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TITLE:

The Power of Interstimulus Interval for the Assessment of Temporal Processing in Rodents

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SUMMARY:

Temporal processing, a preattentive process, may underlie deficits in higher-level cognitive processes, including attention, commonly observed in neurocognitive disorders. Using prepulse inhibition as an exemplar paradigm, we present a protocol for manipulating interstimulus interval (ISI) to establish the shape of the ISI function to provide an assessment of temporal processing.

ABSTRACT:

Temporal processing deficits have been implicated as a potential elemental dimension of higher-level cognitive processes, commonly observed in neurocognitive disorders. Despite the popularization of prepulse inhibition (PPI) in recent years, many current protocols promote using a percent of control measure, thereby precluding the assessment of temporal processing. The present study used cross-modal PPI and gap prepulse inhibition (gap-PPI) to demonstrate the benefits of employing a range of interstimulus intervals (ISIs) to delineate effects of sensory modality, psychostimulant exposure, and age. Assessment of sensory modality, psychostimulant exposure, and age reveals the utility of an approach varying the interstimulus interval (ISI) to establish the shape of the ISI function, including increases (sharper curve inflections) or decreases (flattening of the response amplitude curve) in startle amplitude. Additionally, shifts in peak response inhibition, suggestive of a differential sensitivity to the manipulation of ISI, are often revealed. Thus, the systematic manipulation of ISI affords a

critical opportunity to evaluate temporal processing, which may reveal the underlying neural mechanisms involved in neurocognitive disorders.

INTRODUCTION:

Temporal processing deficits have been implicated as a potential underlying neural mechanism for alterations in higher-level cognitive processes commonly observed in neurocognitive disorders. Prepulse inhibition (PPI) of the auditory startle response (ASR) is a translational experimental paradigm commonly used to examine temporal processing deficits, revealing profound alterations in neurocognitive disorders such as schizophrenia[1], attention deficit hyperactivity disorder[2] and HIV-1 associated neurocognitive disorders[3-4]. Specifically, assessments of temporal processing in preclinical models of HIV-1 have revealed the generality, relative permanence, and suggested the diagnostic utility of PPI across the majority of the animals' functional lifespan[3-6].

Use of an approach varying interstimulus interval (ISI; *i.e.,* the time between the prepulse and the startle stimulus) in the analysis of reflex modification dates back to Sechenov in 1863[7]. The seminal studies of reflex modification, a measure of sensorimotor gating, employed an approach varying ISI to assess flexor response and audition in frogs[7-8], as well as knee-jerk responses in humans[9]. The first clinical application of the reflex modification procedure assessed visual sensitivity in a man with hysterical blindness[10]. Over a century after the first reports of reflex modification, the approach of varying ISI was popularized across a series of seminal papers[11-13]. Despite the inherent differences in the seminal studies on reflex modification (*i.e.,* species, experimental procedures, reflexes), they established a temporal relationship that was strikingly similar between species.

Assessment of prepulse inhibition using an approach varying ISI, as detailed in the present protocol, has multiple advantages over the popularized percent of control approach. First, the approach affords an opportunity to establish the shape of the ISI function, including increases (sharper curve inflections) or decreases (flattening of the response amplitude curve)[3,15] in startle amplitude, as well as shifts in the peak response inhibition[3,5]. Additionally, when an approach varying ISI is employed, startle response is a relatively stable phenomenon[1], suggesting the potential utility of the approach in longitudinal studies examining the progression of neurocognitive deficits[5,15]. Finally, PPI provides a critical opportunity to understand the underlying neural circuitry involved in neurocognitive disorders[16].

In our study, we employed two experimental paradigms (**Figure 1**), including cross-modal PPI and gap prepulse inhibition (gap-PPI), to evaluate the utility of an approach varying ISI to delineate effects of sensory modality, psychostimulant exposure, and age. The cross-modal PPI experimental paradigm utilizes the presentation of an added stimulus (*e.g.*, tone, light, air puff) as a discrete prestimulus prior to an acoustic startling stimulus. In sharp contrast, in the gap-PPI experimental paradigm, the absence of a background (*e.g.*, removal of background noise, light, or air puff) serves as a discrete prestimulus. Here, we describe both experimental paradigms for the assessment of temporal processing, as well as statistical approaches for the analysis of PPI

and gap-PPI. Within the discussion, we compared the conclusions one would draw from the variable ISI approach and the popularized percent of control approach.

PROTOCOL:

All animal protocols were reviewed and approved by the Animal Care and Use Committee at the University of South Carolina (federal assurance number: D16-00028).

1. Defining Parameters and Calibration of the Startle Apparatus

1.1. Set up the startle response system (see **Table of Materials**) according to the manufacturer's instructions.

1.1.1. Enclose the startle platform in a 10 cm-thick double-walled isolation cabinet.

1.2. Calibrate the response sensitivities using the startle calibration system.

1.3. Attach the high-frequency loudspeaker 30 cm above the animal holder.

1.3.1. Measure and calibrate the loudspeaker using a sound level meter by placing the microphone inside the animal holder.

1.4. Affix a white LED light (22 lux) on the wall in front of the animal holder.

1.4.1. Measure the lux presented as a visual prepulse using a light meter.

1.5. Connect a semi-rigid plastic tube (0.64 mm diameter) to a compressed air tank *via* an airline regulator.

1.5.1. Set the air tank to 16 psi for the presentation of tactile prestimuli.

1.5.2. Use a sound level meter to measure the amount of noise being emitted by the tactile stimulus inside the tube, 2.5 cm from the end of the animal holder. If using multiple chambers, ensure that all chambers are calibrated in the same manner.

Note: To prevent the tactile stimulus from being perceived as an acoustic stimulus, the sound of the air puff prepulse must be less than or equal to the white noise background. In the present set-up, the air puff prepulse emitted 70 db(A) inside the tube while the background white noise was also set to 70db(A).

2. Creation of Experimental Programs

2.1. Open the startle response system software.

2.2. Define a pulse-only ASR trial. Select **Definitions** | **Define Trial**.

Note: The pulse-only ASR trial is run during the habituation session, and 6 times at the beginning of every cross-modal PPI and gap-PPI session for habituation.

2.2.1. Type a Trial Name. Hit **Enter**. Record Data.

2.2.2. Set the Analog Level to 720. Define the Wait Length as 20 ms. Introduce Background.

2.2.3. End the Trial. Hit **Accept** to save the trial.

2.3. Create six separate trial definitions for acoustic PPI, including one trial for each ISI (*i.e.,* 0, 30, 50, 100, 200, 4000 ms). Select **Definitions** | **Define Trial**.

2.3.1. Type a Trial Name. Hit **Enter**.

2.3.2. Set the Analog Level to 600 at 0 ms to introduce the prestimulus. Assign the Wait Length to 20 ms to specify the length of the prestimulus. Set the Analog Level to 440 at 20 ms to remove the prestimulus.

2.3.3. Define the Wait Length dependent upon ISI.

Note: Define the wait length as: 10 ms for the 30 ms ISI, 30 ms for the 50 ms ISI, 80 ms for the 100 ms ISI, 180 ms for the 200 ms ISI, and 3980 ms for the 4000 ms ISI. Only one wait length is included for each ISI.

2.3.4. Record Data.

2.3.5. Set the Analog Level to 720. Assign the Wait Length to 20 ms. Introduce Background.

2.3.6. End the Trial. Hit **Accept** to save the trial.

2.4. Create six separate trial definitions for visual or tactile PPI, including one trial for each ISI (*i.e.,* 0, 30, 50, 100, 200, 4000 ms). Select **Definitions** | **Define Trial**.

Note: Visual and tactile cannot be run concurrently because of software and hardware limitations. The modality presented is dependent upon the input into the hardware (*i.e.,* whether the light is connected, or the air puff is connected).

2.4.1. Type a Trial Name. Hit **Enter**.

2.4.2. Turn the Tactile on to introduce the prestimulus.

Note: In this instance, tactile refers to the modality (*i.e.,* either visual or air puff) that is connected to the hardware.

2.4.3. Set the Wait Length to 20 ms. Turn the Tactile off to remove the prestimulus. Set the Analog Level to 440 at 20 ms.

2.4.4. Define the Wait Length dependent upon ISI.

Note: Define the wait length as: 10 ms for the 30 ms ISI, 30 ms for the 50 ms ISI, 80 ms for the 100 ms ISI, 180 ms for the 200 ms ISI, and 3980 ms for the 4000 ms ISI.

2.4.5. Record Data.

2.4.6. Set the Analog Level to 720. Assign the Wait Length to 20 ms. Introduce Background.

2.4.7. End the Trial. Hit **Accept** to save the trial.

2.5. Create six separate trial definitions for acoustic gap-PPI, including one trial for each ISI (*i.e.,* 0, 30, 50, 100, 200, 4000 ms). Select **Definitions** | **Define Trial**.

2.5.1. Type a Trial Name. Hit **Enter**.

2.5.2. Set the Analog Level to 0 at 0 ms to introduce the prestimulus. Assign the Wait Length to 20 ms to specify the length of the prestimulus. Set the Analog Level to 440 at 20 ms to remove the prestimulus.

2.5.3. Define the Wait Length dependent upon ISI.

Note: Define the wait length as: 10 ms for the 30 ms ISI, 30 ms for the 50 ms ISI, 80 ms for the 100 ms ISI, 180 ms for the 200 ms ISI, and 3980 ms for the 4000 ms ISI.

2.5.4. Record Data.

2.5.5. Set the Analog Level to 720. Assign the Wait Length to 20 ms. Introduce Background.

2.5.6. End the Trial. Hit **Accept** to save the trial.

2.6. Create six separate trial definitions for the visual or tactile gap-PPI, including one trial for each ISI (*i.e.,* 0, 30, 50, 100, 200, 4000 ms). Select **Definitions** | **Define Trial**.

2.6.1. Type a Trial Name. Hit **Enter**.

2.6.2. Turn the Tactile on. Set the Analog Level to 0 ms.

2.6.3. Turn the Tactile off. Set the Wait Length to 20 ms.

2.6.4. Turn the Tactile on. Set the Analog Level to 440.

2.6.5. Define the Wait Length dependent upon ISI.

Note: Define the wait length as: 10 ms for the 30 ms ISI, 30 ms for the 50 ms ISI, 80 ms for the 100 ms ISI, 180 ms for the 200 ms ISI, and 3980 ms for the 4000 ms ISI.

2.6.6. Record Data.

2.6.7. Set the Analog Level to 720. Assign the Wait Length to 20 ms. Introduce Background.

2.6.8. End the Trial. Hit **Accept** to save the trial.

2.7. Create a habituation session. Select **Definitions** | **Define Session**.

2.7.1. Set the Background Analog Level to 440, the Number of Record Samples to 100 the Samples per Second to 2000, the Acclimation Period to 5 min, and the Sequence Repetitions to 36.

2.7.2. Type 10 into the intertrial interval (ITI) list box.

2.7.3. Click **Load** and select the pulse-only ASR trial. Click **Save** to save the habituation session.

2.8. Define the session for Cross-Modal PPI. Select **Definitions** | **Define Session**.

2.8.1. Set the Background Analog Level to 440, the Number of Record Samples to 200 the Samples per Second to 2000, the Acclimation Period to 5 min, and the Sequence Repetitions to 1.

2.9. Define the intertrial interval (ITI) list.

2.9.1. Type 10 into the first 6 ITI list boxes.

2.9.2. Type a variable ITI (15-25 s) into the next 72 ITI list boxes, representing trials with a prestimulus.

2.9.3. Click **Load**. Select the pulse-only ASR trial and load it 6 times for Trials 1-6.

2.9.4. Create 6-trial blocks for each prestimulus modality using a Latin Square design **(Table 1)**.

2.9.5. Load the 6-trial blocks in an ABBA counterbalanced order of presentation (*e.g.,* acoustic, visual, visual, acoustic, acoustic, *etc.*) for cross-modal PPI.

Note: Each trial must be loaded individually. Each cross-modal PPI session includes a total of 78 trials.

2.9.6. Click **Save** to save the session.

2.10. Define the session for Gap-PPI. Select **Definitions** | **Define Session**.

2.10.1. Set the Background Analog Level to 440, the Number of Record Samples to 200 the Samples per Second to 2000, the Acclimation Period to 5 min, and the Sequence Repetitions to 1.

2.11. Define the intertrial interval (ITI) list.

2.11.1. Type 10 into the first 6 ITI list boxes.

2.11.2. Type a variable ITI (15-25 s) into the next 72 ITI list boxes, representing trials with a prestimulus.

2.11.3. Click **Load** to load the trials. Select the pulse-only ASR trial and load it 6 times for Trials 1-6.

2.11.4. Create 6-trial blocks for each prestimulus modality using a Latin Square design **(Table 1)**.

2.11.5. Click **Save** to save the session.

Note: Each gap-PPI session includes a total of 42 trials. Each session assesses one sensory modality.

3. Protocol Structure

3.1. Use the F344/N rat strain, the most common inbred rat strain, for assessments.

Note: Cross-modal PPI and gap-PPI can be conducted in animals at a variety of ages, of both sexes, and regardless of hormonal status (*i.e.,* ovariectomized, castrated, intact). Details regarding the animals used in the representative data are presented in the representative results.

3.2. Handle the animals to allow for acclimation across a series of days prior to beginning experimentation.

3.3. Randomize the order of animals for experimentation dependent upon the between-subjects' factors of interest (*e.g.,* biological sex, treatment).

3.4. Open the startle response system software. Click **Run**. Select the session of interest.

Note: Only one session is conducted per day and sessions need to be conducted in a sequential order (*i.e.,* Habituation, Cross-Modal PPI, Gap-PPI)

3.5. Input an Output File Name and click **OK**.

3.6. Enter Subject, Group, and ID information and click **Continue**.

3.7. Place the animal into the startle apparatus using an animal enclosure that is most appropriate for the size of the animal. Click **OK** to begin the session.

3.8. Export data for analysis.

3.8.1. Click **Reports** | **Concatenate Data**. Load the data file and click **Add**. Click **ASCII** to save the data output.

4. Data Analysis

4.1. Calculate an adjusted V. Max for each trial by subtracting the V. Max from the Start value.

Note: The adjusted V. Max creates a measure of mean peak ASR amplitude.

4.2. Graphically visualize results for the habituation session.

4.2.1. Plot group means and standard errors of the mean for each trial. Regression analyses can be conducted and fit with 95% confidence intervals.

4.3. Graphically visualize results for cross-sectional cross-modal PPI and gap-PPI.

4.3.1. Calculate mean values for each ISI by averaging across the 6 trials individually for each animal.

4.3.2. Calculate and graph group means and standard errors of the mean for each ISI and sensory modality.

4.4. Statistically analyze cross-modal PPI and gap-PPI (Optional).

Note: Although the precise statistical approach will be dependent upon the experimental design and research question of interest, a mixed-design repeated measures ANOVA provides one appropriate approach.

REPRESENTATIVE RESULTS:

A prominent non-monotonic ISI function was observed in cross-modal PPI (**Figures 2A, 3A, 4A**) and gap-PPI (**Figures 2B, 3B, 4B**). Baseline startle responses were observed at the 0 and 4000 ms ISIs, included as reference trials within a test session. The importance of the 4000 ms ISI cannot be understated, as it most closely resembles the PPI test trials (*i.e.,* 30, 50, 100, 200 ms ISIs) in that the subject receives both the prepulse and startling stimuli. However, no significant decrease in ASR is observed at the 4000 ms ISI because of the large time interval between the prepulse and startling stimulus. Either the addition (*i.e.,* cross-modal PPI) or removal (*i.e.,* gap-PPI) of a discrete prestimulus produced robust inhibition at the 30, 50, 100, and 200 ms ISIs; inhibition that was dependent upon sensory modality, psychostimulant exposure or age. The power of the ISI approach is revealed by examining these effects on changes in the ISI function (*i.e.,* sharper inflections of the ISI curve, flattening of the ISI curve, and shifts in the point of maximal inhibition).

The utility of an approach varying the ISI to delineate effects of sensory modality in cross-modal PPI are illustrated in **Figure 2A** (F344/N controls between 8 and 10 months of age, n=20). Due to hardware and software limitations, only two prestimulus modalities can be assessed at once. Following habituation, concurrent acoustic and visual prepulse stimuli were used to examine PPI. Next, concurrent acoustic and tactile prepulse stimuli were used to assess PPI. Data for acoustic PPI is presented from the experimental paradigm including the concurrent presentation of acoustic and visual prestimuli (*i.e.,* visual context). A prominent shift in the point of maximal inhibition is dependent upon sensory modality, suggesting a differential sensitivity to the manipulation of ISI. Specifically, maximal inhibition is observed at the 30 ms ISI following the presentation of a discrete acoustic prestimulus, at the 50 ms ISI following the presentation of a discrete visual prestimulus, and at the 200 ms ISI following the presentation of a discrete tactile prestimulus. Additionally, a flatter ISI function, indicative of an insensitivity to the manipulation of ISI, is observed following the presentation of an acoustic prestimulus relative to a visual or tactile prestimulus. A repeated measures ANOVA was conducted to statistically analyze the data, confirming our observations and revealing a significant prestimulus modality x ISI interaction [$F(10,190)=22.8$, $p_{GG}\leq0.001$, $\eta_p^2=0.546$] with a prominent linear-linear component [$F(1,19)=36.1$, $p\leq0.001$, $\eta_p^2=0.655$]. Notably, the interaction accounted for a large proportion of variance within the model, evidenced *via* measures of η_p^2.

Following an animal's experience with each prestimulus in cross-modal PPI, the generalizability of sensory modality effects was assessed in gap-PPI. Acoustic gap-PPI, visual gap-PPI and tactile gap-PPI were each conducted separately. **Figure 2B** demonstrates the generalizability of varying the ISI to delineate effects of sensory modality. A prominent shift in the point of maximal inhibition, suggesting a differential sensitivity to the manipulation of ISI, was observed in tactile gap-PPI (*i.e.,* 30 ms) relative to acoustic gap-PPI and visual gap-PPI (*i.e.,* 50 ms). Additionally, a relative insensitivity to the manipulation of ISI, evidenced by a relatively flatter ISI function was observed in tactile gap-PPI and visual gap-PPI relative to acoustic gap-PPI. As in cross-modal PPI, a significant prestimulus modality x ISI interaction [$F(10,190)=17.6$, $p_{GG}\leq0.001$, $\eta_p^2=0.481$] with a prominent linear-quadratic component [$F(1,19)=58.5$, $p\leq0.001$, $\eta_p^2=0.755$] was revealed; an effect which again, accounts for a significant proportion of the variance.

After the completion of cross-modal PPI and gap-PPI, animals repeatedly orally self-administered methylphenidate (MPH). A post-test assessment of cross-modal PPI with concurrent acoustic and visual prestimuli and acoustic gap-PPI were conducted at approximately 14 months of age following 22-27 days of MPH exposure. The pre-test and post-test ISI functions for acoustic PPI are illustrated in **Figure 3A**. Most notably, at the post-test assessment, a relative flattening of the ISI function is observed, suggesting a relative insensitivity to the manipulation of ISI relative to the pre-test assessment. Additionally, a prominent shift in the point of maximal inhibition is revealed, with inhibition at the 30 ms ISI during the pre-test assessment and the 100 ms ISI at the post-test assessment, suggesting a differential sensitivity to the manipulation of ISI. A repeated-measures ANOVA confirmed these observations, revealing a significant test session x ISI interaction [$F(5,95)=7.4$, $p_{GG}\leq0.003$, $\eta_p^2=0.280$] with a prominent linear-quadratic component [$F(1,19)=10.6$, $p\leq0.004$, $\eta_p^2=0.358$].

Following the post-test cross-modal PPI assessment, acoustic gap-PPI was conducted to assess the generalizability of the effects of psychostimulant exposure on temporal processing. **Figure 3B** illustrates the generalizability of varying the ISI to delineate effects of psychostimulant exposure. The point of maximal inhibition was at the 50 ms ISI during both the pre-test and post-test assessment. However, a significantly flatter ISI function was observed following MPH exposure. A repeated-measures ANOVA confirmed these observations, revealing a significant test session x ISI interaction [$F(5, 95)=3.6$, $p_{GG}\leq0.013$, $\eta_p^2=0.159$] with a prominent linear-cubic component [$F(1,19)=9.1$, $p\leq0.007$, $\eta_p^2=0.325$].

The shape of the ISI function also affords an opportunity to assess the development of temporal processing across age. In a longitudinal study (F344/N controls, male: $n=20$, female: $n=17$), cross-modal PPI with concurrent acoustic and visual prestimuli was conducted every sixty days from postnatal day (PD) 30 to PD 150. The development of temporal processing in visual PPI is illustrated in **Figure 4A**. Within visual PPI, the point of maximal inhibition at all ages is at the 50 ms ISI. However, a sharper inflection of the ISI function is observed across age, suggesting a perceptual sharpening which occurs with development. A repeated-measures ANOVA, with sex as the between-subjects factor and age, ISI, and trial as within-subjects factors, confirmed these observations revealing a significant age x ISI interaction [$F(10,350)=12.6$, $p_{GG}\leq0.001$, $\eta_p^2=0.265$] with a prominent linear-quadratic component [$F(1,35)=32.6$, $p\leq0.001$, $\eta_p^2=0.482$] and a significant ISI x sex interaction [$F(5,175)=4.0$, $p_{GG}\leq0.014$, $\eta_p^2=0.104$] with a prominent quadratic component[$F(1,35)=5.2$, $p\leq0.028$, $\eta_p^2=0.130$].

At every age, acoustic gap-PPI was conducted following cross-modal PPI. The experiences an animal has had have a direct impact on its responses, necessitating the use of a sequential experimental design (*i.e.,* always conducting cross-modal PPI prior to gap-PPI). **Figure 4B** illustrates the development of temporal processing, assessed using acoustic gap-PPI. At PD 30, an insensitivity to the manipulation of ISI was observed, evidenced by a flatter ISI function, relative to PD 90 or PD 150. Observations of the sharpest ISI function at PD 150 suggest a perceptual sharpening that occurs across development. Additionally, a prominent shift in the point of maximal inhibition is revealed, with maximal inhibition occurring at the 30 ms ISI at PD 30 and the 50 ms ISI at PD 90 and PD 150, suggesting a differential sensitivity to the

manipulation of ISI. Statistically, the observation of a significant age x ISI interaction [$F(10,350)=10.4$, $p_{GG}\leq0.001$, $\eta_p^2=0.230$] with a prominent linear-quadratic component [$F(1,35)=70.5$, $p\leq0.001$, $\eta_p^2=0.668$] and an ISI x sex interaction [$F(5,175)=3.8$, $p_{GG}\leq0.010$, $\eta_p^2=0.097$] with a prominent quadratic component [$F(1,35)=11.0$, $p\leq0.002$, $\eta_p^2=0.184240$] confirms our observations.

Figure Legends:

Figure 1. Prepulse Inhibition Experimental Paradigms. A) Animals exhibit a baseline auditory startle response when an acoustic startle stimulus is presented. **B)** During cross-modal prepulse inhibition (PPI), the presentation of a discrete prestimulus (*i.e.,* acoustic tone, light, air puff) 30 to 500 ms[16] prior to an acoustic startle stimulus, produces robust inhibition. **C)** During gap prepulse inhibition (gap-PPI), the removal of a discrete prestimulus (gap in background noise, light, or air puff) 30 to 200 ms[17] prior to an acoustic startle stimulus produces robust inhibition. The image is adapted from Maze Engineers[18].

Figure 2. Cross-Sectional Assessment of Temporal Processing: Sensory Modality. A) Representative analysis of the effect of sensory modality on the interstimulus interval (ISI) function in cross-modal prepulse inhibition (PPI). **B)** Representative analysis of the effect of sensory modality on ISI in gap prepulse inhibition (gap-PPI). Control results from McLaurin *et al.*[6] are reanalyzed in a novel manner to assess the effect of sensory modality. Data are presented as mean ± standard error of the mean.

Figure 3. Cross-Sectional Assessment of Temporal Processing: Psychostimulant Exposure. A) Representative analysis of the effect of psychostimulant exposure (pretest *vs.* posttest) on the interstimulus interval (ISI) function in acoustic prepulse inhibition (PPI). **B)** Representative analysis of the effect of psychostimulant exposure on ISI in acoustic gap prepulse inhibition (gap-PPI). Control results from McLaurin *et al.*[6] are reanalyzed in a novel manner as the pretest component for psychostimulant exposure. Data are presented as mean ± standard error of the mean.

Figure 4. Longitudinal Assessment of Temporal Processing. A) Representative analysis of the effect of age on the interstimulus interval (ISI) function in visual prepulse inhibition (PPI). **B)** Representative analysis of the effect of age on the ISI function in acoustic gap prepulse inhibition (gap-PPI). Data are presented as mean ± standard error of the mean.

DISCUSSION:

The present protocol describes the power of varying ISI for the assessment of temporal processing for studies employing either cross-sectional or longitudinal experimental designs. Examining the effects of sensory modality, psychostimulant exposure, or age on the shape of the ISI function demonstrated its utility in revealing a differential sensitivity to the manipulation of ISI (*i.e.,* shifts in the point of maximal inhibition) or a relative insensitivity to the manipulation of ISI (*i.e.,* sharper inflections of the ISI curve, flattening of the ISI curve). Use of two experimental paradigms, including cross-modal PPI and gap-PPI, demonstrates that the utility of

ISI is independent of the addition (*i.e.,* cross-modal PPI) or removal (*i.e.,* gap-PPI) of a discrete prestimulus.

Critical experimental design considerations for the completion of cross-modal PPI and gap-PPI are included within the protocol. First, a Latin-Square experimental design is implemented for the presentation of ISIs within 6-trial blocks, controlling for variation due to the order of ISI presentation. Second, the use of two control trials, including both the 0 and 4000 ms ISIs, provides reference control trials within the test session. The use of the 4000 ms ISI is particularly critical, as it most appropriately resembles the other (*i.e.,* 30, 50, 100, 200) prepulse + pulse trials, but without the expectation of significant inhibition. Third, a counterbalanced (*i.e.,* ABBA) experimental design is employed within cross-modal PPI to account for the repeated measurement of sensory modalities within a test session. Finally, inclusion of a variable ITI during the prepulse + pulse trials prevents an animal from expecting, and thus preparing for, the start of a trial. Thus, the implementation of a comprehensive number of ISIs in accordance with an appropriate experimental design allows for the determination of relatively precise and defined response functions; functions which provide a critical opportunity to assess the construct of temporal processing.

Methodology described within the present protocol contrasts other contemporary protocols for the analysis of PPI, which have popularized an approach that commonly employs a single ISI[19-20]. The popularized approach is commonly analyzed using percent inhibition, calculated as follows: 100 x {[(startle response amplitude during control trials)-(startle response amplitude during prepulse + pulse trials)] / {startle response amplitude during control trials)}. Two major caveats of the contemporary protocols, including the preclusion of the assessment of temporal processing and inappropriate statistical analyses, are discussed in turn below.

Percent inhibition was calculated for the 100 ms ISI within the representative data to demonstrate the limitations of the popularized approach (**Table 2**). For example, results for the assessment of acoustic gap-PPI and tactile gap-PPI suggest that animals fail to display any significant inhibition. Examination of **Figure 2**, utilizing an approach varying ISI, however, reveals that the animals did not fail to inhibit, but displayed a significant shift in the point of maximal inhibition (*i.e.,* 50 ms in acoustic gap-PPI, 30 ms in tactile gap-PPI). Most notably, however, use of percent inhibition precludes the use of longitudinal experimental designs for assessing the development of temporal processing as a function of age, a well-recognized phenomena[14,21]. Thus, like any percent of control measure, percent inhibition fails to disambiguate changes in PPI from changes in baseline startle response precluding the assessment of temporal processing.

Despite the apparent simplicity of the popularized approach, inferences drawn from statistical analysis must be made with extreme caution. Assumptions of the analysis of variance (*i.e.,* normality of sampling distribution of means, homogeneity of error variance, independence of errors, absence of outliers[22] may not be met with subjectively determined percentage data[23]. Specifically, the error variance for percentage data are not normally distributed[24], but instead are more appropriately described by a Poisson or bimodal distribution[25]. In sharp contrast, a

repeated-measures ANOVA provides one valid and reliable method for the statistical analysis of the ISI function. However, it is vital to account for the potential violation of sphericity, an assumption only present in models involving repeated measures, either using planned orthogonal contrasts or the *post hoc* Greenhouse-Geisser *df* correction factor[26] (p_{GG}).

Utilization of an approach varying ISI, however, is not without limitation. First, hardware and software limitations allow only two prestimulus modalities to be assessed at once. Notably, a differential sensitivity to the manipulation of context (*i.e.,* concurrent visual or tactile stimulus in acoustic PPI) was previously reported in Long-Evans rats[14] and HIV-1 Tg animals[6]. Second, relative to the popularized approach, there is a greater experimental time for the ISI approach (*i.e.,* ~30 minutes for cross-modal PPI; ~20 minutes for gap-PPI).

Thus, an approach varying ISI provides an experimental method for the assessment of temporal processing. In addition to the aforementioned strengths of the approach, the serial neural circuit mediating PPI has been well-established[27-28], allowing for the assessment of neural circuitry alterations in neurocognitive disorders. Additionally, cross-modal PPI and gap-PPI may serve as a diagnostic screening tool for neurocognitive disorders (*e.g.*, HAND[4-6]). Utilization of the variable ISI approach, therefore, may potentially have translational clinical utility for neurocognitive disorders.

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DISCLOSURES:

None of the authors have conflicts of interest to declare.

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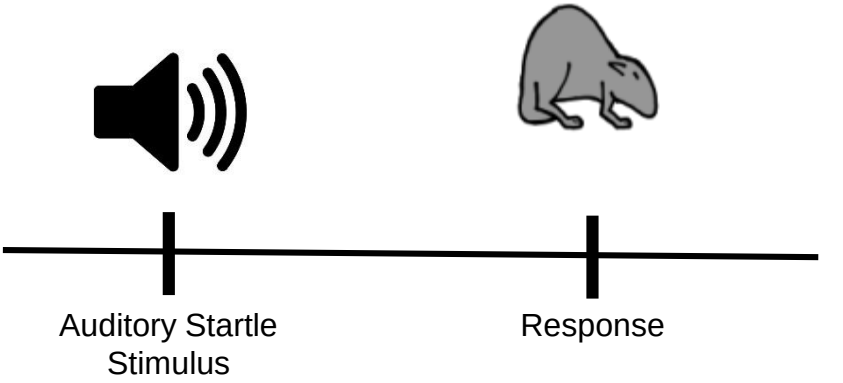
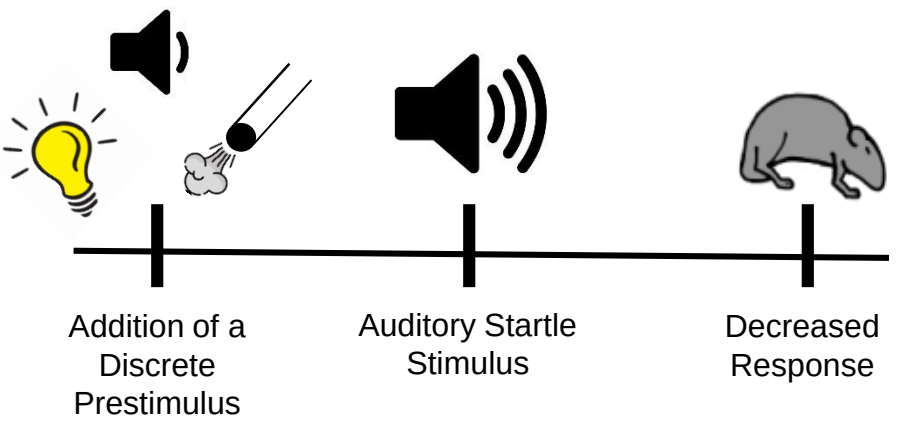
A

Auditory Startle Response



B

Cross-Modal Prepulse Inhibition



C

Gap Prepulse Inhibition

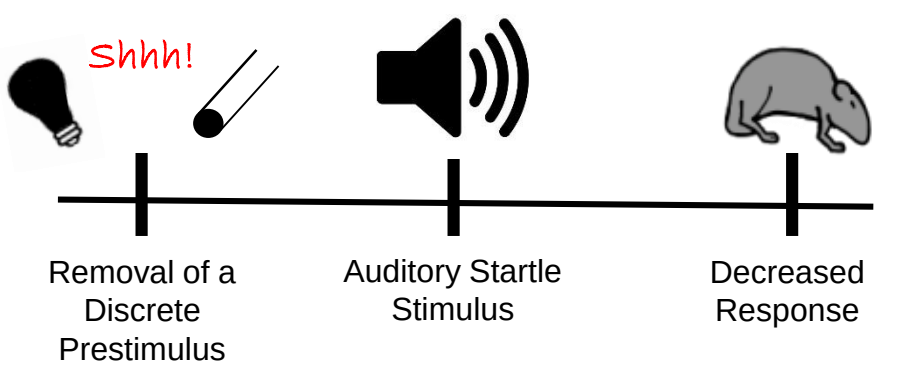


Figure 2

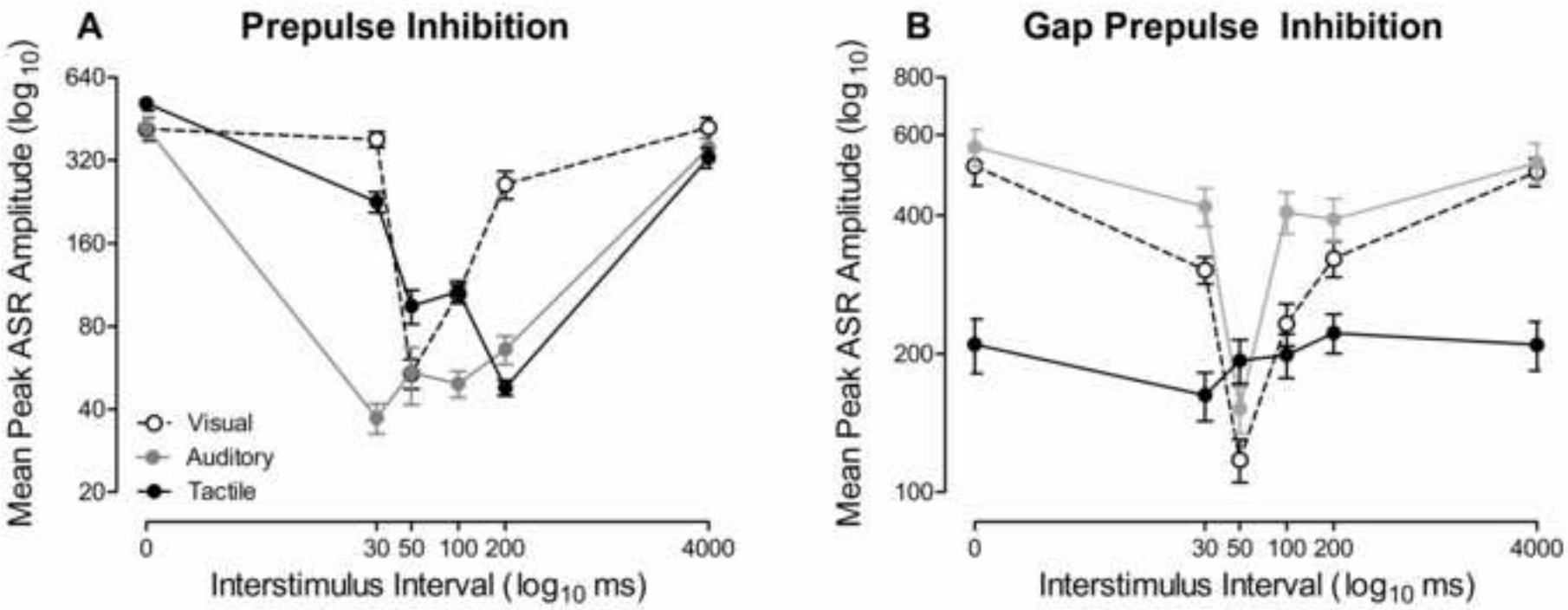


Figure 3

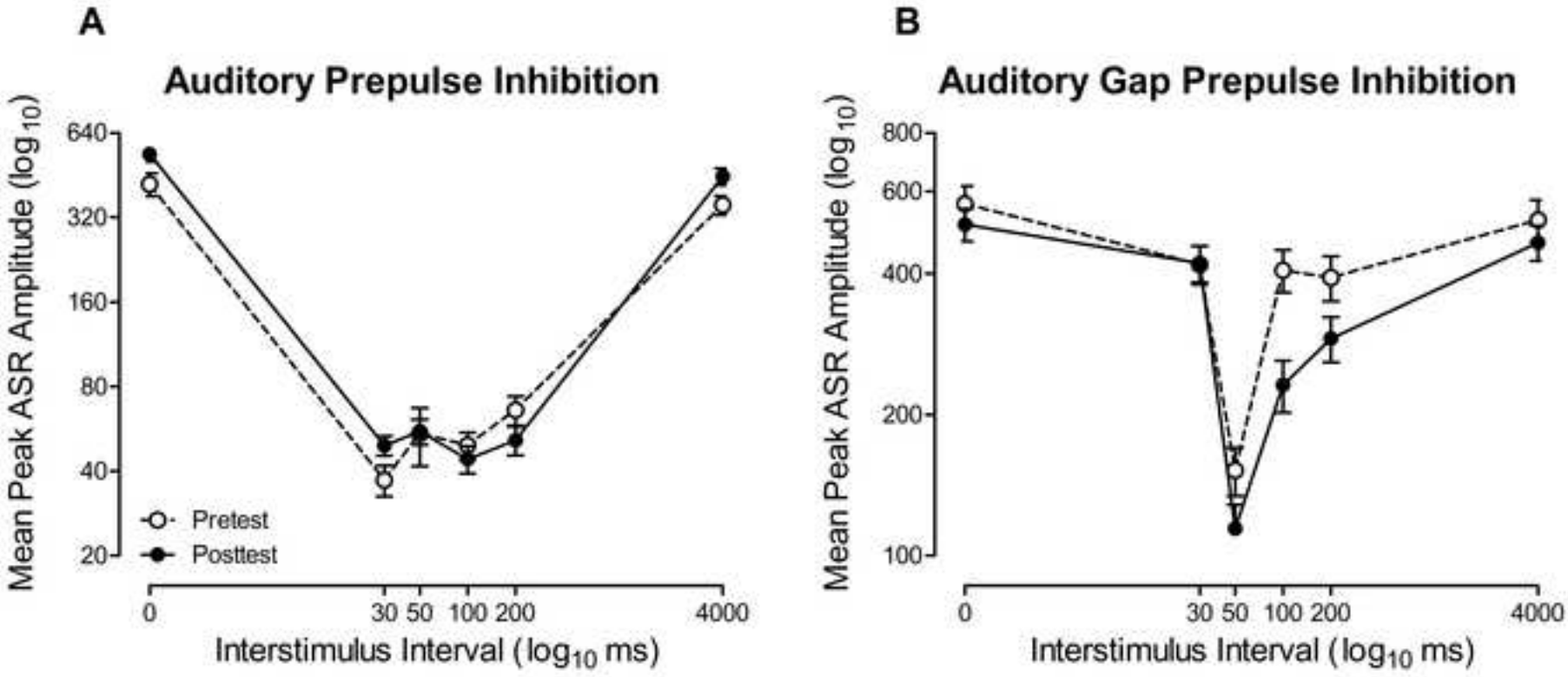


Figure 4

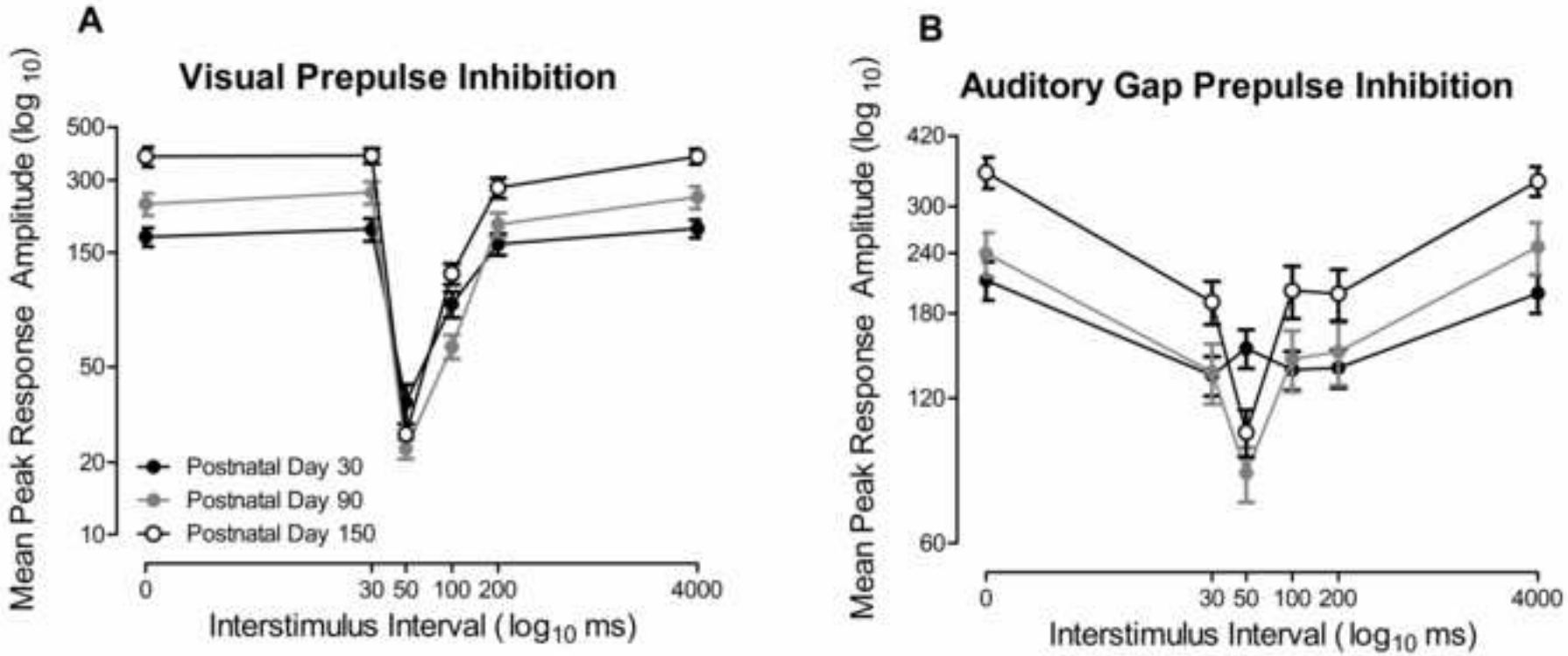


Table 1

Click here to access/download;Table;Table 1 (Latin Square Experimental Design).xlsx

Trial Block	Interstimulus Interval					
1	0	30	50	100	200	4000
2	30	50	100	200	4000	0
3	50	100	200	4000	0	30
4	100	200	4000	0	30	50
5	200	4000	0	30	50	100
6	4000	0	30	50	100	200

Table 2

Sensory Modality	Cross-Modal PPI	Gap-PPI
Auditory	85.7 (2.0)	25.0 (4.3)
Visual	72.6 (2.7)	52.8 (5.3)
Tactile	73.2 (3.0)	-3.6 (8.5)
Psychostimulant Exposure	Cross-Modal PPI	Gap-PPI
Pretest Assessment	85.7 (2.0)	25.0 (4.3)
Posttest Assessment	90.5 (1.3)	52.6 (4.5)
Age	Cross-Modal PPI	Gap-PPI
PD 30	51.3 (3.7)	29.7 (4.4)
PD 90	73.8 (2.2)	39.6 (5.7)
PD 150	66.3 (2.9)	45.0 (3.9)

Name of Material/ Equipment	Company	Catalog Number
SR-Lab Startle Response System	San Diego Instruments	
Isolation Cabinet	Industrial Acoustic Company	
SR-Lab Startle Calibration System	San Diego Instruments	
High-Frequency Loudspeaker	Radio Shack	model #40-1278B
Sound Level Meter	Bruel & Kjaer	model #2203
Perspex Cylinder	San Diego Instruments	
SR-Lab Startle Response System Software	San Diego Instruments	
Light Meter	Sper Scientific, Ltd.	model #840006
Airline Regulator	Craftsman	model #16023
SPSS Statistics 24	IBM	

	Comments/Description
Included with the SR-Lab Startle Response System	
Included with the SR-Lab Startle Response System	
Used for Statistical Analyses (Optional)	

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Editorial Comments:

- All editorial comments have been addressed as requested.

Reviewer #1:

- *While the data is interesting and original, the protocol that the authors propose to calculate "temporal processing" doesn't make sense at all. They propose to integrate the area "above" the curve of absolute startle values as a measure (the indent) for temporal processing, however, all temporal information is in fact gone through this calculation. It is impossible to tell whether changes in this calculated value is due to a general change of PPI across all ISIs, through a shift in maximum PPI to a different ISI, or if PPI changes that are in any way ISI dependent. In fact, it would have been much more interesting to see the entire curves over ISIs across ages in order to assess potential changes in PPI that are ISI dependent (I guess there are none, see below, but even that is an interesting piece of information).*

    - We concur. The primary focus of the protocol is now exclusively on raw data, not any derived measures. The entire curves of ISIs across ages are presented to illustrate changes in PPI that are ISI dependent. The new Figure 4 presents these data.

- *Another huge problem is that the authors use absolute PPI values, not percent PPI. The increase in area "above" the curve across ages is most likely due to the increase in weight of the animals. Heavier animals result in larger baseline startle since they accelerate the startle platform more efficiently. Hence the increase in PPI reported here might in fact reflect a steady percent PPI, while baseline startle increases due to weight gain. This probably also accounts for the difference in males versus females. See Csomor PA, Yee BK, Quednow BB, Stadler RR, Feldon J, Vollenweider FX. Behav Brain Res. 2006 for more detail.*

    - As above, our focus is on raw data (i.e., absolute startle amplitude values). The focus on raw data rather than any derived measures, such as percent PPI, is, in our view, the solution, not the problem.
    - The difficulties with using percent PPI continue to be underappreciated, though well-established decades ago.
        - Despite the apparent simplicity and popularity of that approach, the frequent use of subjectively determined percentage data is not without potential consequence regarding validity of inferences made about meeting the assumptions of analysis of variance (Bliss, 1938). Unfortunately, percentage data have error variances that are a function of the mean and are not normally distributed (Bartlett, 1947); rather they are described by Poisson or bimodal

distributions, depending on whether the data occur over a large portion of the percentage scale (bimodal) or are primarily grouped at either end (Poisson)(Cochran, 1940). The manipulation of ISI guided the present protocol, with the incorporation of a range of ISIs to determine the shape of the PPI response curves. The incorporation of a range of ISIs is fundamental to the establishment of a relatively precise and defined response function, and consequently, a more accurate assessment of response inhibition, as has been employed to examine alterations in the development of PPI as a function of developmental neurotoxin or drug exposure (e.g., Fitting et al., 2006a,b,c; Ison, 1984; Mactutus, Harrod, Hord, Moran, & Booze, 2011; Moran et al., 2014; McLaurin et al., 2017). The plotting of the raw startle amplitude scores, e.g., as in the log–log plot portrayed in Figure 2, 3 and 4 graphically illustrates another advantage of the ISI function approach. Given that PPI is a model of temporal processing, the advantages of incorporating the temporal dimension, and presentation of absolute PPI values, in any assessment of PPI would appear undeniable.

  - The concern that "*heavier animals result in larger baseline startle since they accelerate the startle platform more efficiently"* is puzzling to us. The startle response is indexed by a piezoelectric accelerometer, not a load cell nor stabilimeter. The fact that acceleration is independent of mass was well-established centuries ago according to Newton's second law.
  - The relevance of the Csomor et al. (2006) paper is unclear. Csomor et al. (2006) varied the level of pulse stimulus intensity and prepulse intensity, not ISI. Additionally, the paper is restricted to all male subjects.

- *In summary, many studies vary ISIs when measuring prepulses, and all it takes to do so is changing a small setting in the stimulus protocol (see also Valsamis B and Schmid S, JoVE, 2011). While the concept to use variable ISIs to probe for differences in temporal processing is very compelling, the proposed protocol falls short to do so, in fact, it removes the temporal information. I would love to see the data without this AUC calculations, but the full data set with temporal information present, published in a behavioural Neuroscience journal, with a discussion of its usefulness to probe for temporal processing changes/deficiencies. The use of visual prepulses preceding an acoustic startle pulse also probes for multisensory integration - dependent on the ISI on the level of the brainstem (at ISI <100 ms, or with potential cortical involvement at ISIs > 100ms). The comparisons of the temporal aspects between different prepulse modalities and with gap PPI is also very interesting.*

- As above, the entire curves of ISIs across ages are presented to illustrate changes in PPI that are ISI dependent (Figure 4). Similarly, the entire curves of ISI are presented in all figures.
- We appreciate the recognition of the compelling utility of using variable ISIs to probe for differences in temporal processing.
- The Valsamis and Schmid (2011) paper illustrates and advocates the use of two ISIs (i.e., 30 and 100 ms) for prepulse inhibition. Additionally, Valsamis and Schmid propose the calculation of percent PPI, which, as discussed above, is fraught with consequences. In sharp contrast, our protocol takes that recommendation further into using a range of ISI values and describes the interpretation and analysis of the ISI function.

- *How were tactile prepulses separated from acoustic? The air passing through the tube cause normally a relatively easy to perceive hissing sound, therefore an acoustic as much as a tactile stimulus. If not omitted, this needs to the very least be acknowledged and maybe called an acoustic/tactile stimulus. The low overall startle response during tactile gap PPI at 0 and 4000 msec is probably caused by the constant hissing sound that masks the startle stimulus to some extent - hence the low baseline startle.*

    - We appreciate this comment and the configuration of the protocol recognized that concern. The sound of the air puff prepulse was measured, in the absence of background white noise, as 70 dB(A) inside the tube, 2.5 cm from the end of the test cylinder (at the position of the rodent's ears). Thus, in the presence of 70 dB(A) background noise (again at the level of the rodent's ears), the air puff was effectively a pure tactile stimulus.
    - This information has now been added to the protocol as step 1.5.2, in recognition that they are critical factors in the tactile stimulus parameter definition.

- *The "stimulus interval approach" should be called the "variable stimulus interval approach"*

    - The focus of the protocol is on manipulating ISI across a range of values. We have replaced the phrase "interstimulus interval approach" with "an approach varying interstimulus interval".

- *All "auditory" has to be replaced by "acoustic", since the authors use sound, hence an acoustic stimulus. "Auditory" refers to the neurons processing acoustic information - the auditory neurons. Auditory stimulation would be an electrical stimulation of auditory neurons, not a stimulation by acoustic sound.*

- As requested, we have replaced the term “auditory” with “acoustic.”

- *What animals were used in this study?*

  - Two sets of animals were included in the study. The assessment of sensory modality and psychostimulant exposure was conducted in ovariectomized female F344/N control animals (*n*=20) between 8 to 14 months of age. The longitudinal assessment of cross-modal PPI and gap-PPI was conducted in intact male and female F344/N control animals (male: *n*=20, female: *n*=17) between 1 and 5 months of age (i.e., PD 30 to PD 150).
  - Information regarding the animals used in the study has now been included in the protocol.

- *Reference to Fig. 2A (text page 8): "Auditory PPI is presented within the visual context" - what does this mean??*

  - Hardware and software limitations allow only two prestimulus modalities to be assessed at once. Cross-modal PPI was assessed using concurrently presented acoustic and visual prestimuli, as well as concurrently presented acoustic and tactile prestimuli. The data for acoustic PPI, therefore, is illustrated from the experimental paradigm including the concurrent presentation of acoustic and visual prestimuli.
  - The sentence has been changed to read “Data for acoustic PPI is presented from the experimental paradigm including the concurrent presentation of acoustic and visual prestimuli (i.e., visual context).” to improve clarity.

- *Instead of "punctuated" stimuli, it should maybe say "discrete”*

  - As requested, we have replaced the term “punctate” with “discrete.”

- *The ITI during habituation needs to be 10 sec, not msec.*

  - Thank you for catching this error. It has been corrected.

Reviewer #2:

- The authors of this manuscript describe a new approach to assess temporal processing using the classical PPI chambers used in the literature. As PPI approaches are also used in humans, this protocol can be very interesting for translational research between animals and humans. Although the manuscript is

well-written, the authors should improve the way the protocol is explained in order to help the reproducibility of the protocol for all the readers.

  - We thank the reviewers for their kind comments regarding the translational importance of PPI. We have improved the way the protocol is explained to aid in the reproducibility of the protocol for all readers.

- In the introduction and discussion, the authors should compare this way of assessing temporal processing with other tasks assessing this function. The authors have focused the manuscript 100% in the PPI approach but if the protocol assess temporal processing it will be good to put it in the context of this function. What is known in the literature? Which are the tasks used?

  - Using prepulse inhibition as an exemplar paradigm, we present a protocol for manipulating interstimulus interval (ISI) to establish the shape of the ISI function to provide an assessment of temporal processing.
  - The focus of the present protocol is on the comparison of two methods for conducting and analyzing PPI (i.e., percent PPI vs. a range of ISI values). The discussion of the variety of different tasks that can be used to provide an assessment of temporal processing is beyond the scope of the protocol.

- When explaining the tasks, authors must show schemes/pictures to better define each protocol and apparatus used. Moreover, authors should better define the different modalities (cross-modal PPI and gap-PPI) from the beginning of the manuscript.

  - We concur and have included a schematic of the two experimental paradigms in the Introduction. Additionally, we have enhanced our definitions of the two experimental paradigms, including their similarities and inherent difference, in the introduction.

- In the literature can be good to explain if these modalities are also assessed in humans as the classical PPI is done.

  - Not to our knowledge.