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Nicotinic mechanisms mediating prepulse inhibition of the acoustic startle response (Spine title: Nicotinic mechanisms mediating PPI of the startle response)

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Nicotinic mechanisms mediating prepulse inhibition of the acoustic startle response

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ABSTRACT AND KEY WORDS

Prepulse inhibition (PPI) is the attenuation of a startle response brought-on by a non-startling sensory stimulus (prepulse) presented 5-1000ms before the startle-evoking stimulus. It is a measure of sensory gating that is seen disrupted in schizophrenia, and other mental disorders. Nicotinic acetylcholine receptors (nAChRs) have been implicated in PPI of acoustic startle at both a systemic level and at the level in which the primary startle pathway can receive modulatory input - the caudal pontine reticular nucleus (PnC). This research will help clarify the role that nicotine plays in PPI at both a systemic level and at a level of the PnC. We show that the systemic effect of nicotine is at least partly mediated by non-PnC α7 nAChRs, and that the effect of nicotine in the PnC is mainly mediated by non-α7 nAChRs (likely α4β2 nAChRs). This research helps clarify the role that nicotine plays in sensorimotor gating, and may help in drug development in schizophrenia.

Keywords: prepulse inhibition, PPI, nicotine, nAChRs, sensorimotor gating, acoustic startle response, PnC, schizophrenia.

CO-AUTHORSHIP

All experiments were performed by Tyler Brown with assistance in the following:

My supervisor, Dr. Susanne Schmid assisted in experimental design, data interpretation, thesis planning and editing.

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ABBREVIATIONS

5-HT	- Serotonin
ASR	- Acoustic startle response
BSA	- Basolateral amygdala
CN	- Cochlear nucleus
CS	- conditioned stimulus
DA	- Dopamine
IC	- Inferior colliculus
IC	- Intra-cranial
IP	- Intra peritoneal
ISI	- Interstimulus interval
nAChR	- nicotinic acetylcholine receptor
NMDA	- N-methyl-D-aspartate
MLA	- methyllycaconitine
PAG	- Periaqueductal gray
PnC	- Caudal pontine reticular nucleus
PPI	- Prepulse inhibition
PPTg	- Pedunculopontine tegmental nucleus
SC	- Superior colliculus
SC	- Sub cutaneous
TMPH	- 2,2,6,6-tetramethylpiperidin-4-yl heptanoate
VLL	- Ventral lateral lemniscus
VTn	- Ventral tegmental nucleus

I. INTRODUCTION

The startle response is a protective reflex elicited by a sudden and intense stimulus. This reflex is generally characterized by a whole-body flinch in mammals, but can exist in many different forms across the animal kingdom (Prosser & Hunter, 1936; Wicks et al., 1996; Baird et al., 1993; Currie & Carlsen, 1985; Zotolli, 1978; Russell, 1974; Mongeluzi, 1998). If a minor stimulus (i.e. one that does not elicit a startle reaction) precedes a startle-evoking stimulus, the startle response can be significantly attenuated – this is termed prepulse inhibition (Ison & Hammond, 1971). Prepulse inhibition (PPI) is considered to be an example of automatic sensory gating, which occurs when information is unconsciously filtered-out from an organism's environment. This frees-up precious processing resources for important stimuli, and reduces the cognitive demand incurred by the organism (Graham, 1975; Fendt et al., 2001; Braff & Light, 2004; Geyer, 2006; Ellenbroek, 2004). Since disruptions in sensory filtering (and in PPI) are seen in numerous neurological disorders, most notably schizophrenia (Braff, 1978), understanding the basic mechanisms that mediate PPI could lead to novel compounds that may help treat these disorders.

Tactile, vestibular, or acoustic stimulation can elicit startle in the rat by activating the startle-mediating giant neurons located in the caudal pontine reticular nucleus (PnC) (Lingenhohl & Friauf, 1994; Wu et al., 1988). These neurons have direct projections to cranial, facial, and skeletal motor neurons that, if activated simultaneously, can cause a whole-body flinch. An acoustic prepulse, which does not cause activation of the startle-mediating neurons in the PnC, will travel in a feed forward inhibitory manner that originates with the cochlear root nucleus (CN), and then activates the inferior colliculus

(IC). The IC will then send excitatory input to the superior colliculus (SC) which will cause activation of the pedunculopntine tegmental nucleus (PPTg). If excited, the PPTg will then send powerful inhibitory signals to startle-mediating PnC neurons (for review see Fendt et al 2001; Koch, 1999; Koch & Schnitzler, 1997; Fendt et al. 1994; Fendt, 1999; Li et al., 1998; Li & Yeomans, 2000; Swerdlow & Geyer, 1993). It has been proposed that the inhibitory PPTg projection is largely cholinergic and that inhibition is mediated mainly by muscarinic receptors (M2 and M4 receptors) with a possible role for nicotine as well (Bosch & Schmid, 2006, 2008; Jones & Shannon, 2000).

Other evidence for the role of nicotinic acetylcholine receptors (nAChRs) in PPI comes from the fact that when systemically administered, nicotine improves PPI in rats and in humans (Acri et al., 1994; Smith et al., 2006). Furthermore, the α7 nAChR has been identified as a susceptibility gene in schizophrenia (Lai et al., 2001, Petrovsky et al, 2010). Also, 90% of people suffering from schizophrenia smoke cigarettes, and upon nicotine consumption their PPI and overall cognitive performance improves (Kumari, 1997; Smith et al., 2004). Because of these facts, there has been much research into schizophrenia regarding novel pharmaceutical compounds that specifically target the α7 nAChR, and some have had promising results in phase 2 clinical trials (Kumari et al., 2001; Olincy & Stevens, 2007; Freedman, 2008; for review see Leiser, 2009). Although the α7 nAChR has been strongly implicated in both schizophrenia and PPI, the α4β2 nAChR may play a role as well (Scheiber, 2002; Mizoguchi, 2009). Thus, both major neuronal nAChRs - the α7 and the α4β2 - may be involved in PPI. However, the neural correlates in which these PPI-mediating receptors may reside are yet-to-be elucidated.

Since Bosch and Schmid (2008) have indirectly shown that a role exists for nAChRs in PnC giant neurons and it is known that the PnC contains α4β2 and α7 (Allen

Brain Atlas), the aim of this work is to investigate the role that these two major neuronal nAChRs play in PPI. Therefore we hypothesized that the α7 and/or the α4β2 nicotinic acetylcholine receptors in the PnC are responsible for mediating PPI of acoustic startle in the rat, and that the systemic effect of nicotine is acting through the PnC as well. Briefly, to address this hypothesis, we tested PPI under conditions of systemic of intra-PnC injection of nicotine and two nicotinic antagonists that target α7 and non-α7 containing nAChRs.

II. LITURATURE REVIEW

This research focuses on nicotinic mechanisms of prepulse inhibition of the startle response in rats - an operational measure of sensorimotor gating. This introduction will begin with a general overview of the startle reflex and then expand into modulations of the startle response relevant to my studies, which includes habituation, sensitization, fear conditioning, and prepulse inhibition.

1. The startle reflex and its universality

The startle response can be generally thought of as a reaction to a sensory stimulus that is both sudden and intense. It is a distinct reflex that has been used in literature to express surprise or physical arousal, and was first scientifically described by Sechenov in 1863. In his famous work "Reflexes of the Brain", Sechenov states that "sudden fright, no matter how insignificant the cause (i.e. stimulation of the sensory nerve), always evokes in man pronounced and reflexive movements". More recently, the startle response has been thought of as a protective reflex generally characterised by a whole-body flinch in mammals, but can exist in many different forms across the animal kingdom (Prosser & Hunter, 1936; Pfeiffer, 1962; Russell, 1974; Zotolli, 1978; Currie & Carlsen, 1985; Baird et al., 1993; Wicks et al., 1996; Mongeluzi, 1998). For example, in fish, an escape response exists that can be elicited by tactile stimulation, or by the presence of olfactory cues (Pfeiffer, 1962). A cross species analysis revealed the importance of the Mauthner cells (M-cells), located in the medulla oblongata in Teleosts, for the mediation of this escape response (Zotolli, 1978). In the marine snail *Tritonia*, an escape swim circuit is activated if the tail is given a mild electrical shock. The magnitude of this response can be calculated by the number of circular swim cycles the animal completes (Mongeluzi,

1998). Also, in the nematode *Caenorhabditis elegans*, a withdrawal movement is activated in which the organism will change swim direction in response to a sudden aversive stimulus (Wicks et al., 1996). In 1936, Prosser and Hunter were the first to conduct startle experiments on rats by placing recording electrodes into the gastocnemius muscle of rats while exposing them to an acoustic stimulus. This resulted not only in the contraction of leg muscles, but also caused the back to arch, the neck muscles to bring the head closer to the body and the eyes squinted – changes very similar to the human reaction. Landis and Hunt (1939) examined the human startle response by firing a pistol shot and recording the subject's movements. They found that humans have a whole-body response that included limb, axial, cranial and facial muscle contraction that brought the arms alongside the head in a protective manner.

Although the startle response exists in many species, and comes in many different forms, there are some overarching similarities: *rapid onset* of a *protective movement* that includes *whole body muscular contraction* in response to a *sudden and intense stimulus*.

2. Acoustic startle circuitry in the rat

In the rat, tactile, vestibular, and acoustic stimulation can elicit startle reactions. The acoustic startle response is the most commonly used in startle research, since it is easiest to establish and control in a laboratory environment. Therefore, the following overview will focus on the acoustic startle reflex in the rat.

The first circuit thought to mediate the primary acoustic startle response (ASR) in rats was proposed in 1982 (Davis et al., 1982). By performing electrolytic lesions and *in-vivo* electrical stimulation of various areas of the brainstem, they concluded that the pathway consisted of the auditory nerve, the ventral cochlear nucleus (CN), the ventral lateral lemniscus (VLL), the caudal pontine reticular nucleus (PnC) and finally ending with the motor neurons and their neuromuscular junctions in the face, spine and neck (Davis et al., 1982). Thus, they proposed a tetra-synaptic circuit starting with the auditory nerve, and ending at the muscle endplate. The PnC was further deemed a crucial structure after neurotoxic lesion studies resulted in a decrease of the ASR (Koch et al., 1992). Furthermore, in this study, Koch et al. also found a positive correlation between number of PnC giant neurons lost and percent decrease in the ASR. This study, in combination with collision studies (Yeomans et al., 1993), and tracing experiments in rats (Lingenhöhl & Friauf, 1994), indicated that the PnC is a necessary mediator of the ASR and the involvement of the lateral lemniscus may not be as crucial as originally described. Furthermore, a study by Lee et al. in 1996 reevaluated the original tetra-synaptic hypothesis, proposed by Davis in 1982, by performing more specific electrolytic lesions to the VLL, and concluded that while the VLL receives auditory input and projects to the

PnC, it is not a necessary component for the ASR (Lee et al., 1996). A role for the PnC as an important structure in the ASR was further elucidated using i*n vivo* field recordings in cats (Wu at al., 1988) and in mice (Carlson & Willot, 1998). It should be mentioned that in addition to the CN, clusters of neurons embedded in the auditory nerve, called cochlear root neurons (CRN), receive direct auditory input from the cochlea and project to the PnC (Lee et al., 1996). Thus, both the functional similarities and the anatomical proximity of the CN and the CRN make it appropriate to refer to this segment of the startle circuit as the CN/CRN. In summary, these studies indicate that the fast primary neural circuit of the ASR in rats consists only of three structures that include the CN/CRN, the PnC, and motor neurons (Orange boxes in **Figure 1.1**). Since PnC giant neurons directly project onto facial, cranial, and spinal motoneurons, they represent the fundamental sensorimotor interface of the ASR in rats (Lingenhöhl & Friauf, 1992, 1994).

In addition to this fast primary pathway, there are multiple inputs to the PnC that can relay auditory signals to the PnC and therefore function as secondary, slower startle pathways. In brief, some of these structures include the dorsal and ventral cochlear nucleus (DCN and VCN), the ventral tegmental nucleus (VTN), and the pedunculopontine tegmental nucleus (PPTg) (Davis et al., 1982; Parent et al., 1988; Koch et al., 1993; Herbert et al., 1994, for review see Koch, 1999). Clearly, the PnC is well poised to receive modulatory inputs from higher brain regions. Indeed electrophysiological experiments (both intra- and extra-cellular recordings) indicated that PnC giant neurons have both a high excitation threshold and a long time constant (τ) suggesting that they are suitable candidates to receive modulatory inputs (Wagner & Mack, 1998; Ebert & Koch, 1992).

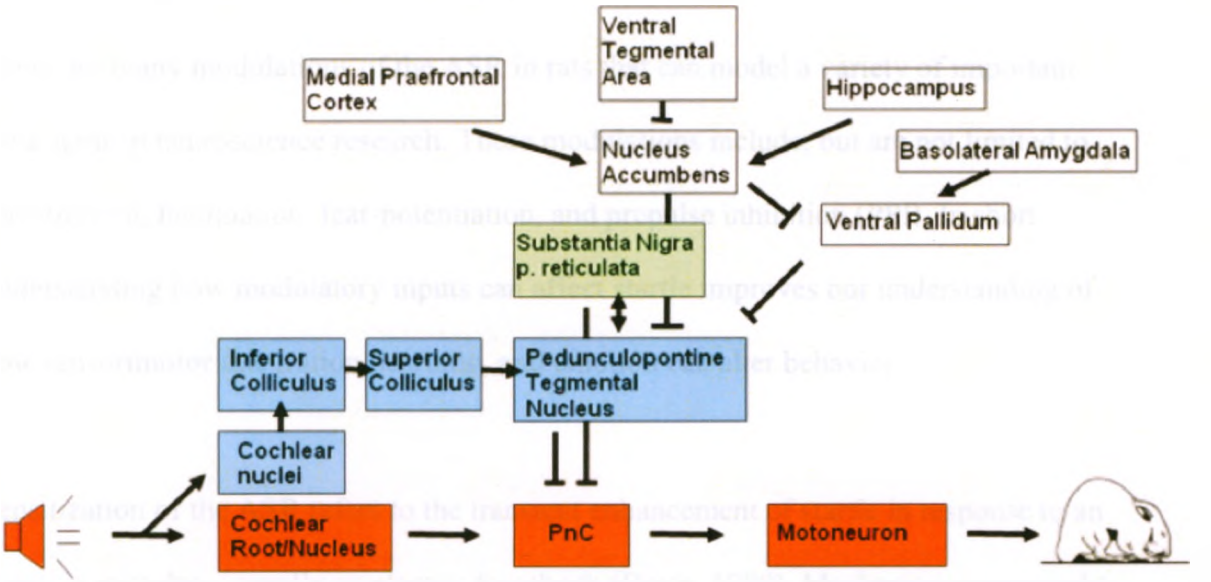


Figure 1.1 Hypothetical primary circuitry for startle and PPI of the ASR. The direct PPI pathway is outlined in orange, whereas an indirect PPI pathway is coloured in blue, which might be modulated by several brain areas. Arrows symbolize excitatory projections whereas straight lines symbolize inhibitory projections. (modified after Koch, 1999)

3. Why study startle? - modulations of the startle response

There are many modulations of the ASR in rats that can model a variety of important paradigms in neuroscience research. These modulations include, but are not limited to sensitization, habituation, fear-potentiation, and prepulse inhibition (PPI). In short, understanding how modulatory inputs can affect startle improves our understanding of how sensorimotor integration, learning, and emotion can alter behavior.

Sensitization of the ASR refers to the transient enhancement of startle in response to an aversive stimulus - usually an electric footshock (Davis, 1989). Mechanisms proposed to underlie sensitization of the ASR include heterosynaptic facilitation, post-synaptic excitation, stress-induced corticotropin release into the startle circuitry and peripheral effects of various systemic hormonal secretions (Kandel, 1976, Birnbaum & Davis, 1998; Swerdlow et al., 1989; Liang et al., 1992; Lee & Davis, 1997). Although the fundamental mechanism(s) that underlie sensitization are unclear, it can be considered a form of non-associative learning. Possible neural substrates thought to be involved in the sensitization of the ASR in rats are the hippocampus and the bed nucleus of the stria terminals either via direct projections to the PnC or through an indirect pathway via the amygdala (Lee & Davis 1997; Walker & Davis 1997). Besides gaining insight into learning mechanisms, sensitization of startle has also been used in studies of general anxiety disorders (Liang et al., 1992).

Habituation, on the other hand, is the attenuation of a stimulus-elicited response upon repeated stimulation. It was first described, as it relates to startle in humans, in

Sechenov's 1863 work entitled "The Reflexes of the Brain", and later described in rats in 1936 by Prosser and Hunter (Prosser & Hunter, 1936). The phenomenon of habituation is important because, like sensitization, it is an example of non-associative learning, and thus provides a means to study non-associative learning. Furthermore, neural disorders, most notably schizophrenia, autism, and Parkinson's disease, show disruptions in habituation (Geyer & Braff, 1982; Putzki, 2008). Mechanisms underlying short-term habituation of the ASR (that is, habituation that occurs in response to repeated stimulation within a test session) are likely to occur at the synapse between the CN and the PnC, since stimulation of the CN induces habituation of startle, but stimulation of the PnC does not lead to startle habituation (David, 1982b, Pilz & Schnitzler, 1997). Long-term habituation of the ASR (habituation that lasts for hours or days across testing sessions) has been shown to involve many neural substrates, such as the mesencephalic reticular formation (Jordan & Leaton, 1983), the ventral periaqueductal gray (Borszcz et al., 1989), and various cortical areas (Groves et al., 1974). Therefore, habituation of the ASR is a very useful assay for investigating mechanisms of implicit, non-associative learning, especially as it is related to disease states such as schizophrenia (Geyer et al. 1990).

Fear potentiation of the ASR occurs when a neutral stimulus (usually a light or tone) is paired with an aversive stimulus (usually a mild foot shock). The conditioned stimulus (CS) is then presented alongside a startle-evoking stimulus, causing an increase in startle magnitude (Brown et al., 1951). Fear potentiated startle can be used as a quantitative measure of fear other than freezing behaviour and hypertension (Davis, 1992; LeDoux, 1996). Lesion studies have shown that the basolateral amygdala (BLA) plays a significant role in forming the connection between the aversive stimulus and the neutral stimulus in

fear potentiation of startle (Miserendino et al., 1990; Davis, 1993; Kim & Davis, 1993; Davis, 1994; Campeau & Davis 1995a, Campeau & Davis, 1995b; Rogan et al, 1997; Shinnick-Gallagher, 1997; Shi & Davis, 1999). Interestingly, if the foot shock intensity is too high, the ASR will not be potentiated at all. This biphasic effect of the magnitude of foot shock on startle potentiation is thought to represent the shift from a more passive fear state to a much more active fight-or-flight state. The more active fight-or-flight state is thought to suppress startle in order to ensure that the animal is not made more vulnerable due to the intense muscular contractions that are present in the startling reaction. This mechanism is thought to work by amygdalar activation of the periaqueductal gray (PAG), triggering the flight-or-flight response and simultaneously suppressing the startle response (Walker et al., 1997; Walker & Davis, 1997). Therefore, it is clear that the amygdala plays a crucial role in the emotional conditioning of fear-potentiated startle in rats. Thus, fear-potentiated startle serves as a logical model for studying the role of emotions in sensorimotor information processing.

In summary, modulations of the startle response include, but are not limited to, sensitization, habituation, and fear-conditioning. Each type of modulation has unique properties that have the potential to enhance our understanding of non-associative learning, anxiety, and emotional control over sensorimotor information processing. One of the important forms of modulation is inhibition of startle by a prepulse stimulus, which will be discussed in the next section.

4. Prepulse inhibition is an operational measure of sensorimotor gating

If a minor stimulus, that does not elicit a startle response, precedes a startle-evoking stimulus, the startle response can be significantly attenuated – this is termed prepulse inhibition (PPI) (Ison & Hammond, 1971). Prepulse inhibition (PPI) is considered to be an example of automatic sensorimotor gating, which occurs when sensory information is "gated-out" from an organism's environment. This gating process frees-up precious processing resources for important stimuli, and reduces the cognitive demand on the animal (Graham, 1975; Fendt et al., 2001; Braff & Light, 2004; Geyer, 2006; Ellenbroek, 2004). PPI of startle has been observed in both vertebrates and invertebrates (Ison & Hammond, 1971; Schall et al., 1999l; Mongeluzi et al., 1998) but not in any single-cell organisms, suggesting that PPI only exists in organisms that have developed some form of neural network. Sensory filtering circuits in animal brains are considered to be important evolutionary mechanisms that allow for the processing of only salient stimuli, and consequently, the unconscious filtering-out of trivial, unimportant sensory information (Graham, 1975).

The attentional capabilities that we enjoy rely heavily upon the ability to unconsciously filter-out unimportant information. The importance of automatic sensory filtering becomes apparent when it is seen disrupted in disorders like attention-deficit hyperactivity disorder (ADHD), Huntington's chorea, and most notably in schizophrenia. The reduced sensory filtering abilities in individuals with these disorders can be measured as deficits in PPI; in fact, in schizophrenia, PPI disruption has become a hallmark symptom (Braff, 1978; Swerdlow et al., 1995; Castellianos et al., 1996). Thus,

understanding the basic mechanisms that mediate PPI will serve two general goals: 1) to enhance our understanding of sensorimotor gating mechanisms; and 2) to understand how disruptions in sensory filtering lead to cognitive impairment. This understanding is crucial for the development of novel pharmaceuticals.

Schizophrenia afflicts approximately 1% of the population and the total costs associated with schizophrenia in Canada amount to approximately CAN$6.85 billion annually (Goeree et al., 2004). Great amounts of time, money and energy have been allocated to investigate the causes of schizophrenia and developing new pharmacotherapies that could treat this disease (Freedman, 2003). Both the positive symptoms (hallucination, delusions, catatonic behaviour etc.) and the negative symptoms (social withdrawal, alogia, avolition etc.) associated with schizophrenia may be at least partially alleviated by typical and atypical antipsychotics (Geyer, 2006). However, there is a third category of symptoms for which there is not yet any effective treatment: cognitive deficits that manifest as problems focussing, disorganized thought, memory loss, and sensory flooding (McGhie & Chapman 1961; Venable, 1960; Geyer, 2006). It is thought that the disruption in PPI in individuals with schizophrenia is a manifestation of being deficient in general sensorimotor gating mechanisms, which could result in sensory flooding and cognitive impairment (Perry et al., 1999). By gaining a better understanding of the mechanisms that mediate PPI, we will be better able to target compounds that could potentially alleviate the debilitating cognitive deficits associated with schizophrenia. To this end, numerous animal models have been created to investigate this disorder. A widely used animal model of schizophrenia is, for example, the early maternal segregation of rat pups - a social isolation rearing paradigm, which results in PPI deficits (Bakshi et al., 1998; Cilia et al.,

2001, 2005a, 2005b; Li et al., 2008). Social isolation rearing is a useful model for studying schizophrenia, particularly to researchers interested in exploring the neurodevelopmental theory of schizophrenia (for review see Weiss & Feldon, 2001). Other popular animal models of schizophrenia include drug-induced PPI deficits via systemic injection of a variety of compounds that include dopamine (DA) agonists, serotonin (5-HT) agonists, muscarinic receptor antagonists, apomorphine, and N-methyl-D-aspartate (NMDA) receptors antagonists (for review see Geyer et al., 2001).

In summary, PPI of the ASR represents a sensorimotor gating mechanism that serves as an example of how organisms automatically filter-out sensory information. Since cognitive deficits are seen in a number of mental disorders, and are believed to be caused by deficits in general sensorimotor gating mechanisms, PPI serves as a useful model for investigating these disorders (including ADHD, Huntington's chorea, and most notably in schizophrenia).

5. Circuitry mediating prepulse inhibition of startle in the rat

The fast excitatory primary startle pathway comprising the CN/CRN and the PnC receives feed-forward inhibition from a parallel pathway that is responsible for the mediation of PPI (Blue boxes in **Figure 1.1**). Like the main startle pathway, the primary PPI pathway also receives acoustic sensory input from the cochlear nucleus. Lesions to the inferior colliculus (IC) will eliminate PPI in rats (Leitner and Cohen, 1985; Li et al., 1998), and electrical stimulations to the IC enhance PPI (Li & Yeomans 2000). From this, it is clear that the prepulse signal passes from the CN to the IC. Since the superior colliculus (SC) is known to receive direct sensory inputs of many modalities as well as inputs from the IC (Meredith, 1992) it was a logical substrate to investigate its involvement in PPI. Indeed, Fendt and colleagues in 1994 showed that fiber-sparing lesions to the SC could attenuate PPI by nearly half. Likewise, SC excitation by both picrotoxin injection and electrical stimulation caused an enhancement in PPI by up to 80% (Fendt, 1999; Li & Yeomans, 2000). Thus it is likely that the SC plays an important role in the PPI of startle. The pedunculopontine tegmental nucleus (PPTg) also plays a crucial role in startle modulation by a prepulse since lesions to this area disrupt PPI (Leitner, 1981), and electrical stimulation of this area significantly enhances PPI (Saitoh et al., 1987). Furthermore, the PPTg becomes activated in response to acoustic stimulation with a short latency of only 13ms after stimulus onset (Ebert & Oswald, 1991). Kodsi and Swerdlow showed that pharmacological inhibition of the PPTg, via the application of a $GABA_A$ agonist, muscimol, had similar effects as the lesion studies performed by Leitner in 1981 (Kodsi & Swerdlow, 1997). Finally, other evidence for the involvement of the PPTg in PPI comes from anatomical tracings showing a direct cholinergic projection from the PPTg to

the startle mediating neurons in the PnC (Semba et al., 1990; Koch et al., 1993). It should be mentioned that none of these manipulations (with the exception of IC lesions) completely blocked PPI, therefore it is hypothesized by some, that other feed-forward inhibitory mechanisms may exists, that could mediate PPI via multiple secondary pathways that run parallel to the primary PPI pathway (for review see Koch, 1999).

In addition to these midbrain and brainstem structures that convey startle and prepulse signalling, other brain areas modulate startle and PPI. These include the hippocampus, amygdala, medial prefrontal cortex, ventral striatum, ventral pallidum, substantia nigra (SN), the ventral tegmental area (VTA) and the nucleus accumbens (NAC) (for a review see Koch, 1999). A model of this is illustrated in **Figure 1.1**. These modulatory areas act on PPI and startle through direct or indirect projections to the PPTg. The PPTg is therefore a structure where much of the top-down control over PPI is funnelled. For example, prepulses that have been associated with fear or reward can improve or inhibit PPI respectively. Also, attentional regulation of prepulses can enhance PPI in rats and humans (for reviews on top-down control, see Li et al., 2009; Koch, 1999). For example, when humans are told to pay attention to the prepulse, this causes enhanced PPI (Filion et al, 1993). Similarly, PPI is enhanced in rats when there is a perceived spacial separation between the prepulse and a background noise (Du et al., 2009).

In summary, the hypothesized PPI pathway for the ASR in rats begins when an acoustic stimulus excites the CN/CRN. Auditory neurons then send excitatory projections to the

IC, which is part of the general ascending auditory pathway. Projections from the IC not only stimulate the auditory thalamus in the ascending auditory pathway, but also stimulates the SC, which can also receive sensory inputs of different modalities. The SC in turn sends excitatory projections to the PPTg. From here, inhibitory projections, presumably cholinergic and GABAergic, innervate the PnC and make contact with startle-mediating giant neurons, which results in the attenuation of startle (Koch et al., 1993; Bosch & Schmid, 2006, 2008; Yeomans et al., 2010). Furthermore, the PPTg is poised to receive top-down modulatory inputs from a myriad of higher neural structures.

6. Evidence for the involvement of nicotine in prepulse inhibition

The cholinergic system and PPI

The main focus of my research is on the cholinergic influence on PPI. Generally, cholinergic systems in the mammalian brain are confined to mainly two cholinergic centres: 1) the basal forebrain and 2) the pontomesencaphalic neurons that are located in the PPTg and the LTD. Both centres have mainly ascending projections, and the PPTg/LTD participates in a variety of activities including arousal, REM sleep/dreaming, attention, reward, addiction, and motor motivation (Lee et al., 1988; Solms, 2000; for review see Steckler et al. 1994). The PPTg cholinergic projections also exist to provide excitatory input into reward-mediating dopaminergic neurons in the VTA and SN, which is significant to startle because it has been shown that rewarding stimuli can inhibit the startle response (Yeomans & Baptista, 1997; Corrigall et al., 1994; Lang et al., 1990; Schmid et al., 1995; Steidl et al., 2001). Most importantly the descending projection from the PPT to the PnC has been implicated to mediate PPI (Semba, 1990; Leitner, 1981; Koch et al., 1993).

Nicotine as a modulator of PPI

Over 90% of schizophrenics consume nicotine by smoking cigarettes, which results in an alleviation of their PPI impairment and cognitive deficits (Lohr & Flynn, 1992). Consuming nicotine by smoking is believed to be a form of self-medication (Kumari et al., 2001; Smith et al., 2006). This supports the notion that there is an interaction between the cholinergic system and PPI (Kumari et al., 2001; Smith et al., 2006; Lagostena et al.,

2008). Evidence implicating specific nAChR subtypes in PPI also comes from genetic linkage analysis of people with schizophrenia. This research shows that a dinucleotide polymorphism at the site of the α7 nAChR gene (CHRNA7) exists in certain schizophrenic patients and it is linked to auditory gating deficits (Chini et al., 1994; Freedman et al., 1997). It has also been shown that people with schizophrenia have significantly reduced density of α7 nAChRs in the mPFC (Guan et al., 1999). Furthermore, both major nAChR subtypes found in the mammalian brain, the α4β2 and the α7 nAChRs, are reported to be involved in PPI and in other types of sensory gating mechanisms (Adler et al., 1992; Schreiber et al, 2002; Leiser et al., 2009). For example, the auditory evoked potential (P50) is an example of an auditory gating mechanism in rats, and is highly dependent on the α7 nAChR since α7 knockout mice show large P50 deficits (Freedman et al., 1997). Finally, both major neuronal nAChRs are found at the site of the startle pathway that receives modulatory input from the PPTg: the PnC (Allen Brain Atlas, http://mouse.brain-map.org).

More direct evidence for the involvement of nicotine in PPI comes from unpublished behavioural studies by Yeomans, Bosch, Dong, and Schmid (2007) showing that the nicotinic antagonist mecamylamine significantly attenuates PPI when injected into the PnC of rats, but only at short ISIs (between 8-50ms) (**Figure 1.2**). The idea that nicotine may only mediate PPI at short ISIs logically follows from the fact that nAChRs are ionotropic and have fast onset and rapid inactivation, whereas muscarinic receptors are G-protein coupled receptors and would therefore mediate PPI at longer ISIs due to their longer-onset and tonic receptor kinetics (Jones & Shannon, 2000a; Jones & Shannon 2000b).

In patch-clamp recordings, Bosch and Schmid (2006) have shown that infusion of a general cholinergic agonist, carbachol, significantly reduces firing of PnC giant neurons. This effect is only partially blocked by the administration of muscarinic antagonists (M2 and M4 antagonists) indicating that nicotinic receptors are likely contributing in part to the cholinergic modulation of startle mediating neurons in the PnC.

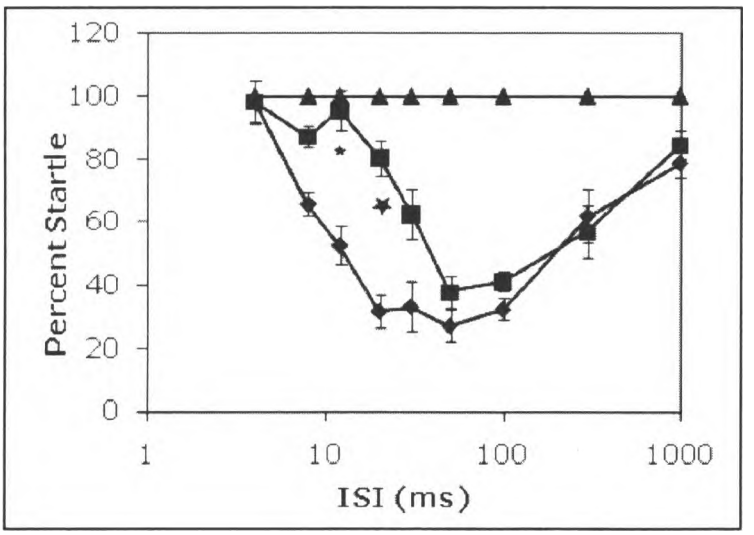


Figure 1.2 Unpublished data from Yeomans, Bosch, Dong, and Schmid showing that the nicotinic antagonist mecamylamine significantly attenuates PPI when injected into the PnC of rats, but only at short ISIs (between 8-50ms). Triangles represent baseline startle values, diamonds represent PPI with PnC injections of saline, and squares represent PnC injections of mecamylamine. Stars represent significant differences ($P<0.05$).

7. Neuronal nicotinic acetylcholine receptors

nAChRs are a large family of pentameric cation channels that allow for the influx of Na^+ and Ca^{2+} and the efflux of K^+ and the simultaneous exclusion of anions due to a ring of negatively charged amino acid residues at the channel entrance (Imoto et al., 1988). In mammals, these excitatory receptors can be assembled from a selection of at least 10 different subunits to form either a heteromeric or homomeric membrane-spanning channel. The endogenous agonist, acetylcholine, generally requires the presence of an α subunit to bind. After binding, the channel takes only 20μs to initiate a conformational shift that rotates the pore-lining domains of all 5 subunits to fully open the channel (Kotzyba-Hilbert et al., 1999). The pore that allows for the passage of cations is no more than 10 Å in diameter when fully open (Unwin, 1995).

Homomeric neuronal type nAChRs include pentamers of the α7, α8, α9 subunits. Heteromeric neuronal type nAChRs generally appear in a 2α3β stoichiometry that consists α2, α3, α4, and the β2 subunits (Conroy et al., 1992; Couturier et al., 1990). The most common neuronal subtypes, and those thought to mediate the behavioral effects of nicotine are the α7 and the α4β2 subtypes (Olale et al., 1997; Hsu et al., 1996). These two receptors subtypes are also the quickest to become upregulated and desensitized after nicotine exposure (Fenster, 1997; Alkondon, 2000). According to the Allen Rat Brain Atlas (http://mouse.brain-map.org), these receptors are present at a relatively low density in the PnC. However, considering the relatively low numbers of startle mediating giant neurons in the PnC (Koch et al., 1992) high densities of nAChRs in the PnC would theoretically not be necessary to elicit large effects.

III. Thesis objectives and hypothesis

We **hypothesize** that the α4β2 and/or the α7 nAChR are activated during prepulse inhibition of the acoustic startle response in rats, at the level of the PnC. We have also hypothesized that the nicotinic mediation of PPI in the PnC occurs only for short interstimulus intervals (12-50ms).

Objectives:

There are three main objectives to this work

1) to characterize the role that nAChRs plays in PPI at a systemic level
2) to determine if nAChRs are involved in the mediation of PPI in the startle circuitry itself
3) to identify possible receptor subtypes that may be mediate these effect at both a systemic and local level

Approach:

To explore this hypothesis, we will perform systemic and intracranial (intra-PnC) injections of nicotine and various nicotinic antagonists. After injections, we will test startle and prepulse inhibition to attain baseline startle values and prepulse inhibition scores.

IV. Methods

1. Animal care and handling

Animals were obtained from Charles River® (Montreal, Quebec) and housed (singly or two per cage) in the animal care facilities at the University of Western Ontario for at least 48 hours before any procedures or handling took place. Animals were allowed food and water *ad libidum,* and were kept on a 12:12 hour, lights on at 7am, day/night cycle in a temperature-controlled room at 23°C. Approximately 3 days before testing, animals were handled to ensure familiarity with the handler and the startle boxes. Animals were socialized for approximately 10 minutes each day for 3 days. On the first handling day, animals were held and petted, and became familiar to the lab environment. On the second handling day, animals were held for 5 minutes and placed in the startle apparatus for another 5 minutes while the constant sound of a 65dB white noise played in the background. On the final handling day, animals were handled and then placed in the startle apparatus for 10 minutes, also under the constant sound of a 65dB background white noise.

2. Stereotaxic surgery

Stereotaxic surgery was performed on 7 week-old male Sprague-Dawley rats in which cannulas, bilaterally targeting the PnC, were inserted into the brain. Rats were anaesthetized using a mixture of 2% isoflorane and 98% oxygen, and a subcutaneous injection of 0.05mg/kg of Buprenorphine and 5mg/kg Ketoprofen was given during surgery for analgesia. Using blunt-ended ear bars, the animals' heads were secured into the stereotaxic frame (Stoelting Co.) as a snout-mask maintained a continuous flow of the isoflorane/oxygen mixture throughout surgery. A mid-sagittal incision of about 3cm in length was made on the scalp, and the skin was retracted, exposing the sagittal and lamdoid sutures. Four, 1mm-wide holes were drilled into the skull and jeweller screws were inserted. The screws protruded from the skull surface by about 1mm to act as anchors for the dental cement cap. The coordinates for the cannulae placements were measured from lambda as follows: +2.5mm in the medial/lateral plane; -8.80mm in the ventral/dorsal plane; -2.1mm in the rostral/caudal plane. Based on these coordinates, two 1mm wide boreholes were drilled and cannulae (PlasticsOne®) were slowly lowered bilaterally and secured with dental cement. Cannulae were kept patent using stainless steel stylets (PlasticsOne®). Silk suture was used to close the wound, and rats were allowed a 7-day recovery period in the animal care facility. All procedures were approved by the University of Western Ontario Animal Use Committee, and conformed to the ethical guidelines of the Canadian Council on Animal Care involving vertebrate animals in research.

3. Drugs

Liquid (−)-Nicotine (Sigma Chemical Co. Ltd., USA), (−)-Cotinine (Sigma Chemical Co. Ltd., USA), methyllycaconitine citrate (MLA) (Tocris Bioscience, Inc., USA) and TMPH hydrochloride (Tocris Bioscience, Inc., USA) were dissolved in physiological saline. All drugs were dissolved into stock solutions and kept at -18°C until used. Drug concentrations were selected based on commonly used concentrations in the literature (nicotine, Acri et al., 1994; Hamman et al., 1992; Schreiber et al., 2002; cotinine, Kyerematen 1988; MLA, Panagis et al., 2000; Chilton et al., 2004; TMPH, Damaj et al., 2005). MLA has high affinity α7 nAChRs but shows some affinity for α4β2 and α6β2 receptors at concentrations 40X higher above its Ki (K_i = 1.4 nM) for α7nAChRs (Ward et al., 1990). TMPH has high affinity for non-α7 neuronal type nAChRs, but has little inhibition for both muscle type, α1β1γδ and α7 receptors (Papke et al., 2005). TMPH displays particular affinity for α4β2 (K_i = 1.4 μM) (Papke et al., 2005).

4. Injections and startle testing

For systemic injections, rats were either injected intraperitoneally (IP) with a 23 G needle or subcutaneaously (SC) with a 25 G needle. For stereotaxic injection 30 G Infusion cannulae were inserted into the guide cannulae and extended 1mm beyond the tip of the guide cannulae. 0.5 μL of drug or vehicle was injected bilaterally in awake animals over 4 minutes using a syringe pump (World Precision Intruments®), and infusion cannulae remained inside the guide cannulae for an extra minute to ensure complete diffusion of drug. Both stereotaxic and systemic injections were pseudo-randomized and balanced with saline controls in order to ensure that multiple injections did not disrupt PPI.

After each injection, animals were placed in startle chambers, and the startle software (Med Associates, Vermont, USA) was used to perform experiments and analyze data. Gain factors were adjusted individually for each animal, and therefore, startle responses represent arbitrary units. Animals were first subjected to 30 startle alone trials (Block 1), then subjected to 60 trials consisting of six different acoustic stimuli (Block 2). These 60 trials consisted of one startle alone trial, and five pseudo-randomized prepulse trials with the following Interstimulus intervals (ISI) between prepulse and startle pulses: 12ms, 20ms, 50ms, 100ms, and 250ms. The startle evoking pulse consisted of a 20ms long burst of white noise at 105 dB. Maximal PPI occurred when a loud, 85dB prepulse was used, and sub-optimal PPI was achieved when lower prepulses of 75dB were used. Sub-optimal and maximal PPI eliciting prepulses were used strategically to either allow for PPI enhancement or PPI disruption by drug. All prepulses consisted of a 4ms long burst of white noise at 85dB. Background noise was 65 dB and trials were 15 sec apart.

A single batch of animals were injection with various doses of a single drug (i.e. to

generate a dose response curves), however, no animals were injection with more than one compound throughout these experiments.

5. Histology and cannulae placement confirmation

Before sacrifice, rats received a high dose of pentobarbital and were injected stereotaxically with a small amount of 3% thionin dye to mark cannulae tip placements. Rats were then sacrificed by CO_2 inhalation, and decapitated. Brains were harvested and fixed by immersion in 4% paraformaldehyde (PFA) for at least 48 hours, and then transferred to 15% sucrose (in buffer) for at least 24hrs. Brains were sliced into 50μm-thick sections by a cryotome. Sections were mounted, dried, and stained using the Haematoxylin and Eosin counterstaining procedure. Cannulae coordinate determination was made using a rat brain atlas by Praxinos and Watson (Praxinos & Watson, 2004). Injection tips that reached or penetrated the boarders of the PnC were deemed as successful hits. All other placements were deemed as misses and therefore their data were discarded.

6. Data analysis

In block 2 of the PPI testing, startle responses that were preceded by a prepulse were divided by startle responses that had no preceding prepulse. These percentages were then subtracted from 1 to yield a "Percent PPI" score. Averages of both percent PPI were taken for every ISI, and two-way, repeated measures ANOVAs were conducted using GB Stat software (GB Stat®). If warranted, a Fischer's Least Significance Difference (LSD) post-hoc test was used to assess points of significance. Baseline startle measurements were calculated by averaging, for each animal, the first 20 startle alone trial responses in block 1. These individual baseline startle scores were analyzed using a two-tailed, paired Student's t-test only after normality of the data was confirmed by a Shapiro-Wilk Normal Distribution test. In both the ANOVAs and the Student's t-tests, differences in the data were deemed significant if p values were less than 0.05 ($\alpha = 0.05$). Standard error of the mean values were calculated for each condition and error bars were generated from these values.

V. RESULTS

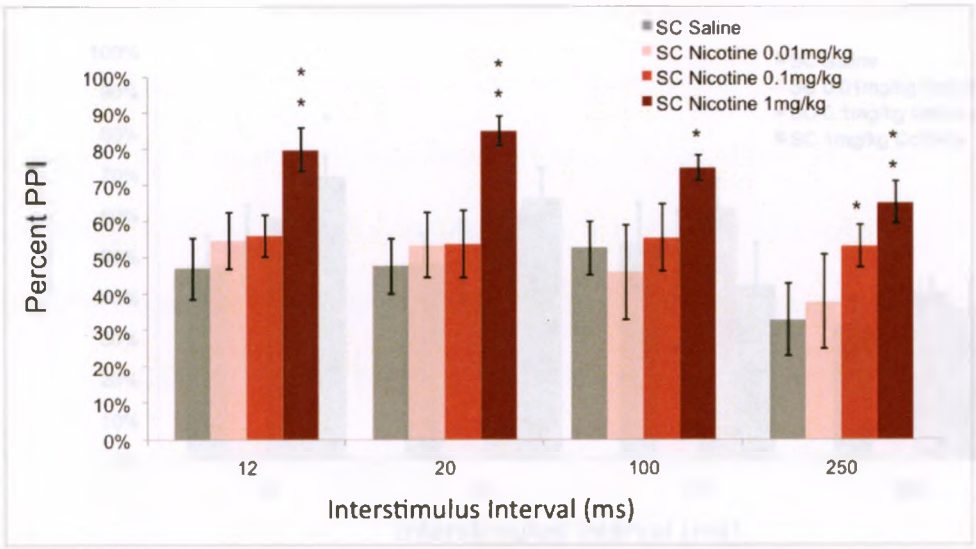
1. Systemic injections

1.1 Effects of systemic nicotine and cotinine

Subcutaneous (SC) injections of nicotine significantly affected PPI (main group effect of drug: ANOVA, $F(3,29) = 14.63$; $p<0.0001$). A *post-hoc* analysis revealed that doses of 0.01mg/kg and 0.1mg/kg had little effect, showing a significant increase in PPI only at an ISI of 250 ms upon a 0. 1mg/kg injection. However, a dose of 1mg/kg significantly increased PPI from ~50% to ~80% for ISIs of 12 ms, 20 ms, 100 ms, and 250 ms. Baseline startle amplitudes (without prepulse) were not affected by SC nicotine, when compared to saline controls [t-test, n=8; $p>0.05$]. (**Figure 2.1**).

Cotinine SC had minimal effects on PPI in all tested doses of 0.01mg/kg and 0.1mg/kg and 1mg/kg [ANOVA, $F(3,29) = 1.67$; $p<0.05$]. A *post-hoc* analysis showed a significant increase in PPI from ~50% to ~70% at an ISI of 12ms for the maximum dose of 1mg/kg. Baseline startle was not affected by systemic cotinine, when compared to saline controls [t-test, n=8; $p>0.05$]. (**Figure 2.2**).

a)



b)

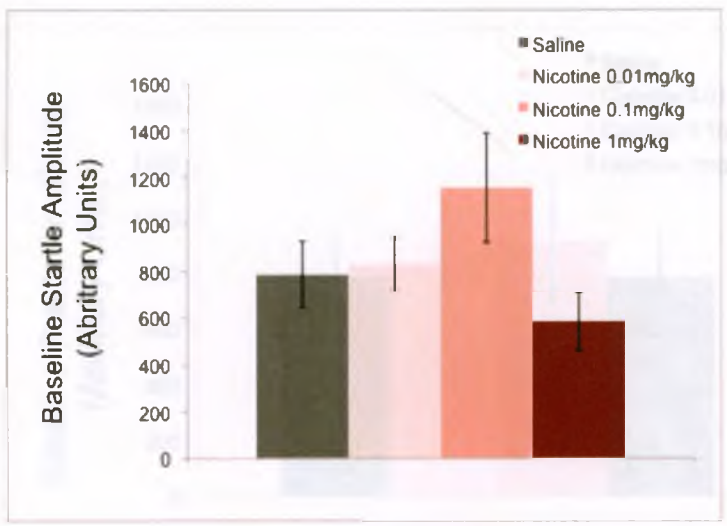
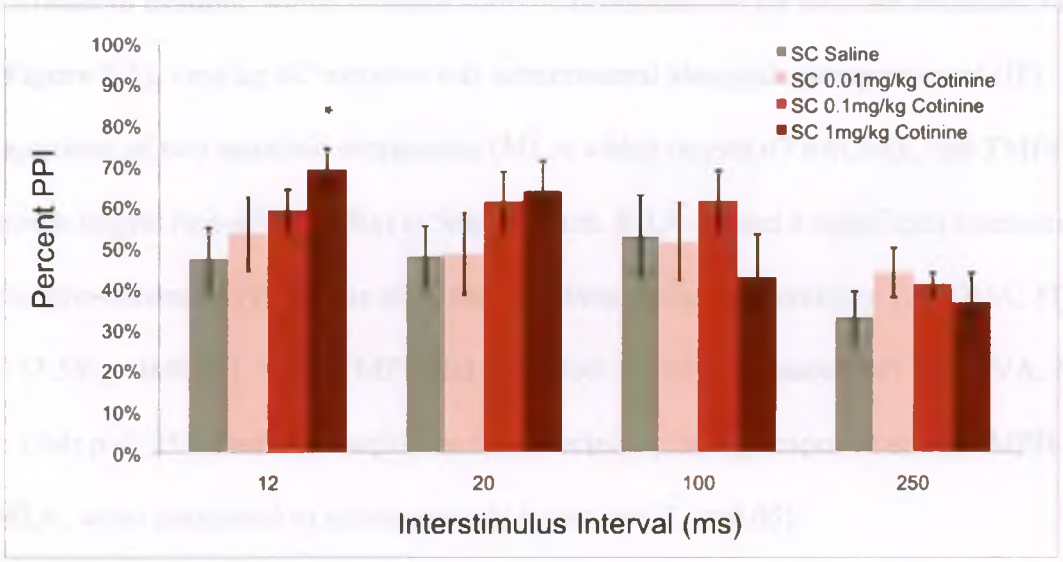


Figure 2.1 Effects of systemic nicotine administration on PPI and baseline startle. One asterisk indicates a significant difference from controls of $p<0.05$ and two asterisks indicate a difference of $p<0.01$.). a) Nicotine significantly enhanced PPI at ISIs of 12 ms, 20 ms, 100 ms, and 250ms [ANOVA, $F(3,29) = 14.63$; $p<0.0001$]. b) There were no significant effects at any dose of nicotine on baseline startle amplitudes, in comparison to saline controls [t-test, n=8; $p>0.05$]. A low prepulse of 75dB was used to allow for PPI enhancement.

a)



b)

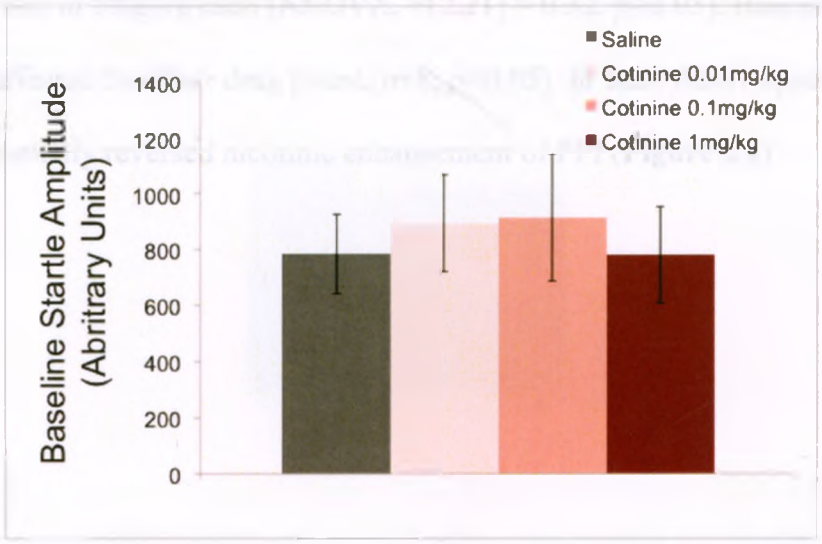


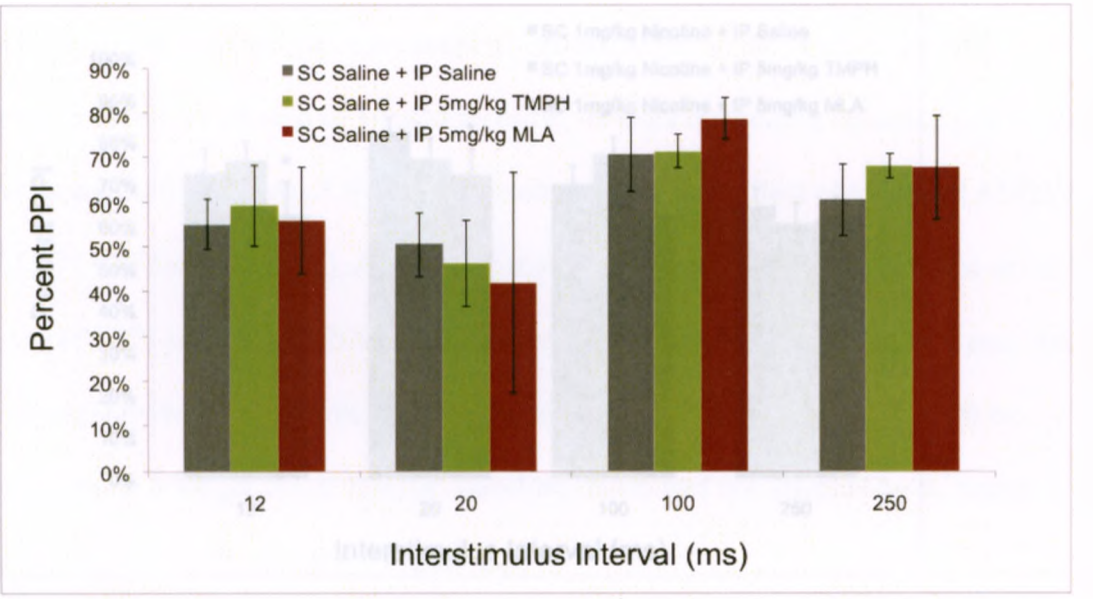
Figure 2.2 Effects of systemic cotinine administration on PPI and baseline startle. One asterisk indicates a significant difference from controls of $p<0.05$. a) Cotinine significantly enhanced PPI at an ISIs of 12ms [ANOVA, $F(3,29) = 1.67$; $p<0.05$]. b) There were no significant effects at any dose of cotinine on baseline startle amplitudes, in comparison to saline controls [t-test, n=8; $p>0.05$]. A low prepulse of 75dB was used here to allow for PPI enhancement by nicotine.

1.2 Effects of systemic nicotinic antagonists on normal and enhanced PPI

In order to examine which receptor subtype is responsible for nicotine enhanced PPI (**Figure 2.3**), 1mg/kg SC nicotine was administered alongside intraperitoneal (IP) injections of two nicotinic antagonists (MLA which targets α7 nAChRs, and TMPH which targets non-α7 nAChRs) at 5mg/kg each. MLA caused a significant attenuation of nicotine-enhanced PPI at ISIs of 12ms and 20ms under this condition [ANOVA, $F(2,33) = 13.59$; $p<0.0001$], while TMPH did not affect nicotine-enhanced PPI [ANOVA, $F(2,33) = 1.04$; $p>0.05$]. Baseline startle was not affected by SC injections of either TMPH or MLA, when compared to saline controls [t-test, n=12; $p>0.05$].

IP injections of TMPH and MLA had no significant effects on normal PPI at a dose of 5mg/kg each [ANOVA, $F(2,21) = 0.32$; $p>0.05$]. Baseline startle also was not affected by either drug [t-test, n=8; $p>0.05$]. In sum, the α7-specific antagonist MLA, partially reversed nicotinic enhancement of PPI (**Figure 2.4**).

a)



b)

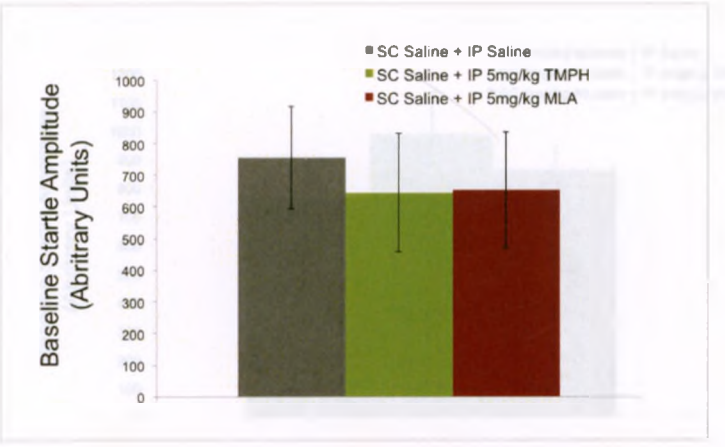
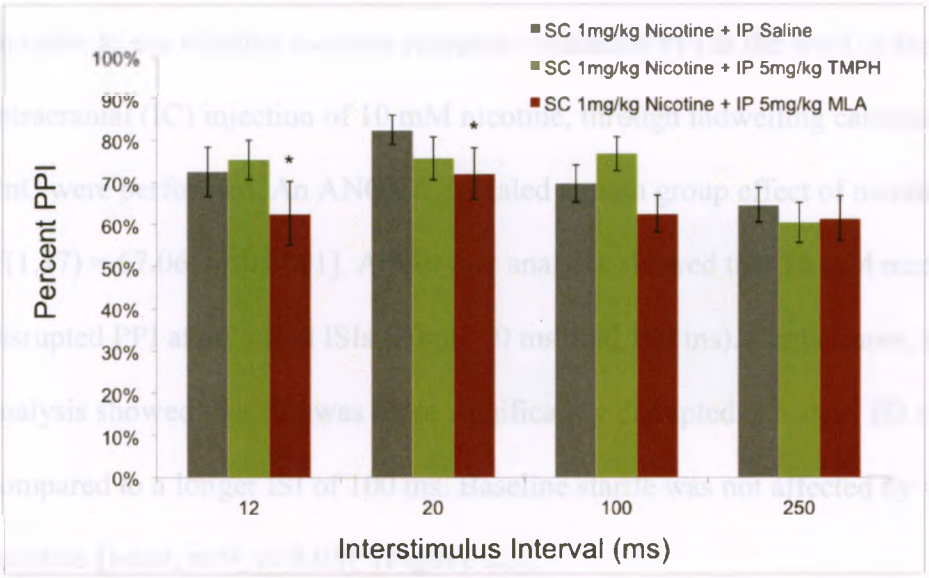


Figure 2.3 Effects of SC injections of saline administered alongside IP injections of MLA and TMPH (both 5mg/kg) on PPI and baseline startle. a) No significant effects of either TMPH or MLA were found [ANOVA, $F(2, 21) = 0.32$; $p>0.05$]. b) There were no significant effects of either TMPH or MLA on baseline startle amplitudes, in comparison to saline controls [t-test, n=8; $p>0.05$]. A low prepulse of 75dB was used to leave room for PPI enhancement.

a)



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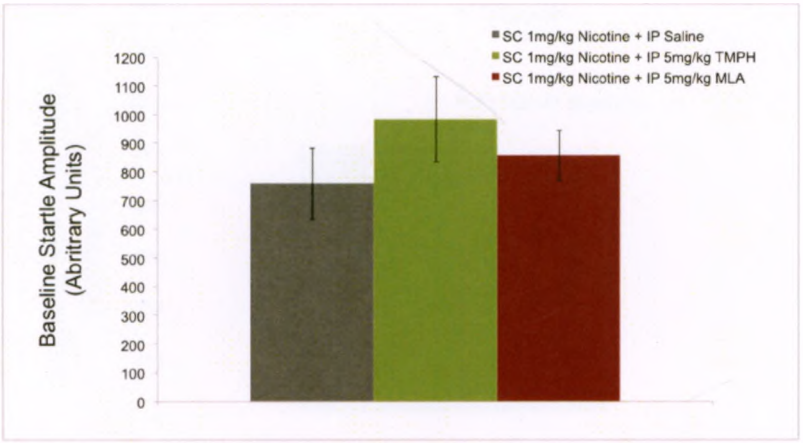


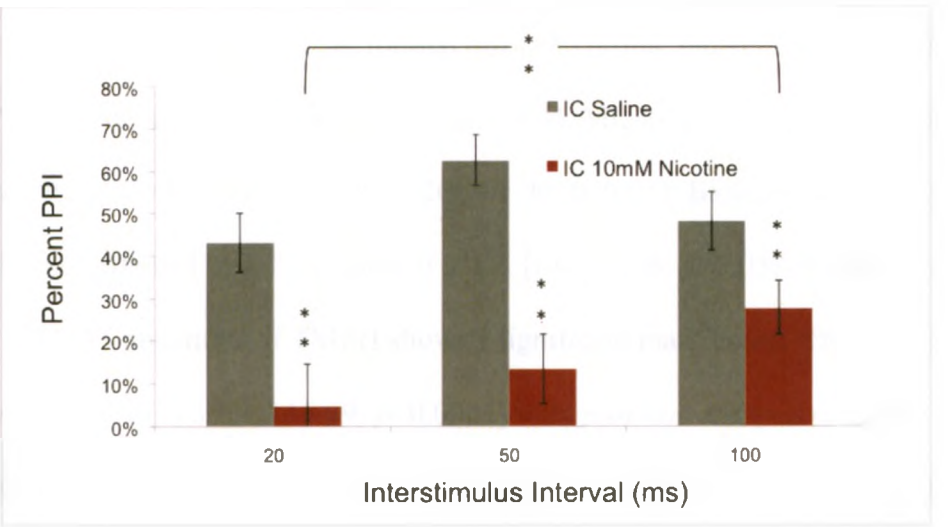
Figure 2.4 Effects of SC injections of nicotine (1mg/kg) administered alongside IP injections of MLA and TMPH (both 5mg/kg) on PPI and baseline startle. One asterisk indicates a significant difference from controls of $p<0.05$. a) MLA significantly attenuated nicotine-enhanced PPI at only ISIs of 12ms and 20ms [ANOVA, $F(2,21) = 50.27$; $p<0.0001$] b) There were no significant effects of either TMPH or MLA on baseline startle amplitudes, in comparison to saline controls [t-test, n=8; $p>0.05$]. A low prepulse of 75dB was also used here to allow for PPI enhancement by either TMPH or MLA.

2. Intracerebral injections

2.1 Effects of local application of nicotine

In order to see whether nicotine receptors influence PPI at the level of the PnC, intracranial (IC) injection of 10 mM nicotine, through indwelling cannulae targeting the PnC were performed. An ANOVA revealed a main group effect of nicotine [ANOVA, $F(1,17) = 67.06$; $p<0.0001$]. A *post-hoc* analysis showed that 10 mM nicotine severely disrupted PPI at all tested ISIs (20ms, 50 ms, and 100 ms). Furthermore, the *post-hoc* analysis showed that PPI was more significantly disrupted at a short ISI of 20 ms compared to a longer ISI of 100 ms. Baseline startle was not affected by IC injection of nicotine [t-test, n=9; $p>0.05$]. (**Figure 2.5**).

a)



b)

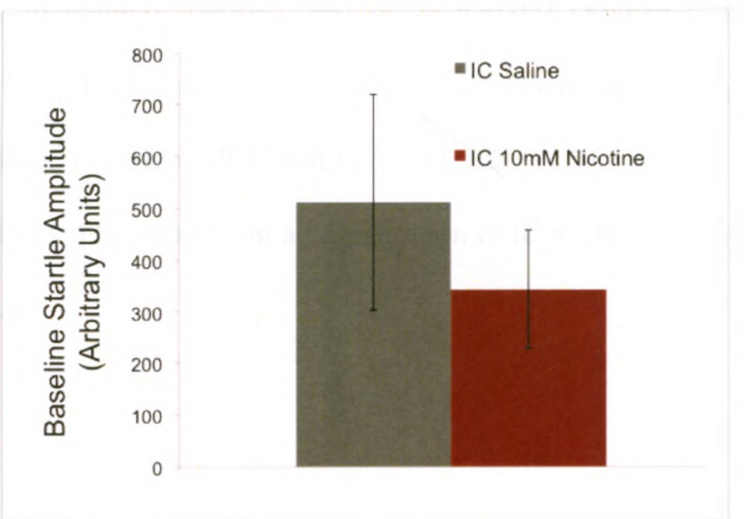


Figure 2.5 Effects of IC 10 mM nicotine administration on PPI and baseline startle. Two asterisks indicate a significant difference of $p<0.01$. a) IC 10 mM nicotine significantly disrupted PPI at ISIs of 20 ms, 50 ms, and 100 ms, with most severe disruption occurring at the short ISI of 20 ms [ANOVA, $F(1,19) = 67.06$; $p<0.0001$]. b) There were no significant effects of nicotine on baseline startle amplitudes [t-test, n=9; $p>0.05$]. A low prepulse of 75dB was used to allow for PPI enhancement by nicotine, although the reverse was observed.

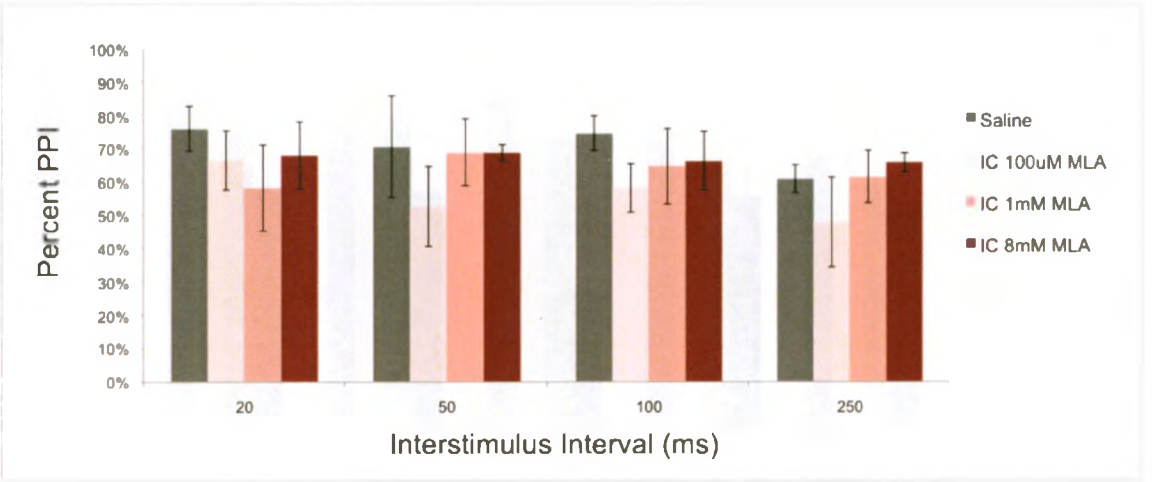
2.2 Effects of local application of MLA and TMPH

In order to asses the role of different PnC nicotine receptor subtypes in PPI, IC injections of TMPH and MLA were performed through chronic indwelling cannulae targeting the PnC. At doses of 0.1 mM, 1 mM, and 8 mM, MLA did not have any significant effects on PPI at any ISI [ANOVA, $F(3, 20) = 1.90$; $p>0.05$]. Baseline startle amplitudes were also not affected by IC injection of MLA [t-test, n=6; $p>0.05$]. (**Figure 2.6**).

IC injections of TMPH showed significant main group effects of drug on PPI [ANOVA, $F(3, 36) = 115.89$; $p<0.0001$] with *post-hoc* analysis revealing significant attenuation of PPI for the 10mM dose of TMPH at short ISIs of 12 ms, 20 ms, and 50 ms, and no significant effect at ISIs of 100ms and 250ms. Baseline startle was not affected by IC injection of TMPH [t-test, n=10; $p>0.05$]. (**Figure 2.7**).

Finally, IC injections of 8mM MLA alongside 1mg/kg nicotine showed no main effect of drug on PPI [ANOVA, $F(1, 23) = 2.21$; $p>0.05$]. Baseline startle was not affected by concurrent administration of IC 8mM MLA and SC 1mg/kg nicotine. (**Figure 2.8**)

a)



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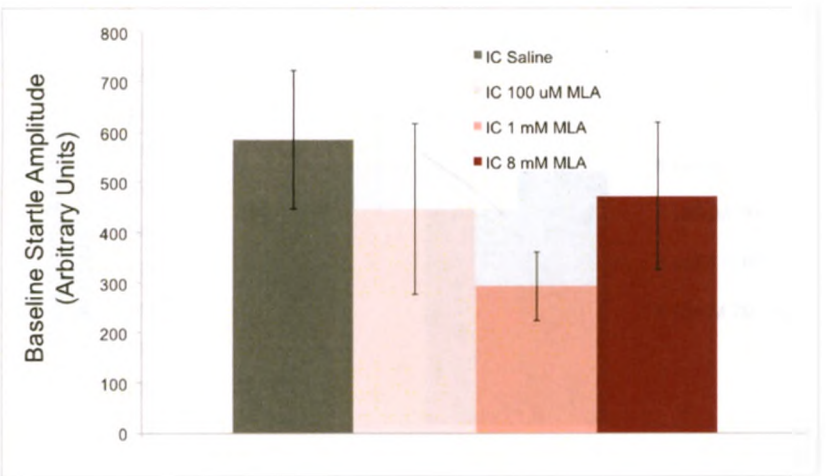
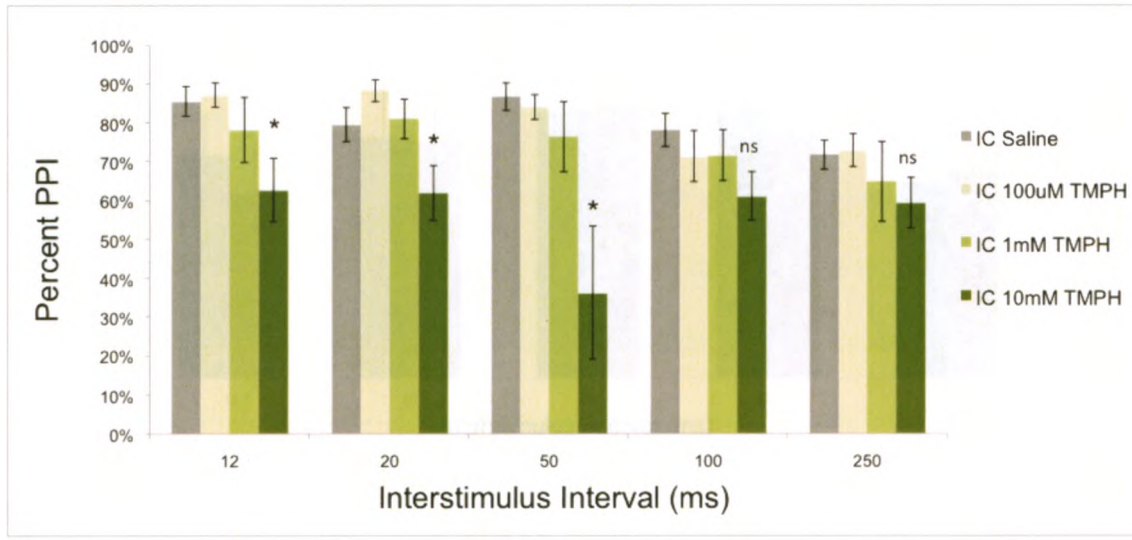


Figure 2.6 Effects of local injection of MLA into the PnC on PPI and baseline startle amplitudes. a) An ANOVA showed no significant effect of drug on PPI at any ISI for any concentration [ANOVA, $F(3, 20) = 1.90$; $p>0.05$]. b) There were no significant effects at any dose of MLA on baseline startle amplitudes, in comparison to saline controls [t-test, $n=6$; $p>0.05$]. A prepulse of 85dB was used to obtain maximal PPI scores.

a)



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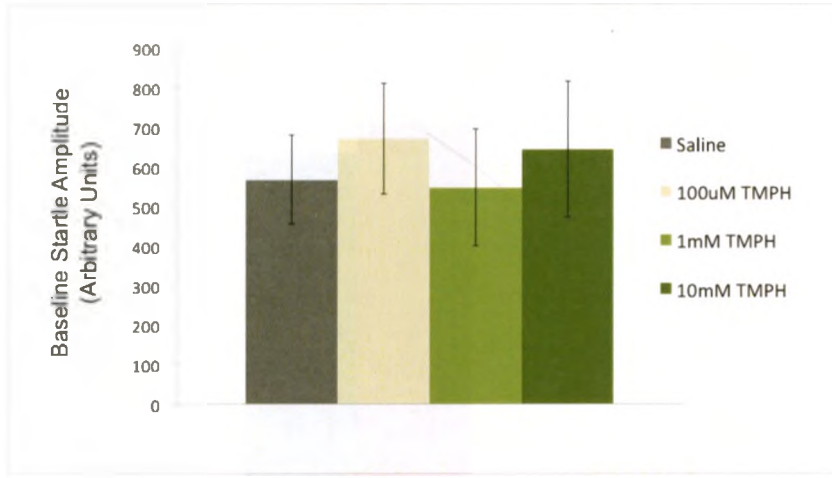
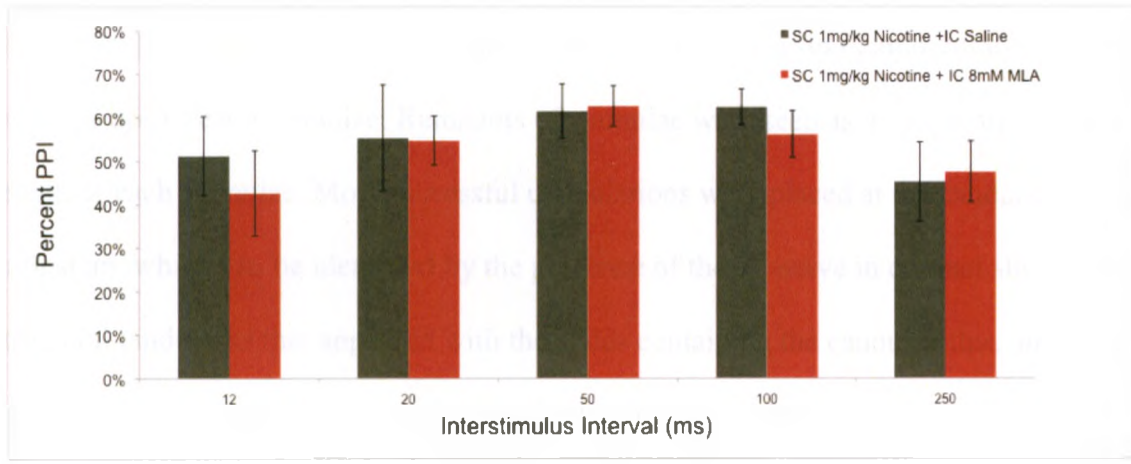


Figure 2.7 Effects of IC TMPH on PPI and baseline startle amplitude. One asterisk indicates a significant difference from controls of $p<0.05$. a) 10mM TMPH significantly disrupted PPI at the short ISIs of 12ms, 20ms, and 50ms and no significant differences appearing in the longer ISIs of 100ms and 250ms [ANOVA, $F(3, 36) = 115.89$; $p<0.0001$]. b) There were no significant effects at any dose of TMPH on baseline startle amplitudes, in comparison to saline controls [t-test, n=10; $p>0.05$]. A prepulse of 85dB was used to obtain maximum PPI.

a)



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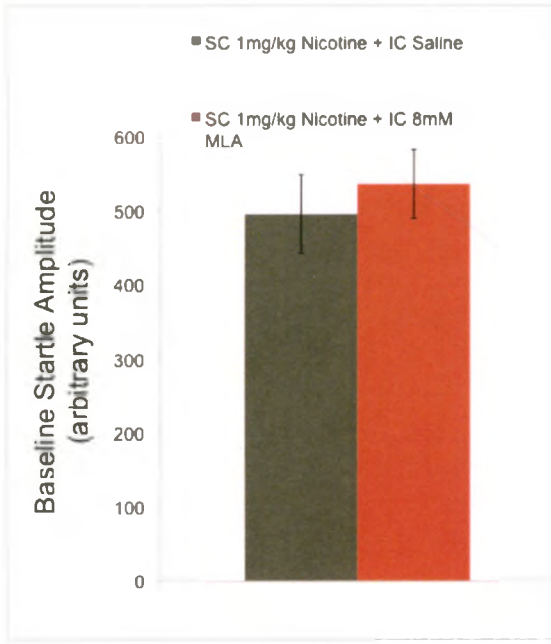

Figure 2.8 Effects of SC 1mg/kg nicotine + IC 8mM MLA on PPI and baseline startle amplitude. a) 8mM MLA did not significantly disrupt PPI at any ISIs [ANOVA, $F(1, 23) = 2.21$; $p>0.05$]. b) There were no significant effects at any dose of TMPH on baseline startle amplitudes, in comparison to saline controls [t-test, n=12; $p>0.05$]. A prepulse of 75dB was used to leave room for PPI enhancement.

2.3 Injection of thionine – confirmation of cannulation

Thionine stain was injected while rats were being sacrificed. A typical example of a histological section can be seen in **Figure 2.9.** We observed a small amount of dye spread from the tips of each cannulae. Remnants of cannulae were seen as minor scarring along the length of each cannulae. Most successful cannulations were placed at the caudal end of the brainstem, which can be identified by the presence of the 7^{th} nerve in coronal slices. Other structural landmarks that appeared with the slices containing the cannulae tips, and were used to confirm placement of the tips within the PnC, were the shape of the 4^{th} ventricle and the presence of the lateral superior olive.

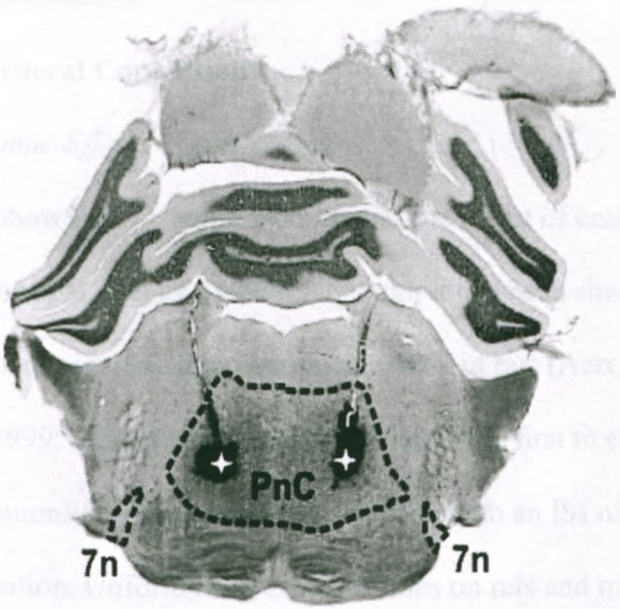

Figure 2.9 A micrograph of a 50um coronal rat brain section showing the PnC and the 7th nerve. The slice was stained using a common Haematoxylin and Eosin counterstaining procedure. The PnC and 7th nerve (a landmark structure to the PnC) are outlined with black dashes. White stars indicate tips of cannulae.

VI. Discussion

1. General Conclusions

Systemic Effects

We show that systemic nicotine has the effect of enhancing PPI of the acoustic startle response at a range of ISIs. Although it has been shown before in some studies that systemic doses of nicotinic agonists enhance PPI in rats (Acri, 1994; Curzon et al., 1994; Faraday et al., 1999; Schrieber et al., 2002), ours is the first to explore this question at multiple ISIs. Commonly, PPI is assessed in humans with an ISI of 100 ms, which elicits maximum inhibition. Unfortunately, most studies on rats and mice use the same ISI of 100ms, although maximum PPI can be observed around 50 ms ISI in rats and 30 ms ISI in mice (Yeomans at el. 2010). It is evident from our data that a lower dose of nicotine is effective at an ISI of 250ms while a 10X higher dose was more effective at shorter ISIs. By testing PPI at an ISI of 100 ms only, it is possible that some studies miss significant effects of drugs.

To our knowledge, this was also the first study to show significant PPI-enhancing effects of nicotine's main metabolite, cotinine, at a short ISI. Based on recent reports of the cognitive-enhancing effects of cotinine, it has been speculated by some that nicotine's cognitive enhancement may be due in part to the action of its main metabolite (Kyerematen et al., 1988; Drasdo et al., 1992; Sastry et al., 1995; Buccafusco & Terry, 2003; Terry et al., 2006). To more fully explore this hypothesis, higher doses of this neuroactive metabolite must be used to more accurately simulate actual blood-plasma concentrations that would occur after *repeated* nicotine consumption.

In this study, nicotine was injected a single time and PPI-enhancement was observed. The PPI-enhancing dose of cotinine given here (1mg/kg) would theoretically yield a plasma

concentration that would be much higher than the plasma concentration of cotinine resulting from the metabolism of a 1mg/kg dose of nicotine (the dose primarily used here). It can therefore safely be concluded that cotinine is not the major mediator in the nicotinic enhancement of PPI in this particular paradigm. The PPI-enhancement caused by cotinine may be due to the partial activity that cotinine has at nicotinic receptors (Dwoskin, 1999). This would be in accordance with the effects that have been found after acute administration of cotinine on information processing in humans (Herzig et al., 1998).

MLA, the α7 nAChR antagonist, partly reversed the PPI-enhancement caused by nicotine, suggesting a role for the α7 nAChR in this type of sensorimotor gating enhancement. However, since neither systemic MLA nor TMPH had any detectable effect on PPI alone, it can be hypothesized that different brain areas that modulate PPI and are affected by nicotine, may have opposing effects that result in zero-sum PPI modulation. For example, activation of the mPFC and the SN, which both receive cholinergic input and modulate PPI, have opposing effects on the PPTg which would result in the simultaneous increase and decrease of PPI (see Koch, 1999).

Intra-PnC Effects

Nicotine has a clear role in the mediation or modulation of PPI within the startle circuitry, as evidenced by its PPI-disruptive effects when injected into the PnC. It seems contradictory to our hypothesis at a first glance that local nicotine would inhibit PPI. However, the persistent presence of a high concentration of nicotine following local injection would lead to the inactivation of nicotine receptors in the PnC (Revah et al., 1991), and therefore to the occlusion of the effect of cholinergic transmission during PPI. Nicotine would therefore

mimic nicotinic antagonists. Furthermore, this disruptive effect was more pronounced at an ISI of 20 ms compared to an ISI of 100ms, suggesting that nAChRs mediate PPI at shorter ISIs.

In addition, intra-PnC injections of MLA had no effect of PPI, but injection of 10mM TMPH had significantly disruptive effects at all tested ISIs below 100ms. Therefore, we can conclude that non-α7 nAChRs are involved in the mediation of PPI, at short interstimulus intervals, at the level of the PnC. TMPH antagonizes the majority of subtypes found in non-α7 containing neural nAChRs, which include the α3, α4, β2, and β4 subunits (Damaj et al., 2005). Since the α4β2 subtype is the most common non-α7 neuronal nAChR, this is the most likely player here.

To our knowledge, this is the first study to show that nAChRs can have a specific role in the mediation of PPI at the level of the primary startle circuit. Furthermore, this study adds to the body of evidence implying that different mechanisms mediate PPI of the ASR at different ISIs (Yeomans et al., 2010; Jones & Shannon 2000b).

2. Methodological Issues

Drug specificity and kinetics

In rats, the metabolic half-life of nicotine is a 55-65 minutes (Kyerematen et al., 1988). This suggests that it is methodologically challenging to establish stable plasma concentrations of nicotine throughout behavioral testing, since testing takes approximately 45 minutes. One way to overcome this limitation was create a "rapid PPI" program with shorter acclimatization phases and inter-trial intervals. However this only mitigates the problem, and does not provide steady-state plasma concentration throughout testing. It is possible that by the end of a test session, especially with systemically applied nicotine, plasma concentrations will have dropped from original levels.

Although MLA shows high selectivity towards α7 nAChRs, it can also antagonize α4β2 and α6β2 nAChRs in very high doses (Drasdo et al., 1992). Since it is rather difficult to predict drug concentrations in neural areas that receive intracranial injections (due to the volume of diffusion, tissue metabolism, and blood perfusion), it is impossible to discern if the concentration of MLA in the PnC exceeded the dose that is selective to α7 nAChRs.

3. Role of nicotinic versus muscarinic receptors in PPI

Muscarinic and nicotinic AChRs are involved in PPI

Jones and Shannon have shown that at a systemic level, PPI is enhanced by muscarinic agonists and disrupted by variety of muscarinic antagonists (Levin et al., 1989; Jones & Shannon, 2000; Ukai et al., 2004). PPI disruptions by muscarinic antagonists has been termed anti-muscarinic syndrome and has become another animal model of sensorimotor gating (for review see Barak, 2009). Results produced here, and results from other studies have shown that systemic nicotine enhances PPI (Acri et al., 1994; Curzon et al., 1994; Faraday et al., 1999; Schrieber, 2002). Intra PnC injections of muscarinic antagonists have also been shown to disrupt PPI in rats (Fendt & Koch, 1999). Patch clamp recordings in the PnC by Bosch and Schmid further support a role for muscarinic receptors in PPI, and indirectly implicated a role for nicotinic receptors as well (Bosch and Schmid, 2008). Thus, there is strong indication that both muscarinic and nicotinic acetylcholine receptors play an important role in the mediation of PPI at a systemic level and at the level of the PnC.

Cholinergic modulation of PPI

Multiple brain areas that have been shown to modulate both startle and PPI receives cholinergic input or are cholinergic centers themselves. These areas include the basal lateral amygdala, the substantia nigra (SN), the hippocampus, the striatum, the medial prefrontal cortex (mPFC), the PPTg and the PnC (**Figures 1.1 and 2.11**). Any of these areas could be responsible for the systemic nicotinic and muscarinic effects on PPI. nAChRs have been found in high numbers on dopamine cells or terminals in some of these regions (i.e. the

substantia nigra and striatum) (Clarke & Pert 1985; Schwartz, 1986). Furthermore, it has been shown that nicotine stimulates dopamine release in the striatum, excites dopamine cells in the SN, and modulates dopamine transport function in the mPFC (Lichtensteiger et al. 1982; Clarke et al. 1985; Imperato et al. 1986; Rapier et al. 1988, Middleton et al, 2004). D2 dopamine receptor agonists such as apomorphine and quinpirole are shown to significantly modulate both startle and PPI. Mecamylamine, a nAChR antagonist causes PPI deficits and these deficits are reversed by D2 agonists (Levin et al., 1989) and potentiated by D2 antagonists (McGurk et al., 1987). Ichikawa et al. (2002) have shown that oxotremorine and scopolamine module dopamine release in the mPFC. Furthermore, it has been well established that cholinergic input from the PPTg mediates dopamine release in the SN in a calcium dependent manner (Marchi et al., 1991; for review see Zhou et al, 2003) and that this activity is controlled by muscarinic receptors (Scroggs et al., 2001). All this suggests there exists a strong interaction between the cholinergic and dopamine systems in the rat brain, and that dopamine may be involved in the nicotinic modulation of PPI. However, the precise neural area(s) involved and what mechanism(s) may mediate this systemic effect has not been fully resolved.

Differential role of nicotine and muscarinic receptors in PPI

Systemic (IP) injections of muscarinic antagonists disrupt PPI at long interstimulus intervals, but these antagonists do not disrupt PPI at short ISIs (Jones & Shannon, 2000b; Ukai et al., 2004). Conversely, we show here that nAChRs at the level of the PnC mediate PPI at short ISIs, and are not involved in PPI at longer ISIs. The temporal dissociation of PPI over two classes of receptors makes sense considering the activation and inactivation kinetics of the

respective receptor types. Nicotinic receptors are fast-onset ion channels and are therefore well-poised to mediate PPI at shorter ISIs. Since nicotinic channels are also very quick to desensitize, long-lasting inhibition by the prepulse would require longer-lasting effects. Muscarinic receptors are metabotropic receptors, therefore much slower to activate, and their activation leads to a biochemical process that is longer lasting. The fast onset of prepulse inhibition might therefore be mediated at least partly by nicotine receptors, while muscarinic receptors take over upon inactivation of nicotine receptors. In many ways, the cholinergic system is an ideal mechanism for the mediation of PPI since it has the benefit of consisting of two temporally distinct receptors that are both activated by the same ligand.

4. Potential mechanism mediating the nicotinic effect on PPI in the PnC

Nicotinic receptors are excitatory cation channels, however the data presented here suggests that nAChRs provide inhibition to startle mediating neurons in the PnC. Therefore, the involvement of inhibitory interneurons provides a likely explanation to the inhibitory role nAChRs play in the PnC. There is some evidence for the existence of inhibitory interneurons containing nAChRs in areas important to cognitive performance (Alkondon et al., 1998; Frazier, 1998; Azam et al., 2003). For example, the hippocampus contains GABAergic interneurons that receive cholinergic input and expresses the α7 nAChR on its presynaptic terminal (Alkondon et al., 1998; Frazier et al., 1998). Striatal interneurons have also been shown to express α4β2 heteromers on their presynaptic terminals (Azam et al. 2003). Furthermore, the human cerebral cortex possesses inhibitory interneurons that contain both α7 and α4β3 nAChRs at their presynaptic terminals (Alkondon et al. 2000).

Recent patch clamp recordings showed that hyperpolarization of PnC giant neurons induced by GABA agonist muscimol can be reversed by GABA antagonist bicuculline (Yeomans et al., 2010). Furthermore, unpublished data from our laboratory show that glycine strongly inhibits PnC neurons. This suggests that GABAergic and/or glycinergic inhibitory interneurons may be present in the PnC and that they may be activated by nicotine. Thus, a hypothetical PPI pathway outlining the mechanics of the PPTg-PnC inhibitory projection has been proposed below (**Figure 2.12**). It should be said that this is a hypothetical pathway on which future studies may be based to test the proposed pathway.

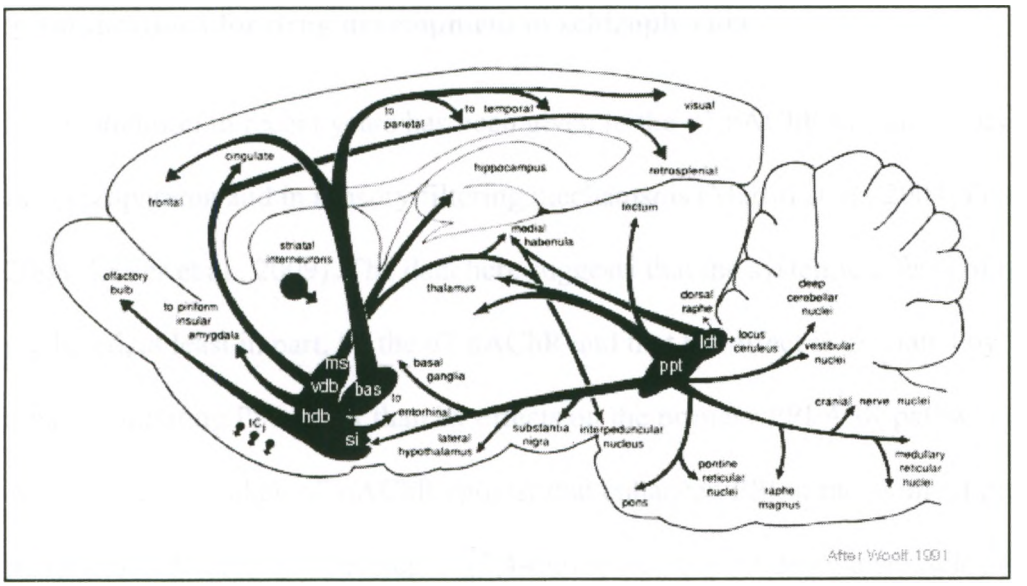

Figure 2.10 Cholinergic centers of the rat brain (after Woolf, 1991).

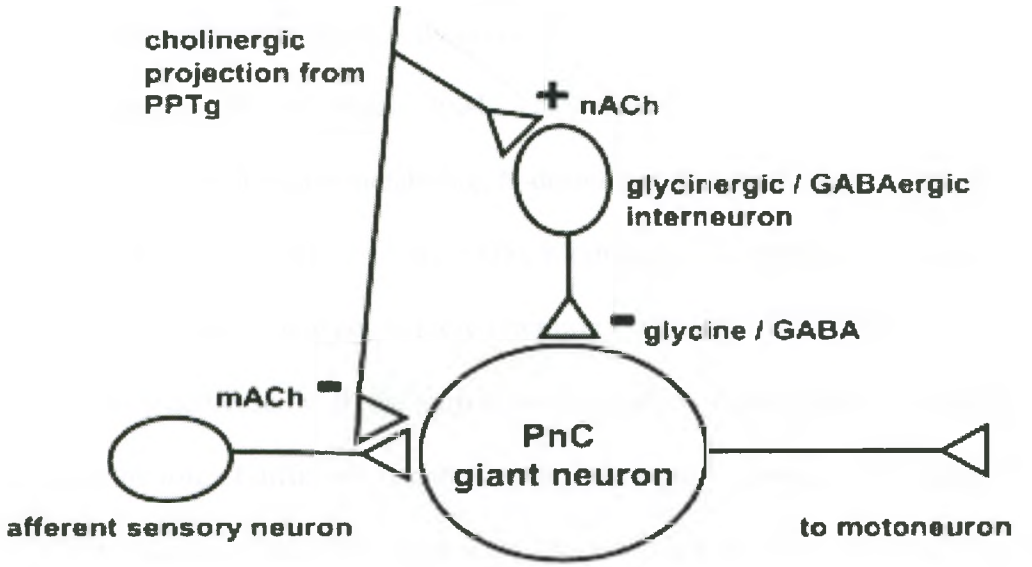

Figure 2.11. Hypothetical cholinergic mechanisms mediating PPI in the PnC. Muscarinic acetyl choline receptors = mACh, nicotinic receptors = nACh.

5. Implications for drug development in schizophrenia

Much attention in recent years has been given to the α7 nAChR because of its implications in schizophrenia and in sensory filtering mechanisms (Martin et al., 2004; Freedman et al., 2008; Leiser et al., 2009). The data here suggests that the systemic effects of nicotine are mediated, at least in part, by the α7 nAChR and that the effect is mediated by higher brain areas modulating PPI rather than by effects on the primary PPI/ASR pathway. In 2000, Adams et al. found an α7 nAChR agonist that enhanced PPI in rats without producing any peripheral side effects. The drug, 3-(2,4-dimethoxy-benzylidene) anabaseine (DMXB-A), has entered phase 2 clinical trials and it has shown to improve attention, visual learning, working memory, and problem solving compared to placebo in schizophrenic patients (Freedman et al., 2008). In addition to this, a well-known and often used atypical antipsychotic, clozapine, is the only known medication that partially relieves cognitive deficits in patients (Weiner et al., 2004). The reason for this is not clear, however it may be due to the fact that its main metabolite, N-desmethylclozapine, activates muscarinic receptors (Sur et al., 2003; Li et al., 2005). Modulation of cholinergic receptors provide a potential to alleviate some cognitively crippling symptoms of schizophrenia that are insufficiently addressed with the current medications available. More research is needed to elucidate the role of different receptor subtypes in sensory gating, so that drug developers can tailor drugs more precisely. The work presented here may help to shed some light on what receptor subtypes mediate and modulate PPI in specific areas of the brain.

VII. FINAL CONCLUSIONS

1. Summary

Here we investigated the role nicotinic acetylcholine receptors (nAChRs) play in prepulse inhibition (PPI) of the acoustic startle response (ASR) in rats. The results gathered here can be summarized in point form:

1. No effect of MLA or TMPH systemically on PPI
2. Systemic Nicotine enhances PPI
3. Systemic MLA reverses systemic nicotine enhancement of PPI but TMPH does not
4. Intra-PnC nicotine disrupts PPI
5. Intra-PnC MLA has no effect on PPI
6. Intra-PnC TMPH disrupts PPI
7. Intra-PnC MLA has no effect on systemic nicotine enhancement of PPI

Systemic injections of nicotine (a potent nAChR agonist) enhanced PPI. Systemic methyllycaconitine (MLA) significantly reversed nicotine-enhanced PPI suggesting a role for the specific nAChR subtype that MLA targets - the α7 nAChR. Systemic administration of 2,2,6,6-tetramethylpiperidin-4-yl heptanoate (TMPH) had no effect on PPI in either nicotine-enhanced or normal conditions suggesting no significant involvement of non-α7 nAChRs (TMPH targets most non-α7 nAChR subtypes).

This data is counter-pointed with intra-cranial injections of nicotine and the above mentioned antagonists. These injections targeted the main area of the startle circuit, the PnC (caudal pontine reticular nucleus). Intra-PnC injections of nicotine significantly disrupted

PPI, especially at shorter interstimulus intervals (ISIs) (below 100ms). This suggests that the effects of systemic nicotine are not mediated by the PnC. Intra-PnC injections of MLA yielded no significant differences in PPI scores, whereas intra-PnC injections of TMPH disrupted PPI significantly at shorter ISIs. This suggests that α7-nAChRs do not mediate PPI at the level of the PnC, but rather non-α7 nAChRs (perhaps the α4β2 subtype) are responsible.

2. Implication for the current model of PPI

The current model of PPI has only muscarinic and GABAergic inhibition coming from the pedunculopontine tegmental nucleus (PPTg) to the PnC. Furthermore, muscarninic and GABAergic receptors are thought to mediate PPI at longer ISIs. Our work adds to the current model of the PPI circuitry by suggesting that non-α7 nAChRs may play a direct role in PPI at the level of the PnC, especially at shorter ISIs. Although it is less clear what mechanisms are mediating the systemic effect of nicotine on PPI, this data suggests that α7 nAChRs are involved, perhaps, they may play a role in modulating dopamine release. Our findings are important because they may help in the development of pharmaceuticals that could be used to treat cognitive deficits in schizophrenia and a variety of other psychiatric disorders that impair cognition.

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Western

March 2, 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:
Mechansims Underlying Habituation and Prepulse Inhibition of Startle
Funding Agency: NSERC Discovery Grant - Applied For

has been approved by the University Council on Animal Care. This approval is valid from **March 2, 2008 to March 31, 2008**. The protocol number for this project is **2008-010-03**.

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	2-8 months Male	D	860
Rat	Wistar	2-12 months M/F	D	4
Rat	Wistar	2-8 months M/F	D	160

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
330 Post-Operative Care/Rodent
343 Surgical Prep/Rodent/Recovery Surgery

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 Protocol - S. Schmid, W. Lagerwerf
Approval Letter - W. Lagerwerf

03/12/08

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

April 1, 2009
*This is the **1st Renewal** of this protocol
*A Full Protocol submission will be required in **2012**

Dear Dr. **Schmid:**

Your Animal Use Protocol form entitled:

Mechanisms underlying habituation and prepulse inhibition of startle

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

This approval is valid from **April 1, 2009** to **March 31, 2010**

The protocol number for this project remains as **2008-010**

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.

c.c. Approved Protocol - S. Schmid, W. Lagerwerf
Approval Letter - S. Schmid, W. Lagerwerf

The **University** *of* **Western Ontario**
Animal Use Subcommittee / University Council on Animal Care
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