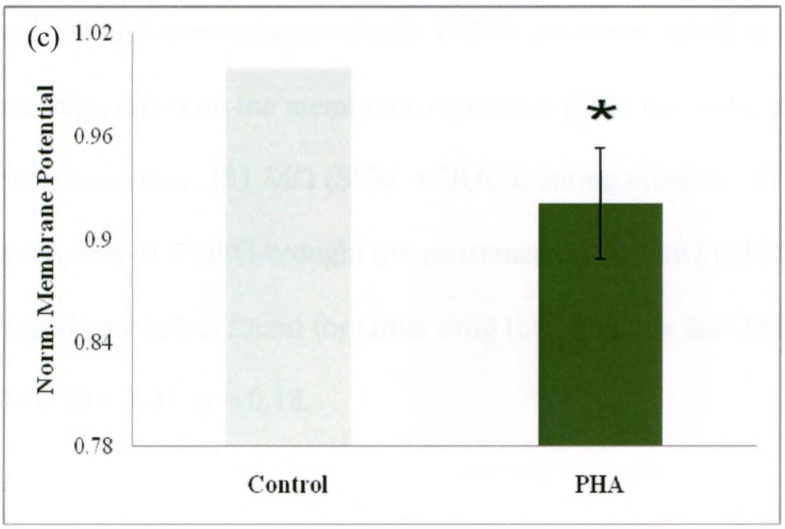


Figure 21a - c: *Effect of the α_7 agonist, PHA 543-613 on PnC giant neurons*. (a – b) Perfusion of PHA 543-613 (30 μM) did not significantly affect cells' (n = 6) leak current (p = 0.60.) or membrane resistance (p = 0.69). (c) PHA 543-613 significantly depolarized the resting membrane potential of PnC giant neurons (p < 0.03).

Results: Effects of a non-α_7 nAChR antagonist on PnC giant neurons

4.5 The effects of the addition of TMPH, a non-α_7 nAChR antagonist, to the nicotine perfusion

Low dose of the non α_7-preferring antagonist TMPH reversed the nicotine effect

To test the involvement of non-α_7 nAChRs we used TMPH, a non-competitive antagonist with a high affinity for most nAChRs and a very low affinity for α_7. We first confirmed the nicotine (10 μM) effect on leak current, membrane resistance and resting membrane potential as reported above. TMPH (100 nM) reversed the effect of nicotine on leak current ($p = 0.007$); under the control condition leak was -173 pA (SEM = 44.21), in the presence of nicotine the leak increased to -474 pA (SEM = 130.24) and the addition of TMPH decreased the leak current to -201 pA [SEM = 43.72; $F(2, 5) = 8.42$, $p = 0.007$]. In addition, TMPH fully reversed the nicotine effect on the resting membrane potential: Nicotine depolarized the resting membrane potential [control: -48 mV (SEM = 3.51) and nicotine: -42 mV (1.32)], TMPH brought neurons back to their initial potential [-50 mV (SEM = 1.72); $F(2, 4) = 6.42$, $p = 0.01$]. See Figures 22 – 24 for drug effects on leak current and membrane potential. TMPH, however, failed to significantly reverse the nicotine effect on the membrane resistance [$F(2, 4) = 5.98$, $p = 0.06$]; during the control resistance was -131 MΩ (SEM = 20.62), during nicotine -85 MΩ (SEM = 17.61) and perfusion of TMPH brought the resistance to -115 mΩ (SEM = 25.07). There was not a significant effect found for either drug (i.e., nicotine and TMPH) on EPSC amplitude, $F(2, 3) = 2.35$, $p = 0.18$.

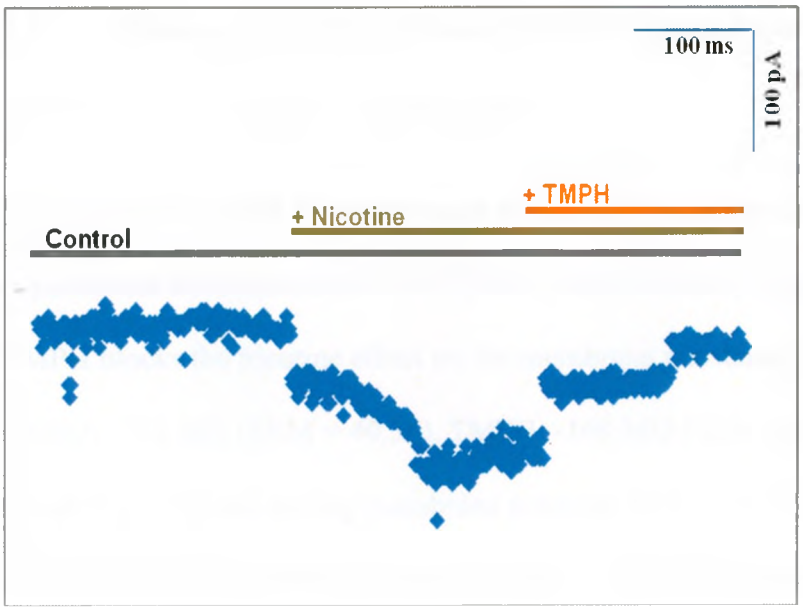


Figure 22: *Effect of TMPH on nicotine- induced increase in leak current*. A sample leak current trace illustrating that nicotine (10 μM) increased leak current in PnC giant neurons and TMPH (a non-α_7 nAChR antagonist) reversed this nicotine effect. Breaks within the graph indicate that other protocols were measured prior to drug perfusion.

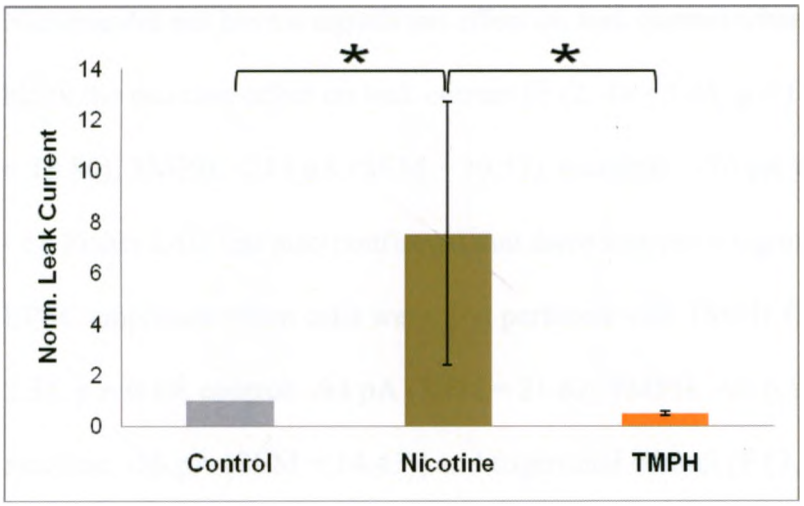


Figure 23: *Effect of TMPH on nicotine-induced increase in leak current*. Nicotine significantly increased leak current, which was reversed by TMPH (100 nM) to control levels ($n = 6$; $p < 0.007$).

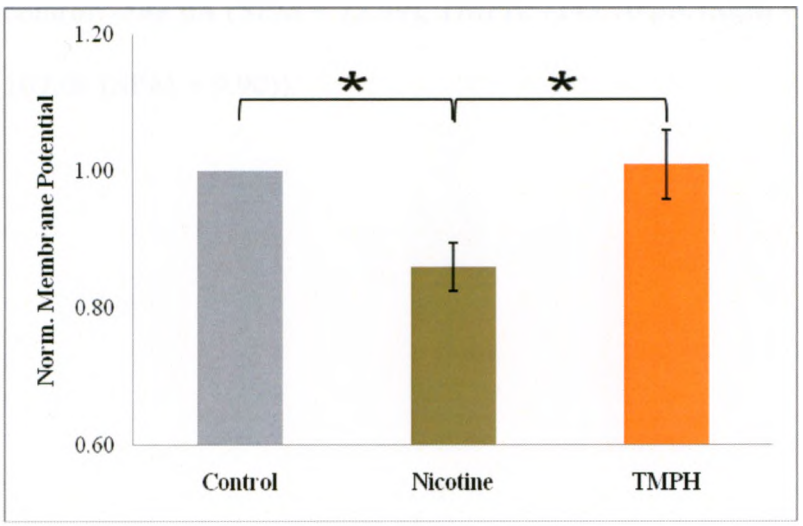
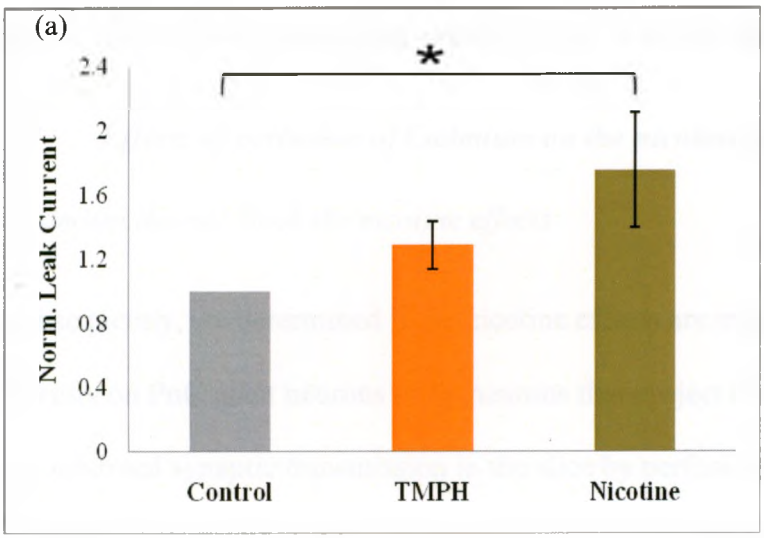


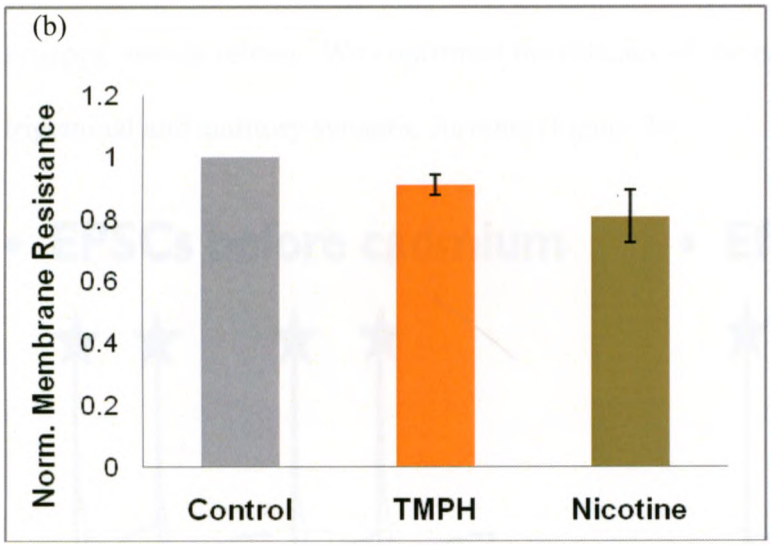
Figure 24: *TMPH effect on nicotine-induced membrane potential depolarization*. TMPH reversed the nicotine effect on neurons' membrane potential, bringing cells back to a more negative potential ($n = 5$; $p = 0.01$).

4.6 Effects of perfusing cells with TMPH followed by perfusion of nicotine

TMPH blocked most of the nicotine effects

To ensure that TMPH did not have an effect on the measured parameter, control experiments were carried out with TMPH perfused before nicotine. Pre-application of TMPH blocks the nicotine effect on the membrane resistance [F (2, 4) = 2.41, p = 0.15; control: -179 MΩ (SEM = 40.34), TMPH: -166 MΩ (SEM = 41.56); Nicotine: -142 MΩ (SEM = 31.77)] and resting membrane potential [F(2, 3) = 3.19, p = 0.11; control: -48 mV (SEM = 7.97), TMPH: -45 mV (SEM = 2.74) and nicotine: -42 mV (SEM = 1.18)]. Nicotine did not have a significant effect on leak current when compared to does not fully block the nicotine effect on leak current [F (2, 4) = 5.05, p = 0.01; control: -161 pA (SEM = 18.86), TMPH: -213 pA (SEM = 39.57), nicotine: -276 pA (SEM = 51.12); Figures 25a - c]. Fisher LSD test also confirmed that there was not a significant effect of nicotine on EPSC amplitude when cells were first perfused with TMPH for both auditory [F(2,3) = 2.31, p = 0.19; control: -94 pA (SEM = 21.62, TMPH: -94 pA (SEM = 10.19) and nicotine: -56 pA (SEM = 14.43)] and trigeminal stimuli [F (2, 3) = 3.19, p = 0.11; control: -148 pA (SEM = 25.99), TMPH: -143.70 pA (SEM = 10.64) and nicotine - 107.08 (SEM = 9.90)].





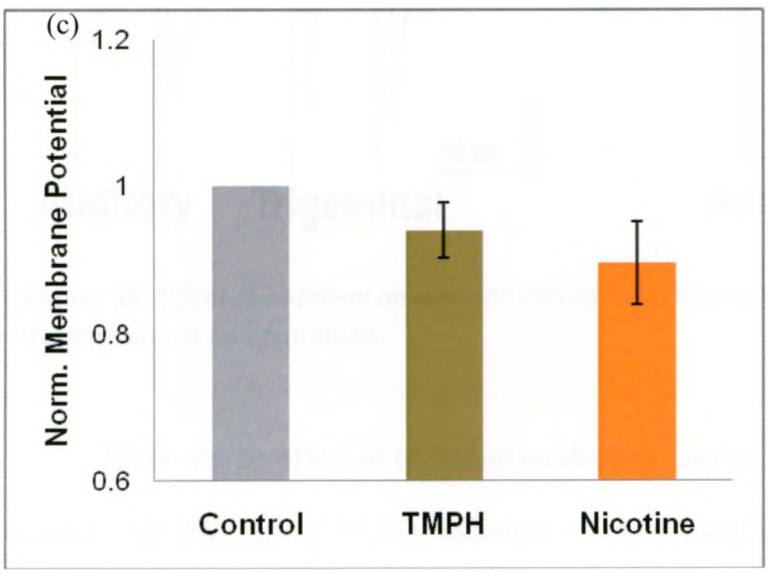


Figure 25a - c: *Pre-application of TMPH on the nicotine effect on PnC giant neurons*. (a) Nicotine did not have a significant effect on leak when compared to TMPH (n = 5; p = 0.012), but nicotine was different than the control (p = 0.01). (b – c) Perfusion of TMPH prior to nicotine blocks the nicotine effect on the membrane resistance (n = 5; p = 0.15) and resting membrane potential (n = 4; p = 0.11).

Results: Effects of blocking synaptic transmission on the nicotine effect

4.7 Effects of perfusion of Cadmium on the nicotine effect

Cadmium did not block the nicotine effects

Subsequently, we determined if the nicotine effects are mediated by nAChRs expressed directly on PnC giant neurons or by neurons that project onto PnC giant neurons. Thus, we inhibited synaptic transmission in the slice by perfusion of 100 μM cadmium chloride. Cadmium ions block voltage-gated calcium channels that are required for synaptic vesicle release. We confirmed the efficacy of the cadmium block by monitoring trigeminal and auditory synaptic currents (Figure 26).

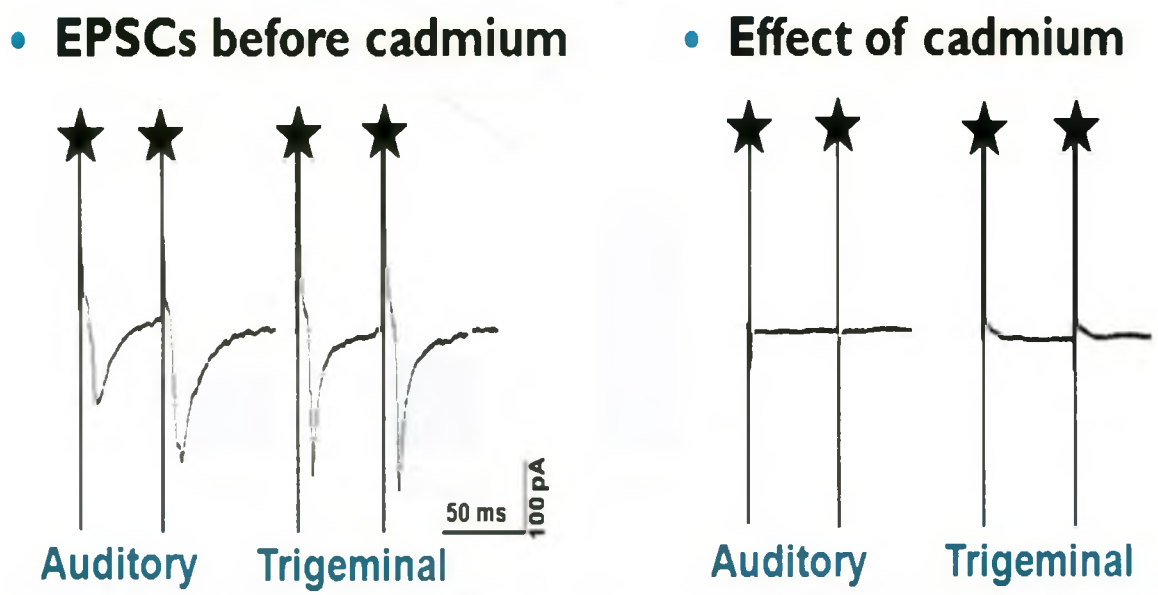


Figure 26: *Effect of cadmium on synaptic currents*. Cadmium completely blocked synaptic transmission in rat brain slices.

There was no effect of cadmium on the leak current [F (2, 4) = 19.72, p = 0.45; control: -128 pA (SEM = 31.21), cadmium = -145 pA (SEM = 35.16)] or on the

membrane resistance [F(2, 4) = 4.94, p = 0.75; control: -157 MΩ (SEM = 20.56), cadmium: -149 MΩ (SEM = 9.65)] or the resting membrane potential [F(2,5) = 17.25, p = 0.14; control: -48 mV (SEM = -2.70) and cadmium: -43 mV (SEM = 4.79)]. After blocking synaptic transmission with cadmium, nicotine still had an effect on PnC giant neurons for all measured parameters: leak current [-250 pA (SEM = 35.80), p = 0.001], membrane resistance [-126 MΩ (SEM = 10.47), p = 0.03], and resting membrane potential [-35 mV (SEM = 4.28), p = 0.005]. Thus, synaptic transmission is not necessary for the nicotine effects reported here, suggesting that nAChRs are expressed by PnC giant neurons. Figures 27 – 29 show the cadmium plus nicotine effect on cells' leak current, membrane resistance and resting membrane potential.

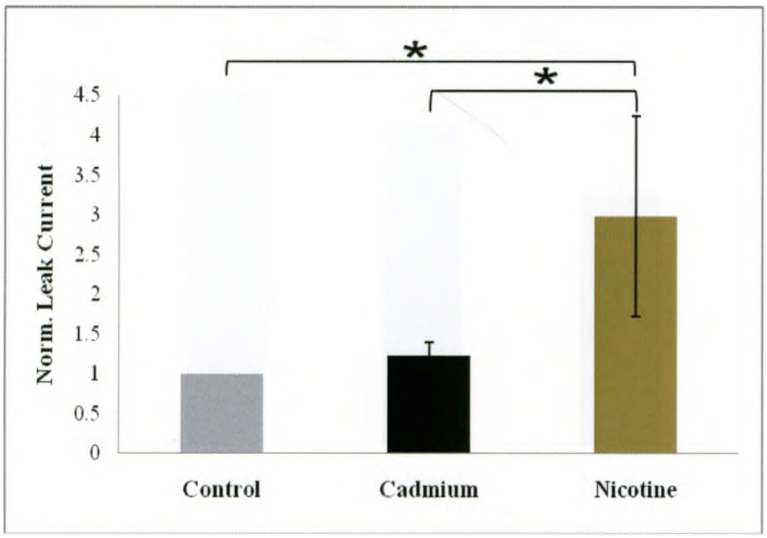


Figure 27: *Effect of nicotine on leak current in the presence of the calcium channel blocker cadmium.* Cadmium was perfused onto brain slices to block synaptic transmission. Subsequently, nicotine was added to the perfusion and the data indicated a significant increase in leak current (n = 5; p = 0.001). Therefore, synaptic transmission is not necessary for the nicotine effect on startle-mediating PnC neurons. Nicotine also significantly differed from the control condition, $p < 0.001$.

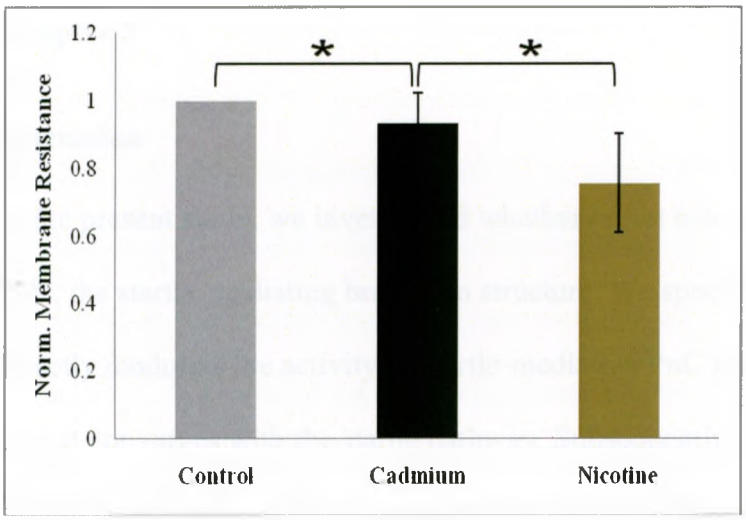


Figure 28: *Effect of nicotine on membrane resistance after blocking synaptic transmission with cadmium*. Nicotine significantly decreased cells' membrane resistance even after blocking synaptic transmission (n = 5; p = 0.03).

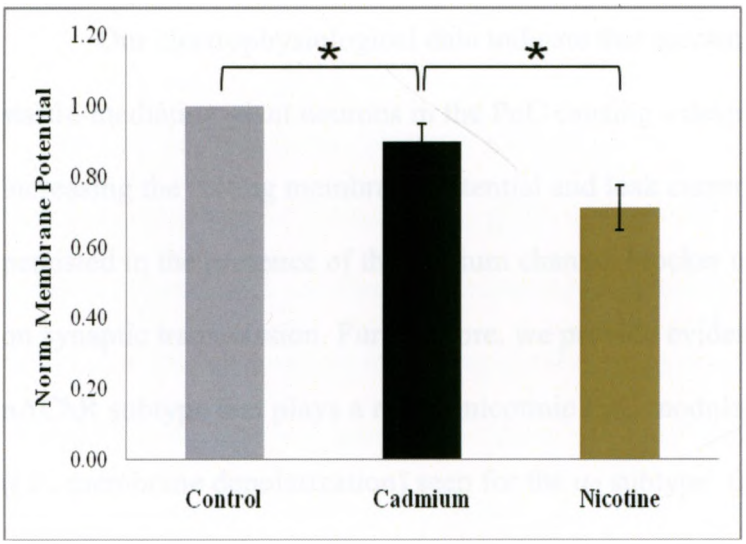


Figure 29: *Effect of nicotine on resting membrane potential in the presence of cadmium*. In spite of the perfusion of cadmium, nicotine still depolarized PnC giant neurons (n = 6; p = 0.005).

Chapter 5

Discussion

In the present study, we investigated whether or not nicotine has a direct effect in the PnC, the startle-mediating brainstem structure. We specifically assessed if nicotine could directly modulate the activity of startle-mediating PnC giant neurons where the PPI circuit converges with the startle pathway. Subsequently, we tried to identify the receptor subtype involved as well as examine if the startle-mediating neurons expressed nAChRs or if synaptic transmission was necessary for nicotine to elicit its effect.

Our electrophysiological data indicate that nicotine has a direct effect on the startle-mediating giant neurons in the PnC causing a drop in membrane resistance, increasing the resting membrane potential and leak current. The effect of nicotine persisted in the presence of the calcium channel blocker cadmium and thus does not rely on synaptic transmission. Furthermore, we provide evidence that it is mainly the non-α_7 nAChR subtype that plays a role in nicotinic PnC modulation, with only minimal effects (i.e., membrane depolarization) seen for the α_7 subtype. Our results suggest both excitatory and inhibitory effects of nicotine. Here, we will first discuss the excitatory effect of nicotine caused by membrane depolarization in relation to the PPI literature. Subsequently, we demonstrate that the drop in cells' membrane resistance and the occurrence of receptor desensitization elicit inhibitory effects of nicotine. These findings will also be examined in light of the extant body of literature on PPI.

The mixed excitatory and inhibitory effects of nicotine found here have been previously reported for other brain areas. Similar to our findings, it was revealed that nicotine had a direct effect on neurons in the medial pontine reticular formation (mPRF), an important region responsible for REM (rapid eye movement) sleep. In fact, nicotine decreased membrane resistance and increased leak current depolarizing cells even in the presence of TTX (tetrodotoxin; a sodium channel blocker; Stevens, Birnstiel et al. 1993). Curro Dossi and colleagues similarly demonstrated that nicotine-induced depolarizations within the mesopontine (peribrachial and laterodorsal tegmental (LDT)) nuclei also elicited decreased membrane resistance (Curro Dossi, Pare et al. 1991). In this way, our data lends further support for these mixed excitatory and inhibitory nicotine effects.

Our findings of an excitatory effect of nicotine causing depolarization of the membrane potential is not surprising given that nAChRs are cation channels. However, in order to enhance PPI, nicotine was expected to inhibit PnC giant neurons. The excitatory effect of nicotine on startle-mediating giant neurons would lead to an increase in baseline startle while counteracting an enhancement of PPI of startle unlike hypothesized. There is some variability in the reported effects of nicotine on the baseline startle amplitude or on PPI with several studies demonstrating enhancing effects of nicotine while others find disruptions. Systemic nicotine administrations, particularly at low (nanomolar) concentrations, can increase ASR amplitude (Acri, Grunberg et al. 1991; Acri, Morse et al. 1994; Faraday, O'Donoghue et al. 1999). Furthermore, systemic administration of nicotine can further enhance stress-induced increase in the startle amplitude (Acri 1994; Faraday, O'Donoghue et al. 1999).

Nicotine has been reported to have a dual effect on PPI. For instance, consumption of cigarettes with a high nicotine content has been shown to decrease PPI due to exciting mesolimbic dopaminergic neurons that tend to disrupt activation of the PPI pathway (Hutchison, Niaura et al. 2000). Conversely, there is significant evidence for a nicotine-enhancing effect on PPI. The positive effects of nicotine can be found in its ability to ameliorate sensorimotor gating in healthy individuals, clinical populations and in animal models of PPI deficits (Acri, Grunberg et al. 1991; Curzon, Kim et al. 1994; Kumari, Cotter et al. 1997; Swerdlow, Light et al. 2006; Hong, Wonodi et al. 2008).

In order to reconcile these diverse findings, pharmacological data have revealed that low and high doses of nicotine act on different receptors that may have opposing roles. For instance, nicotine tends to act on $\alpha_4\beta_2$ nAChRs at low doses whereas at higher concentrations it can activate the low affinity α_7 nAChRs involved in sensorimotor gating; thus enhancing PPI of startle (see Schreiber, Dalmus et al. 2002). Evidently, there is significant evidence to illustrate that nicotine can activate different receptor subtypes and differentially modulate PPI depending on the dosage.

Our data suggest that both α_7 and non-α_7 nAChRs might be involved in mediating the direct effect of nicotine on giant neurons of the PnC. We found a significant effect of the α_7 nAChR specific antagonist MLA on reversing the nicotine effect on leak current, membrane resistance and resting membrane potential. However, the 10 μM concentration of MLA applied to the bath-perfusion was very high. At concentrations above 100 nM

MLA was found to be non-selective and can antagonize several non-α_7 nAChR subtypes (Drasdo, Caulfield et al. 1992; Mogg, Whiteaker et al. 2002). Therefore, our MLA data show that the nicotine effect is mediated by nAChRs, but cannot give further information about the subtype involved due to the high dosage used.

In order to assess the receptor-subtype mediating the nicotine effect we perfused the α_7-specific agonist PHA 543-613 and the non-α_7 preferring antagonist TMPH. It is worth noting that the EC_{50} for PHA 543-613 is 65 nM for α_7 nAChRs with 30 μM gives maximal synaptic currents during electrophysiological recordings from rat Hippocampal neurons (Wishka, Walker et al. 2006); our concentration of 30 μM is within the dosage that has been shown to elicit a profound effect only on α_7 nAChRs. Furthermore, the 100 nM concentration used for TMPH is highly specific for non- α_7 nAChRs only (Papke, Buhr et al. 2005). The α_7 agonist affected the membrane potential only, while most of the other nicotine effects seem to involve non-α_7 nAChRs since they were reversed by TMPH. Although nicotine receptor inactivated during the perfusion of nicotine, there was still an observed additional effect of TMPH in blocking the nicotine effect. Most importantly, the perfusion of TMPH before nicotine blocked the nicotine effect demonstrating that non-α_7 nAChRs mediate the main portion of the nicotine effect on PnC giant neurons. There are different non-α_7 nAChRs expressed in the brain, thus future experiments need to further elucidate which non-α_7 nAChR subtypes are involved. It is possible that the $\alpha_4\beta_2$ nAChRs which are also involved in cognitive processing, may be implicated (Changeux 2010).

Apart from the acute effects of nicotine on the excitability of PnC neurons, tonic application of nicotine leads to receptor desensitization. In this study nicotine was perfused with the bath solution. Leak current and membrane resistance were continuously monitored so that the effect of nicotine perfusion could be measured from the onset of the nicotine effect. Recordings for examining the effect of nicotine on EPSC amplitude were commenced five minutes after the onset of nicotine perfusion instead of immediately after the onset of the observed effect of nicotine on leak current. The problem with this design of continued nicotine bath perfusion is that nAChRs desensitize rapidly which may have masked the main effect of nicotine on EPSC amplitude (Katz and Thesleff 1957; Pidoplichko, DeBiasi et al. 1997; Frazier, Rollins et al. 1998; Quick and Lester 2002; Wooltorton, Pidoplichko et al. 2003; Wang and Sun 2005). In fact, we initially found that nicotine significantly reduced EPSC amplitude in a few cells where recordings of synaptic currents began immediately after detection of the nicotine effect on leak current (data not shown). All nicotine effects reported here, especially the effect on EPSC amplitudes, might therefore underestimate the full (transient) nicotine effect on PnC neurons given the experimental design. In any event, tonic nicotine acts more or less as a nicotine receptor antagonist given that it keeps the receptors in a desensitized state. It may be that the nicotine effect through smoking lies in this antagonistic nicotine effect rather than in the acute effect of nicotine that largely desensitizes within milliseconds/seconds.

Nonetheless, it may be that while nicotine depolarizes PnC giant neurons it also decreases neuronal response to synaptic input from the trigeminal and auditory pathways

due to the drop in input resistance of the neurons. Indeed bath-perfusion of nicotine has been shown to decrease EPSP (excitatory postsynaptic potential) amplitude in another study, although the mechanism of action seemed to require postsynaptic NMDA receptors (Levy, Reyes et al. 2006). The input resistance of PnC giant neurons was considerably decreased by nicotine in our study, but the EPSC amplitude data reported here was not significantly reduced during nicotine perfusion, although there was a general trend towards reduced amplitudes. The low input resistance may represent an inhibitory effect whereby stronger stimulations would be required to elicit an action potential as compared to the control condition. This effect of nicotine suggests that nicotine can potentially enhance PPI of startle by making cells less responsive to sensory stimuli (i.e., auditory and trigeminal stimulations). The fact that EPSC amplitudes were not significantly reduced might be attributed to the above mentioned design of the experiments.

We hypothesized that nicotine may have a direct effect on PnC giant neurons or may necessitate inhibitory interneurons to elicit its effect. Thus far, the data suggest a direct effect, but we cannot rule out that inhibitory interneurons may be involved. Although the blockage of synaptic transmission did not occlude the nicotine effect but instead provided strong evidence of a postsynaptic effect, presynaptic α_7 nAChRs can increase transmitter release due to increase in intracellular Ca^{2+} (Gray, Rajan et al. 1996; Griguoli, Scuri et al. 2009). High Ca^{2+} permeability is an inherent trait of α_7 nAChRs and may result in inhibitory transmitters being released if they are located on presynaptic interneurons. Nonetheless, it is safe to conclude that the nicotine effect does not require

inhibitory interneurons since the data only showed minimal effect of the α_7 agonist and specifically no reduction in EPSC amplitude (data not reported). It may be that bath perfusion of nicotine can cause rapid desensitization of the α_7 nAChRs and thus not allow for appropriate detection of α_7 nAChRs (Sargent 1993; Frazier, Rollins et al. 1998). In order to detect a possible effect of α_7, future studies would need to include in their methodological design electrophysiological experiments that apply a brief puff of nicotine to brain slices rather than bath-superfusion, although this is a very challenging technique.

Our data suggests a possible role of nicotine not only in exciting PnC giant neurons, but also in inhibiting neuronal activity potentially facilitating PPI disruption or enhancement, respectively. We have demonstrated that nicotine does not seem to require synaptic transmission in order to elicit its effects on PnC giant neuron excitability. Furthermore, both α_7 and the non- α_7 nAChRs seem to mediate the nicotine effect on startle-mediating giant neurons in the PnC; although non-α_7 receptor subtypes seem to have a more substantial effect. Behavioural pharmacological experiments are required whereby nicotine and specific nAChRs antagonists are infused locally into the PnC to assess to what extent their activation affect prepulse inhibition of startle.

There has been significant research aiming to further understand the neural mechanisms underlying sensory gating. Gaining insight into the neural circuitry of sensory gating could aid in ameliorating cognitive dysfunctions among afflicted clinical

populations. Evidence that cognitive disruptions measured as PPI deficits can be significantly improved following nicotine consumption has led to the self-medication hypothesis and fuelled substantial interest in brain areas targeted by nicotine (Hughes, Hatsukami et al. 1986; Fendt, Li et al. 2001; Kumari, Soni et al. 2001; Ucok, Polat et al. 2004; Kumari 2005; George, Termine et al. 2006; Swerdlow, Light et al. 2006; Hong, Wonodi et al. 2008). To date, the limbic cortex, ventral pallidum, VTA and substantia nigra are among the regions proposed to be involved in nicotine modulation of PPI of startle (see Fendt, Li et al. 2001 for a review). Our results demonstrate that nAChRs may in fact be implicated in sensorimotor gating also directly within the startle-circuit by regulating PnC giant neuron activity.

Chapter 6

Summary and Conclusion

The result of our electrophysiological experiments provides important information about the potential of nicotine in modulating the activity of startle-mediating giant neurons within the PnC. These experiments were particularly designed to examine whether or not nicotine could affect PnC giant neurons directly as well as characterize the receptor subtype mediating any nicotine effects found. To our knowledge, the data reported here is the first systematic electrophysiological study conducted that suggests that nicotine does in fact have a direct effect on startle-mediating PnC giant neurons. We demonstrated a significant effect of nicotine on leak current, membrane resistance and resting membrane potential that persisted when synaptic transmission was blocked. More specifically, nicotine increased leak current, decreased membrane resistance and depolarized cells' resting membrane potential. Furthermore, our data also suggests a minimal role of the α_7 nAChR in depolarizing the membrane potential and a more significant involvement of non- α_7 nAChR in mediating the reported nicotine effect on PnC giant neurons.

When nicotinic receptors are activated there is an initial inhibitory effect causing decreased input resistance while the neurons are simultaneously depolarized. Subsequently, the nicotine effect inactivates leaving the nicotine receptors desensitized. Prolonged nicotine perfusion has been previously shown to first activate and subsequently inactivate nAChRs by desensitization (Katz and Thesleff 1957; Pidoplichko, DeBiasi et al. 1997; Frazier, Rollins et al. 1998; Quick and Lester 2002; Wooltorton, Pidoplichko et al. 2003; Wang and Sun 2005). Once nAChRs are

desensitized nicotine can no longer elicit its inhibitory effects unless the concentration is substantially increased by 20 percent (see Wang and Sun 2005 for review). The PnC neuron depolarization by activation of nicotine receptors by the PPI pathway leads to a higher excitability of PnC neurons and would increase startle, while the drop in input resistance would counteract this. Future experiments will need to address whether or not nicotine has a net inhibitory or excitatory effect on PnC giant neurons as well as its mechanism of action.

Nonetheless, our data does suggest some involvement of nicotinic receptors in PnC inhibition. However, nAChRs have a fast onset of activation and desensitization which cannot explain the lasting inhibition of PnC giant neurons to fully mediate PPI found at several interstimulus intervals. Previously, metabotropic muscarinic receptors have been implicated in PPT cholinergic inhibition of PnC giant neurons (Bosch and Schmid 2006; Bosch and Schmid 2008). Given that muscarinic receptors are activated slowly and have a long lasting effect, it may be that nAChRs on PnC giant neurons activate first mediating the initial suppression of PnC giant neuron activity (Jones and Shannon 2000; Jones and Shannon 2000; Yeomans, Bosch et al. 2010). Subsequently, mAChRs activate soon after and maintain the inhibition. Our electrophysiological data clearly suggests the functional expression of nAChRs in the PnC that may play some role in PnC inhibition. However, these findings necessitate subsequent experiments to corroborate the behavioural role of nAChRs in the PnC. Collectively these findings would be very beneficial in not only advancing our current understanding of basic

cognitive processing such as sensorimotor gating, but could also benefit certain clinical populations.

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Appendices

Ethics Approval

Part 2 - closure

Western

January 24, 2008

This is the Original Approval for this protocol
A Full Protocol submission will be required in 2012

Dear Dr. Schmid:

Your Animal Use Protocol form entitled:
Cellular and Molecular Mechanisms Underlying Behaviour and Learning in Mammals
Funding Agency NSERC

has been approved by the University Council on Animal Care. This approval is valid from **January 24, 2008 to January 31, 2009.** The protocol number for this project is **2008-006-01**

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

ANIMALS APPROVED FOR 1 YR.

Species	Strain	Other Detail	Pain Level	Animal # Total for 1 Year
Rat	Sprague Dawley	9-12 days M/F	B	300
Rat	Sprague Dawley	adult Female	B	36
Rat	Sprague Dawley	adult Male	B	36

STANDARD OPERATING PROCEDURES
Procedures in this protocol should be carried out according to the following SOPs. Please contact the Animal Use Subcommittee office (661-2111 ext. 86770) in case of difficulties or if you require copies.
SOP's are also available at http://www.uwo.ca/animal/acvs
310 Holding Period Post-Admission
320 Euthanasia
321 Criteria for Early Euthanasia/Rodents

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

c.c. Approved Protoc :rwerf
Approval Letter

Jan 25/08

The University *of* Western Ontario
Animal Use Subcommittee/University Council on Animal Care
Health Sciences Centre, • London, Ontario • CANADA - N6A 5C1
PH: 519-661-2111 ext. 86770 • F: 519-661-2028 • www.uwo.ca/animal

Western

02.01.10
*This is the 2nd Renewal of this protocol
*A Full Protocol submission will be required in 2012

Dear Dr. **Schmid**

Your Animal Use Protocol form entitled:

Cellular and molecular mechanisms underlying behaviour and learning in mammals

has had its yearly renewal approved by the Animal Use Subcommittee.

This approval is valid from **02.01.10 to 01.31.11**

The protocol number for this project remains as **2008-006**

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.
The holder of this *Animal Use Protocol* is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

c.c. W. Lagerwerf

The University *of* Western Ontario
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre. • London, Ontario • CANADA – N6A 5C1
PH: 519-661-2111 ext. 86770 • FL 519-661-2028 • www.uwo.ca / animal