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# Midbrain Glutamatergic Neurons Modulate the Acoustic Startle Reflex and Prepulse Inhibition in Mice

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**Midbrain Glutamatergic Neurons Modulate the Acoustic Startle Reflex and Prepulse Inhibition in Mice**

**Abbreviated title:** Midbrain Glutamatergic Neurons Modulate ASR and PPI

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## Abstract

Prepulse inhibition (PPI) of the acoustic startle reflex is a widely used operational measure of sensorimotor gating that is disrupted in several neuropsychiatric disorders. PPI deficits are a hallmark feature of schizophrenia, often associated with attentional and cognitive impairments. Although PPI is extensively used in translational and preclinical research, the cellular and circuit mechanisms underlying this phenomenon remain incompletely understood, even under physiological conditions. Recent evidence suggests that non-cholinergic projections from the pedunculopontine tegmental nucleus (PPTg) to the caudal pontine reticular nucleus (PnC) contribute to PPI. Here, we investigated the role of PPTg glutamatergic neurons in acoustic startle and PPI. Tract-tracing, immunohistochemical analyses, and *in vitro* whole-cell recordings in wild-type male and female mice demonstrated that PPTg glutamatergic neurons innervate the PnC and establish excitatory synapses onto PnC neurons. Optogenetic inhibition of PPTg-PnC glutamatergic synapses in vivo increased PPI across interstimulus intervals without altering baseline startle responses. In contrast, optogenetic activation of this pathway did not modify PPI. However, activation of PPTg–PnC glutamatergic projections instead of an acoustic prepulse bidirectionally modulated startle responses in a time-dependent manner, suppressing startle at short interstimulus intervals while enhancing startle at longer intervals. Additionally, while PPTg glutamatergic inputs target PnC glycinergic neurons, our *in vitro* whole-cell recordings combined with optogenetic stimulation at PPTg-PnC synapses revealed that PPTg glutamatergic inputs also activate PnC glutamatergic giant neurons. Together, our findings identify a novel temporally dependent

excitatory mechanism within the brainstem startle circuit, through which PPTg glutamatergic neurons modulate sensorimotor gating.

## Significance Statement

The pedunculopontine tegmental nucleus (PPTg) contributes to prepulse inhibition (PPI) of startle, a translational measure of sensorimotor gating that is impaired in neuropsychiatric disorders, including schizophrenia. While PPTg cholinergic neurons have been shown not to contribute to PPI, the mechanisms by which non-cholinergic PPTg neurons modulate PPI remain incompletely understood. Here, combining tract-tracing, optogenetics, *in vitro* electrophysiology, and in vivo startle testing in mice, we show that PPTg glutamatergic neurons that innervate startle-mediating neurons of the caudal pontine reticular nucleus (PnC) play a critical role in PPI. Their activation modulates startle responses in a time-dependent manner, either decreasing or increasing startle magnitude. These findings refine our understanding of sensorimotor gating neural mechanisms and may inform therapeutic strategies.

## Introduction

Prepulse inhibition (PPI) of startle responses is the gold standard operational measure of sensorimotor gating, a pre-attentive mechanism by which sensory events inhibit motor outputs (Swerdlow et al., 2000; Braff et al., 2001). PPI occurs when the presentation of a non-startling acoustic, visual or tactile stimulus prior to a startling stimulus reduces the subsequent startle response (Hoffman and Fleshler, 1963). PPI deficits are associated with and predictive of attention impairments, psychotic symptoms and motor/speech dysfunctions. These deficits occur in several neurological disorders: schizophrenia (Mena et al., 2016; Ding et al., 2023), obsessive compulsive disorder (Swerdlow and Geyer, 1993; Kohl et al., 2015), Tourette syndrome (Castellanos et al., 1996; Buse et al., 2016), and post-traumatic stress disorder (Grillon et al., 1996). PPI exhibits high translational value because it can be elicited in human populations (Takahashi and Kamio, 2018) and other species (Koch, 1999; Burgess and Granato, 2007; Aguilar et al., 2018) and can predict psychotic symptom severity (Graham, 1975; Koch, 1999; Burgess and Granato, 2007). While PPI and the reversal of PPI deficits by antipsychotic drugs have been investigated in animal models of disease (Swerdlow and Geyer, 1993; Sinclair et al., 2017; Zheng et al., 2023), the circuit-level mechanisms underlying PPI remain unknown. Despite extensive study, common antipsychotics show inconsistent effects on PPI and cognitive symptoms in affected individuals (de Bartolomeis et al., 2023), highlighting a crucial knowledge gap. Therefore, it is essential to identify the cellular mechanisms underlying PPI to understand the impact of disease and develop targeted therapeutic interventions.

In the mammalian acoustic brainstem startle circuit, auditory information activates cochlear root neurons which subsequently activate contralateral startle-mediating giant glutamatergic neurons located in the caudal pontine reticular nucleus (PnC). Interneurons expressing the glycine transporter type 2 (GlyT2; Zeilhofer et al., 2005) are intermingled between these PnC giant neurons which directly innervate cervical and spinal motoneurons and therefore represent the sensorimotor interface of the startle pathway (**Fig. 1**; Lingenhohl and Friauf, 1992; Yeomans and Frankland, 1996).

The pedunculopontine tegmental nucleus (PPTg) is a midbrain region known to inhibit PnC-mediated startle responses (Koch et al., 1993). It was initially suggested that PPI requires PPTg cholinergic neuron activity. This was supported by studies showing that PPTg cytoarchitectural abnormalities are observed in patients with PPI deficits, that PPTg lesions reduce PPI, and that PPTg cholinergic inputs inhibit PnC giant neurons (Swerdlow and Geyer, 1993; Kodsi and Swerdlow, 1997; Bosch and Schmid, 2008). This initial hypothesis was recently ruled out by rat in vivo studies showing that optogenetic or chemogenetic manipulation of PPTg cholinergic neurons does not impact PPI (Azzopardi et al., 2018; Fulcher et al., 2020). Since the PPTg is also comprised of substantial populations of glutamatergic and GABAergic neurons (Wang and Morales, 2009; Luquin et al., 2018), these results suggest that non-cholinergic PPTg neurons play a crucial role in PPI. While the contribution of PPTg glutamatergic neurons to PPI remains unexplored, PnC-projecting PPTg glutamatergic neurons modulate locomotion in rodents (Martinez-Gonzalez et al., 2014; Huang et al., 2024b; Roseberry et al., 2016; Josset et al., 2018). Additionally, PPTg glutamatergic neurons can selectively target inhibitory striatal interneurons to enable feedforward inhibition (Assous et al., 2019).

We recently described a feedforward inhibitory mechanism by which glutamatergic neurons located in the central nucleus of the amygdala (CeA) activate GlyT2$^+$ PnC glycinergic neurons in mice (Cano et al., 2021; Huang et al., 2024a). Our optogenetic experiments further confirmed that both CeA glutamatergic neurons and GlyT2$^+$ PnC neurons modulate PPI.

Building on these results, here we tested the hypothesis that PPTg glutamatergic neurons also modulate PnC neuronal activity during startle and PPI. Using a combination of anatomical, *in vitro* optogenetic and electrophysiological approaches in mice, we report the existence of glutamatergic projections from the PPTg that innervate both PnC giant and inhibitory neurons. We also functionally characterized PnC-projecting PPTg glutamatergic neurons using in vivo optogenetic manipulations during startle and PPI.

## Materials and Methods

All experimental procedures were performed in accordance with the National Institute of Health Guidelines, and were conducted in compliance with the UMass Institutional Animal Care and Use Committee (IACUC).

***Animals.*** Adult C57CBL/6J male and female mice aged between 5-6 months (Jackson Laboratories) were used for all procedures. Following surgery, animals were singly housed at the University of Massachusetts Amherst (UMass) vivarium with controlled temperatures and a 12hour light/dark cycle with *ad libitum* access to food and water.

***Study design.*** To label the glutamatergic projections between the PPTg and PnC, we performed unilateral viral tracing experiments. For retrograde labeling experiments, mice received unilateral injections of the retrograde virus AAVrg-DJ-CAG-tdTomato (Addgene # 59462; N = 4) in the **PnC**. Viral expression constructs (AAVs) used the CAG promoter (i.e., CMV immediate-early enhancer/chicken β-actin promoter with exon 1 and intron 1/rabbit β-globin splice acceptor) to drive transgene expression. tdTomato (i.e., tandem dimer Tomato) was used as reporter. For anterograde tracing, mice (N = 8) were injected unilaterally with AAV-DJ-CaMKII-ChR2-mCherry (i.e., monomeric Cherry; Addgene #26975) targeted at the **PPTg**. Two to five weeks later, we immunostained for the appropriate fluorescent reporter (eGFP or mCherry) along with either choline acetyltransferase (ChAT) and GAD67 for slices containing the PPTg or postsynaptic density marker PSD-95, NeuN, and parvalbumin (PV) for slices containing the PnC.

To optogenetically inhibit PPTg glutamatergic axons in the PnC during behavior, we unilaterally injected WT mice (N = 8) with AAV-DJ-CaMKIIα-NpHR3.0-eYFP (i.e., Enhanced Yellow Fluorescent Protein; Addgene #26971) targeted at the **PPTg**. To optogenetically activate PPTg glutamatergic axons in the PnC during behavior, the same animals used for anterograde tracing were used for behavioral assessment (N = 7). All mice undergoing behavioral assessment (N = 15) received a fiberoptic implant ipsilateral to the injection site over the PnC. Behavioral experimental paradigms used a unilateral approach due to prior evidence that unilateral manipulations were sufficient to produce PPI deficits (Forcelli et al., 2012) and the clinical relevance demonstrating that unilaterally impacted brain regions can lead to PPI deficits (Li et al., 1998; Silva et al., 2005; Sonnenberg et al., 2006). Four weeks following fiberoptic implant, animals were tested on acoustic startle response and prepulse inhibition behavioral assays in the absence and presence of yellow or blue light.

To investigate electrophysiological properties of PPTg glutamatergic neurons and their postsynaptic targets in the PnC, mice (N = 10) received injections of AAV-DJ-CaMKII-ChR2mCherry (Addgene #26975) targeted at the **PPTg**. After allowing for viral expression for 4-5 weeks, mice brains were collected for electrophysiology experiments.

***Surgery.*** Mice were sedated by inhaling 5% isoflurane vapors (Vedco, Saint Joseph, Missouri), placed in a stereotaxic apparatus, and immobilized using ear bars and a nose cone. Mice were maintained under anesthesia (1.5–3% isoflurane) for the duration of the surgery, and the head was leveled in all three axes. All injections were performed using a pressure micro-injector (Quintessential Stereotaxic injector, Stoelting Co., IL).

Anterograde unilateral injections consisted of 0.3 µL total volume (2 injections of 0.15 µL each) targeted at the PPTg at the following coordinates relative to Bregma: **#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. Retrograde unilateral injections consisted of 0.1 µL total volume aimed at the **PnC** (coordinates: AP: -5.4 mm, ML: +0.5 mm, DV: -5.15 mm; Paxino's and Franklin, 2004). To do so, a craniotomy was drilled ipsilateral to the PPTg injection site, but at the PnC level. A cannula guide with a 200-µm core optical fiber (Thorlabs, Newton, NJ) was then implanted over the PnC (coordinate: AP − 5.35mm, ML + 0.7mm, DV − 4.5 mm using StereoDrive Robot Stereotax by Neurostar) and fixed to the skull with dental cement (Parkell, Edgewood, NY). Following surgery, mice recovered for two-weeks, allowing for maximal transport of viral particles, or four-weeks to allow for opsin expression prior to behavioral testing.

***Behavior.*** Mice underwent the PPI task in a startle response system (PanLab System, Harvard Apparatus, Holliston, MA). Behavioral testing trials were designed, and data were recorded using PACKWIN V2.0 software (Harvard Apparatus, Holliston, MA). Sound pressure levels were calibrated using a standard SPL meter (model 407730, Extech, Nashua, NH). Mice were placed on a movement-sensitive platform. Vertical displacements of the platform induced by startle responses were converted into a voltage trace by a piezoelectric transducer located underneath the platform. Startle amplitude was measured as the peak maximum startle magnitude of the signal measured during a 1s window following the presentation of the acoustic stimulation.

Mice underwent two consecutive testing days where animals performed the startle reactivity task and the PPI task (with and without optogenetic manipulation) once per day in a pseudorandomized order. Prior to any testing session, animals were first handled and acclimatized to the testing chamber, where the mice were presented to a 65dB background noise, for 10 min. This acclimatization period was used to reduce the occurrence of movement and artifacts throughout testing trials. Following the acclimatization period, an input/output (I/O) assay was performed to test startle reactivity. This I/O test began with the presentation of a 40 ms sound at different intensities (in dB: 70, 80, 90, 100, 110, and 120) every 15s, in a pseudorandomized order with 65dB background noise present throughout. A total of 35 trials (i.e., 7 sound intensities, each sound presented 5 times) were acquired and quantified. Startle reactivity, derived from this I/O assay, allowed the gain of the movement-sensitive platform to be set. This gain allowed the startle responses to be detected within a measurable range. Once determined, the gain for each experimental subject was kept constant throughout the remaining of the experiment. As we previously published, startle magnitude was monitored throughout sessions and animals with >15% startle decline across blocks were excluded (Cano et al., 2021, Huang et al., 2024).

PPI testing consisted of two different components: (1) startling “pulse alone” stimulations and (2) combinations of a non-startling prepulse followed a 120dB startling pulse at 8 different interstimulus intervals (ISIs; in ms): 10, 30, 50, 100, 200, 300, 500, and 1000 (end of prepulse to onset of startle pulse). Mice were presented with 7 startle pulses (120 dB sound, 40ms) every 29 s (interspersed with 65 dB background noise) to achieve a stable baseline startle response. This was followed by 5 presentations of each prepulse+startle pulse condition (i.e., 40 trials – 5 times each ISI condition) in a

pseudorandom order every 29s (also interspersed with 65dB background noise). Prepulse+startle pulse trials were also interspersed with 5 “startle pulse” trials to ensure that the startle response did not diminish throughout the entirety of the task.

For combined optogenetic manipulations and PPI testing, animals injected with viral vectors AAV- DJ-CamKIIa-NpHR3.0-eYFP (N = 8 mice) or AAV-DJ-CamKIIa-ChR2-mCherry (N = 7 mice) were tested in the startle chamber. These animals were closely monitored to ensure that they were comfortably tethered to an optic fiber, which exited through a small opening from the roof of the startle chamber. The optic fiber (200 μm diameter, Thorlabs, Newton, NJ) was connected to the animal’s head via a cannula implanted on the head of the mouse with a zirconia sleeve (Thorlabs, Newton, NJ). Animals were tethered ~ 15min in the startle chamber to acclimate while 65 dB background noise was presented. Optogenetic stimulation was triggered by a signal from the Packwin software (PanLab System; Harvard Apparatus, Holliston, MA), which was transformed into a Transistor-Transistor Logic (TTL) pulse. This TTL pulse triggered a waveform generator (DG1022, Rigol Technologies), which was used to modulate light stimulation. Photostimulation used a blue 473-nm laser (Opto Engine LLC, Midvale, UT) for ChR2 activation, while photoinhibition used a yellow 593.5-nm laser (Opto Engine LLC, Midvale, UT) for NpHR3.0 activation. During PPI trials paired with optogenetic inhibition or activation, a train of light stimulation (1 ms light ON, 199ms light OFF) was delivered at 5 Hz and was either (1) delivered 400ms prior to and concurrent to the pulse-alone stimulation, or (2) delivered 400ms prior to and concurrent to the prepulse stimulation, lasting the entire interstimulus interval. During PPI trials with optical stimulation used as a prepulse, a 5Hz stimulation train (3 pulses of 15ms) was delivered

214 at 400ms prior to and directly before various ISI (10, 30, 50, 100, 200, 300, 500, and 1000
215 ms) prior to the startling pulse. At the end of each experiment, histological analyses were
216 performed to confirm that (1) the injected viral particles were confined to the PPTg, and
217 (2) the cannula guide placement was successfully aimed at the PnC. If these criteria were
218 not met, the subject was excluded from the study.
219
220 ***In vitro electrophysiology***. Brainstem slices for electrophysiological recordings were
221 prepared using protocol described previously (Ting et al., 2018), with modifications. Mice
222 (N = 10) were anesthetized in an induction chamber with 5% isoflurane in an oxygenated
223 environment for 1 min or until completely unresponsive to toe pinch. Mice were removed
224 from induction chamber, tested for lack of reflex response, and quickly decapitated. The
225 brain was exposed and swiftly removed from the skull and submerged in ice-cold NMDG-
226 HEPES cutting solution (in mM) : NMDG (92), KCl (2.5), $NaH_2PO_4$ (1.25), $NaHCO_3$ (30),
227 HEPES (20), glucose (25), thiourea
228 (2), Na-ascorbate (5), Na-pyruvate (3), $CaCl_2$ (0.5), and $MgSO_4$ (10) (bubbled with 95 %
229 $O_2$ / 5
230 % $CO_2$ and pH to 7.3–7.4 with HCl). Then, using a Leica VT 1200s vibratome (Buffalo
231 Grove,
232 IL), brainstem were sliced in coronal sections using zirconium ceramic injector blade
233 (EFINZ10, Cadence Blades, Staunton, VA). PnC and PPTg containing slices were
234 collected and transferred to interface chamber containing slice recovery buffer,
235 maintained at 35 °C, containing
236 (mM): NaCl (92), KCl (2.5), $NaH_2PO_4$ (1.25), $NaHCO_3$ (30), HEPES (20), glucose (25),
237 thiourea (2), Na-ascorbate (5), Na-pyruvate (3), $CaCl_2$ (0.5), and $MgSO_4$ (10) (bubbled

with 95 % O2 / 5 % CO2 and pH to 7.3–7.4 with NaOH). Slices were subject to “Na spike-in,” according to protocol and age, as previously described (Ting et al., 2018). Upon completion of Na-spike-in, slices were then transferred to artificial cerebral spinal fluid (aCSF) maintained at room temperature (22 °C) containing (in mM): NaCl (124), KCl (2.5), NaH2PO4 (1), NaHCO3 (25), glucose (10), MgSO4 (1), CaCl2 (2) (bubbled in 95 % O2 / 5% CO2) for minimum of one hour prior to electrophysiological recordings.

Patch-clamp electrophysiological recordings were performed in brainstem slice preparations, under the whole-cell mode at room temperature with either EPC10/2 amplifier

(HEKA Elektronic, Lambrecht, Germany) or Multiclamp 700B (Molecular Devices, San Jose,

CA), and data were acquired with a PC computer running either Patchmaster (HEKA) or pClamp 10 (Molecular Devices) acquisition systems. Brain slices were placed in a recording chamber where ACSF was fed by gravity at a rate of 2-3 mL/minute. Cells were impaled with patch pipettes pulled from borosilicate glass capillaries (1B150F-4, World Precision Instruments, Sarasota, FL) using a P-97 horizontal pipette puller (Sutter Instruments, Novato, California). The resistance of patch pipettes was typically 3-6 MΩ when filled with an internal solution containing (in mM): KMeSO4 (125), KCl (10), HEPES (10), NaCl (4), EGTA (0.1), MgATP (4), Na2GTP (0.3), Phosphocreatine (10), Biocytin (0.1%) (pH = 7.3; osmolarity = 285–300 mosm).

Neurons were compensated for capacitance and series resistance online to 90% using software driven capacitance control circuit. In voltage clamp experiments cells were held at 70mV to record spontaneous activity and during optogenetic stimulation. For

current clamp experiments, cells were rejected if current compensation to maintain -70mV holding voltage exceeded 200pA. Access resistance was monitored continuously and cells with >20% change were excluded.

In the PPTg, neurons expressing channelrhodopsin (ChR2) were targeted for patch clamp recordings, and were visualized guided by the coexpression of the fluorescent marker eYFP. In the PnC, mCherry-fluorescent axon fibers from PPTg Glutamatergic neurons transfected with AAV-CaMKII-ChR2-mCherry were targeted by photostimulations for post-synaptic patch clamp recordings. Patched PPTg or PnC neurons were allowed to equilibrate with pipette solution for minimum of 15 minutes prior to optogenetic stimulation. Optogenetic stimulation of ChR2-expressing PPTg axons was performed by TTL pulses to Plexon, PlexBright LD-1 single channel LED driver (Plexon, Dallas, TX) connected to an LED module (470 nm; Thor Labs) and an optic fiber cable. Using a micromanipulator, the LED optic fiber was closely positioned (~ 1 mm) at an angle of 30 degrees over the brain section and the light was aimed at the patched cell which was visualized using a water-immersion objective. A small number of electrophysiological traces showed a fast transient that reflected a brief LED light-onset artifact rather than an electrode-derived electrical stimulus artifact. This transient was present in only a few number of $ChR2^{+}$ and $ChR2^{-}$ recordings and was not time-locked to synaptic currents. No electrical stimulation was applied during these experiments. For single pulse and paired pulse stimulation for ChR2 activation, the LED light pulse duration lasted 1 ms, unless stated otherwise. Light pulse train stimulation templates were generated in Igor Pro 6.0 (WaveMetrics, Lake Oswego, OR) through template feature in Patchmaster. Data were analyzed with either Igor Pro 6.0 or Clampfit (Molecular Devices).

***Immunohistochemistry***. 24-hours following the conclusion of behavioral testing, mice (N = 15) were anesthetized with isoflurane and exsanguinated by transcardial perfusion with approximately 50 mL chilled 0.9% saline solution followed by chilled 4.0% paraformaldehyde (PFA) solution in phosphate buffered saline (PBS). After perfusion, animals were decapitated and the brains were extracted and harvested followed by post-fixing in PFA overnight at 4°C for approximately 12-18 hours and cryoprotection in 30% sucrose at 4°C for approximately 36-48 hours. 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 1-in-6 series throughout the extent of the PnC and PPTg at a thickness of 30µm in the coronal plane of section 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% 0.05M phosphate buffer, 3-& ethylene glycol, 20% glycerol) at ⁻20°C - ⁻80°C until stained and/or plated for visualization.

The viral constructs contained the reporter gene mCherry or eYFP which are translated into fluorescent proteins which were enhanced via immunohistochemistry for optimal visualization/labelling (chicken anti-mCherry, 1:1000, Abcam, ab205402; or chicken anti-GFP, 1:1000, Abcam, ab13970). Additional antibodies were used to double-label cells expressing specific proteins to reveal their identities. The brain slices were collected and separated into two groups depending on whether they contained the PPTg or the PnC. The brain sections were rinsed off of cryoprotectant solution for 3 times for 5 minutes each using Tris-buffered saline (TBS; pH 7.4 at room temperature) and incubated

in Blocking solution (NDS) (3% 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 1 hour at room temperature. The PPTg sections were treated with goat anti-ChAT antibody (1:200; Millipore, AB144P) since labeling PPTg Cholinergic neurons still constitutes the best way to delineate the anatomical border of the PPTg. The PnC sections were treated with either a guinea pig anti-NeuN antibody (1:1000; Sigma Aldrich, ABN90P), a marker of mature neurons, or a rabbit anti-parvalbumin antibody (1:1000; Abcam, ab11427), a marker of inhibitory neurons, and a goat anti-PSD95 antibody (1:500; Abcam, ab12093), a marker of excitatory synapses. Slices were incubated in primary antibodies for 1-2 days at 4°C. The slices were then washed of the primary antibody in TBS for 3 times for 10 minutes each at room temperature and incubated in combinations of secondary antibodies used at a concentration of 1:500 (donkey anti-guinea pig DyLight405, Jackson IRL, 706-475-148; donkey anti-goat Cy3 or AF488, Jackson IRL; donkey anti-rabbit DyLight405, Jackson IRL, 711-475-152; donkey anti-chicken Cy3 or AF488, Jackson IRL) for 3-4 hours at room temperature, then rinsed again. The trays with the tissue were covered by aluminum foil to prevent photo-bleaching during the antibody treatment. The slices were then mounted on super frost slices, air-dried and cover-slipped.

***Statistical analysis***. Statistical analyses were performed using SigmaPlot (Systat Software, Inc., San Jose, CA) and GraphPad Prism (GraphPad Software, La Jolla, CA).

Normality and equal variance of the data were first tested, and data transformations were made before performing further statistical analyses. We determined the significance of the interaction between the factors assessed using ANOVA. For the results of whole-cell patch clamp recordings with receptor antagonists, one-way repeated measures (RM) ANOVA and Sidak post hoc testing were used to assess the effect of the receptor antagonists on the light-evoked events. PPI was defined and measured as [1– (startle amplitude during “Prepulse+Pulse” trials/startle amplitude during “Pulse” trials)] × 100. Two-way Repeated Measures (RM) ANOVA was used to assess the effect of the light, sound intensity/inter-stimulus interval (ISI), and light interaction and the interaction among groups. For optical stimulation experiments where the photo stimulation of neurons fibers was used as a prepulse in vivo, two-way RM ANOVA was used to assess the effect of the stimulation, ISI, ISI and stimulation interaction and interaction among groups. Then, Sidak testing was applied for post hoc comparisons. A confidence level of $p < 0.05$ was considered statistically significant. Sample sizes were chosen based on expected outcomes, variances, and power analyses. Data are presented as means ± SEM. N indicates total number of animals; n indicates total number of brain slices or testing trials. Adobe Illustrator and BioRender were used to create figures.

## Results

### *PPTg glutamatergic neurons innervate the PnC*

To visualize putative PPTg glutamatergic neurons that project to the PnC, we first injected a retrograde AAV that transduced the fluorescent reporter tdTomato under the control of the CAG promoter (AAVrg-CAG-tdTomato) into the PnC of WT mice (N = 4). As previously reported in rats (Wang and Morales, 2009; Luquin et al., 2018), we identified retrogradely labeled tdTomato$^+$ PPTg neurons that were ChAT-immunonegative in mice (**Fig. 2**). Such ChAT-negative PnC-projecting PPTg neurons are likely glutamatergic since they were labeled with tdTomato but not co-labeled with the inhibitory neuron GAD67 antibody (**Fig. 2D**).

Using *in situ* hybridization, we previously showed that CaMKIIα is exclusively expressed by PPTg glutamatergic neurons (Cano et al., 2021). Therefore, to track how PPTg glutamatergic fibers course within the PnC, we took an anterograde approach and successfully targeted CaMKIIα$^+$ PPTg neurons by injecting AAV-DJ-CaMKIIα-ChR2-mCherry (**Fig. 3**) in the PPTg of WT mice (N = 8). Following PPTg injection (**Fig. 3 B; also see Figure S1**), mCherry$^+$ PPTg glutamatergic fibers were seen across both ventrolateral and ventromedial areas of the PnC delineated by the 7th cranial nerve (**Fig. 3 C, D**). We then used immunohistochemistry in combination with confocal microscopy to evaluate the apposition between mCherry$^+$ PPTg glutamatergic fibers and PnC sections stained for PSD95, an adaptor protein involved in clustering postsynaptic receptors at glutamatergic synapses (Cho et al., 1992; Kornau et al., 1995) in the entire extent of the PnC (**Fig. 3 D**). PSD95-positive puncta were observed in close apposition to PPTg

glutamatergic fibers within the PnC, consistent with putative PPTg excitatory synaptic contacts with PnC neurons.

***Silencing PPTg-PnC glutamatergic synapses enhances PPI without affecting baseline startle***

Next, we aimed to determine the contribution of PPTg glutamatergic neurons projecting to the PnC during acoustic startle responses and PPI, in vivo. To do so, we conducted optogenetic experiments to silence PPTg-PnC glutamatergic synapses in WT mice by transducing PPTg glutamatergic neurons with the yellow-light sensitive inhibitory optogenetic tool Halorhodopsin (NpHR3.0). Mice were injected with the AAV-DJ-CaMKIIα-NpHR3.0-eYFP viral vector unilaterally in the PPTg. Following the intracranial viral injection, an optic fiber was implanted ipsilateral to the injection site at the level of the PnC, to photo-inhibit PPTg fibers/terminals expressing NpHR3.0 with yellow light (**Fig. 4 A;** N = 8).

To determine if PPTg-PnC glutamatergic synapses contribute to baseline acoustic startle responses, we assessed how photo-inhibiting PPTg-PnC glutamatergic synapses with yellow light alters startle reactivity at increasing sound levels in mice expressing NpHR3.0 in PPTg glutamatergic neurons. In these mice, in the absence of yellow light photo-inhibition, sound intensities of 90 dB and beyond elicited an acoustic startle reflex (**Fig. 4 B, D**), measured as an involuntary innate defensive response occurring within milliseconds after the onset of startling stimulus and characterized by a whole-body flexor muscle contraction (Davis et al., 1982; Lee et al., 1996; Swerdlow et al., 1999).

Interestingly, photo-inhibition of PPTg-PnC glutamatergic synapses concurrent with such acoustic startling stimuli did not affect startle amplitude (**Fig. 4 D**; [Two-Way RM ANOVA , light: ($F(1, 7) = 0.506$, $p = 0.4998$); sound intensity × light interaction: ($F(1.96,13.76) = 1.269$, $p = 0.3113$). These data confirm that PPTg glutamatergic neurons projecting to the PnC do not contribute to baseline startle reactivity.

We then tested whether silencing the PPTg-PnC glutamatergic synapses alters PPI in vivo. To do so, we photo-inhibited this pathway starting from the beginning of the prepulse until the end of the interstimulus intervals (**Fig. 4 C, E**). Photo-inhibition of PPTg-PnC glutamatergic synapses resulted in a significant effect of light, increasing PPI (**Fig. 4 E**; Two-Way RM ANOVA, light: ($F(1, 7) = 6.160$, $p = 0.0421$). These photo-inhibition results suggest that PPTg glutamatergic neurons provide an excitatory drive to the PnC startle circuit during PPI.

***Activating PPTg-PnC glutamatergic synapses modulates acoustic startle and PPI***

Next, we tested whether photo-activating PPTg-PnC glutamatergic synapses concurrent with an acoustic prepulse impacts baseline startle (**Fig. 5**). To do so, WT mice were injected with the blue-light sensitive excitatory optogenetic vector AAV-DJ-CaMKIIα-ChR2-mCherry in the PPTg and implanted with an optic fiber positioned above the PnC. In these mice, PPTg-PnC glutamatergic synapses were photo-activated with short trains of blue light at 5Hz, concurrent with an acoustic startling (pulse-alone) stimulation. Photo-activation of $ChR2^+$ PPTg-PnC glutamatergic synapses concurrent with acoustic startling

stimulations did not significantly impact baseline startle (**Fig. 5 E**; Two-Way RM ANOVA, light: ($F(1, 6) = 3.299$, $p = 0.1192$).

Then, to test whether stimulating this glutamatergic connection could alter PPI, PPTg-PnC glutamatergic synapses were photo-activated during PPI using blue-light. Photo-activation started at the beginning of the prepulse and lasted until the end of the interstimulus intervals (**Fig. 5 C, F**). Our results show that photo-activating PPTg-PnC glutamatergic synapses did not alter PPI (**Fig. 5 F**; Two-Way RM ANOVA, light: ($F(1, 6) = 0.3677$, $p = 0.5665$). These results suggest that artificially activating PPTg glutamatergic neurons does not further alter acoustic PPI.

We next aimed to test whether PPTg-PnC glutamatergic synapses alone are sufficient to modulate subsequent baseline startle responses (**Fig. 5 D, G**). To do so, we assessed the effect of photo-activating PPTg-PnC glutamatergic synapses alone, prior to an acoustic startling stimulation, in the absence of an acoustic prepulse. PPTg-PnC glutamatergic synapses were photo-activated with a 20 ms blue light pulse prior to the startling stimulation, at intervals relevant to acoustic PPI (**Fig. 5 D, G**). Interestingly, our results show that photo-activating PPTg-PnC glutamatergic synapses in the absence of an acoustic prepulse had two effects: **1)** it reduced subsequent startle responses, mimicking an acoustic prepulse inhibition effect (or PPI) at *short* ISIs (≤100 ms), but **2)** it enhanced subsequent startle responses, mimicking an acoustic prepulse facilitation effect (potentiation or “negative PPI”) at *longer* ISIs (>100 ms) (Fig. 5 G). Statistical analysis revealed an overall trend effect of light stimulation (two-way RM ANOVA: light effect, $F(1,5) = 4.893$, $p = 0.0779$), with Sidak’s multiple comparisons showing significant effects

435 at the 200 ms (adjusted $p$ = 0.0137) and 500 ms ISIs (adjusted $p$ = 0.0201), and a trend
436 towards significance at 30 ms (adjusted $p$ = 0.0651). These results suggest that PPTg
437 glutamatergic neurons can bidirectionally modulate subsequent startle responses,
438 possibly by providing an excitatory drive to the startle circuit and activating inhibitory or
439 excitatory elements within the PnC depending on the timing of their activation.
440
441 ***Electrophysiological properties of PPTg glutamatergic neurons***
442 Before further investigating how PPTg glutamatergic neurons modulate the PnC
443 startle circuit, we took advantage of the fact that CaMKIIα-mCherry$^+$ PPTg neurons
444 expressing ChR2 are sensitive to blue light photostimulation to record their basic electrical
445 properties (N = 10 mice; n = 10 slices from which 26 neurons were recorded). We
446 performed *in vitro* patch clamp recordings in acute PPTg slices of WT mice previously
447 injected with AAVDJ-CaMKIIα-ChR2-mCherry in the PPTg. This transduced PPTg
448 glutamatergic cells with ChR2 and mCherry (**Figure S2**). From these ChR2-mCherry$^+$
449 PPTg glutamatergic neurons held at −70 mV, mean amplitude of spontaneous excitatory
450 post-synaptic currents (sEPSC) were recorded and not significantly different than the
451 sEPSC amplitude of neighboring ChR2-mCherry-negative PPTg neurons (mCherry-
452 negative: 18.46 ± 1.85 pA. mCherry$^+$ : 16.55 ± 1.31 pA; T-test, $p$ = 0.4059; **Figure S2 A,**
453 **B**). While the half width of the action potentials did not differ between cell types (**Figure**
454 **S2 B,** ***Inset;*** mCherry$^+$: 1.41 ± 0.164 ms vs. mCherry-negative: 1.62 ± 0.265 ms; T-test,
455 $p$ = 0.4812), current injections elicited action potentials firing at a maximum rate of 9.4 ±
456 1.91 Hz in ChR2-mCherry$^+$ PPTg glutamatergic neurons vs. 12.4 ± 1.01 Hz in ChR2-

457 mCherry-negative PPTg neurons (**Figure S2 C**). Other intrinsic properties such as input
458 resistance, I/V curves and rheobase were not different.

459 Importantly, photostimulation induced large current responses in ChR2-mCherry$^+$
460 PPTg neurons (**Fig. 6 A**), confirming that our stimulation protocol can successfully
461 activate these PPTg glutamatergic neurons. Next, to quantify the blue-light sensitivity of
462 ChR2-mCherry$^+$ PPTg glutamatergic neurons, we recorded their activation pattern in
463 response to short photostimulation trains consisting of blue light pulses of 0.1 ms, 0.3 ms
464 and 1.0 ms in duration, applied at 10, 20 and 40 Hz (**Fig. 6 B, C**). Photostimulation trains
465 using 0.1 ms light pulses were sufficient to elicit few action potentials but inefficient at
466 eliciting large current responses in ChR2-mCherry$^+$ PPTg neurons, at the three
467 frequencies tested. In contrast, photostimulation trains using 0.3 ms and 1.0 ms pulses at
468 10, 20 and 40 Hz induced several action potentials and large light-evoked current
469 responses (**Fig. 6 B, C**). These light-evoked current responses reliably followed the
470 stimulation frequency, and the cumulative charge was correlated with the pulse duration
471 (**Fig. 6 D**).

472 Next, to ensure that the optically evoked currents recorded in ChR2-mCherry$^+$
473 PPTg neurons resulted from activating non-synaptic membrane channels located on the
474 soma of these

475 PPTg neurons, we delivered pairs of blue light pulses, using standard short-term synaptic
476 plasticity stimulation protocols (**Figure S2 D-F;** Zucker and Regehr, 2002). Light-induced
477 paired current (voltage clamp) or voltage (current clamp) responses were recorded in
478 ChR2-mCherry$^+$ PPTg neurons, before and after the bath-application of the glutamate

receptor blockers, CNQX and APV (**Figure S2 D**). The pharmacological blockade of glutamate receptors did not affect the individual responses recorded in PPTg neurons or the paired-pulse ratios, indicating that the light-evoked responses recorded in ChR2-mCherry$^+$ PPTg neurons resulted from light-activated membrane channels (**Figure S2 E-F;** RM ANOVA, $p > 0.05$). These findings are aligned with previous tracing studies (Kroeger et al., 2017) showing that PPTg glutamatergic neurons send projections outside the PPTg without forming local synaptic connections within the PPTg.

***PPTg glutamatergic neurons activate PnC giant neurons***

Next, we aimed to investigate how PPTg glutamatergic neurons activate PnC neurons within the startle circuit. To do so, we injected the AAV viral vector AAV-DJ-CaMKIIα-mCherry into the PPTg of WT mice. *Post-hoc* immunohistochemical analysis confirmed that the PPTg was successfully targeted. ChR2-mCherry$^+$ PPTg glutamatergic fibers were observed in acute PnC slices obtained from the same mice (**Fig. 7**). Using these PnC slices, we then wanted to determine whether photostimulation of ChR2-mCherry$^+$ PPTg glutamatergic fibers could elicit excitatory synaptic responses in PnC giant neurons identified by their size > 35 µm (**Fig. 7**). These neurons displayed spontaneous currents of 23.33 ± 3.6 pA in amplitude and spontaneous action potentials with ½ width of 0.91 ± 0.185 ms. In these giant PnC neurons held at −70 mV, blue light photostimulation pulses evoked large currents (**Fig. 8 A**), as well as excitatory postsynaptic potentials (**Fig. 8 B**, *top traces*). These excitatory responses showed facilitation at short ISI (**Fig. 8 B**, *middle and bottom traces*; EPSP Paired-Pulse Ratio: 50

ms = 1.6, 100 ms = 1.2; EPSC Paired-Pulse Ratio: 50 ms = 1.70, 100 ms = 1.17). These results confirm that PPTg glutamatergic neurons establish excitatory synapses with PnC giant neurons and that they can display facilitation.

Aside from giant neurons, the PnC also contains GlyT2$^+$ PnC inhibitory neurons previously shown to contribute to PPI (Cano et al., 2021; Huang et al., 2024a). Therefore, we next tested if PPTg glutamatergic neurons also innervate GlyT2$^+$ PnC neurons. To do so, we used WT mice injected with the AAVDJ-CaMKIIα-ChR2-mCherry viral vector in the PPTg to transduce PPTg glutamatergic cells with mCherry and a fluorescent parvalbumin (PV$^+$) antibody in PnC sections to label PnC inhibitory neurons. Interestingly, mCherry$^+$ PPTg glutamatergic fibers were found within the PnC in close apposition to PV$^+$ neurons (**Fig. 9**), suggesting that PPTg neurons could activate inhibitory neurons within the PnC to induce PPI at certain ISIs.

## Discussion

During aging and in various psychiatric disorders, PPI deficits are linked to cognitive symptoms, but the cellular mechanisms remain ill defined. While the PPTg is clearly involved, here we specifically investigated the role of PPTg glutamatergic neurons. Our tract tracing and immunohistochemical analyses confirmed that these neurons project to the PnC. Optogenetic inhibition of PPTg-PnC glutamatergic synapses increased acoustic PPI. Although optogenetic activation of PPTg–PnC glutamatergic projections did not alter acoustic PPI, stimulation alone was sufficient to bidirectionally modulate subsequent startle responses in a timing-dependent manner. *In vitro* optogenetic and electrophysiological single cell recordings revealed that PPTg glutamatergic inputs can activate PnC giant neurons but interestingly, *post hoc* staining also shows that these glutamatergic inputs course in proximity to PnC inhibitory neurons. These findings support a model in which PPTg glutamatergic neurons can activate both excitatory and inhibitory PnC elements, with temporal recruitment dynamics determining the net behavioral outcome. Understanding how the PPTg contributes to PPI is important for sensorimotor gating disruptions in neuropsychiatric disorders and improving cognitive treatments.

### *Neuroanatomical properties of the PPTg and its glutamatergic neurons*

Research has increasingly focused on the PPTg due to its key role in cognitive and sensorimotor functions disrupted in disorders such as Parkinson's disease, schizophrenia, and Tourette syndrome (Swerdlow et al., 2016). Accordingly, the PPTg has emerged as a potential target for deep brain stimulation to improve cognitive

symptoms. Its extensive connectivity with basal ganglia circuits enables relay to thalamic, brainstem, and spinal effectors, supporting motor regulation and PPI. Ascending projections target cortical and subcortical regions, including the basal ganglia, thalamus, hypothalamus, VTA, amygdala, and frontal cortex, while descending projections influence brainstem motor nuclei (Ryczko and Dubuc, 2013; Roseberry et al., 2016; Lavoie and Parent, 1994a, c; Dautan et al., 2014; Saper and Loewy, 1982; Edley and Graybiel, 1983; Jackson and Crossman, 1983; Rye et al., 1987; Woolf and Butcher, 1989). Recent studies highlight descending PPTg glutamatergic neurons, particularly Vglut2$^{+}$ neurons, in locomotion and muscle tone regulation (Ryczko et al., 2016). These neurons project ipsilaterally to brainstem motor nuclei, forming excitatory synapses in the PnC (Liang et al., 2012; Caggiano et al., 2018; Huang et al., 2024b). Consistent with this, here we show PPTg glutamatergic fibers that project predominantly ipsilaterally to the PnC, where PSD95-positive puncta were observed.

Altogether, the PPTg contains cholinergic, glutamatergic, and GABAergic neurons (Lavoie and Parent, 1994b; Martinez-Gonzalez et al., 2012), with some cholinergic neurons co-releasing glutamate (Wang and Morales, 2009). Therefore, further anatomical studies are needed to define PPTg postsynaptic targets in the PnC and their alterations in disorders associated with PPI deficits.

***Electrophysiological properties of PPTg neurons***

The firing properties of PPTg cholinergic, GABAergic, and glutamatergic neurons are incompletely defined. Electrophysiological and immunohistochemical studies in rats classify

PPTg neurons into three types: Type I neurons are small-to-medium fusiform or triangular cells (3–5 dendrites), likely glutamatergic, exhibiting T-type $Ca^{2+}$ channel-dependent bursting; Type II neurons are medium-to-large polygonal cells (5–7 dendrites), ~50% $ChAT^+$, displaying A-type $K^+$ currents and tonic firing; and Type III neurons are ChAT-negative with mixed T-type and $I_A$ currents (Kang and Kitai, 1990; Leonard and Llinas, 1990; Takakusaki et al., 1996, 1997). Consistent with Type I neurons, our data show that $CaMKII\alpha^+$ glutamatergic neurons exhibit spontaneous bursting and reliably follow 20 Hz stimulation, similar to $vGluT2^+$ neurons (Huang et al., 2024b). Notably, intrinsic properties were similar between $CaMKII\alpha^+$ and CaMKIIα-negative neurons, likely reflecting heterogeneity within the CaMKIIα-negative population, including neurons that co-release acetylcholine and glutamate (Clarke et al., 1996, 1997). Their firing properties also resemble recently described cholinergic PPTg neurons (Chen and Evans, 2024). Further studies are needed to refine definitions of PPTg neuronal subpopulations.

Our *in vitro* data also align with prior studies showing that PPTg glutamatergic neurons primarily project outside the PPTg rather than forming local synapses, targeting regions such as the striatum and PnC to modulate locomotion (Kroeger et al., 2017; Assous et al., 2019; Caggiano et al., 2018). While striatum-projecting neurons are well characterized, less is known about PnC-projecting neurons. In the striatum, optogenetic activation of PPTg glutamatergic axons drives feedforward inhibition by activating fast-spiking GABAergic interneurons. Consistent with this, our data suggest that PPTg glutamatergic neurons may similarly engage feedforward inhibition and contribute to PPI

via $PV^+$ PnC inhibitory neurons, while also directly exciting PnC giant neurons, thereby shaping the balance of excitation/inhibition within the startle circuit.

The PnC contains giant glutamatergic neurons and $PV^+$ inhibitory neurons, including GABAergic and glycinergic populations in rodents and non-human primates (Gradwell et al., 2024). We previously showed that $GlyT2^+$ (glycinergic) neurons modulate PPI and can be activated by glutamatergic inputs originating from the central nucleus of the amygdala (CeA; Cano et al., 2021; Huang et al., 2024a). Given that PPTg inputs also target the PnC, future studies should determine how these projections differentially target postsynaptic populations to affect PPI.

***The contribution of PPTg neurons to locomotion and startle modulation***

Previous mouse studies show that $vGluT2^+$ PPTg neurons can enhance (Caggiano et al., 2018; Masini and Kiehn, 2022), inhibit (Josset et al., 2018; Dautan et al., 2021), or bidirectionally modulate locomotion (Carvalho et al., 2020) depending on their projection targets. This variability reflects the activation of distinct pathways involving PPTg glutamatergic neurons, each with specific downstream targets: projections to the substantia nigra suppress locomotion, while those to the spinal cord or medulla promote movement (Ferreira-Pinto et al., 2021; Goñi-Erro et al., 2023). Similarly, activation of PnC-projecting $vGluT2^+$ PPTg neurons inhibits locomotion, whereas projections to the zona incerta facilitate it (Huang et al., 2024b), supporting distinct PPTg glutamatergic subpopulations.

Ample evidence also supports the contribution of the PPTg to PPI in rodents. Early studies demonstrated that PPTg cholinergic neurons play a key role in mediating PPI, as

cholinergic-specific lesions in the PPTg disrupt PPI (Koch et al., 1993). Consistent with this, ex vivo studies show that activation of mesopontine inputs produces a delayed inhibition of synaptic transmission in startle-mediating brainstem neurons that is reversed by muscarinic receptor antagonists, indicating a presynaptic muscarinic mechanism underlying this effect (Bosch and Schmid, 2008). However, recent research challenged this view as optogenetic activation and chemogenetic inhibition of PPTg cholinergic neurons in rodents did not alter acoustic PPI responses but rather they were shown to facilitate startle responses (MacLaren et al., 2014; Azzopardi et al., 2018; Fulcher et al., 2020). Additionally, PPTg cholinergic boutons observed in close proximity to both glutamatergic and GABAergic cells in the inferior colliculus, suggest that ACh may modulate both excitatory and inhibitory circuits (Noftz et al., 2020). However, the postsynaptic targets of PPTg cholinergic neurons in the PnC remain unclear, and the mechanism by which these cholinergic neurons enhance startle is still unknown.

A limitation of the present study is the use of unilateral, rather than bilateral, optogenetic manipulations. Although bilateral manipulations would provide more complete circuit engagement, prior work demonstrates that unilateral brainstem perturbations are sufficient to alter prepulse inhibition (Forcelli et al., 2012), and complementary rat studies show that unilateral collicular lesions reliably impair PPI (Li et al., 1998; Silva et al., 2005). While bilateral PPTg manipulations have been successfully performed in rats (Azzopardi et al., 2018; Fulcher et al., 2020), anatomical scaling and technical constraints in mice limited the feasibility of bilateral approaches in the present study. Accordingly, unilateral manipulation provided a feasible strategy for probing PPTg–

PnC circuitry. Future mouse studies incorporating improved methods will be important to extend these findings bilaterally.

The apparent discrepancy between the rapid inhibitory effects of PPTg–PnC glutamatergic activation *in vitro* and the longer latencies observed in vivo likely reflects differences in circuit recruitment. *In vitro*, monosynaptic PPTg inputs may rapidly recruit highly excitable feedforward glycinergic interneurons in the PnC (Brill et al., 2021; Cano et al., 2021), suppressing giant neuron output within tens of milliseconds. In contrast, modulation at longer interstimulus intervals in vivo may depend on polysynaptic circuits and metabotropic signaling. Because electrophysiological recordings were performed at room temperature and used artificial activation of a direct PPTg–PnC pathway, such slower network mechanisms were likely bypassed. Previous studies show that PPTg cholinergic inputs suppress excitatory transmission onto PnC giant neurons through M2/M4 muscarinic receptors (Bosch and Schmid, 2006, 2008), while $GABA_B$ receptors contribute to modulation at longer intervals (Yeomans et al., 2010). Metabotropic glutamatergic signaling may even play a similar role. Thus, our recordings likely captured rapid ionotropic effects, whereas the longer-latency modulation observed in vivo likely involves complex network connectivity with longer integration times.

Finally, given the limited focus on non-cholinergic PPTg populations in PPI, we examined PPTg glutamatergic neurons and found that they project to both PnC giant excitatory neurons and $PV^+$ inhibitory neurons. The data from our in vivo photo-inhibition experiments indicate that PPTg glutamatergic neurons provide an excitatory drive to the PnC during PPI. Photo-activation experiments further revealed time-dependent effects: activation at longer interstimulus intervals enhanced startle, likely via PnC giant neurons,

649 whereas activation at shorter intervals reduced startle (mimicking PPI), consistent with
650 recruitment of $PV^+$ inhibitory neurons. Different neuronal circuits are likely targeted by
651 distinct PPTg glutamatergic subpopulations during PPI.
652

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## Figure Legends

**Figure 1. Proposed neural circuits involved in the acoustic startle response (ASR) and prepulse inhibition (PPI).**

The primary acoustic startle pathway (orange pathway) begins with auditory stimulation of the cochlear nuclei (CN), which relay auditory signals to giant neurons in the caudal pontine reticular nucleus (PnC) of the brainstem which also contains parvalbumin$^+$ (PV) interneurons. PnC giant neurons directly excite spinal cord motor neurons (MN). The PPI pathway (blue pathway) responds to low-decibel acoustic prepulses and can inhibit a subsequent startle response through activation of the inferior colliculus (IC), superior colliculus (SC), and pedunculopontine tegmental nucleus (PPTg). The PPI pathway is further modulated by midbrain and cortico-limbic structures (green pathway), including the basolateral amygdala (BLA), central amygdala (CeA), nucleus accumbens (NAcc), and ventral pallidum (VP). Together, these structures form the cortico-striato-pallido-pontine (CSPP) network. Our study focuses on how PPTg glutamatergic neurons modulate PnC activity (dashed red pathway) during startle and PPI.

**Figure 2. Retrograde labeling of PnC-projecting PPTg neurons.**

**(A)** Schematic illustrating unilateral injection of AAVrg-DJ-CAG-tdTomato into the PnC of WT mice. **(B)** Representative PnC injection site of AAVrg-DJ-CAG-tdTomato shown at 10× magnification. **(C)** Retrogradely labeled PPTg neurons express tdTomato (red), and a subset co-expresses choline acetyltransferase (ChAT; green) or GAD67 (cyan), shown at 10× magnification. **(D)** Higher-magnification view of labeled PPTg neurons from the

boxed region in (C). Images are representative of N = 4 animals. Scale bars: B, C = 1 mm; D = 100 μm.

**Figure 3. Anterograde labeling of PnC-projecting PPTg glutamatergic neurons.** **(A)** Schematic illustrating unilateral injection of AAV-DJ-CaMKIIα-mCherry into the PPTg of wild-type mice. **(B)** Representative AAV microinjection site in the PPTg shown at 20× magnification. **(C)** Labeled PPTg glutamatergic axon fibers (red) in the PnC at 20× magnification. **(D)** Higher-magnification view of the region outlined by the yellow box in (C), showing glutamatergic axon fibers (red) closely apposed to PnC neurons labeled with the neuronal marker NeuN (blue) and the excitatory postsynaptic marker PSD95 (green) at 40× magnification. Images are representative of N = 8 mice. Scale bar = 30 μm in (D).

**Figure 4. Silencing PPTg-PnC glutamatergic synapses increases PPI without affecting baseline startle.**
(**A**) Schematic illustrating the location of AAV injection of Halorhodopsin (NpHR3.0) and yellow light delivery for the optogenetic silencing of PPTg-PnC glutamatergic synapses. (**B**)
Schematic illustrating the acoustic startle reflex protocol performed using mice injected with Halorhodopsin, in the absence (top) or presence (bottom) of yellow light. (**C**) Schematic illustrating the PPI protocol performed using mice injected with Halorhodopsin, in the absence (top) or presence (bottom) of yellow light. (**D**) Graph showing that silencing PPTg–PnC glutamatergic synapses with yellow light did not affect baseline startle

amplitude elicited by 70–120 dB acoustic pulses (two-way RM ANOVA: F(1,7) = 0.1946, p = 0.6724). **(E)** Graph showing that silencing PPTg–PnC glutamatergic synapses with yellow light significantly increased PPI when delivered during acoustic prepulses and interstimulus intervals (ISIs) (two-way RM ANOVA: F(1,7) = 6.160, p = 0.0421). N = 8 mice. Data are represented as mean ± SEM. Statistical comparisons were performed using two-way repeated-measures ANOVA followed by Sidak's post hoc testing where appropriate. *$p < 0.05$.

**Figure 5. Optogenetic activation of PPTg glutamatergic projections to the PnC bidirectionally modulates startle responses in a time-dependent manner.**
(**A**) Schematic illustrating the unilateral PPTg injection of ChR2 and ipsilateral optic fiber placement above the PnC. (**B**) Schematic illustrating the acoustic startle reflex protocol performed using mice injected with ChR2, in the absence (top) or presence (bottom) of blue light prior to and during 70–120 dB acoustic pulses. (**C**) Schematic of the PPI protocol performed using mice injected with ChR2, in the absence (top) or presence (bottom) of blue light paired with acoustic prepulses and interstimulus intervals (ISIs). (**D**) Schematic illustrating the PPI Light Mimic protocol performed using mice injected with ChR2, where blue light was present as a prepulse at various ISIs (replacing the acoustic prepulse). (**E**) Graph showing that optical activation of PPTg–PnC glutamatergic projections concurrent with acoustic startling stimuli did not significantly alter baseline startle amplitude. (Two-Way RM ANOVA: F(1,6) = 3.299, $p = 0.1192$). (**F**) Graph showing that optical activation during acoustic prepulse trials did not significantly alter PPI (Two-Way RM ANOVA: F(1,6) = 0.3677, $p = 0.5665$). (**G**) Graph showing that

optical activation alone prior to startling stimuli showed a trend of reduced startle responses at short interstimulus intervals (ISIs ≤ 100 ms) and enhanced startle responses at longer intervals (ISIs > 100 ms; Two-Way RM ANOVA: $F(1,5) = 4.893$, $p = 0.0779$; Sidak's multiple comparisons show significant enhancement of startle at 200ms ($p = 0.0137$) and 500ms ($p = 0.0201$) and a trend towards reduced startle at 30ms ($p = 0.0651$). N = 7 mice per group. Data are represented as mean ± SEM. Statistical comparisons were performed using two-way repeated-measures ANOVA followed by Sidak's post hoc testing where appropriate. *$p < 0.05$, **$p < 0.01$.

**Figure 6. Light-evoked electrophysiological characterization of CaMKIIα-ChR2-mCherry$^+$ PPTg neurons.**

**(A)** Mean current responses recorded in PPTg neurons, expressed as a fraction of the maximal peak response, at increasing blue-light photostimulation intensities (arbitrary units). *Inset*, representative light-evoked inward currents recorded from a ChR2-mCherry$^+$ PPTg neuron in whole-cell voltage-clamp mode in response to 1-ms blue light photostimulation delivered at increasing intensities. **(B)** Representative traces showing blue light-evoked firing patterns and action potential fidelity of CaMKIIα-ChR2-mCherry$^+$ PPTg neurons in response to trains of light photostimulation of 0.1, 0.3, and 1.0 ms duration at stimulation frequencies of 10, 20, and 40 Hz. **(C)** Representative traces showing blue light-evoked currents recorded in CaMKIIα-ChR2-mCherry$^+$ PPTg neurons in response to trains of light photostimulation of 0.1, 0.3, and 1.0 ms duration at stimulation frequencies of 10, 20, and 40 Hz. **(D)** Mean cumulative charge transfer (integral of current over time) during the stimulation trains shown in (C) increased with

pulse duration and stimulation frequency. Within each frequency, 0.1-ms blue light pulses elicited the smallest current responses, followed by 0.3-ms pulses, whereas 1.0-ms blue light pulses elicited the largest current responses. N = 10 mice; n = 10 slices. Data are presented as mean ± SEM. Statistical comparisons were performed using two-way repeated-measures ANOVA followed by Sidak's post hoc test where appropriate.

**Figure 7. PPTg glutamatergic fibers innervate PnC giant neurons.**

**(A)** Schematic of the *in vitro* patch clamp recording experiment of CaMKIIα-ChR2-mCherry$^+$ PPTg fibers and whole-cell recordings of giant neurons in PnC sections of WT mice. **(B, C)** Image of a patched PnC neuron (white arrow) innervated by CaMKIIα-ChR2-mCherry$^+$ PPTg fibers (red), viewed under IR-DIC (B) or fluorescence (C) mode. Representative of N = 10 mice. Scale bars: B, C = 50μm

**Figure 8. Photostimulation of PnC-projecting CaMKIIα-ChR2-mCherry$^+$ PPTg fibers evokes excitatory responses in PnC giant neurons *in vitro*.**

**(A)** Sample traces showing evoked EPSCs recorded by whole-cell patch clamp from a PnC giant neuron in response to the blue light photostimulation of CaMKIIα-ChR2-mCherry$^+$ PPTg fibers in PnC sections. **(B)** Sample traces showing evoked EPSPs recorded in a PnC giant neuron in response to the blue light photostimulation of CaMKIIα-ChR2-mCherry$^+$ PPTg fibers (top traces), and in response to paired photostimulations separated by 50 ms (middle traces), or paired photostimulations separated by 100 ms (bottom traces). Red traces represent the sum of the black traces. Representative of N = 10 mice; n = 10 slices.

**Figure 9. PnC-projecting PPTg fibers course within parvalbumin-postive ($PV^+$) PnC neurons.**

In a WT mouse unilaterally injected with AAV-DJ-CaMKIIα-mCherry in the PPTg, $mCherry^+$ PPTg fibers (red) course through a region containing PnC $PV^+$ neurons (blue) and are closely apposed to excitatory synapses (PSD95; green), suggesting direct innervation. The image was acquired at 60× magnification and is representative of N = 3 mice.