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Functional Investigation Of A Brainstem Excitatory Connection Relevant To Sensorimotor Gating

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Dedicated to my Mother, Lorena A. Aguirre Orpinel.

FUNCTIONAL INVESTIGATION OF A BRAINSTEM EXCITATORY CONNECTION RELEVANT TO SENSORIMOTOR GATING

By

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THESIS

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Abstract

To focus attention, our brain has to "gate" or block irrelevant sensory information that could lead to brain overload. This is done by way of a neuronal pre-attentive mechanism termed sensorimotor gating (SG). Therefore, deficits in the SG mechanism prevent patients from focusing attention. SG deficits have been observed in patients suffering from various neurological disorders, and it is a hallmark of schizophrenia. Previous work has identified key brain areas, such as the pedunculopontine tegmental nucleus (PPTg), that send inputs to the brainstem to regulate SG. However, there is still a knowledge gap concerning what cell types are involved and what other brain areas could potentially contribute to SG. Our objective in this study is to further identify the cell types located in the PPTg that contribute to SG. The caudal pontine reticular nucleus (PnC) is the brainstem area at the center of the SG circuitry. It has been long known that the PPTg contains cholinergic, glutamatergic and GABAergic neurons and sends direct inputs to the PnC, modulating SG. Recently, the contribution of cholinergic neurons to SG has been shown to be minimal. Therefore, it is not known whether other PPTg neurons project to the PnC and whether they contribute to SG. We Investigated the role of the PPTg glutamatergic inputs onto the PnC in the context of SG, which had not been demonstrated before.

To test our hypothesis, we used neuronal dyes to label cellular pathways, immunohistochemistry to reveal cellular neurochemistry and *in vivo* optogenetic to functionally study the contribution of the PPTg-PnC glutamatergic connection to SG. Our data show for the first time that there is a direct glutamatergic connection between the PPTg and the PnC, and that it does contribute to SG *in vivo.*

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Chapter I: Introduction

I.1. Sensorimotor Gating

Sensorimotor gating (SG) is a neuronal mechanism that helps us to filter out irrelevant sensory information before we consciously focus attention (Logan, 1992; Swerdlow, 1996; Swerdlow 1999). Deficits in SG have been reported in people suffering from various neurological disorders such as schizophrenia, obsessive compulsive disorder (OCD), attention-deficit/hyperactivity disorder (ADHD), Huntington's disease (HD), Tourette's syndrome, post-traumatic stress disorder (PTSD), and autism spectrum disorders (ASD) (Geyer and Braff, 1987; Swerdlow et al., 1993; Swerdlow and Geyer, 1998; Swerdlow et al., 2001; Kohl et al., 2013; Sinclair et al., 2017). This pre-attentive mechanism deficit is a true problem since there is no known cure for it, meaning that people's social insertion and cognitive performance will be impaired for an undefined amount of time. SG deficits are a hallmark of schizophrenia, which affects 21 million people worldwide currently, according to the World Health Organization. Since SG deficits are a burden for millions of people, efforts must be done in order to better understand the neuronal pathways and elements that underpin SG.

I.2. The Acoustic Startle Response and Prepulse inhibition

Both in laboratory and clinical settings, SG can be measured by the prepulse inhibition (PPI) of the startle reflex task (Ison and Hammond, 1971; Geyer and Braff, 1982; Koch et al., 1993; Swerdlow et al., 2001). As an operational measure of SG, PPI can be measured in both humans and animal models (Graham, 1975; Braff et al., 1978; Swerdlow et al., 2001; Bosch and Schmid, 2006). As seen in **Figure 1.1**, The PPI task is performed by presenting a non-startling sensory stimulus (also termed "pre-pulse") prior to a startling sensory stimulus ("pulse"), to a subject. The non-startling stimulus can diminish the startle response elicited by the startling stimulus. The diminished startle response is what is known as prepulse inhibition of the startle response. PPI can be measured using different sensory modalities (Fendt et al., 2001), and since it is an operational measure of SG, it is impaired in people suffering from schizophrenia. Thus, in disease states, a reduction in PPI is observed and results in a significant startle response even when a non-startling stimulus precedes the startling stimulus. Since PPI can be measured across species, many animal models of disease have been used to study SG deficits. Despite this, there are still gaps in the understanding of the SG neuronal circuitry and efforts are still needed towards developing

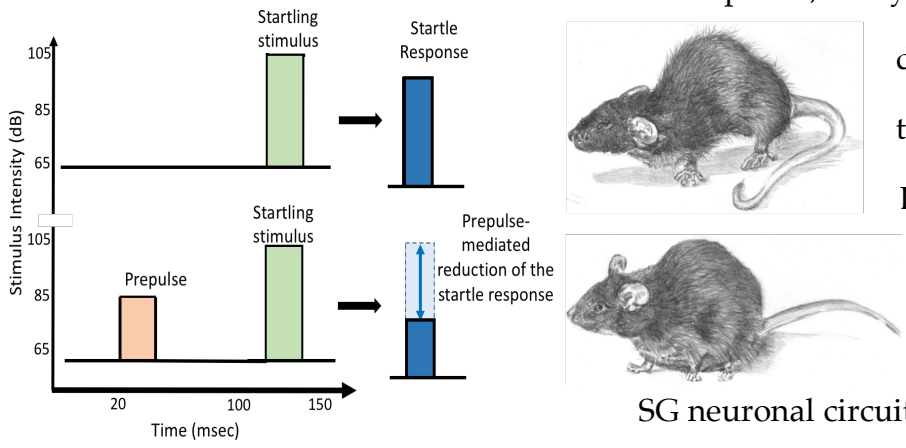

Figure 1.1. **The Prepulse inhibition of the startle reflex task.** The Top panel shows the startle reflex when only the startling stimulus is being presented, and the Bottom pane shows the prepulse before the startling pulse and showing a % reduction in PPI.

therapeutic strategies for pre-attentive deficits.

The previous PPI behavioral studies mostly employed tactile or auditory sensory stimuli. Previous published work using acoustic sensory stimulations allowed us to better understand the neural circuitry mediating SG. The acoustic startle reflex (ASR) is elicited when a loud startling sound enters the ear, stimulates the hair cells in the cochlea activating the cochlear nuclei. The cochlear nuclei then innervate the giant glutamatergic neurons in the brainstem caudal pontine reticular nucleus (PnC) which then activate the spinal cord, cranial, and facial motor neurons causing a startling response as seen in **Figure 1.2** (Lingenhöhl and Friauf, 1994; Swerdlow et al., 2001; Fendt et al., 2001; Bosch and Schmid, 2006). This pathway is short; it only takes a few milliseconds for the stimulus to travel from cochlear sensory receptors to the startling response. During PPI, a non-startling stimulus is presented a few milliseconds prior to the startling stimulus. This non-startling stimulus will activate different brain regions before ultimately converging to the startling stimulus pathway. That is, a non-startling stimulus will first activate the cochlear nuclei, but then, the inferior and superior colliculi will be activated which will lead to the activation of the pedunculopontine tegmental nucleus (PPTg). Once activated, the PPTg was thought to be responsible for the inhibition of the PnC giant glutamatergic neurons as seen in **Figure 1.2** (Davis et al., 1982; Swerdlow et al., 2001; Fendt et al., 2001). Once inhibited by the PPTg, the subsequent activation of PnC by a startling stimulus leads to a smaller startle reaction compared to the startle response induced by the pulse presented alone (Koch et al., 1993). Even though much is known about the brain regions underlying PPI, there is a

large knowledge gap in regards of the chemical nature of the anatomical connection between the PnC and the PPTg, which is thought to be at the heart of SG.

I.3. The PPTg-PnC connection and neurotransmitters involved

The PPTg is a major cholinergic center in the lower brain (Saper and Loewy, 1982; Armstrong et al., 1983; Rye et al., 1987), but also contains glutamatergic (Clements and Grant, 1990; Koch et al., 1993; Wang and Morales, 2009) and GABAergic neurons (Swerdlow et al., 2001; Fendt et al., 2001; Wang and Morales, 2009; Kroeger et al., 2017; Mena-Segovia and Bolam, 2017). The PPTg has been mainly studied due to his involvement in arousal and modulation of sleep/wale cycles, sensorimotor gating, but

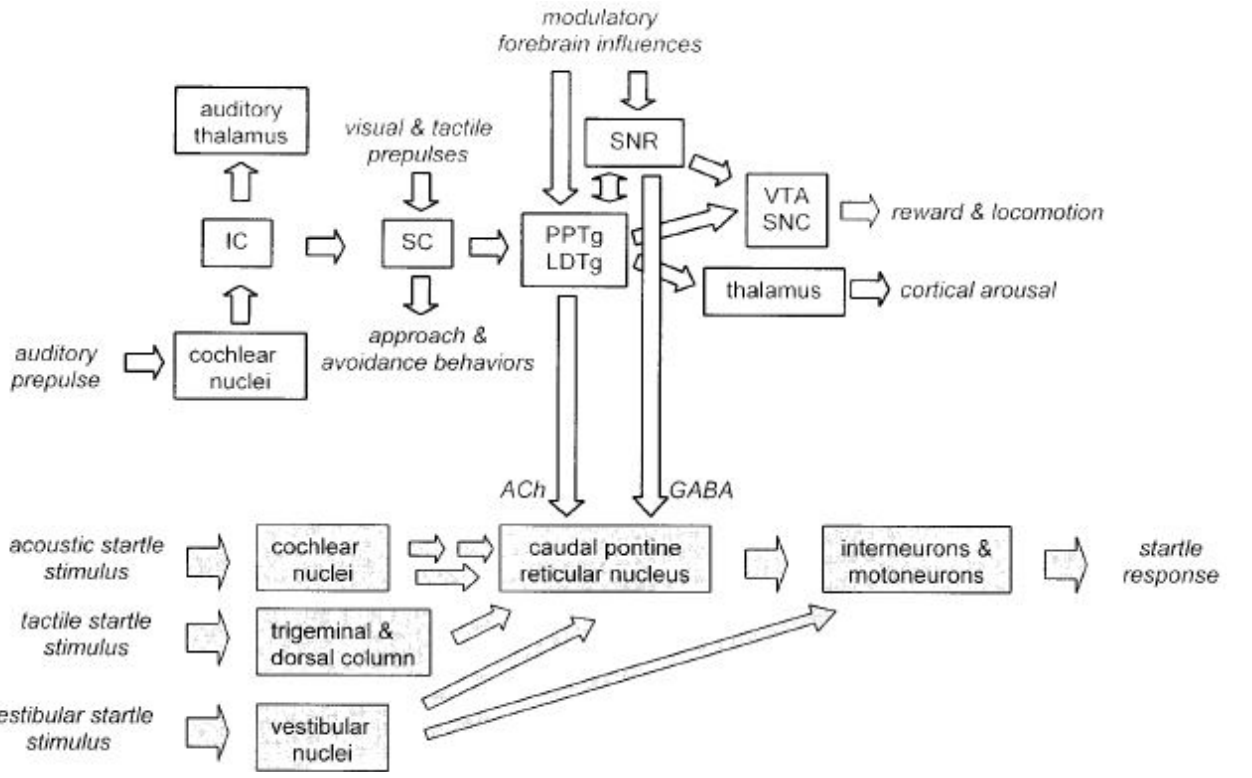

Figure 1.2. **The Acoustic Startle Pathway, the PPI pathway, and the areas that modulate and regulate PPI.** The Top of the figure shows the brain regions that are activated by the prepulse who mediate PPI. The regions send their inputs to the PPTg/LDTg or the substantia nigra pars compacta (SNR) which send cholinergic and GABAergic projections to the PnC, respectively. The Bottom of the figure shows the pathways that the ASR follows through different startling stimuli. If a prepulse reaches the PPTg before a startling stimulus had the time to reach the PnC, then the prepulse-induced inhibition of the PPTg will lead to a decreased startle response. (Fendt, et al., 2001)

not much of the PPTg is studied in its involvement on motor systems. Some studies have shown that the PPTg sends has direct connections with the mesencephalic locomotor region (MLR), first described by Shik et al., (1966) and following studies showed that activation of the PPTg in decerebrate cats elicited movement (Garcia-Rill et al., 1987). Several studies done in rats by Bosch and Schmid have been performed to understand how the PPTg inhibits the PnC. Initially, these studies suggested that the cholinergic neurons are the only neurons responsible of the inhibition of the startle response by activating muscarinic receptors (M2 and M4) on PnC neurons (Bosch and Schmid, 2006, 2008). In fact, during the PPI protocol performed on an *in vitro* slice preparation, the activation of PPTg fibers led to the inhibition of PnC neuronal activity. Interestingly, under these *in vitro* conditions, PPI only occurred when the prepulse and the pulse were separated by the specific inter-stimulus intervals (ISIs) of 300 and 1000 ms. To confirm the contribution of the muscarinic receptors to PPI induced by the PPTg stimulation, the muscarinic receptor antagonist scopolamine was added to the PnC slice preparation. Surprisingly, scopolamine abolished the *in vitro* PPI phenomenon only at the 1000 ms ISI, but not at the 300 ms ISI inhibition. From these results, it was thought that different neurotransmitters and receptors might contribute to PPI, depending on the ISI separating the pulse from the prepulse (Bosch and Schmid, 2008). Another argument supporting this hypothesis was the fact that disrupting the cholinergic projections linking the PPTg to the PnC only decreased PPI, without abolishing it. The results from these earlier studies made us suggest that there must be other PPTg neurons (and neurotransmitters) and/or other brain areas contributing to the inhibition of the PnC during PPI.

Recently, elegant studies further demonstrated the contribution of other neurotransmitters to PPI, such as glutamate. First, it was shown that a non-selective

damage to all the neuronal types within the PPTg reduces PPI. In contrast, the selective disruption of the PPTg cholinergic neurons did not affect PPI (MacLaren et al., 2014). Then, in a gene sequencing study, it was found that the zebrafish *Gsx1* gene, which is essential for proper PPI, is present in PPTg glutamatergic neurons in mice. Such studies demonstrated that the neurons expressing the *Gsx1* gene are equivalent to the PPTg glutamatergic neurons in mice, and that they synapse onto the zebrafish Mauthner neurons, which are equivalent to the PnC giant glutamatergic neurons (Bergeron et al., 2015). This study used a simple animal model to suggest a cross-species occurrence where a PPTg-PnC glutamatergic connection that is involved in PPI occurs. Other studies showed that the activation of group III metabotropic glutamate receptors in the PnC *in vivo* and *in vitro* with ₗ-AP4 strongly inhibits the giant glutamatergic neurons (Schmid et al., 2010). The glutamatergic contribution to PPI was shown to come from the medial pre-frontal cortex (mPFC) (Valsamis et al., 2014). In addition to glutamate, the neurotransmitter GABA was also suggested to play a role during PPI. A rodent study showed a significant GABAergic contribution to PPI at the level of the PnC by applying different GABA receptor antagonists. Indeed, following bicuculline *in vivo* injection, a decrease of PPI was observed at different ISIs (Yeomans et al., 2010). Furthermore, many other studies have been done to identify other brain areas that have a regulatory influence in PPI (Davis and Gendelman, 1977; Fendt et al., 2001). Such studies have demonstrated that areas such as the PFC, the hippocampus (HPC), the basolateral amygdala (BLA), and the nucleus accumbens (NAC) among others, are areas that modulate PPI as seen in Figure 2 (Swerdlow et al., 2001; Fendt et al., 2001; Miller et al., 2010; Kohl et al., 2013). The prefrontal cortex has shown to send glutamatergic inputs to the PnC involved in PPI that have been studied in rats. The study reports performing NMDAR antagonist injections in the PnC, and the disrupted

PPI was reversed with NMDAR agonist injections in the mPFC *in vitro* (Valsamis et al., 2014).

While no direct anatomical connection has been described between the HPC and the PnC, it has been shown that HPC is a modulator of PPI via different neurotransmitters. Studies performed in rats showed the cholinergic receptor agonist carbachol into the HPC and it affected PPI (Caine et al., 1991), in addition to showing a glutamatergic component where NMDAR agonists applications in the vHPC inhibited PPI (Wan et al., 1997). It has been known that the Amygdala is involved in modulating PPI, and emerging research is showing a direct anatomical connection between the basolateral amygdala (BLA) and the PnC (preliminary data). The modulation shown has been described by lesion studies in rats with quinolinic acid in the BLA showing an inhibited PPI. In addition, glutamatergic, dopaminergic and GABAergic components from the BLA have been described by applying $GABA_A$ receptor antagonist picrotoxin and NDMAR antagonists dizocilpine into the BLA and inhibiting PPI, with a reversal effect seen with a systemic injection of the dopamine antagonist haloperidol (Fendt et al., 2001).

I.4. Why should we study the PPTg-PnC glutamatergic pathway?

Even though it is clear that more research efforts are needed to understand the contribution from all the previously mentioned areas into PPI, the focus of my project is to understand how the PPTg inhibits the PnC and to identify if glutamatergic neurons from the PPTg might play a role in the inhibition of the PnC during PPI. The PPTg was first described in humans in 1909 (Jacobsohn, 1909; Rye et al., 1987) and even though much is known about it, there are currently no studies that have focused on the function of the glutamatergic PPTg neurons in the context of SG. The PPTg forms part of the

extrapyramidal motor system (Saper and Loewy, 1982) and plays a key role in the modulation of arousal and behavioral states in higher brain centers (Fendt et al., 2001; Eban-Rothschild et al., 2016; Kroeger et al., 2017; Mena-Segovia and Bolam, 2017). The PPTg contributes to the modulation of PPI of auditory and tactile sensory modalities. As mentioned before, damages to the PPTg cause PPI disruptions, and patients with schizophrenia have been found to have an increased number of PPTg cholinergic neurons in humans (Karson et al., 1991). Furthermore, emerging research is suggesting that the cholinergic neurons from the PPTg projecting to the PnC might not contribute to PPI at all (Maclaren et al., 2014; Valsamis et al., 2014). As mentioned above, the PPTg contains several neuronal subtypes (Wang and Morales 2009) and, from tracing studies, it was shown that the different PPTg neuronal subpopulations likely target similar sites.

Therefore, we have hypothesized that excitatory glutamatergic neurons from the PPTg might project to the PnC and contribute to PPI by activating, among other PnC glycinergic interneurons. We could then speculate that once activated, these PnC glycinergic interneurons could inhibit the PnC giant glutamatergic neurons and reduce startle *in vivo*. If such PPTg-PnC glutamatergic connection is present and contributes to PPI, a new way to study SG could emerge and potentially lead the way to the development of therapeutic interventions to treat diseases associated with SG deficits.

I.5. Specific Aims

The proposed *Specific Aims and experiments* were designed to further investigate the glutamatergic connection between the PPTg and the PnC using WT mice:

I.5.1. Specific Aim 1

To characterize the glutamatergic connection between the PPTg and the PnC. Here, the goal was to identify and precisely locate the PPTg non-cholinergic (likely glutamatergic) cell bodies in the caudal portion of the PPTg that sends projections to the PnC. To do so, I injected a retrograde neuronal tracer and a retrograde virus into the PnC and immuno-labelled the traced cell bodies within the PPTg. I also labeled and visualized the PPTg neuronal fibers terminating within the PnC.

I.5.2. Specific Aim 2

To establish how PPTg-PnC glutamatergic synapses underpin SG *in vivo*. Here, using a cannula-guided fiber optic in mice injected with inhibitory opsins, I performed the prepulse inhibition (PPI) task *in vivo*. In some trials, I photo-silenced the PPTg-PnC glutamatergic connection already identified in Aim1. Following the behavioral testing, the brain was extracted and analyzed using immunohistochemistry assays to double-label the targeted neurons and confirm the extent of the viral injection and cannula placement.

Chapter II: Experimental Approach

II.1. Specific Aim 1 Experiments

Specific aim 1: To characterize the glutamatergic connection between the PPTg and the PnC.

Experiment 1.1: I injected the retrograde neuronal tracer Fluorogold (FG) or the adeno-associated retrograde viral particles driving the expression of the tdTomato protein under the control of the CAG promoter (AAVrg-CAG-tdTomato) into the PnC, where the PPTg axon terminals are present and take up the tracer molecules or virus and back-fill the cell bodies within the PPTg. The labeling strategy can be seen in **Figure 2.1**.

Experiment 1.2: In a subset of mice, I injected either the tracer cholera toxin sub-unit B (CTB) (can be used both as an antero-and retrograde tracer) or the adeno-associated viral particles driving the expression of the mCherry protein under the control of the CaMKIIα promoter (AAV-DJ-CaMKIIα-mCherry) into the PPTg. Because these tracers travel in an anterograde fashion, they should label the PPTg neuronal cell bodies, and also the extent of the axonal fibers/terminals expressing mCherry within the PnC. The labeling strategy can be seen in **Figure 2.1**.

Experiment 1.3: I performed immunohistochemistry on the extracted brain tissue cut into thin sections. More precisely, I treated these sections with the antibodies of the proteins that we wished to target (described in more details below) to double-label the cell bodies that were already made fluorescent by the neuronal tracers injected.

II.1.1. Objective and Overview

Neuronal labeling studies in rats previously identified a direct anatomical connection between the PPTg cholinergic fibers and the PnC (Koch et al., 1993), and such connection was initially thought to be the central contributor of sensorimotor gating. Subsequent rodent *in vitro* studies demonstrated that this connection provides an inhibitory influence on PnC neurons, but that other neurotransmitter systems also contribute to PPI (Bosch and Schmid 2006; Bosch and Schmid 2008; Schmid et al., 2010; Yeomans et al., 2010; Valsamis et al., 2014). Importantly, *in situ* hybridization and immunohistochemical analyses provided evidence for the presence of glutamatergic neurons within the PPTg (Clements and Grant, 1990; Wang and Morales, 2009). Although PPTg glutamatergic neurons have also been shown to encode locomotor state and speed (as a puitative part of the mesencephalic locomotor region), their function has mainly been studied in the context of arousal (Roseberry et al., 2016; Kroeger et al., 2017; Mena-Segovia and Bolam, 2017). Therefore, whether the PPTg glutamatergic neurons are anatomically connected to the PnC and contribute to PPI is still unknown.

The objective of Specific Aim 1 was to identify and characterize a PPTg-PnC glutamatergic connection. I first injected retrograde and anterograde neuronal tracers/viral particles in the PnC and PPTg, respectively, and analyzed the fluorescence of fibers and cell bodies in both regions as seen in **Figure 2.1**. Secondly, I used immunohistochemistry to identify the chemical nature of the labeled neurons. The data generated from experiments in *Specific Aim 1* was needed to allow me to pursue my second Aim, consisting of evaluating the functional contribution of this connection to PPI *in vivo*. Such anatomical connection provides information and can fill a gap in the understanding of the modulation of PPI and sensorimotor gating by the PPTg. Moreover, this knowledge can potentially give us an understanding of this faulty mechanism characterized in people with neurological disorders.

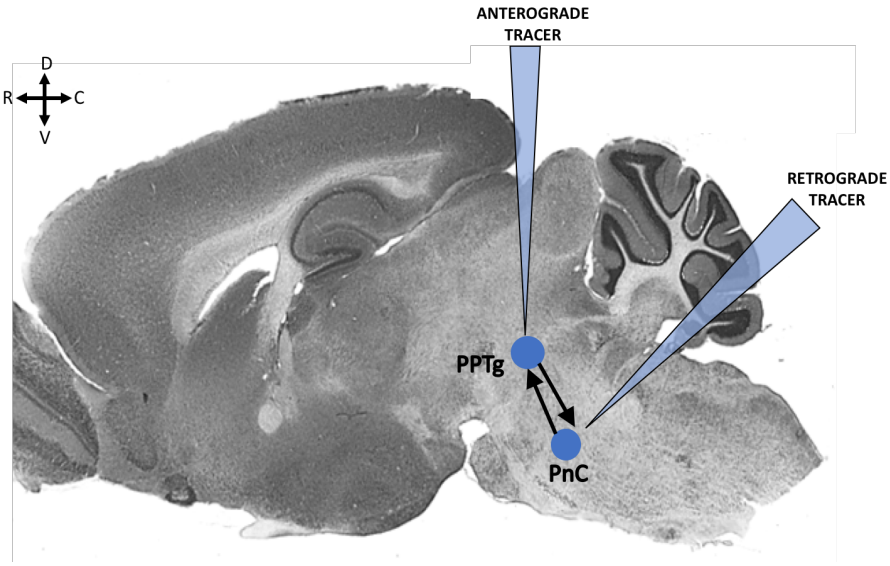

Figure 2.1. **Neuronal labeling strategy.** The injection sites of each neuronal tracer used and the targeted regions, including the tracing direction are seen in the scheme. This is the technique that was used for *Specific Aim 1.*

II.1.2. Materials and Methods

Animals: Adult C57CBL/6 male mice aged between 1-2 months (Jackson Laboratories) housed in the University of Texas at El Paso (UTEP) vivarium with controlled temperatures and a 12-hour light / dark cycle have been used for these

experiments. All protocols were conducted in compliance of the UTEP Institutional Animal Care and Use Committee (IACUC).

Surgery / neuronal tracer injections: All injections were done with a pressure micro-injector (Quintessential Stereotaxic injector, Stoelting Co., IL). For the retrograde labeling experiments, mice were injected unilaterally with 0.1 μL of the tracer Fluorogold (FG; 2% in 9% Saline, Fluorochrome, Denver, CO) or the retrograde virus AAVrg-CAG-tdTomato (Addgene viral prep # 59462-AAVrg) using pulled glass micropipettes targeted at the **PnC** with the following stereotaxic coordinates relative to Bregma: AP: -5.34 mm, ML: +7.0 mm, DV: -5.35 mm (Paxino's and Franklin, 2004). For the anterograde labeling experiments, mice were injected unilaterally with either 0.3 μL total volume (2 injections of 0.15 μL each) of the Cholera Toxin Subunit B (CTB; dilution: 0.5 μg/μL, Millipore) tracer or the AAV-DJ-CaMKIIα-mCherry viral particles using pulled glass micropipettes targeted at the **PPTg**: coordinates are: **#1** AP: -4.84 mm, ML: +1.3 mm, DV: -3.75; and **#2** AP: -4.84 mm, ML: +1.3 mm, DV: -3.5mm. The mice were anesthetized with 1.5% isoflurane (Vedco, Saint Joseph, Missouri) via inhalation through a nose cone attached to the stereotaxic platform head holder for the entire duration of the surgery. A seven days recovery period allowed maximum transport of FG and CTB and a four week-period was used for the viral particles proliferation. The mice were then anesthetized with isoflurane and exsanguinated by transcardial perfusion with about 200 mL 9.0% saline solution followed by chilled 4.0% paraformaldehyde (PFA) solution in phosphate buffered saline (PBS). After the perfusion, the animals were decapitated and the brains were extracted and harvested by post-fixing it in 30% sucrose in PFA for 12-18 hr. The brains were drained of excess sucrose, and frozen in super cooled hexanes and stored at -80°C. Then, the brains were

sectioned on a Leica CM3050 S Cryostat (Leica, Wetzlar, Germany). The sections were cut into five 1-in-5 series throughout the extent of the PnC and PPTg at a thickness of 30 μm in the coronal plane of section in order to survey the whole area in which the PPTg and PnC areas are present, since the areas are in a short distance from one another. Sections were stored in cryoprotectant solution (50% 0.05 M phosphate buffer, 3-& ethylene glycol, 20% glycerol) at -20°C until plated for visualization.

Histological Analyses: The viral constructs contained the reporter gene mCherry, eYFP or tdTomato which are translated into fluorescent proteins. However, using immunohistochemistry, I enhanced these protein's fluorescence for optimal visualization/labelling. The brain sections that contained CTB had their fluorescence enhanced since the tracer works stronger as a retrograde tracer and weaker as an anterograde one. Additional antibodies were used to double-label the cells expressing the dyes and reveal their identities. Appendix 2 lists the primary and secondary antibodies, and the reagents used to enhance fluorescence. Fluorogold is not considered in the table since it already expresses fluorescent properties and does not require antibodies for visualization. The brain sections were rinsed off the cryoprotectant solution for 5 times for 5 minutes each using Tris-buffered saline (TBS; pH 7.4 at room temperature) and incubated in Blocking solution (NDS) (2% normal donkey serum; EMD-Millipore, Billerica, MA; catalog #S30-100ML; lot NG1827420 and 0.1% Triton X-100; Sigma-Aldrich, St. Louis, MO; catalog #T8532 in TBS) for 2-3 hr at room temperature. The brain slices were collected and separated into two groups depending on whether they contained the PPTg or the PnC. The PPTg sections were treated with the antibody against Choline Acetyltransferase (ChAT) since until now, labeling PPTg Cholinergic neurons with such antibody still constitutes the best way to delineate its borders. The PnC sections were treated with the antibody against the Vesicular

Glutamate Transporter type 2 (VGluT2) in an attempt to label and visualize excitatory synapses. The antibodies used were dissolved to their working dilutions within the blocking solution (NDS). Slices were incubated in combination of the primary antibodies for 3 Days at 4°C. The slices were then washed off the primary antibody in TBS for 5 times for 5 minutes each and incubated in combinations of secondary antibodies for 6-8 hrs at room temperature, then rinsed again. The trays with the tissue were covered under aluminum foil to prevent photo-bleaching during the antibody treatment. The slices were then mounted on super frost slices, air-dried and cover slipped. Nissl staining was performed on one of the tissue series to parcellate the nuclei in the areas of interest. Cells in the slices were demyelinated and then rehydrated and incubated in Thionin (Sigma-Aldrich, St. Louis, MO**)** solution and differentiated in 0.4% glacial acetic acid in deionized water, then dehydrated and cleared with xylenes, before being cover slipped with DPX (VWR, Radnor, PA catalog #360294H)

Microscopy: The slides were examined under the Zeiss Observer.Z1 equipped with needed filter sets for Alexa 488, UV (for FG), and Texas Red. Sections were further analyzed using the Keyence BZ-X710 All-in-one Fluorescence Microscope (Keyence, Inc. Elmwood Park, NJ) with filter sets of DAPI, Alexa 488, Texas Red/Cy3, and UV (for FG) to visualize the fluorescence from the injected dyes and the secondary antibodies in the tissue.

II.2. Specific Aim 2 Experiments

Specific aim 2: To Establish how PPTg-PnC glutamatergic synapses underpin SG *in vivo*.

Experiment 2.1: An optogenetic approach was used to selectively infect the excitatory neurons of the PPTg by injecting the AAVDJ-CaMKIIα-eArch 3.0-eYFP

(Archaerhodopsin, proton pump, sensitive to green light) or AAVDJ-CaMKIIα-eNpHR2.0- eYFP (Halorhodopsin, chloride pump, sensitive to orange/yellow light) (Yizhar et al., 2011) viral particles into the PPTg. The excitatory glutamatergic projections from the PPTg were photo-inhibited within the PnC using green or orange/yellow light, depending on the virus injected, while performing PPI *in vivo*. Both types of opsins were used to further confirm the behavioral results, and as their activation should yield similar outcomes.

Experiment 2.2: At the end of the behavioral testing sessions, the brain was extracted, sectioned and analyzed through immunohistochemical assays. Since the viral construct contained a reporter gene encoding a fluorescent protein, the additional immunohistochemical experiments allowed me to double-label the neurons that were infected by the viral particles and verify the chemical identity of such neurons. In addition, the brain sections of the animals used for the behavioral experiments allowed me to confirm the correct placement and implantation of the optic fiber/cannula.

II.2.1. Objective and Overview

Previous *in vivo* and *in vitro* rodent studies demonstrated that the PPTg is one of the major modulators of PPI, though its cholinergic input to the PnC (Koch et al.,1993; Bosch and Schmid, 2006; Bosch and Schmid, 2008). Interestingly, the PPTg cholinergic neurons inhibited the PnC neuronal activity during PPI *in vitro* at inter-stimulus intervals of 300 and 1000 ms (Bosch and Schmid, 2008). In January 2017, during the BBRC seminar series, we were excited to host Dr. Susanne Schmid (from the University of Western Ontario), a pioneer in the field. Her recent results using state-of-the art chemogenetic and Optogenetic approaches further supported a minor contribution of the PPTg cholinergic projections during PPI *in vitro* or *in vivo* (also see Valsamis et al.,

2014 and MacLaren et al., 2014). Her results actually indirectly supported our hypothesis that a PPTg-PnC glutamatergic connection might contribute to PPI.

The objective of Specific Aim 2 was to test the contribution of the PPTg-PnC glutamatergic connection to PPI *in vivo*. An optogenetic approach was used to selectively express light-sensitive channels in excitatory neurons from the PPTg that hyperpolarize the cell in response to green or orange/yellow light, depending on the virus that was used. The cells infected with archaeorhodopsin express proton pumps in their membranes and open with green light leading to hyperpolarization of the membrane (through an outward proton current). The cells infected with halorhodopsin express chloride pumps in their membranes, also leading to hyperpolarization (through an inward Cl^- current). We decided to use two different inhibitory opsins to confirm the effects of inhibition due to photo-inhibition. We were also able to visualize the channel-expressing neurons due to the presence of the reporter gene eYFP contained in the viral constructs and expressed in the excitatory neurons infected. At the end of the behavioral testing, I extracted the brains of these mice and I performed immunohistochemistry to reveal the chemical identity of the targeted PPTg neurons and visualize the neuronal fibers and terminals within PnC slices. The data generated from these experiments furthered our understanding of the modulation of PPI and identified a potential new target for the development of treatments for patients suffering from SG impairments.

II.2.2. Materials and Methods

Animals: Adult C57CBL/6 male mice aged between 1-2-month-old mice (Jackson Laboratories) housed in the University of Texas at El Paso (UTEP) vivarium with controlled temperatures and a 12-hour light / dark cycle have been used for these

experiments. All protocols were conducted in compliance of the UTEP Institutional Animal Care and Use Committee (IACUC).

Surgery / Viral injections: All injections were done with a pressure micro-injector (Quintessential Stereotaxic injector, Stoelting Co., IL) 4-6 week-old-mice were injected unilaterally. The following viral particles were injected intracranially and contained: archaeorhodopsin, halorhodopsin, or only mCherry (i.e., AAV-DJ-CaMKIIα-mCherry, a "control" viral construct devoid of opsin gene). A recent review by Mena-Segovia & Bolam (2017) states that the glutamatergic neurons in the PPTg are more densely concentrated in its caudal portion, hence we targeted that area for injection. Two injections of 0.15 μL each (0.3 μL total) were administered using pulled glass micropipettes in the following coordinates relevant to Bregma targeting the **PPTg**: **#1** AP: -4.84 mm, ML: +1.3 mm, DV: -3.75 mm, and **#2** AP: -4.84 mm, ML: +1.3 mm, DV: -3.5 mm. The mice were anesthetized with 1.5% isoflurane via inhalation through a nose cone attached to the stereotaxic platform head holder for the entire duration of the surgery.

Optic Fiber Implants: Three weeks post-injection, the mice were anesthetized with 1.5% isoflurane via inhalation through a nose cone attached to the stereotaxic platform head holder for the implantation duration of the optic fiber/ cannula (Thor Labs, Newton, NJ). The optic fiber was held with an adapter to the stereotaxic platform arm and the tip of the optic fiber measures a length of 5.0 mm and was lowered into the following **PnC** coordinates: AP (Bregma) -5.34 mm, ML +0.7 mm, DV - 5.0. The coordinates were chosen guided by the light dispersal equation given in Yizhar et al., 2011. Once the optic fiber was positioned inside the brain, a mixture of C&B Metabond (Parkell, Edgewood, NY) was spread across the skull and air-dried until it hardened.

The adapter was then raised, and the optic fiber was left on the mice's head for one week (for recovery) until the behavioral tests were performed. The mice were allotted a total time of four weeks post-injection for the virus to proliferate before the *in vivo* behavioral studies.

In vivo Optogenetics Behavioral studies*:* The mice were quickly anesthetized with isoflurane in an anesthesia chamber and the doric fiber was coupled to the tip of the cannula. The light sources were either a PlexBright LD-1 single channel LED Driver (1200 mA max) with a PlexBright green LED green emitting module (525 nm) (Plexon, Dallas, TX) for the animals injected with archaeorhodopsin or an orange/yellow laser source (P/N: MGL-F-593.1 ± 1 nm-100mW; Opto Engine LLC, Midvale, UT) for the mice injected with halorhodopsin. Once the mouse fully recovered, it was let to walk freely in its cage for 10 min and placed inside the startle testing chamber (Harvard Apparatus, Holliston, MA) for another 5-10 min for acclimation before the experiments. The load cell coupler module (Harvard Apparatus, Holliston, MA) detects the movement of the mouse when it startles, which is connected to the main computer (HP Pro Desk 600, Intel i5-6500@ 3.20 GHz, 4GB RAM; Hewlett Packard, Palo Alto, CA) and fed into the Packwin v2.0 software (Panlab) (Harvard Apparatus, Holliston, MA), who correlates the sound stimulus to the startle response and presents the data in an excel file, as well as live traces. The following four different protocols were used for the behavioral experiments: **#1**: Acoustic startle response with sound only, **#2** Acoustic startle response with "light on" (during the startling sound), **#3** PPI only, and **#4** PPI with "light on" during the task. The intensities of the startling stimuli were delivered randomly at different times to prevent habituation. We chose to use 75 db for the prepulse intensity since it is the highest intensity at which none of the mice startled. The non-startling prepulses and startling pulses of the PPI protocol were delivered randomly at different

Inter-stimulus intervals (ISIs) to prevent adaptation of the animal. The experimental procedure is summarized in **Figure 2.2**. The animals were taken out of the chamber for 10 min after each protocol and placed in their cages for a 10-minutes resting period before starting the next protocol. This prevented a decreased response due to adaptation of the animal to the startling chamber. Once the behavioral testing completed, the mice were perfused as described previously in the Specific Aim 1 *Surgery/neuronal tracer injections* method section. Briefly, I extracted and fixed the brains in 4% PFA and I sliced them in 30 μm sections encompassing from the caudal PnC to the rostral PPTg.

Histological analyses*:* The brain slices collected containing the PPTg were treated with an antibody against ChAT (1:100, which labels cholinergic neurons) to delineate the cytoarchitectonic borders of the PPTg. The slices containing the PnC were treated with an antibody against VGluT2 (1:300) in an attempt to label and visualize excitatory synapses between the PPTg fibers/terminals and the PnC neurons, as described previously in *4.1.3b.* Briefly, the slices were incubated in NDS and a combination of primary and secondary antibody solutions. In addition, the tissue was Nissl-stained to further delineate cell bodies and anatomical borders.

Microscopy*:* The slides were examined under the Keyence BZ-X710 All-in-one Fluorescence Microscope (Keyence, Inc. Elmwood Park, NJ) with filter sets of DAPI, Alexa 488, Texas Red/Cy3, and FG to visualize fluorescence from the eYFP expressed in the neurons and from the secondary antibodies used. The slices were further examined under the Zeiss Observer.Z1 equipped with needed filter sets for Alexa 488, Cy5 to visualize terminals in more detail.

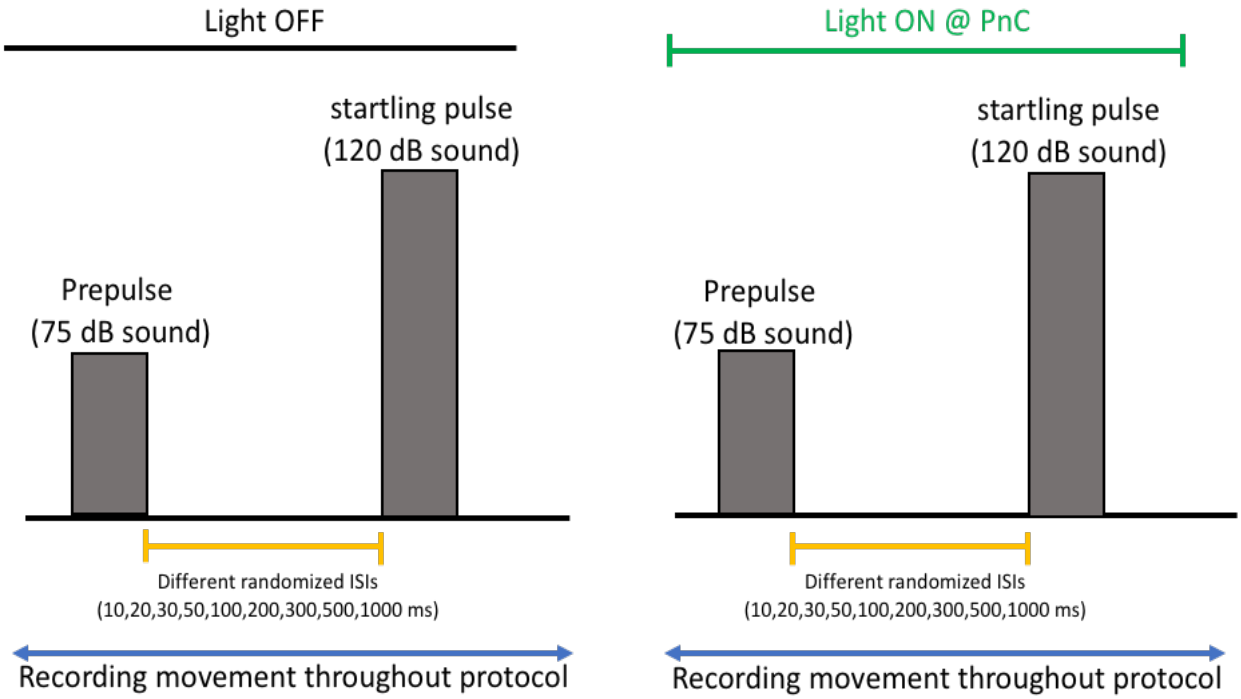

Figure 2.2. **Schematic representation of the PPI protocol *in vivo*.** *Left,* PPI protocol under control conditions (ie, NO light). *Right,* PPI protocol with the photoinhibition of the PPTg-PnC excitatory synapses with green or yellow-orange light (ie., light ON) at the level of the PnC. The light was turned on 1 ms before the prepulse and stayed on for 2 seconds. All the shown ISIs were performed 7 times in a randomized order.

Chapter III: Results

Understanding the PPTg-PnC connection and its functional role in SG requires both visualizing the neuronal projections and assessing SG behaviorally. To visualize the PPTg neurons connected to the PnC, I used both retrograde and anterograde tracing methods. Then, to investigate the connection's behavioral contribution to SG, I assessed PPI behaviorally *in vivo* while photo-inhibiting the connection. The results from the mentioned experiments are described below.

III.1. PPTg-PnC glutamatergic connection

To visualize and characterize the PPTg projections to the PnC, I used a total of 28 mice that I injected with the retrograde tracer FG and with the retrograde virus AAVrg-CAG-tdTomato (N=4). I used another subset of mice that I injected with either CTB(N=2), AAVDJ-CaMKIIα-eArch3.0-eYFP(N=6), AAVDJ-CaMKIIα-eNpHR3.0-eYFP(N=6) or AAVDJ-CaMKIIα-mCherry(N=4) viral particles. Moreover, to confirm the identity of the neurons that expressed the retrograde or anterograde tracers or viruses, antibody staining against ChAT, GAD67 and VGluT2 were performed at either the injection site or projection sites. An injection summary for both retrograde and anterograde labeling experiments is in Appendix 1.

III.1.1. PPTg projections to the PnC | Retrograde labeling

Both FG and the retrograde viral particles were injected in a location to the right side of the midline in the PnC for all mice as seen in **Figure 3.1 A** and **B**, which is a representative example of the retrograde tracing experiments. The injection sites were evaluated using the Paxinos and Franklin brain atlas (Paxinos & Franklin, 2014).

Adjacent sections were further treated with Nissl staining which allowed me to delineate the brain region boundaries. In addition, the sections containing the PPTg were treated with ChAT and GAD67 antibody staining helping me to visualize the cholinergic and GABAergic neurons confined within the PPTg. In order to evaluate if an injection targeted the PnC, I analyzed the injection site and its size from different sections. When the injection was contained within the PnC boundaries dorso-ventrally and rostro-caudally, I could confidently determine that the back-filled cells observed within the PPTg projected to the PnC.

My results show that in sections that contained the PPTg, retrogradely labeled neurons were observed within the PPTg as seen in **Figure 3.1 C-G**, which is a representative example of the retrograde back-filled cells in the PPTg. Interestingly, two observations were made. The first is that cell bodies were back-filled on both the ipsilateral and contralateral sides of the injection side as seen in **Figure 3.2**. Also, some cell bodies from both sides co-localize the expression of td-tomato and the ChAT, showing that some of those back-filled cells are cholinergic, and they cross the midline to the contralateral side. Also, some cell bodies expressing tdTomato do not co-localize with cells stained with ChAT nor GAD67, suggesting a glutamatergic identity of the back-filled cell bodies.

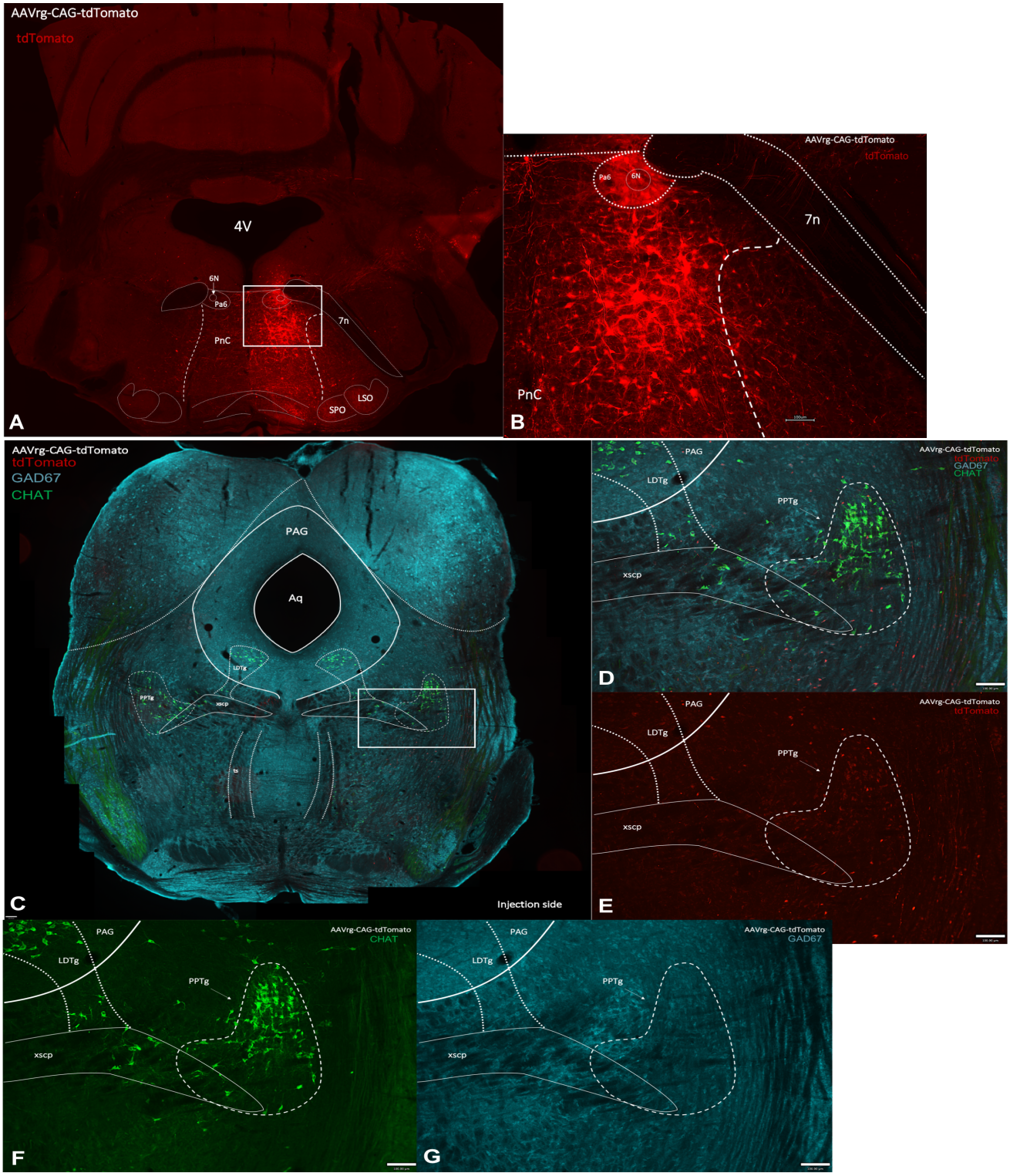

Figure 3.1. **Retrograde tracing**.
(A) Wide-field image of a PnC containing slice of a WT animal (ID 919) injected with the AAVrg-CAG-tdTomato retrograde virus in the PnC. The fibers expressing the reporter gene tdTomato are seen with a Cy3 filter. The injection site is inside the white rectangle in the dorsal part of the PnC. (B) A close-up image of the injection site in the dorsal part of the PnC slice shows a retention within the PnC boundaries. (C) A wide-field image of a PPTg containing slice of the same animal injected in the PnC stained immunohistochemically for ChAT and GAD67 and showing tdTomato expressing fibers and cell bodies. The PPTg of the injection side is enclosed with a white rectangle. (D) A close-up image of the PPTg of the injection side revealing the boundaries of the PPTg by the abundance of cholinergic neurons revealed by the ChAT stain and showing back-filled cell bodies expressing tdTomato within the PPTg. (E, F, G) The same close-up image as D but showing the image in the Cy3, GFP, and Cy5 filter, respectively, to show individual labeling.

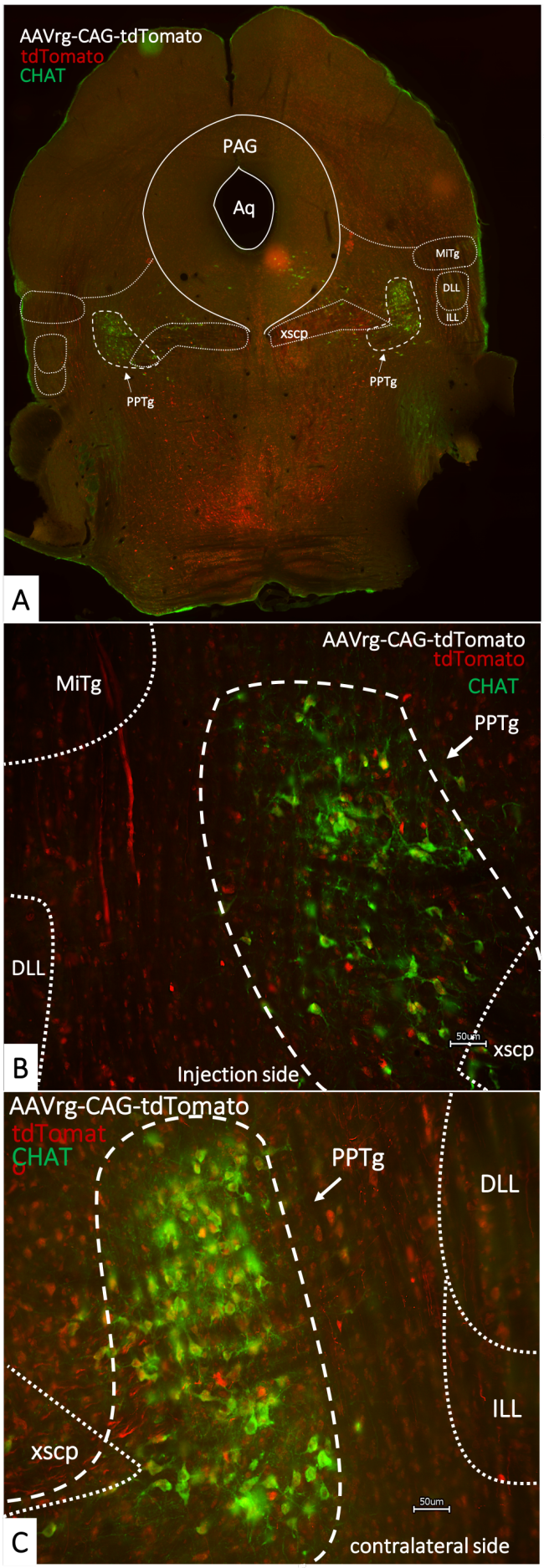

Figure 3.2. **Contralateral retrograde filling.** (A) Wide-field image of a PPTg containing slice of a WT animal (ID 923) injected with the AAVrg-CAG-tdTomato retrograde virus in the PnC. The fibers expressing the reporter gene tdTomato are seen with a Cy3 filter and the PPTg on both sides delineated by ChAT immunostaining. (B) A close-up image of the injection side on the left-side of the PPTg showing cell bodies expressing tdTomato and co-staining with ChAT within the PPTg. (C) A close-up image of the PPTg of the contralateral side of the injection containing slice of the same animal injected in the PnC showing tdTomato expressing fibers and cell bodies and co-staining within the PPTg.

III.1.2. PPTg projections to the PnC | Anterograde labeling

As previously mentioned, I used a subset of animals for the anterograde tracing experiments, using the animals that had performed the behavioral experiments. CTB and all the viruses used for these experiments were injected in the caudal PPTg of the right hemisphere of the brains in WT mice. The caudal PPTg was chosen due to a recent review by Mena-Segovia and Bolam, 2017, mentioning the concentration of glutamatergic neurons in the caudal PPTg. The injection and projection sites were evaluated using the Paxinos and Franklin brain atlas (Paxinos & Franklin, 2014). The brain sections containing the PPTg and the PnC were stained in a similar fashion as those described in the retrograde labeling section. The slices containing the injection site in the PPTg were stained for ChAT and some with Nissl. The slices containing the PnC were analyzed for the expression of fluorescent fibers within the PnC, which suggest that neurons injected within the PPTg send fibers to the PnC. After enhancing the fluorescence of CTB in a series of slices, it was evident that an accurate injection was performed in the PPTg, but it was quite difficult to find fibers in the PnC, as seen in **Figure 3.3.** Due to the faint fluorescence of CTB, we decided to use the viral particles as anterograde tracers. Fibers expressing eYFP were found in the PnC 4-6 weeks post-injection as seen in **Figure 3.4**.

From the viral construct used, eYFP expression was driven under the control of the CaMKIIα promoter, suggesting that the eYFP-expressing neurons are excitatory. My results show that some cells within the PPTg seemed to express both eYFP and ChAT, suggesting some PPTg cells can co-release ACh and glutamate as neurotransmitters. It is also possible that cholinergic and glutamatergic cells were very closely intermingled. Other PPTg cells were only expressed eYFP, strongly suggesting a glutamatergic

identity **as seen in Figure3.4 B**. There were several areas within PnC sections in which the PPTg eYFP-expressing fibers could be observed. The expression of eYFP was more predominantly seen at the ventro-medial part of the PnC, with some fluorescence on the contralateral side as seen in **Figure 3.4 D.** I also performed VGluT2 staining in order to identify the chemical nature of the fibers expressing eYFP. Since there is a major abundance of glutamatergic synapses within the PnC, with many areas projecting to it and its nature of having the giant glutamatergic neurons, the VGlutT2 stain showed major fluorescence all along the PnC and it was impossible to identify if the fibers were truly co-localizing with the stain or adjacent tissue was causing background fluorescence (not shown).

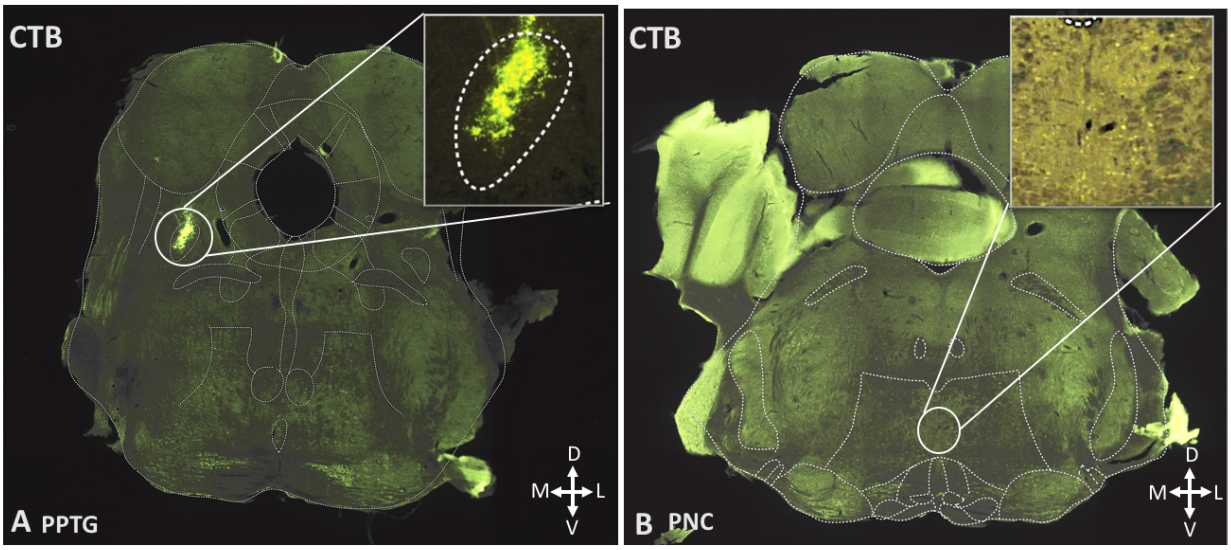

Figure 3.3. **CTB Injection.** Coronal sections showing the injection site in the PPTg (*A*), and the CTB-fluorescent PPTg fibers within the PnC (*B*). (A) *Inset,* High-magnification of the injection site at the PPTg with labeled cell bodies. (B) *Inset,* High-magnification of the projection site at the PnC with labeled axonal terminals.

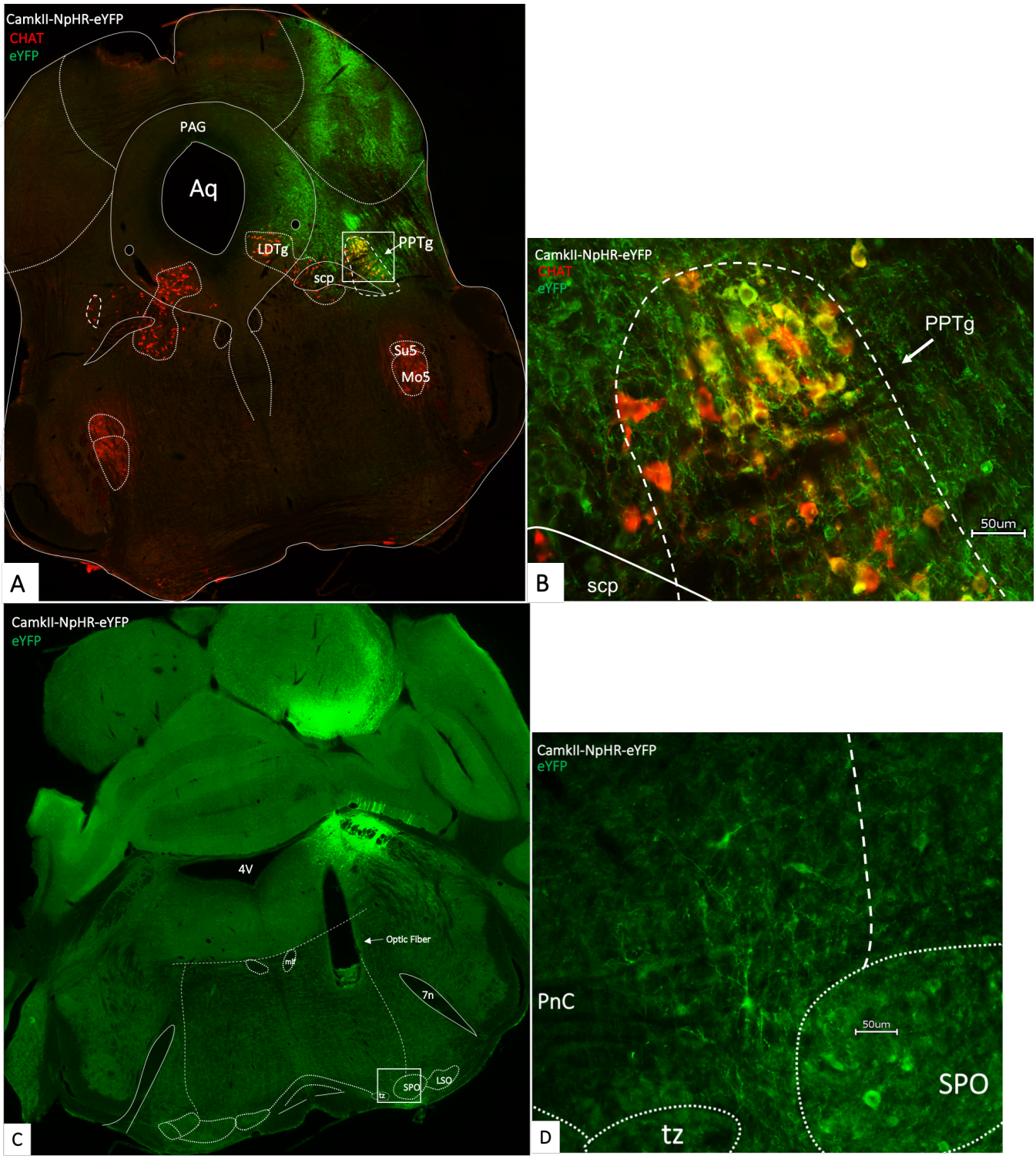

Figure 3.4. **Anterograde Labeling and optic fiber implantation.**
(A) Wide-field image of a PPTg containing coronal slice of a WT animal (ID 724) injected with AAVDJ-CamKIIα-eNpHR3.0- eYFP (halorhodopsin) in the PPTg. The cell bodies and fibers expressing eYFP were enhanced immunohistochemically and visualized with a GFP filter. The tissue was treated immunohistochemically for ChAT (Cy3) to reveal PPTg boundaries and identify neuronal identities. (B) A close-up image of the injection site in the PPTg showing cell bodies expressing eYFP and some co-staining with ChAT within the PPTg. (C) A wide-field image of a PnC-containing slice showing fibers expressing eYFP projecting from the PPTg. A tract from the optic fiber implantation can be seen ventro-laterally to the 4th ventricle with the tip terminating within the boundaries of the PnC. (D) A close-up image of some PPTg fibers expressing eYFP within the ventro-lateral portion of the PnC.

III.2. Behavioral assessment of the PPTg-PnC synapse

To determine the contribution of our characterized glutamatergic PPTg-PnC connection to SG *in vivo,* I photo-inhibited the connection during the PPI task. A total of 16 mice were injected with either AAVDJ-CaMKIIα-eArch3.0-eYFP (archaeorhodopsin, N=9, n=6), AAVDJ-CaMKIIα-eNpHR3.0-eYFP (halorhodopsin, N=9, n=6), and AAVDJ-CaMKIIα-mCherry viruses (no opsin, N=5, n=4) and used for the behavioral experiments. Some mice could not be used for the behavioral experiments and were only perfused and the tissue used for visualization. The main purpose of archaeorhodopsin and halorhodopsin is to inhibit those PPTg fibers expressing eYFP in the PnC while assessing ASR and PPI *in vivo*. I also used a viral construct devoid of opsin gene, as a "control" injection. As mentioned before, the fluorescence from these experiments was also used as the anterograde labeling technique for *Specific Aim 1*. To confirm the identity of the neurons targeted by the viral injections, antibody stains with ChAT and VGluT2 were performed at either the injection site or projection sites. In addition, I visualized and confirmed proper optic fiber implantation with the brain slices using immunohistochemistry. A summary of the injections performed for these experiments is included in Appendix 1.

III.2.1. Acoustic Startle Response

In order to assess PPI, the acoustic startle response (ASR) has to be assessed first to evaluate the animal's basal startle levels and find the sound level needed to elicit a "non-startling" response (or "prepulse"). The ASR was measured using the first two protocols described in the *II.1.2. Materials and Methods* section of Chapter II. The first protocol was used to measure basal startle levels, and the second protocol was used to

confirm that photo-inhibition of the PPTg fibers in the PnC does not affect the acoustic startle response, since the PPTg is not involved in the ASR pathway. The experiments were performed using the mice I injected with either one of the three viral particles mentioned above, 4 weeks post injection and one week following cannula implantation.

Behavioral experiments began with a period of five minutes of exploration, then the ASR was assessed by presenting the mice with pulses of different intensities in a randomized order with the light off during the whole experiment. Following the "Light-off" protocol, the second protocol was performed in a similar manner, but now with the "Light-on" condition where the light, either green (for archaeorhodopsin injected mice) or yellow/orange (for halorhodopsin injected mice), turned on, one second before the pulse was presented and turned off one second after. My results show an Increased startle reaction as a function of sound Intensity. Interestingly and as expected, I was happy to confirm that the sound intensity-dependent increase in the ASR did not differ between the "light-on" and "light-off" protocols across all the conditions tested (i.e. by the two inhibitory opsins and control virus), as seen in **Figure 3.5.**

III.2.2. PPTg-PnC Synapse silencing during PPI

The ASR protocol allowed us to select the sound intensity low enough to be used as a prepulse (i.e., a detectable sound that did not startle the animal) as well as a sound intensity loud enough to be used as a startling pulse (i.e., a sound level at which all animals startle). The values chosen were 75db for the prepulse and 120db for the startling pulse. Protocols #3 and #4 were designed to measure changes in PPI. Protocol #3 was the "PPI light-off" and Protocol #4 was the "PPI light-on" condition where the light turned on one second before the prepulse and turned off one second after the

startling pulse for any given inter-stimulus interval (ISI). The prepulse and the subsequent startling pulse were presented every 29 seconds at different ISIs in a randomized fashion for both protocols. The basal startle level was also evaluated throughout the entire extent of the experiment, as described by Diamantopoulou et al., 2017. I therefore used this basal startle level to calculate the PPI values. Each ISI was presented seven times and averaged for analysis purposes.

Both Protocols, #3 and #4 were compared to each other to analyze any change in PPI induced by silencing the PPTg-PnC connection, as shown in **Figure 3.6**. The control mice showed no significant change in the basal startle responses between the protocols. Since these mice were only Injected with a viral vector devoid of Opsin gene, these mice still exhibited PPI, but the "Light On" condition did not produce any change in PPI. These results were expected since the cells infected with this "control" viral construct do not express any Opsin protein (i.e., no inhibitory channel in their membrane) but only express mCherry. These data suggest that if any change in PPI is observed by the shining light on the PPTg fibers projecting onto the PnC, this change will likely be due to the photoinhibition of the PPTg fibers and not due to an undesired effect of the injection or optic fiber implants themselves.

The mice injected with archaeorhodopsin showed no significant change in the basal startle responses when the "Light Off" and "Light On" protocols were compared. Regarding PPI, these mice exhibited normal PPI under "Light Off" conditions. Interestingly, when the PPTg-PnC excitatory connection was inhibited by the green light (i.e., "Light On" condition), a lowered startle response was elicited, which resulted in an increased inhibition of the startle response, in other words, an increased PPI. These exciting results were contrary to what we were expecting (as discussed below)

and the difference in PPI was statistically significant at the 500 ms ISI ($p = 0.027$) as seen in **Figure 3.6, middle.**

To confirm the Archaerhodopsin effects on PPI, we also used another Opsin, i.e., Halorhodopsin, for comparison purposes. The mice injected with halorhodopsin did show a decrease in the basal startle responses when the PPTg-PnC excitatory connection was inhibited by the orange light compared to when the light was off. An explanation for these results is provided in the *IV.2.2 ASR and PPI* section of Chapter IV. Moreover, the photoinhibition of the PPTg-PnC excitatory connection with this Opsin also affected PPI efficiently, and the mice also showed a lowered startle response when the light was on, as seen in **Figure 3.6, bottom.** The results of these experiments show a trend on PPI similar to those obtained with archaeorhodopsin. Altogether, these results strongly suggest that the inhibition of the PPTg-PnC glutamatergic connection by different opsins increases the inhibition of the startle response or PPI.

After the completion of the behavioral experiments, the mice were transcardially perfused, and the brains sliced and treated with different antibodies to confirm neuronal identities and a correct optic fiber implantation targeting the PPTg fibers in the PnC with light. All of the mice included in the results section had a correct optic fiber implantation as seen in **Figure 3.4 C,** showing a PnC section with labeled PPTg fibers, closely positioned to the optic fiber tract. As also discussed in the anterograde tracing section of the results, the VGluT2 stain could not be used to identify the chemical nature of the opsin expressing fibers since it could not be distinguished from the high background expression. In addition, from the slices containing PPTg, I have evidence confirming that the injection was confined within the borders of the PPTg as seen in **Figure 3.4 A,** which is a representative image of all the injections targeting the PPTg used for the behavioral tests of this project**.**

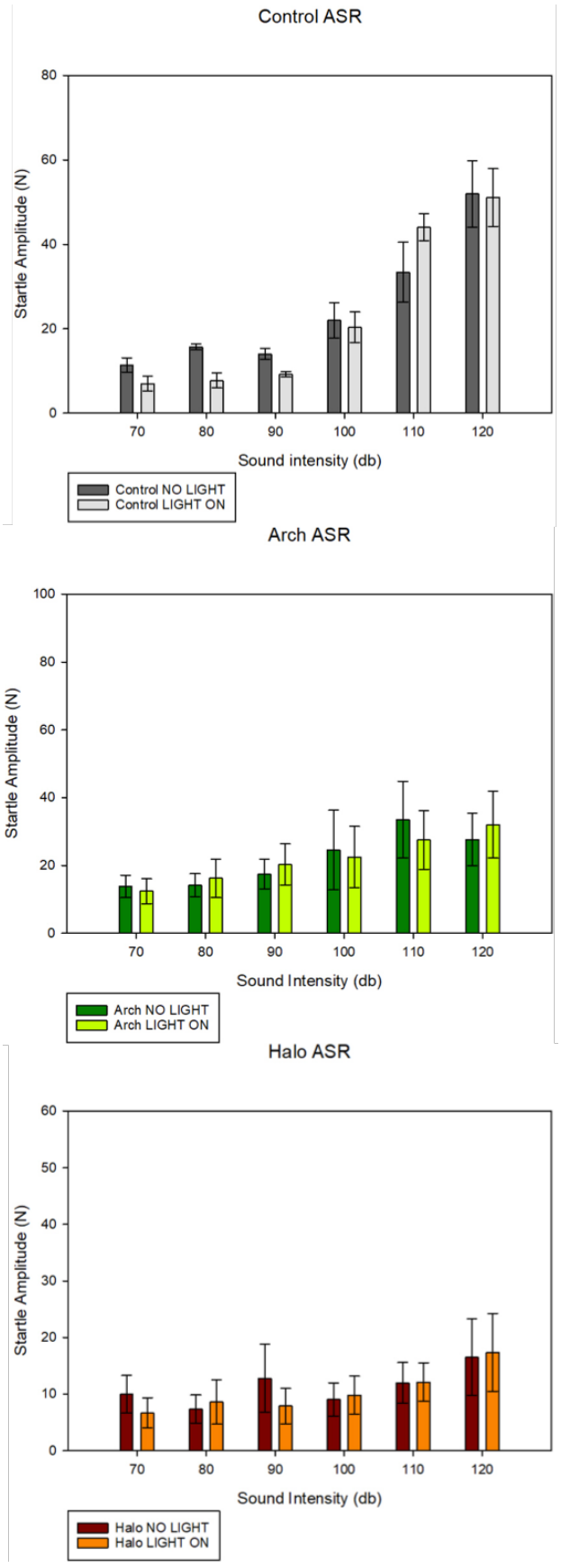

Figure 3.5. **Acoustic Startle Response.** Shows the different intensities at which the experiments were performed and the startle responses of the animals. (Top) The responses of the Animals injected with AAVDJ-CaMKIIα-mCherry (N=5, n=4) (Control animals). (Middle) The responses of the animals injected with AAVDJ-CaMKIIα-eArch3.0-eYFP (N=9, n=6) .(Bottom) The responses of the Animals injected with AAVDJ-CaMKIIα-eNpHR3.0-eYFP (N=9, n=6).

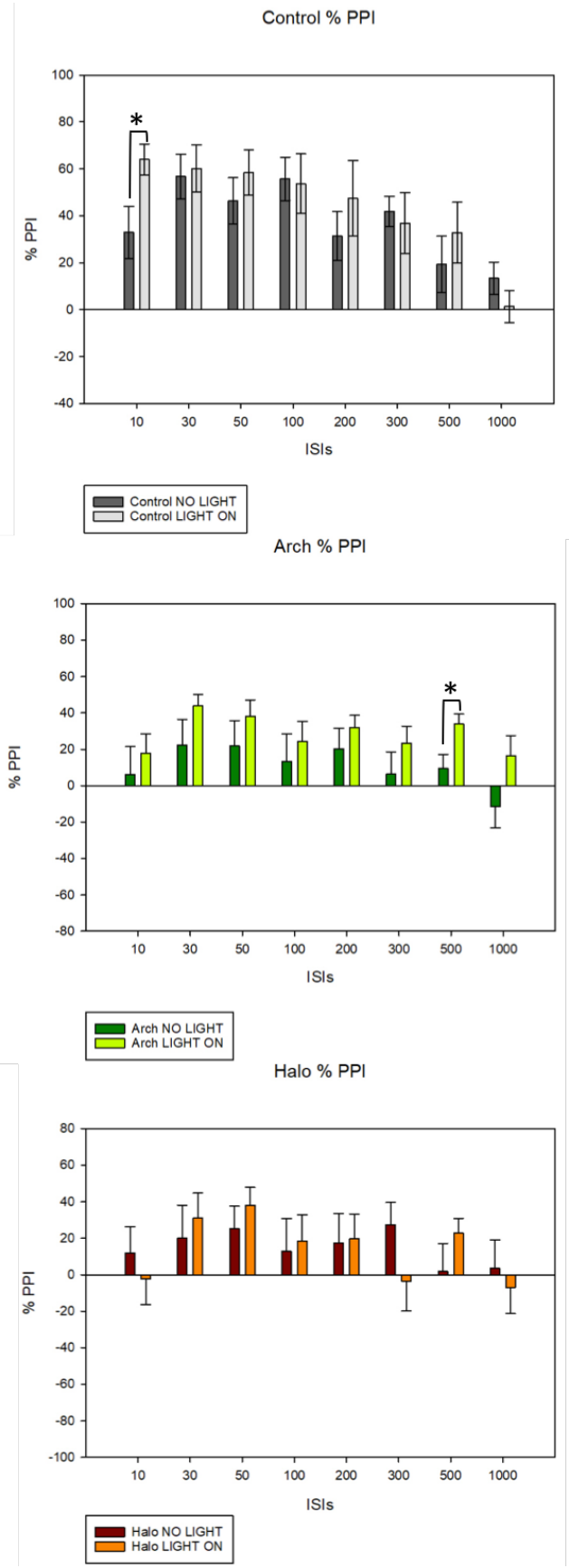

Figure 3.6. **Prepulse Inhibition.**
Shows the assessment of PPI of the animals manipulated with different opsins and silencing the PPTg-PnC glutamatergic synapses. (Top) Shows the PPI of the animals injected AAVDJ-CaMKIIα-mCherry (N=5, n=4) (Control animals) which show a significant difference in the 10ms ISI. (Middle) The responses of the Animals injected with AAVDJ-CaMKIIα-eNpHR3.0-eYFP (N=9, n=6) which shows a significant difference in the 500ms ISI. (Bottom) The responses of the animals injected with AAVDJ-CaMKIIα-eArch3.0-eYFP (N=9, n=6) showing no significant difference between the conditions. All the results show a trend of potentiated PPI when the light is on vs. when the light is off conditions.

Chapter IV: Discussion

The goal of this project was to determine a PPTg-PnC glutamatergic connection that contributes to PPI. This was done first by characterizing and confirming a direct anatomical connection between the PPTg and the central PnC and second, by assessing its functional contribution to SG. The approaches taken to study this neuronal connection can be seen in Chapter II. Briefly, tract tracing coupled to immunostaining experiments were performed in parallel to *in-vivo* optogenetics experiments to address these goals. For the tracing experiments, FG and the AAVrg-CAG-tdTomato virus were used for retrograde labeling while CTB, the AAVDJ-CaMKIIα-eArch3.0-eYFP (archaeorhodopsin), AAVDJ-CaMKIIα-eNpHR3.0-eYFP (halorhodopsin), and AAVDJ-CaMKIIα-mCherry ("control" vector) viruses were used for anterograde labeling. Early studies had focused on and described a cholinergic connection between the PPTg and the PnC (Koch et al., 1993; Swerdlow et al., 2001) and was initially thought to be the sole contributor to SG. However, recent studies have suggested that the PPTg-PnC cholinergic connection plays a modest role in SG (MacLaren et al., 2014), that the cholinergic involvement in PPI could be originating from a different part of the brain (Schmid et al., 2008) and that perhaps a glutamatergic connection could have a bigger impact on SG assessed using PPI (Bergeron et al., 2015). Moreover, most studies were performed in rats, without focusing on glutamate neurotransmission. Our research efforts aimed at focusing specifically on a PPTg-PnC glutamatergic connection in mice in efforts to understand its involvement in SG by being at the core of the PPI circuitry. In addition, many SG deficits can be induced by blocking or altering glutamate neurotransmission (Valsamis et al., 2014).

Our tract tracing results show that a PPTg-PnC glutamatergic connection does exist, and that it contributes to SG *in vivo*, in mice. Injections of the retrograde tracer/virus in the PnC show back-filled cells in the PPTg, and injections of anterograde tracer/viruses in the PPTg show terminals in the PnC. A glutamatergic identity of this connection was confirmed with immunohistochemistry experiments. Moreover, photo-inhibition of the PPTg-PnC glutamatergic synapse while assessing SG *in vivo* showed an increase in PPI, suggesting a functional contribution of this synapse in SG.

These experiments provide information about a novel synapse, supporting previous evidence showing that the PPTg is involved in SG but through a glutamatergic connection, instead of a cholinergic connection. It would be interesting to test the properties of the PPTg-PnC glutamatergic synapses and their involvement in SG using translational animal models of disease, specifically those exhibiting SG deficits. Then, subsequent genetic studies would provide a more molecular insight about the causes of SG deficits in such disorders and thus identify potential targets for therapeutic interventions and treatments.

IV.1. PPTg-PnC glutamatergic connection characterization

IV.1.1. Injecting FG and AAVrg in the PnC

FG and AAVrg were used to trace the PPTg-PnC projections retrogradely in our experiments. Initially, only FG was going to be used due to previous studies using it to characterize the PPTg-PnC connection. Although FG is a great tracer and yields great fluorescence within a short time post-injection, as explained by Schmued and Fallon, 1986, FG contains several caveats. FG is taken up by axonal terminals and up to the cell body via kinesins that are found all along the axons. One of the problems with this is that FG is easily taken up by damaged fibers of passage, which are commonly caused due to the pipette entry damage on the tissue. Moreover, the tracer is a heavy and viscous fluid when diluted, which tends to clog pipette tips that generally leads to the need of using wider tips, thus resulting in larger injection sites. Such large injections are hard to retain within the boundaries of the PnC, creating the possibility of back-filling other cells with axons ending in close proximity to the PnC, but not within it.

The PPTg lies rostral to the PnC in the brainstem. Fortunately, it has been described that the PPTg sends most of its projections rostrally, and only a few caudally, going either to the PnC, or straight to the spinal cord ventrally to the PnC. Due to the nature of the injections, when the tracer spills out of the boundaries, they are either lateral or dorsal to the PnC, where PPTg has not been described to have projections passing by other than those projecting into the PnC. A spilled injection in the PnC could

lead to back-filled cell bodies in other areas, but here we focused on back-filled cells in the PPTg, as our main interest.

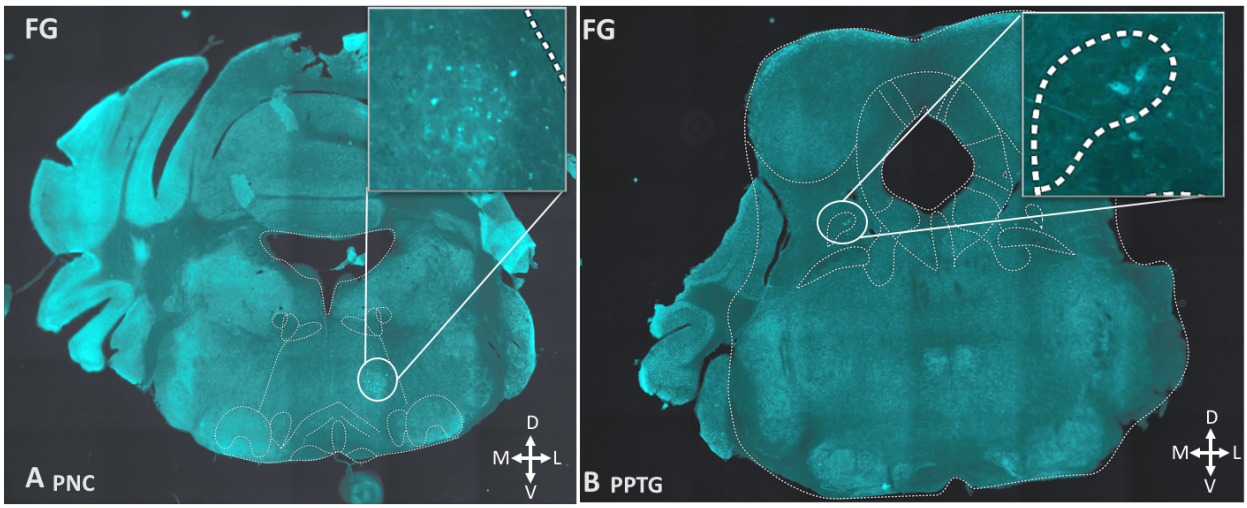

Figure 4.1. **FG- expressing neurons in the PPTg.** Coronal sections showing the injection site (*A*, PnC) and the back-filled neurons (*B,* PPTg) with FG (light blue) tracer. (A) *inset,* High-magnification of injection site at PnC. (B) *inset,* High-magnification image of Back-filled cell bodies in the PPTg.

I achieved a few successful FG injections confined within the PnC borders as seen in **Figure 4.1**. However, due to the difficulty of the FG injections, we decided to use the retrograde virus AAVrg-CAG-tdTomato. The first type of this adeno-associated retrovirus (AAV2-retro) that infects in a retrograde fashion was first described by Tervo et al., 2016. The group reports that in a set of experiments labeling cortical neurons, with the injection site in the basal pontine nuclei, showed that the density of the neurons labeled by the retrograde virus was comparable to that achieved with FG, which is a synthetic tracer that labels robustly. We opted to use the retro virus created by the team of Dr. Edward Boyden (MIT) that contains the CAG promoter, which is non-selective in the mammalian genome and yields high levels of expression of the tdTomato fluorescent protein in neurons. Further studies are necessary to determine of the function of these new viruses in terms of *in vivo* Optogenetic manipulations (Tervo et al., 2016; Kobayashi et al., 2018; Sun & Schaffer, 2018).

The PnC is an area in the ventral portion of the brainstem that expands in a quite large area in the rostro-caudal plane. When trying to find the PnC in some sections, it was difficult to distinguish it from the oral pontine reticular nucleus (PnO), since they are very similar and occur adjacently. The PnO lies rostral to the PnC, which is closer to the PPTg. However, it was not very difficult to find the PnC after using the Nissl stained tissue to locate the injection site, which gives us features that we can compare in the Paxinos & Franklin mouse Brain atlas, such as the 7th nerve.

The brainstem is known for regulating various vital physiological roles, such as breathing. In addition, many motor centers are contained within it and damage to these areas can cause great damage to the animals, even death. One of the greatest difficulties with the injections in the PnC was the survival of the animals. Due to the limited control of the FG injection volumes, a massive amount of the heavy tracer would be injected and expand the tissue, causing damage and pressure on vital areas. Due to this, many animals would wake up with evident muscular contractions or seizures and eventually stop breathing and die within a short time after the injection. Some animals did show these reactions to the injections but recovered successfully.

This type of tracing experiments using FG have been performed since the mid-late 1900s and are still used. An alternative procedure to the FG pressure injections could be implemented by using iontophoretic injections instead (Schmued & Heimer, 1990). Iontophoretic injections lead to small injection sites by ejecting the tracer of the pipette via an electrical current, thus having more control over the volume injected. Alternatively, we chose to use the state-of-the-art AAVrg which has been recently developed and available, allowing for high expression and fluorescence of tdTomato in axons and cell bodies. As mentioned, our interest in the PnC is due to its core

involvement in the PPI neural circuitry and is still highly uncharacterized. The injections of the retrograde tracers are vital in order to have a larger understanding of the core circuitry of PPI while coupling them with anterograde tracing experiments and confirming the neuroanatomy of this connection.

IV.1.2. Observation of back-filled neurons in the PPTg

As seen in the results section, the injections of the retrograde virus were contained within the boundaries of the PnC and showed high expression of the tdTomato protein along the fibers. Nissl and ChAT stainings were performed on this tissue to identify the boundaries of the PPTg. The ChAT staining served two purposes: 1- to further characterize the identity of the cells expressing (or devoid of) tdTomato, and 2- to delineate the PPTg is by identifying it by its high density of cholinergic neurons.

In the PPTg containing slices, tdTomato expressing ascending fibers are seen along the slice and interestingly in cell bodies within the PPTg on the ipsilateral and contralateral side of the injection. The ChAT stain identifies the presence of cholinergic neurons, and it co-stains a few td-tomato expressing cell bodies on both sides as seen in **Figure 3.2**. It was expected that the cell bodies co-stained with the ChAT, since the neurons expressing tdTomato are under the control of the CAG promoter, which is non-specific to the mammalian genome, and it is known that cholinergic neurons project to the PnC and cross the midline (Rye et al., 1987; Koch et al., 1993). In addition, we also performed a GAD67 stain, which did not show co-localization with the back-filled cells as seen in **Figure 3.1 D, G**. The fact that they did not co-localize in our experiments does not rule out the possibility that a PPTg-PnC GABAergic projection could be present since the cell bodies could be present at a different location within the PPTg. Also, the

injection of the retrograde virus does not cover the whole PnC meaning that these terminals could be present at a part of the PnC that was not injected with the virus. Moreover, most importantly, there were also cell bodies within the PPTg expressing tdTomato that did not co-localize with ChAT nor GAD67, strongly supporting our hypothesis of the presence of a PPTg-PnC glutamatergic connection.

IV.1.3. Injecting CTB and AAVDJ-CaMKIIα in the PPTg

The first anterograde tracer injections for this project were done with CTB. CTB is normally used as a retrograde tracer, but it can also serve as an anterograde tracer (Angelucci et al., 1996). The tracer can be seen with an Alexa488 filter (green), but due to low expression we decided to enhance the fluorescence with antibodies and make the anterograde terminals more evident. Although CTB is a good tracer, we did not see much expression, even with the enhanced fluorescence as seen in **Figure 3.3.** As an alternative experiment, we decided to take advantage of the injections performed for the behavioral section of the project (Specific Aim 2). The injections targeted the PPTg, where the cell bodies are infected with the viral construct containing the opsin gene and/or the reporter gene (mCherry or eYFP) and the translated proteins are expressed in the membrane of the axons and terminals in an anterograde fashion.

The PPTg is involved in many functions and is very important in arousal. It encompasses from -4.16 mm through -4.96 mm from bregma in mice (Paxinos & Franklin, 2014). The PPTg is ventrolateral and in close proximity to the cerebral periaqueductal gray (PAG) and can be sometimes confused with the laterodorsal tegmental nucleus (LDTg) since they are also in very close proximity and almost adjacent in more caudal parts of the PPTg. Due to the small area of the PPTg, it is sometimes difficult to retain the injection within its boundaries, and it sometimes spills to the LDTg. It has been demonstrated that the LDTg also projects to the PnC but is

involved in locomotor activity, rather than startle (Takakusaki et al., 2016). Therefore, injections that spilled into the LDTg were not considered for the results section since inhibition of these fibers could alter our results during the PPI tests. In addition to the literature showing that there are no other areas within the plane of the PPTg, other than the LDTg, that project to the PnC, we analyzed injections that missed the PPTg and targeted the lateral parts of it, close to the medial and inferior lemniscus and showed no fibers expressing eYFP in the PnC. With this observation, we decided to consider the injections that hit the PPTg and spilled over laterally and dorsally since they would not affect the results for Specific Aim 1 or Specific Aim 2.

The PPTg is known to be part of the biggest cholinergic center in the brain and it is strongly characterized by containing a high density of cholinergic neurons. In order to delineate its borders, a ChAT antibody is commonly used to stain the cholinergic neurons (Rye et al., 1987). The viruses injected in the PPTg contained the CaMKIIα promoter which targets the neurons expressing the Ca^{2+}/calmodulin-dependent protein kinase II, seen specifically at excitatory neurons. In addition to the cholinergic neurons, the PPTg also contains GABAergic and glutamatergic neurons (Wang & Morales, 2009), and it is known that the cholinergic neurons relevant to SG are inhibitory in the PPTg-PnC synapses (Swerdlow et al., 2001; Fendt et al., 2001; Schmid et al., 2006) but not all the cholinergic neurons project to the PnC. This means that the other cholinergic neurons in the PPTg are not necessarily inhibitory.

As expected, after immunostaining the tissue for ChAT and enhancing the fluorescence of the cells infected in the PPTg slices, we confirmed that there are excitatory cell bodies that are stained with ChAT, and there are also cell bodies that only express eYFP within the PPTg, which suggest being glutamatergic as seen in **Figure 3.4 A, B**. Ideally, we should use an antibody to label glutamatergic cell bodies and ultimately test their synaptic contribution *in vitro*, using electrophysiology in brain slices.

IV.1.4. Observation of PPTg fluorescent terminals in the PnC.

Four weeks post-injection, resting period during which the virus can proliferate, my results show that there are fibers in the PnC that express eYFP and that emerge from the PPTg. Since the fibers are only expressing eYFP, we were not able to determine their chemical identity solely by labeling. Therefore, we decided to use antibodies to target VGluT2 since it is used to target the vesicular glutamate transporter 2 which is found almost exclusively on axons and terminals. The PnC slices were treated to enhance the eYFP fluorescence and to label glutamatergic axons and terminals, but the labeling too dense, preventing the clear distinction between fibers within the PnC that originated from the PPTg. The PnC is a large area receiving inputs from various sources, largely glutamatergic (reviewed in Fendt et al., 2001). In fact, past and current projects from our laboratory show that there are glutamatergic projections from the medial pre-frontal cortex (mPFC) and the basolateral amygdala (BLA) in mice. In addition, the PnC contains a great abundance of glutamatergic receptors and neurons that could have synapses within the same area, being easily labeled by the antibody against VGluT2.

Since it was shown that the cholinergic neurons from the PPTg had an inhibitory effect on the PnC giant neurons, we proposed that the excitatory fibers expressing eYFP in the PnC that are involved in PPI must be glutamatergic. This was further confirmed by the behavioral experiments with the light targeting all the eYFP expressing fibers in the PnC, but only the glutamatergic ones had an effect on PPI, as seen in the results section. There have been recent studies proposing that the PPTg cholinergic neurons are not involved in PPI, which what has led us to perform this study, and supports our motive to postulate that the excitatory neurons seen in the PnC emerging from the PPTg are the ones involved in PPI.

Most of the fluorescence from the PPTg fibers on the PnC was seen on the ipsilateral side of the injection, but there were also some fibers seen along the midline

and on the contralateral side supporting what was seen in the retrograde injections section. It would be interesting to study if there is a specific contribution from the glutamatergic neurons in PPI by synapsing onto the different sides of the PnC.

IV.2. Behavioral tests and optogenetic inhibition

IV.2.1. ASR and PPI

The primary goal of this project was to identify if PPTg-PnC glutamatergic synapses are involved in SG and were assessed using the PPI task. To do so, I made the PPTg-PnC synapses express archaeorhodopsin (a proton pump) and I silenced them with green light. I could have shined light on the whole PPTg and silenced all the excitatory neurons within it to assess PPI, but this could probably yield different results since the PPTg is involved in many other systems like sleep/wake cycles, arousal and rate of locomotor output. Affecting the behavioral state of the animal (i.e. induce drowsiness/tiredness) could affect the startle levels and yield other results. Since we were interested in this specific connection, I implanted the optic fiber, so the tip could be on the dorsal portion of the PnC and the light reach most of the PnC. A correct implant placement was confirmed imaging fiber tract made by the canula and optic fiber in PnC brain slices. My behavioral results also show that the PPTg glutamatergic fibers on the PnC neurons indeed contribute to PPI. In these type of studies, a laser light source is generally used to reach deeper into the tissue with higher power, but due to the overwhelming cost of such equipment, we used a green LED light source which yielded significant results.

For all of the animals tested, we recorded startle responses with the light turned off and compared them to the responses when the light was turned on, using the same animal (as internal control). In order to control for the physical and unspecific effects

that the actual injections, optic fiber implants, or light could cause, I injected different animals with the AAV-DJ-CaMKIIa-mCherry vector which do not affect neurons activity. This design was performed in order to confirm the actual effects of inhibiting the glutamatergic PPTg-PnC synapses in PPI. There were no significant changes seen in the control subjects in neither the ASR tests, but there was a significant difference in the 10ms ISI for PPI. Since there are only a few samples to average for the control experiments, this might have caused this significant difference. In order to confirm that this effect is due to the low sample number, or the manipulations, more experiments need to be performed to decrease the standard error. This is needed to show that the unspecific manipulations of the injections or light penetration do not account for the changes seen during the inhibition of the synapses with a different vector, since this should not be a factor contributing to the results seen.

To confirm the results seen with one inhibitory opsin, we decided to use the same approach but with a different opsin (ie., halorhodopsin instead of archaeorhodopsin). The ASR results for the animals injected with both viruses showed no difference between the dependence of the startle levels on the sound intensity, which is expected since the PPTg is not involved in the ASR pathway. The PPI protocols compare the startle of presenting a prepulse before the starling pulse (yielding to prepulse inhibition) to a pure startle response elicited by the startling sound spread throughout the protocol. Since the PPTg is not involved in the ASR pathway, the startle response within the PPI protocol should not change significantly within the same animal when the light is turned off, compared to when the light is on. In contrast to my archaeorhodopsin results, in the animals injected with halorhodopsin, the light altered the startle responses. Surprised by this unexpected and obviously wrong result, we uncovered the cause of this error: the gain of the software (but not that of the load cell coupler) was lowered, hence giving a lower startle response. The gain that is normally manipulated is the one of the load cell coupler, but due to software issues the settings

were reset to factory, and we did not account that the gain from the software had to be re-adjusted back to the level in which the rest of the animals were tested on. This could explain the lowered startle response in the halorhodopsin injected animals. Despite the difference in startle levels, the trend of PPI seen in the halorhodopsin injected animals was similar to the one seen in archaeorhodopsin injected animals. All the ISIs showed a trend of lower PPI values when the light was on (I.e. when the PPTg-PnC excitatory synapse was silenced), but the 500ms ISI was the one that showed the most robust PPI change. In order to confirm these results and be able to show differences with confidence, the experiments using the inhibitory opsins have to be performed on more mice, and the ones with halorhodopsin with the gain adjusted to the original setting.

It was previously mentioned that the results obtained were not expected since theoretically the silencing of this connection should lead to a decreased PPI, which is normally seen in people suffering from disorders characterized by SG. Interestingly, the silencing of the PPTg-PnC glutamatergic connection has yielded results similar to those seen in a mouse model of autism (Stark et al., 2009). In this model, a decrease in PPI was seen in the 100ms ISI and at different prepulse intensities. It is interesting to see that the silencing of this PPTg-PnC glutamatergic connection expresses a response very similar to the impaired SG of a model of disease. To further test this, this model of disease could be analyzed to look at the PPTg glutamatergic neurons and identify if there is any difference compared to a wild type and possibly identify a strong involvement in the impairment of SG.

As stated initially, we were expecting that the PPTg glutamatergic neurons had an effect on PPI, and since the PPTg contribution to PPI is inhibitory, we were expecting for them to synapse onto the PnC glycinergic interneurons and inhibit the giant glutamatergic neurons. If this would be the case, inhibiting the PPTg glutamatergic neurons would lead to a decreased PPI, but this was not what was seen. Contrary to what we were expecting, by inhibiting these neurons the PPI is potentiated, suggesting

that other components could be contributing to this mechanism. These data are suggesting that the PPTg-PnC glutamatergic synapses could possibly directly excite the giant glutamatergic PnC neurons, and when these synapses are inhibited, a different input would be unmasked leading to an enhanced inhibition (PPI). We have proposed a PPTg-PnC circuit relevant to SG seen in **Figure 4.2**, where there is also a GABAergic projection emerging from the PPTg to the PnC and when the glutamatergic neurons are silenced, a contribution from such neurons is unmasked and they directly inhibit the PnC giant glutamatergic neurons that mediate the startle. In order to test this, the next procedures would be to try to evaluate the contribution of the PPTg GABAergic neurons to PPI at the level of the PnC.

Since we have proposed a GABAergic component into the PPTg-PnC synapses, a possible explanation to the altered results seen in the animals injected with halorhodopsin could be due to the nature of the channel allowing the selective flow of chloride ions. The translation of archaeorhodopsin leads to the expression of proton pumps in the neuronal membranes, leading to inhibition of the cells by expelling protons out of the cells and thus hyperpolarizing the membranes. On the other hand, the translation of halorhodopsin leads to a chloride pump in the membranes of the cells, leading to inhibition by taking chloride ions into the cells and hyperpolarizing the membranes. The terminals of the glutamatergic neurons expressing halorhodopsin are inhibited at the level of the PnC, where the synapses affecting PPI must occur. Based on our proposed PPTg-PnC circuit, the glutamatergic and the GABAergic processes on the PnC are in close proximity synapsing onto the giant glutamatergic neurons, the use of halorhodopsin could affect the concentrations of chloride ions in the extracellular space altering the natural function of the GABAergic neurons when the light is shined. We only saw an abnormal startle response when the light was on in the halorhodopsin injected animals, and not on the archaeorhodopsin animals and a chloride ion

concentration manipulation could be an explanation, but as mentioned before, more experiments need to be performed to confirm this effect, or to rule out this possibility.

Another possible explanation to these results could be a presence of group II metabotropic glutamatergic receptors (mGluR2/3) in the PPTg-PnC connection relevant to SG as seen in a similar connection relevant to SG (Valsamis et al., 2014). These receptors happen in the pre-synaptic terminals and have an inhibitory effect by preventing neurotransmission, and not activating the subsequent cells. Further studies also have to be performed in order to confirm this proposed involvement.

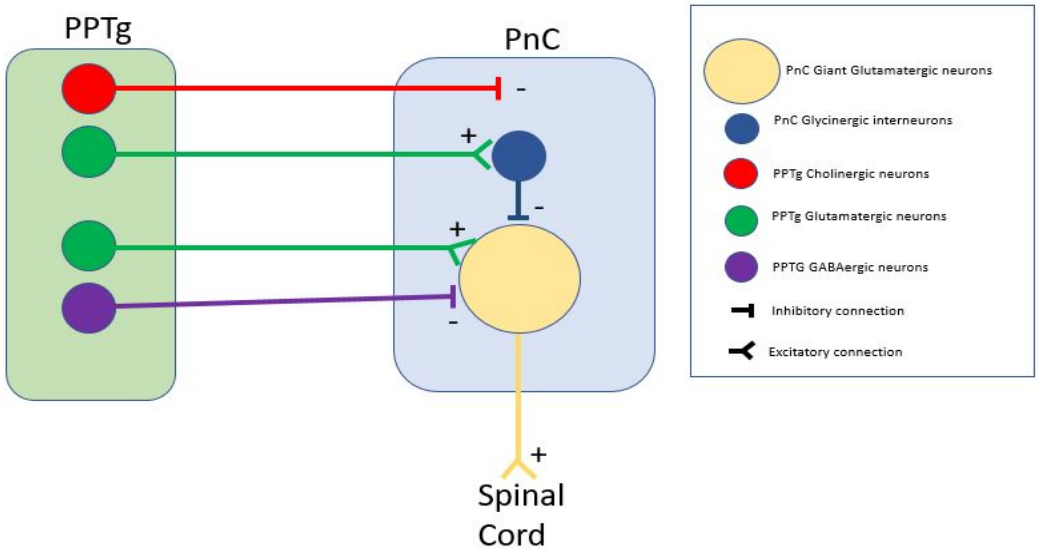

Figure 4.2. **Proposed PPTg-PnC circuit relevant to SG.** The diagram shows a proposed PPTG-PnC circuit. The (Left) panel represents the PPTg, containing cholinergic, glutamatergic, and GABAergic neurons that are projecting onto the PnC. The (Right panel) shows the PnC containing the PPTg afferents synapsing onto glycinergic interneurons, or the giant glutamatergic neurons, who then project to the spinal cord. It is proposed that the (inhibitory) cholinergic neurons don't contribute to SG in this circuit, (excitatory) glutamatergic neurons synapse onto (inhibitory) glycinergic neurons and giant glutamatergic neurons, and the (inhibitory) GABAergic neurons project onto the giant glutamatergic neurons.

IV.3. Future Studies

The main purpose of this project has been to identify a putative connection at the core of PPI and test its involvement functionally. The results of this project have demonstrated a circuit that has not been described before. The techniques that have been used are tract-tracing experiments and in-vivo optogenetic silencing while the animals perform the PPI tasks. What these results have revealed is that there is a glutamatergic involvement in PPI at a specified ISI, but more experiments need to be performed to see if they are involved in more than one ISI. Now that we have shown this type of involvement, many more questions can be asked about this circuit and the mechanism. Future studies for an in-depth understanding of this mechanism and newly described circuit can be performed to look at the electrical and molecular characteristics of the glutamatergic PPTg-PnC synapses.

The following studies to further support our initial hypothesis and results could be done by obtaining *in vitro* extracellular recordings with an optogenetic approach. The virus AAVDJ-CaMKIIα-ChR2-eYFP could be injected directly to the PPTg and selectively make the glutamatergic neurons excitable with blue light. To test this connection in vitro, we could mimic the PPI task in PnC slices with electrophysiological techniques, as previously published (Bosh and Schmid, 2008). After the viral proliferation period, the brain would then be extracted and 300 μm-thick brain slices would be obtained at the level of the PnC, which would contain the terminals of the PPTg glutamatergic neurons that would be photo-excitable with blue light. The protocol to follow would be to shine blue light at the PnC level, where the PPTg terminals are located and excite them, simulating the prepulse. Subsequently, we would electrically stimulate the auditory fibers to simulate a startling stimulus while performing electrophysiological recordings. Then, a cocktail of glutamatergic receptor blockers would be added; CNQX for AMPA receptors and AP5 for NMDA receptors to confirm the glutamatergic nature of the synaptic inputs. We would expect to see a diminished

PPI response after the drug treatment and recover the response by a washout. In addition, a similar approach of analyzing SG *in vivo* while recording could be performed in order to look at electrical activity and pair it with behavior.

It would be interesting to look at different aspects of this connection like what types PPTg neurons specifically target the PnC. As described in Leinweber et al., 2017, a combination of viruses can be used to specifically locate projection neurons at very specific synapses. The experiment would be to inject AAV2/1-Ef1a-TVA-T2A-CVS11G into the PnC to express the rabies virus G protein and TVA receptors in the local PnC neurons. Then, the retrograde monosynaptic rabies virus would be injected 2 days after the AAV injection to specifically infect and label the cell bodies in the PPTg synapsing onto specific PnC neurons. The brains of these animals would be extracted and to further visualize this connection they would be treated immunohistochemically to be able and identify their chemical nature. The AAV fluorescence would reveal the cell bodies in the PnC and the rabies virus would label the PPTg-PnC projections. In addition, it would be interesting to test if all the PPTg glutamatergic neurons are involved in the modulation of PPI, or if there is a subset of them involved in other activities.

Many studies and reviews have shown the areas that project to the PPTg and regulate PPI, but they have not shown what is their contribution to the PPTg in function to PPI. To identify these areas, injections of the monosynaptic rabies virus could be performed targeting the PPTg and seeing which other areas project to them. After these have been identified, a series of optogenetic manipulations could be performed on these different areas while the animal is doing the PPI behavioral tasks *in vivo* to see the identified area's contribution to the PPTg.

IV.4. Conclusion

The inability to focus attention due to brain overload becomes devastating when it is involved in your daily life. People suffering with many neurological disorders suffer from such attention deficit as a result of a faulty SG mechanism, which reduces their daily life quality. Many research efforts have been made throughout the years to understand the nervous system, but due to the lack of understanding and the late emergence of new techniques, there are still disorders that are yet to be understood. It is imperative that many efforts are made to understand and be able to treat nervous systems disorders due to its complete control over the body and develop possible treatments.

For this project, I have identified a novel synapse at the core of the circuitry modulating SG. This connection was identified by tract-tracing techniques and its involvement was characterized by performing PPI *in vivo*, which allowed us to identify the synapse's function. More research has to be done to understand this connection's molecular involvement, in addition to the electrical activity leading to the decrease in PPI seen when the connection was silenced. Moreover, it would be interesting to determine if a problem in this connection leads to the impairments in SG, or if it is part of it at least. The manipulation of this synapse has shown a similar effect of PPI as that seen in a model of disease of autism. The analysis of this disease model could give more insights to the determinants of the impairment of SG in neurological disorders. Therefore, many more research efforts must be made in order to be able to develop possible treatments and therapeutic interventions of this life-impairing deficit.

References

Angelucci, Alessandra, Francisco Clascá, and Mriganka Sur. 1996. "Anterograde Axonal Tracing with the Subunit B of Cholera Toxin: A Highly Sensitive Immunohistochemical Protocol for Revealing Fine Axonal Morphology in Adult and Neonatal Brains." *Journal of Neuroscience Methods* 65 (1): 101-112. doi:10.1016/0165-0270(95)00155-7.

Armstrong, DE, CB Saper, AI Levey, BH Wainer, and Rd Terry. 1983. "Distribution of Cholinergic Neurons in the Rat Brain: Demonstrated by the Immunocytochemical Localization of the Choline Acetyltransferase." *Journal of Comparative Neurology* 216: 53-68.

Bergeron, SA, N. Carrier, GH Li, S. Ahn, and HA Burgess. 2015. "*Gsx1* Expression Defines Neurons Required for Prepulse Inhibition." *Molecular Psychiatry* 20 (974): 985.

Bosch, D. and S. Schmid. 2006. "Activation of Muscarinic Cholinergic Receptors Inhibits Giant Neurones in the Caudal Pontine Reticular Nucleus." *European Journal of Neuroscience* 24: 1967-1975.

Bosch, D. and Schmid S. 2008. "Cholinergic Mechanism Underlying Prepulse Inhibition of the Startle Response in Rats." *Neuroscience.*155 (1): 326-35.

Braff, D., C. Stone, E. Callaway, M. Geyer, and I. Glick. 1978. "Prestimulus Effects on Human Startle Reflex in Normals and Schizophrenics." *Psychophysiology* 5 (4): 339-43.

Caine, S. B., M. A. Geyer, and N. R. Swerdlow. 1991. "Carbachol Infusion into the Dentate Gyrus Disrupts Sensorimotor Gating of Startle in the Rat." Psychopharmacology 105 (3): 347-354. doi:10.1007/BF02244429.

Clements, JR and S. Grant. 1990. "Glutamate-Like Immunoreactivity in Neurons of the Laterodorsal Tegmental and Pedunculopontine Nuclei in the Rat." *Neuroscience Letters* 120: 70-73.

Davis, M. 1988. "Apomorphine, D-Amphetamine, Strychnine and Yohimbine do Not Alter Prepulse Inhibition of the Acoustic Startle Reflex." *Psychopharmacology* 95 (2): 151-6.

Davis, M., DS Gendelman, MD Tischler, and PM Gendelman. 1982. "A Primary Acoustic Startle Circuit: Lesion and Stimulation Studies." *Journal of Neuroscience* 2 (6): 791-805.

Davis, M. and PM Gendelman. 1977. "Plasticity of Acoustic Startle Response in the Acutely Decerebrate Rat." *Journal of Comparative and Physiological Psychology* 91 (3): 549-63.

Diamantopoulou, A., Z. Sun, J. Mukai, B. Xu, K. Fenelon, M. Karayiorgou, and JA Gogos. 2017. "Loss-of-Function Mutation in Mirta22/Emc10 Rescues Specific Schizophrenia-Related Phenotypes in a Mouse Model of the 22q11.2

Deletion." *Proceedings of the National Academy of Sciences of the United States of America* 114 (30): E6136.

Eban-Rothschild, A., G. Rothschild, WJ Giardino, J. R. Jones, and L. de Lecea. 2016. "VTA Dopaminergic Neurons Regulate Ethologically Relevant Sleep-Wake Behaviors." *Nature Neuroscience* 19: 1356-1366.

Fendt, M., L. Li, and JS Yeomans. 2001. "Brain Stem Circuits Mediating Prepulse Inhibition of the Startle Reflex." *Psychopharmacology* 13 (3): 216-24.

Garcia-Rill, E., C. R. Houser, R. D. Skinner, W. Smith, and D. J. Woodward. 1987. "Locomotion-Inducing Sites in the Vicinity of the Pedunculopontine Nucleus." Brain Research Bulletin 18 (6): 731-738. doi:10.1016/0361-9230(87)90208-5.

Geis, HR and S. Schmid. 2011. "Glycine Inhibits Startle-Mediating Neurons in the Caudal Pontine Reticular Formation but is Not Involved in the Synaptic Depression Underlying Short-Term Habituation of Startle." *Neuroscience Research* 71: 114-123.

Geyer, MA and DL Braff. 1982. "Habituation of the Blink Reflex in Normal and Schizophrenic Patients." *Psychophysiology* 19 (1): 1-6.

Geyer, M., and Graff, D. 1987. "Startle Habituation and Sensorimotor Gating in Schizophrenia and Related Animal Models." *Schizophrenia Bulletin* 13 (4): 643-68.

Graham, FK. 1975. "The More or Less Startling Effects of Weak Prestimulation." *Psychophysiology, Presidential Address 1974* 12 (3): 238-248.

Ison, JR and GR Hammond. 1971. "Modification of the Startle Reflex in the Rat by Changes in the Auditory and Visual Environments." *Journal of Comparative and Physiological Psychology* 75 (3): 435-52.

Jacobsohn, L. 1909. "Uber Die Kerne Des Menschlichen Hirnstamms." *Berlin: Verlag Der Konigl Akademie Der Wisenschaftern*.

Karson, CN, E. Garcia-Rill, J. Biedermann, R. E. Mrak, MM Husain, and RD Skinner. 1991. "The Brain Stem Reticular Formation in Schizophrenia." *Psychiatry Research* 40 (1): 31-48.

Kobayashi, Kenta, Shigeki Kato, and Kazuto Kobayashi. 2018. "Genetic Manipulation of Specific Neural Circuits by use of a Viral Vector System." *Journal of Neural Transmission* 125 (1): 67-75. doi:10.1007/s00702-016-1674-7.

Koch, M., M. Kungel, and H. Herbert. 1993. "Cholinergic Neurons in the Pedunculopontine Tegmental Nucleus are Involved in Mediation of Prepulse Inhibition of the Acoustic Startle Response in the Rat." *Experimental Brain Research* 97 (1): 71-82.

Kohl, S., K. Heekeren, J. Klosterkötter, and J. Kuhn. 2013. "Prepulse Inhibition in Psychiatric Disorders-- Apart from Schizophrenia." *Journal of Psychiatric Research* 47 (4): 445-52.

Kroeger, D., LL Ferrari, G. Petit, CE Mahoney, PM Fuller, E. Arrigoni, and TE Scammell. 2017. "Cholinergic, Glutamatergic, and GABAergic Neurons of the

Pedunculopontine Tegmental Nucleus have Distinct Effects on Sleep/Wake Behavior in Mice." *Journal of Neuroscience* 37 (5): 1352-1366.

Lingenhöhl, K. and E. Friauf. 1992. "Giant Neurons in the Caudal Pontine Reticular Formation Receive Short Latency Acoustic Input: An Intracellular Recording and HRP-Study in the Rat." *Journal of Comparative Neurology* 325 (4): 473-92.

Logan, GD. 1992. "Attention and Preattention Theories of Automaticity." *The American Journal of Psychology* 105 (2): 317-339.

MacLaren, DA, T. Markovic, and SD Clark. 2014. "Assessment of Sensorimotor Gating Following Selective Lesions of Cholinergic Pedunculopontine Neurons." *European Journal of Neuroscience* 40: 3526-3537.

Marcus Leinweber, Daniel R Ward, Jan M Sobczak, Alexander Attinger, and Georg B Keller. 2017. "A Sensorimotor Circuit in Mouse Cortex for Visual Flow Predictions." *Neuron* 95 (6): 1420. doi:10.1016/j.neuron.2017.08.036.

Mena-Segovia, J. and PJ Bolam. 2017. "Rethinking the Pedunculopontine Nucleus: From Cellular Organization to Function." *Neuron* 94: 7-18.

Miller, EJ, LR Saint Marie, MR Breier, and NR Swerdlow. 2010. "Pathways from the Ventral Hippocampus and Caudal Amygdala to Forebrain Regions that Regulate Sensorimotor Gating in the Rat." *Neuroscience* 165 (2): 601-11.

Paxinos, G. and KB Franklin. 2014. *The Mouse Brain in Stereotaxic Coordinates*. compact second edition ed.

Roseberry, Thomas K., A. Moses Lee, Arnaud L. Lalive, Linda Wilbrecht, Antonello Bonci, and Anatol C. Kreitzer. 2016. "Cell-Type-Specific Control of Brainstem Locomotor Circuits by Basal Ganglia." Cell 164 (3): 526-537. doi:10.1016/j.cell.2015.12.037.

Rye, DB, CB Saper, HJ Lee, and BH Wainer. 1987. "Pedunculopontine Tegmental Nucleus of the Rat: Cytoarchitecture, Cytochemistry, and some Extrapyramidal Connections of the Mesopontine Tegmentum." *Journal of Comparative Neurology* 259: 483-528.

Saper, CB and A. D. Loewy. 1982. "Projections of the Pedunculopontine Tegmental Nucleus in the Rat: Evidence for Additional Extrapyramidal Circuitry." *Brain Research* 252: 376-372.

Schmid, S., T. Brown, N. Simons-Weidenmaier, M. Weber, and M. Fendt. 2010. "Group III Metabotropic Glutamate Receptors Inhibit Startle-Mediating Giant Neurons in the Caudal Pontine Reticular Nucleus but do Not Mediate Synaptic Depression/Short-Term Habituation of Startle." *Journal of Neuroscience* 30 (31): 10422-30.

Schmued, L. C. and L. Heimer. 1990. "Iontophoretic Injection of Fluoro-Gold and Other Fluorescent Tracers." *Journal of Histochemistry and Cytochemistry* 38 (5): 721-723. doi:10.1177/38.5.2332627.

Schmued, Laurence C. and James H. Fallon. 1986. "Fluoro-Gold: A New Fluorescent Retrograde Axonal Tracer with Numerous Unique Properties." *Brain Research* 377 (1): 147-154. doi:10.1016/0006-8993(86)91199-6.

Shik, M. L., F. V. Severin, and G. N. Orlovskiĭ. 1966. "Control of Walking and Running by Means of Electric Stimulation of the Midbrain." Biofizika 11 (4): 659

Sinclair, D., B. Oranje, KA Razak, SJ Siegel, and S. Schmid. 2017. "Sensory Processing in Autism Spectrum Disorders and Fragile X Sundrome-- from the Clinic to Animal Models." *Neuroscience & Biobehavioral Reviews* 76 (B): 235-253.

Stark, Kimberly L., Rachel A. Burt, Joseph A. Gogos, and Maria Karayiorgou. 2009. "Analysis of Prepulse Inhibition in Mouse Lines Overexpressing 22q11.2 Orthologues." *The International Journal of Neuropsychopharmacology*12 (7): 983-989. doi:10.1017/S1461145709000492.

Sun, Sabrina and David V. Schaffer. 2018. "Engineered Viral Vectors for Functional Interrogation, Deconvolution, and Manipulation of Neural Circuits." *Current Opinion in Neurobiology* 50: 163-170. https://doi.org/10.1016/j.conb.2017.12.011.

Swerdlow, N. R., DL Braff, and MA Geyer. 1999. "Cross-Species Studies of Sensorimotor Gating of the Startle Reflex." *Annals of the New York Academy of Sciences* 877: 202-16.

Swerdlow, NR, V. Bakshi, and MA Geyer. 1996. "Seroquel Restores Sensorimotor Gating in Phenyclidine-Treated Rats." *Journal of Pharmacology and Experimental Therapeutics* 279 (3): 1290-9.

Swerdlow, NR and MA Geyer. 1993a. "Prepulse Inhibition of Acoustic Startle in Rats After Lesions of the Pedunculopontine Tegmental Nucleus." *Behavioral Neuroscience* 107 (1): 104-17.

Swerdlow, NR, and Geyer MA. 1993b. "Prepulse Inhibition of Acoustic Startle in Rats After Lesions of the Pedunculopontine Tegmental Nucleus." *Behavioral Neuroscience* 107 (1): 104-17.

Swerdlow, NR, MA Geyer, and DL Braff. 2001. "Neural Circuit Regulation of Prepulse Inhibition of Startle in the Rat: Current Knowledge and Future Challenges." *Psychopharmacology* 156 (2-3): 194-215.

Takakusaki, Kaoru, Ryosuke Chiba, Tsukasa Nozu, and Toshikatsu Okumura. 2016. "Brainstem Control of Locomotion and Muscle Tone with Special Reference to the Role of the Mesopontine Tegmentum and Medullary Reticulospinal Systems." *Journal of Neural Transmission* 123 (7): 695-729. doi:10.1007/s00702-015-1475-4.

Tervo, D. Gowanlock R, Bum-Yeol Hwang, Sarada Viswanathan, Thomas Gaj, Maria Lavzin, Kimberly D Ritola, Sarah Lindo, et al. 2016. "A Designer AAV Variant Permits Efficient Retrograde Access to Projection Neurons." *Neuron*92 (2): 372-382. doi:10.1016/j.neuron.2016.09.021.

Valsamis, B., M. Chang, M. Typlt, and S. Schmid. 2014. "Activation of mGluR2/3 Receptors in the Ventro-Dorsal Prefrontal Cortex Reverses Sensorimotor Gating

Deficits Induced by Systemic NMDA Receptor Antagonists." *International Journal of Neuropsychopharmacology* 17 (2): 303-12.

Wan, FJ., and Swerdlow, NR. 1997. "The Basolateral Amygdala Regulates Sensorimotor Gating of Acoustic Startle in the Rat." Neuroscience 76 (3): 715-724.

Wang, H. L. and M. Morales. 2009. "Pedunculopontine and Laterodorsal Tegmental Nuclei Contain Distinct Populations of Cholinergic, Glutamatergic and GABAergic Neurons in the Rat." *European Journal of Neuroscience* 29 (2): 340-58.

Yeomans, JS, D. Bosch, N. Alves, A. Daros, RJ Ure, and et al. 2010. "GABA Receptors and Prepulse Inhibition of Acoustic Startle in Mice and Rats." *European Journal of Neuroscience* 31 (11): 2053-61.

Yizhar, O., LE Fenno, TJ Davidson, M. Mogri, and K. Deisseroth. 2011. "Optogenetics in Neural Systems." *Neuron* 71 (1): 9-34.

Appendix 1: Injections Summary

AIM 1 INJECTIONS			AIM 2 INJECTIONS			
ID	TRACER	INJECTION AREA	ID	VIRUS	IN RESULTS?	INJECTION AREA
5296	FG	PnC	963	AAV-DJ-CaMKIIa-eArch 3.0-eYFP	Y	PPTg
5297	FG	PnC	1569	AAV-DJ-CaMKIIa-eArch 3.0-eYFP	Y	PPTg
5070	FG	PnC	1818	AAV-DJ-CaMKIIa-eArch 3.0-eYFP	Y	PPTg
5071	FG	PnC	1570	AAV-DJ-CaMKIIa-eArch 3.0-eYFP	Y	PPTg
5072	FG	PnC	223	AAV-DJ-CaMKIIa-eArch 3.0-eYFP	Y	PPTg
5073	FG	PnC	721	AAV-DJ-CaMKIIa-eArch 3.0-eYFP	Y	PPTg
5074	FG	PnC	634	AAV-DJ-CaMKIIa-eArch 3.0-eYFP	N	PPTg
5294	FG	PnC	962	AAV-DJ-CaMKIIa-eArch 3.0-eYFP	N	PPTg
5295	FG	PnC	1819	AAV-DJ-CaMKIIa-eArch 3.0-eYFP	N	PPTg
5293	FG	PnC	NA	AAV-DJ-CaMKIIa-eArch 3.0-eYFP	N	PPTg
5031	FG	PnC	NA	AAV-DJ-CaMKIIa-eNpHR 3.0-eYFP	N	PPTg
5032	FG	PnC	220	AAV-DJ-CaMKIIa-eNpHR 3.0-eYFP	N	PPTg
5023	FG	PnC	222	AAV-DJ-CaMKIIa-eNpHR 3.0-eYFP	Y	PPTg
5025	FG	PnC	221	AAV-DJ-CaMKIIa-eNpHR 3.0-eYFP	Y	PPTg
5024	FG	PnC	723	AAV-DJ-CaMKIIa-eNpHR 3.0-eYFP	Y	PPTg
7668	FG	PnC	724	AAV-DJ-CaMKIIa-eNpHR 3.0-eYFP	Y	PPTg
7667	FG	PnC	975	AAV-DJ-CaMKIIa-eNpHR 3.0-eYFP	Y	PPTg
7669	FG	PnC	977	AAV-DJ-CaMKIIa-eNpHR 3.0-eYFP	Y	PPTg
7272	FG	PnC	976	AAV-DJ-CaMKIIa-eNpHR 3.0-eYFP	Y	PPTg
7273	FG	PnC	926	AAV-DJ-CaMKIIa-mCherry (Control)	Y	PPTg
31	FG	PnC	922	AAV-DJ-CaMKIIa-mCherry (Control)	Y	PPTg
33	FG	PnC	782	AAV-DJ-CaMKIIa-mCherry (Control)	Y	PPTg
32	FG	PnC	783	AAV-DJ-CaMKIIa-mCherry (Control)	Y	PPTg
34	FG	PnC	234	AAV-DJ-CaMKIIa-mCherry (Control)	N	PPTg
500	FG	PnC	501	AAV-DJ-CaMKIIa-mCherry (Control)	N	PPTg
499	FG	PnC				
235	FG	PnC				
236	FG	PnC				
7677	CTB	PPTg				
7269	CTB	PPTg				
919	AAVrg	PnC				
923	AAVrg	PnC				

Appendix 2: Antibody List

TYPE	ANTIBODY	SOURCE	CATALOG/LOT #s	DILUTION
Sera				
Used for CTB	Normal Donkey Serum	Millipore	S30-100ML/NG1827420	2%
1°	Goat anti- CTB	Millipore	DO0176250/227040	(1:200)
2°	Biotin Donkey anti-Goat	Jackson	711-065-152/92433	(1:500)
Conjugate	Streptavidin Alexa Fluor 488	Invitrogen	N21483/927003	(1:200)
Used for Archaeorhodopsin and Halorhodopsin				
1°	Chicken anti-GFP (both)	Abcam	ab13970	(1:500)
1°	Goat anti-ChAT (PPTg)	Millipore	AB144P/2713234	(1:100)
1°	Rabbit anti-VGluT2 (PnC)	Millipore	41-7800/QC215902	(1:300)
2°	A488 Donkey anti-Chicken	Jackson	703-545-155	(1:500)
2°	Cy3 Donkey anti-Goat (PPTg)	Jackson	705-165-147/134527	(1:500)
2°	Cy3 Donkey anti-Rabbit (PnC)	Jackson	711/165-152/126883	(1:500)
Used for AAVrg				
1°	Rabbit anti-GAD67 (PPTg)	Thermo Fisher	PA5-21397	(1:200)
1°	Goat anti-ChAT (PPTg)	Millipore	AB144P/2713234	(1:100)
2°	A647 Donkey anti-Rabbit (PPTg)	Jackson	711-605-152	(1:500)
2°	A488 Donkey anti-Goat (PPTg)	Jackson	105-545-147/125100	(1:500)
Used for CaMKII-mCherry				
1°	Chicken anti-mCherry (PPTg)	Abcam	AB205402	(1:500)
1°	Goat anti-ChAT (PPTg)	Millipore	AB144P/2713234	(1:100)
2°	Cy3 Donkey anti-Chicken (PPTg)	Jackson	703-165-155/130328	(1:500)
2°	A488 Donkey anti-Goat (PPTg)	Jackson	105-545-147/125100	(1:500)

Vita

Luis Enrique Martinetti was born in El Paso, Texas in 1994 as the third child to Luis B. Martinetti and Lorena A. Aguirre. He graduated from Thomas Jefferson High School in 2012 and obtained a bachelor's degree in Biological Sciences from the University of Texas at El Paso (UTEP) in May, 2016. Luis joined Dr. Karine Fénelon's laboratory in March 2014 and was part of the Research Initiatives for Scientific Enhancement (RISE) program as an undergraduate since fall 2014. Luis's undergraduate project involved analyzing memory impairments caused by low blood levels of environmental lead exposure in mice, relevant to those in children from at-risk neighborhoods in the El Paso-Ciudad Juárez border region. He presented his research at various conferences like SFN, ABRCMS and SACNAS. Luis interned at the Minority Health and Health Disparity International Research Training (MHIRT) summer program in 2015 in Gondar, Ethiopia with Howard university under the supervision of Drs. Vernon Morris and Ayele Gugssa analyzing airborne microflora in the dry-season to wet-season transition in an open-air hospital in efforts to locate the sources of cross-contamination.

Excited to be accepted into the Biology M.S program at UTEP in 2016, Luis started the project leading to this thesis and had the opportunity to attend the optogenetics workshop at the world-renowned Optogenetics Innovations Laboratory in Stanford University in 2017. Close to graduation, Luis was honored to be chosen as the College of Science Graduate Student Marshall of Students at Commencement and also received the Academic and Research Excellence Award in Spring 2018. Luis has been accepted to the PhD. Program in Neuroscience at Michigan State University starting in July 1st and will continue to work in sensory processing deficits.

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This thesis was typed by Luis Enrique Martinetti.