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**STANDARD OPERATING PROCEDURE NO. 11**

**ACOUSTIC STARTLE AND PRE-PULSE INHIBITION**

**PROTOCOLS**

The Acoustic Startle Response (ASR) Paradigm is a widely utilized behavioral means of assessing

integrity of the sensorimotor neural system and reactivity/anxiety in rodents as well as humans. Generally a very brief intense sound stimulus is presented to a subject and the latency and magnitude of a startle response is measured via an automated system. In the Pre-pulse Inhibition version of the ASR paradigm, the magnitude of the ASR is dramatically reduced by an imperceptible brief auditory stimulus that

precedes the startle stimulus by less than 500msec (1/2 second). Failure of the pre-pulse to inhibit the startle is indicative of an abnormal or altered sensorimotor system for processing incoming sensory stimuli.

**Basic Protocol:**

The basic ASR and ASR PPI procedures for use with rodent species that has been approved by the WC IACUC is described in:

Geyer, M.A. & Swerdlow, N.R. (1998) Measurement of startle response, prepulse inhibition, and habituation. In Gerfen C., Holmes, A., Sibley D., Skolnick, P., Wray, S. (Eds.) *Current Protocols in Neuroscience*, pp. 8.7.1-8.7.15. Wiley & Sons, Inc.: New York. <http://onlinelibrary.wiley.com/book/10.1002/0471142301>

Any differences or modification from this protocol must be reviewed by the IACUC. January 2013

**Measurement of Startle Response, Prepulse**

**Inhibition, and Habituation**

The startle response is comprised of a constellation of reflexes elicited by sudden, relatively intense stimuli. It offers many advantages as a behavioral measure of central nervous system (CNS) activity and can be measured in numerous species, including humans, when elicited by acoustic (noise bursts), electrical (cutaneous), tactile (air puff), or visual (light flash) stimuli.

The startle reflex has served as a tool for studying fundamental properties of nervous function ranging from neurophysiological and anatomical relationships within the pons and reticular formation to forebrain regulation of complex behavioral states and cognitive processes. The primary acoustic startle circuit is located at or below the pons, while the forebrain modulates several forms of startle plasticity, including the two forms discussed in this unit: habituation and prepulse inhibition (PPI). Changes in startle magnitude through repeated stimulus presentations—habituation and sensitization—represent the simplest forms of learning, and have been used to identify the neural basis of learning in invertebrates. However, quantifying startle habituation and sensitization in rats may have more physiological relevance to human CNS function than, for example, studying the gill withdrawal reflex of *Aplysia*.

Procedures presented include measurement of the startle response in rats to acoustic stimuli (see Basic Protocol 1), along with modifications encompassing an experimental manipulation such as drug treatment (see Alternate Protocol 1) and measures of habitu- ation of startle (see Alternate Protocol 2); a method for measuring prepulse inhibition (PPI) of startle (see Basic Protocol 2); and specialized rat handling and calming techniques that supplement the startle protocols (see Support Protocol).

*NOTE:* All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

**STRATEGIC PLANNING**

Startle measures are used to study a wide range of phenomena including drug toxicity, auditory system physiology, neurodevelopment, and behavioral genetics. Therefore the materials and procedures needed to study the startle response vary enormously depending on the particular goals of the study. The specific application must determine decisions related to the particular startle apparatus; animal species, strain, sex, and age; stimulus and test session characteristics; and experimental design.

Commercially available systems for measuring the startle response lend themselves to studies of a variety of small mammals, including mice, hamsters, guinea pigs, rats, ferrets, and some lower primates (e.g., marmosets). Interchangeable chambers of different sizes for different species or ages of animals make it possible to change quickly from small cylinders suitable for mice to larger cylinders for rats or even larger ones for guinea pigs. Rodent studies are the most convenient and efficient for screening drug effects on or assessing the neurobiological mechanisms of startle plasticity. For typical drug or surgical manipulations, cohorts of eight to twelve animals provide adequate power for most statistical comparisons.

***UNIT 8.7***

**Contributed by Mark A. Geyer and Neal R. Swerdlow**

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**Behavioral**

**Neuroscience**

**8.7.1**

The SR-LAB system (San Diego Instruments) recommended for these protocols com- prises from one to eight lighted and ventilated chambers, each containing a stabilimeter consisting of an 8.2-cm diameter Plexiglas cylinder mounted on a Plexiglas base. A tweeter mounted 24 cm above the animal provides the background noise, prepulse stimuli, and startle stimuli, which are controlled by the SR-LAB. Startle responses are transduced by a piezoelectric accelerometer mounted below the cylinder, digitized (0-4095), rectified, and recorded as 100 1-msec readings, starting at the onset of each startle stimulus. The average of the 100 readings is used as the dependent measure.

**Gender**

Both male and female rats have been used in measures of startle reflex magnitude and plasticity. The effects of rat gender on many variables and drug responses have not been studied systematically, so it is important to match the gender of the rats used previously when studies are replicated or extended.

**Age**

Startle magnitude and PPI are influenced by rat age. Prepubescent rats exhibit smaller startle responses and less robust, and more variable, PPI compared to adults. Aged rats may experience frequency-dependent hearing loss, which can alter startle response characteristics.

**Animal Care and Housing**

Because of the effects of stress on startle behavior, animal care procedures should be designed to limit exposure of rats to stress. Consideration should be given to the means of shipment from the supplier, and the amount of time the animals must spend within confined and poorly ventilated shipping boxes before they reach their home cages.

Isolation or extremely crowded housing can significantly affect startle variables. Animals should be housed two to three per cage, unless the effect of different housing conditions on startle is a test parameter. Animals should be kept in a housing facility offering conditions appropriate for other sensitive behavioral measures, including constant ambi- ent temperature and a regular circadian lighting pattern. Startle is best measured in the dark phase of a rat’s circadian cycle, when it is most robust and least variable; effects of circadian cycle on startle plasticity have not been studied completely.

It is important to control the ambient noise level and assess whether sound insulation is needed in the housing area. The sound-attenuated testing room must be separated from any hallway or heavily used thoroughfare. Floor vibrations, such as those triggered by pedestrian traffic or doors, can contaminate the results, as any phasic sensory events perceived by the test subjects can affect startle measures by virtue of prepulse inhibitory or dishabituation effects.

Because of the sensitivity of startle to stressors, begin animal handling procedures within three days of shipment arrival, and at regular intervals thereafter throughout the course of the experiment (see Support Protocol 1). Health checks are particularly important because of the possible impact of infectious processes on the auditory system, as well as the effects of generalized debilitation on reflex responsiveness. Inspect the animals daily, and handle thoroughly (at least 1 min per animal) every 3 to 4 days.

**Startle Response**

**8.7.2**

**Drug Effects**

Startle testing is commonly performed after drug administration via systemic injection, oral drug delivery, drug delivery via chronic indwelling pumps or drug pellets, or

Supplement 3 Current Protocols in Neuroscience

intracerebral drug delivery. In the interpretation of the effects of a drug on startle habituation, the time course of the drug effect is a potential confounding factor, as it may produce altered levels of reactivity late in the trial series not evident initially. Such a pattern could indicate either a delayed drug effect per se or a drug effect on habituation.

**Data Analysis**

The practical steps required for analyzing startle data are dictated by the nature of the data files created by the test system. Assemble all the data from an entire experiment into a single ASCII file for use by a standard statistical package such as SAS, SYSTAT, SPSS, or BMDP, condensing the data by averaging trials for each animal prior to actual analysis. While this step can be accomplished using spreadsheet programs, it is more efficient to use programs that condense the data automatically and rapidly, such as SAS or SYSTAT. The software provided with the San Diego Instruments system combines all the raw data generated from the experiment into one ASCII file using a utility program. The authors reduce these data to the descriptive variables of interest for each animal and sort them using SYSTAT, and conduct inferential statistical analyses using SYSTAT, SPSS, or BMDP (PC systems) or SuperANOVA (MAC systems).

**BASIC TEST OF ACOUSTIC STARTLE REACTIVITY**

The most basic startle experiment assesses the level of reactivity in groups of rats manipulated in a particular manner, such as by drug treatment. For this purpose, startle reactivity is defined as the magnitude of the startle response on either the initial stimulus presentation or over a relatively small number (e.g., 10) of startle trials. The following protocol measures acoustic startle reactivity in naive rats as a prelude to a subsequent study of prepulse inhibition (PPI) in the same rats. Data obtained from Basic Protocol 1 are used to match groups of rats for the PPI experiment described in Basic Protocol 2.

Both within- and between-subject comparisons are possible in startle experiments. This matching procedure reduces the large subjective variation typical of startle reactivity measures. Because these differences tend to be stable over days or weeks, animals can serve as their own control by being tested on two different days.

Programming of all aspects of the stimuli and test sessions is accomplished by the menu-driven SR-LAB software.

***Materials***

Forty naive male Sprague-Dawley rats weighing 250 to 300 g

Drugs and placebos

Sound-attenuated testing room

SR-LAB startle apparatus with digitized electronic signal output (San Diego

Instruments) or equivalent

Calibrated sound level meter (Quest) Drug delivery system

1. Obtain and prepare animals. Handle all forty rats (see Support Protocol 1) and acclimate them to the vivarium and light cycle for 1 week before testing.

*Delay startle testing after a surgical procedure for at least 1 week to minimize the potential effects of surgical stress on startle response measures. Continue handling during that postoperative week. A suitable light cycle is lights on at 8:00 a.m. and off at 8:00 p.m., with testing conducted between 9:00 a.m. and 7:00 p.m.*

***BASIC PROTOCOL 1***

**Behavioral**

**Neuroscience**

**8.7.3**

Current Protocols in Neuroscience Supplement 3

2. Select and define testing parameters: for example, plan to present one initial and ten subsequent trials of a single acoustic stimulus to each rat. To provide a consistent acoustic environment and to mask external noises, maintain a continuous background noise level of 65 dB within each startle chamber.

*Conduct testing during the dark phase of the animals’ diurnal cycle, no closer than 1 hr to either light change. For the acoustic startle stimulus, use a fast-rise-time (<1 msec) burst of noise presented for 40 msec at an intensity of 120 dB. Set the intertrial intervals (ITIs)*

*to average 15 sec, with a range from 8 to 23 sec. Thus, the test session lasts* ∼*8 min per rat.*

*Stimulus rise-time, duration, and intensity are variables that will affect startle reflex magnitude (Graham, 1975; Hoffman and Searle, 1968). The use of a variable ITI is intended to minimize habituation of startle across the eleven trials, although some habitu- ation will still occur.*

3. Calibrate the stimulus delivery and response recording systems.

*For multiple test stations, calibrate all stations to the same standards and program them to deliver the startle stimuli simultaneously. Measure sound levels with continuous tones and experimental chambers closed.*

*Measure stabilimeter sensitivity with the SR-LAB dynamic startle response calibration unit.*

4. Clean and dry the animal enclosure before testing each rat.

5. Weigh and test each of the forty rats, beginning the test session with a 5-min period of acclimation to the background noise.

6. Collect the peak or average response from each rat on each of eleven trials. Record the initial response value separately, and average the remaining ten responses together for each rat.

*Only the initial startle response provides a pure measure of startle reactivity uninfluenced by habituation and sensitization, but single response measures are too variable for most practical purposes. Therefore, the standard practice is to average the responses across blocks of five to ten trials (after the initial response) for each subject.*

7. Define four matched groups of ten rats each by sorting the forty startle reactivity values to give each group the same mean and variance. For multiple test stations, include the same number of rats from each station in each group.

*Ensure that the four groups match by the less contaminated but more noisy measure of the initial startle response.*

***ALTERNATE PROTOCOL 1***

**BETWEEN-SUBJECTS TESTS OF STARTLE REACTIVITY**

Basic Protocol 1 can be modified easily to test the effects of a manipulation on startle reactivity where the expected effect size is likely to have adequate power between subjects. With manipulations such as prenatal exposure to a noxious agent, where animals cannot be tested before the manipulation, use control subjects and a between-subjects design.

In selecting manipulations to study the startle reflex, consider factors such as the route of drug administration, the expected drug effect, and dose-response characteristics. Certain drugs produce ceiling or floor effects (i.e., plateau or threshold measurements) in startle testing that complicate data interpretation. In cases where this sort of effect is anticipated, select a range of startle pulse intensities to delimit submaximal to maximal startle magnitude characteristics.

**Startle Response**

**8.7.4**

Supplement 3 Current Protocols in Neuroscience

Carry out the basic test of acoustic startle reactivity (see Basic Protocol 1) with the following alterations:

1. Modify steps 1 and/or 5 as appropriate for the manipulation being studied. For example, if a dose-response study of a drug is the goal, treat each of the rats with the appropriate vehicle or dose at a suitable time before introducing the animal into the chamber in step 5.

2. Modify step 7 by including the independent variable (e.g., vehicle or drug treatment) as a factor in analyses of variance (ANOVA) on the two dependent measures (initial response and average of trials 2 through 11).

**BETWEEN-SUBJECTS TESTS OF STARTLE HABITUATION**

A modification of Alternate Protocol 1 to test the effect of a manipulation on startle habituation as well as on startle reactivity is simply to increase the number of trials to demonstrate adequate levels of habituation.

Carry out the basic test of acoustic startle reactivity (see Basic Protocol 1) with the following alterations:

1. Modify steps 1 and/or 5 of Basic Protocol 1 as appropriate for the manipulation being studied (see Alternate Protocol 1).

2. Modify step 2 to include more trials.

*With acoustic startle stimuli, a total of 121 trials is often presented to ensure that adequate habituation occurs.*

3. Modify step 7 by including the independent variable (e.g., vehicle or drug treatment) as a between-subjects factor and blocks of trials as a within-subjects factor in ANOVA.

*If the response to the initial trial is excluded from this analysis as being a unique event (see Basic Protocol 1, step 6), the principal dependent measure becomes the repeated blocks of trials. Average the data for each subject as 24 blocks of five trials each or 12 blocks of ten trials each, as dictated by the rapidity of habituation observed (evidenced by a statistically significant effect of trial block in the ANOVA). Treatment-induced changes in startle habituation are evidenced by a statistically significant treatment-by-trials interaction in the ANOVA. Alternatively, assess habituation as the percent decrease in startle reactivity between the first and last blocks of trials.*

***ALTERNATE PROTOCOL 2***

**TESTING PREPULSE INHIBITION (PPI) OF STARTLE**

In combination with pharmacological and neurosurgical procedures, this protocol allows for the systematic investigation of the neurochemical and neuroanatomical systems that modulate sensorimotor inhibition. Startle magnitude is reduced when the pulse stimulus is preceded 30 to 500 msec by a weak prepulse. This inhibition (“gating”) of a motor response elicited by a weak sensory event, termed prepulse inhibition (PPI), provides an operational measure of sensorimotor gating. Prepulse stimuli of 3, 6, or 12 dB above the

65 dB background noise inhibit the startle response elicited by 120-dB pulse stimuli.

Holding the interval between the prepulse and pulse stimuli constant at 100 msec typically yields suitable levels of PPI, ranging from 20% to 80% inhibition.

Three types of prestimuli used in intramodal studies of sensorimotor gating of acoustic startle are: (1) delivery of a discrete acoustic prepulse several msec before the startle pulse, with an intensity below startle threshold; (2) a variation of this design, the peak-on-ped- estal stimulus, in which the prepulse is a continuous elevation of the background noise

***BASIC PROTOCOL 2***

**Behavioral**

**Neuroscience**

**8.7.5**

Current Protocols in Neuroscience Supplement 3

(the pedestal) beginning at a set interval prior to delivery of the pulse (the peak); and (3) gap inhibition, where a discrete reduction (gap) in background noise precedes the startle pulse by a prescribed interval. In all these cases, the interval length, intensity, and duration of the prepulse significantly influence the amount of PPI.

This protocol describes a useful set of parameters for demonstrating a variety of experi- mental effects, but it is by no means the only appropriate procedure.

***Additional Materials*** *(also see Basic Protocol 1)*

Apomorphine⋅HCl for the administration of 0.1, 0.3, and 1.0 mg/kg injections at a

volume of 1.0 ml/kg, prepared fresh

Vehicle solution of sterile isotonic saline containing 0.1 mg/ml ascorbic acid, purged of oxygen by sparging with nitrogen gas for 10 min, prepared fresh

Sterile 1-cc syringes

Sterile 25-G syringe needles

1. Follow the basic acoustic startle reactivity test (see Basic Protocol 1) to define four matched groups of ten rats each.

*Omitting the baseline matching step described in Basic Protocol 1 permits proceeding directly to assessment of PPI in animals previously untested in startle, as in studies of development.*

2. Define test parameters as follows:

A pulse-only trial containing only a 40-msec burst of 120-dB noise; Variable ITIs to average 15 sec;

Three different trial types containing prepulse stimuli preceding the 120-dB

pulse stimulus by 100 msec (onset to onset);

Defined sequence of presentation of the four trial types;

Recording of the startle response (i.e., the recording window) to begin at the onset of the pulse stimulus;

Test session beginning with a 5-min acclimation period and then six presen-

tations of the pulse-only trial (these trials will not be used in the assess- ment of PPI);

Defined pseudorandom sequence in which the pulse-only trial is presented

ten times and each of the three prepulse trial types is presented five times; Conclusion with an additional set of five pulse-only trials.

**Startle Response**

**8.7.6**

*This is a total of 36 trials.*

*The three prepulse stimuli should be 3, 6, or 12 dB above the 65 dB background noise (i.e.,*

*68, 71, or 77 dB) with a duration of 20 msec.*

3. Clean and calibrate the equipment (see Critical Parameters).

4. Assign each of the four matched groups of rats to receive either vehicle or one of the three doses of apomorphine.

5. Bring rats from the vivarium to the laboratory 60 min prior to testing, shielding them from any sounds from startle testing of other animals.

6. Weigh each rat and prepare appropriate syringes for drug administration.

7. Administer the vehicle and apomorphine injections subcutaneously 5 min prior to introducing the rats to the startle chambers.

8. Test the rats using the test session defined in step 2 above.

Supplement 3 Current Protocols in Neuroscience

9. For each rat, define the following descriptive statistics: Response amplitude on the first trial;

Average response magnitude on pulse-only trials 2 to 6 and 32 to 36;

Average response magnitude in each of the four trial types between trials 7 and 31 inclusively (i.e., ten pulse-only trials and five each of the three prepulse variations).

*Analyze the first response and the first block of pulse-only trials as measures of startle reactivity. Analyze the first and last blocks of pulse-only trials together in a repeated measures ANOVA to assess habituation of acoustic startle across the test session, or use the percent habituation score for this purpose.*

10. Using the four values (3, 6, or 12 dB above background) derived from trials 7 to 31 to assess PPI, calculate for each rat:

*Percentage score:*

*PPI = 100* × *{[pulse-only units* − *(prepulse + pulse units)]/(pulse-only units)}*

*Thus, if a startle magnitude is 50 units in the absence of a prepulse, and 20 units in the presence of a prepulse, % PPI = 100* × *(50* − *20)/50 = 100 (30/50) = 60% inhibition.*

*Difference score:*

*PPI = pulse-only units* − *(prepulse + pulse units)*

*In this example, if a startle magnitude is 50 units in the absence of a prepulse, and 20 units in the presence of a prepulse, a difference score of PPI = 50* − *20 = 30 units of inhibition.*

*Although the most widely accepted measure of PPI is the percent score, measure both percent and difference scores.*

**RAT HANDLING**

Handling techniques vary greatly from laboratory to laboratory. This specialized proce- dure aids in reducing variation in behavioral results arising from stressful ambient conditions.

1. Bring animals to the test laboratory, three per cage, within 24 hr of shipment arrival.

2. Place three animals at a time on a large, open table surface, and move them quickly and gently so that they experience a significant amount of contact with each other and with the experimenter’s hand.

3. Hug the three animals together between the experimenter’s hands gently but firmly

(see Fig. 8.7.1A).

*Kicking or vocalizations suggest the handling is not being performed correctly.*

4. After the animals become completely calm, usually within 3 min, return them to their cage.

5. Remove each animal individually and place it on the table with one hand covering the head in a gentle fashion to shade any light, and the other hand on its hindquarters to prevent perambulation (see Fig. 8.7.1B).

6. Grip the animal gently between the hands, again firmly but not tightly, and lift it off the table with the hands moving in a massaging fashion.

*Grip the rat’s head so that the thumb and forefinger support the bottom of the neck and upper chest, and squeeze together the forepaws so that they cross over the underside of the*

***SUPPORT PROTOCOL***

**Behavioral**

**Neuroscience**

**8.7.7**

Current Protocols in Neuroscience Supplement 3

*neck and jaw region; this position prevents the rat from accidentally biting the experimenter, or scratching with the forepaws (see Fig. 8.7.1C).*

7. Massage the animal for 2 min: gently twist the torso by rotating each hand in an opposite direction for a total displacement of ∼30°, and then rotate in the opposite

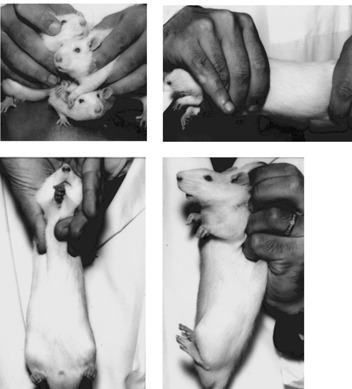
direction.

*At this point, the animal is usually ready to return to its cage. Verify this by lifting the rat by the scruff of its neck, gripped between the thumb and several fingers. A fully relaxed rat reacts via a primitive relaxation reflex, having a limp body with all four paws extended but lying limp against the body. A fully relaxed rat does not struggle in this position and can actually be turned over without any protest, vocalizations, or kicks (see Fig. 8.7.1D). This posture recreates that of a rat pup being moved by the scruff of the neck by its mother.*

A B

C D

**Figure 8.7.1** Technique to reduce acute stress in response to pretest handling of rats. (**A**) Hug three animals gently but firmly between the experimenter’s hands. (**B**) Place an animal individually on the table surface with one hand covering the head in a gentle fashion to shade it from light and causing the forepaws to cross over the underside of the neck and jaw region, and the other hand placed on its hindquarters to prevent perambulation. Gently, but firmly, massage the animal between



the hands. (**C**) After ∼2 min of massage in position B, the rat relaxes completely and goes limp, and

is allowed to dangle in the hand that continues to secure the forepaws across the midline. (**D**) Verify

that the animal is ready to be returned to the cage by lifting the rat by the scruff of its neck. A fully relaxed rat reacts via a primitive relaxation reflex that leaves the body lax with all four paws extended but limp against the body (not fully achieved in this illustration).

**Startle Response**

**8.7.8**

Supplement 3 Current Protocols in Neuroscience

**COMMENTARY Background Information**

***Startle response***

For over 50 years, the startle reflex has been studied systematically as a means of under- standing the neural control of behavior. A major advantage of startle response paradigms is that the same phenomena can be studied across species. In humans, the blink reflex component of the startle response elicited by acoustic or tactile stimuli is measured using electromyo- graphy of the orbicularis oculi muscle. In rats, a stabilimeter chamber measures the whole- body flinch elicited by acoustic or tactile stim- uli similar to those used in humans. In 1982, Davis and colleagues reported a primary mam- malian acoustic startle circuit (Davis et al.,

1982), now thought to consist of three synapses linking the auditory nerve with the spinal motor neuron. The relatively simple startle reflex can be modified by more complex forms of behav- ioral plasticity, including habituation and PPI, that are regulated by forebrain circuitry. These malleable processes exhibit striking similari- ties across species, from rodents to humans.

A typical animal study assesses the effect of a controlled manipulation—environmental, contextual, surgical, or pharmacological—on startle. After the desired experimental manipu- lation, startle reactivity (defined as the magni- tude of the startle response either on initial stimulus presentation or over a number of star- tle trials), habituation, and/or PPI can be meas- ured in rodents using particular patterns of startle-eliciting stimuli and specific forms of prestimuli. Startle reflex amplitude is predict- ably modified by changes in startle-eliciting stimuli and prestimuli. Alterations in stimu- lus/response characteristics after specific ex- perimental manipulations elucidate the rela- tionship of the brain with particular behaviors. These manipulation-induced changes in startle or startle plasticity can be compared with startle abnormalities in specific human clinical popu- lations, and may be useful in animal models of human neuro- or psychopathology.

Basal levels of the startle reflex elicited by acoustic or tactile stimuli in the absence of prestimulus or conditioned effects are also studied in animals, for many reasons. Startle reactivity can provide useful information per- taining to drug effects, and also can be used as a dependent measure for characterizing physi- ological and anatomical properties of sensory and cardiovascular systems, and/or brainstem

and pontine neural circuits. Some studies have employed intracerebral microdialysis meas- ures during acoustic startle in rats (e.g., Humby et al., 1996) to elucidate brain-behavior rela- tionships.

A major advantage of the startle reflex over other behavioral measures is the exquisite sen- sitivity of this behavior to stimulus parameters directly controlled by the experimenter. This tight stimulus control affects each aspect of the experimental design, and the presence or ab- sence of an effect of a specific drug or experi- mental manipulation under one set of condi- tions does not necessarily predict their presence or absence under another set of conditions.

***Habituation***

A fundamental form of startle plasticity, considered to be the simplest form of learning, is habituation, defined as the exponential dec- rement in response when an initially novel stimulus is presented repeatedly at speeds too slow to produce sensory adaptation or receptor fatigue. While the observed habituation of un- conditioned responses theoretically arises by two opposite behavioral processes, habituation and sensitization, there are still no accepted methods for distinguishing these underlying processes experimentally in mammals. Re- searchers typically examine only the manifes- tation of habituation, not the hypothetical un- derlying process. As with PPI, habituation can be conceptualized as a fundamental gating mechanism that filters sensory information and inhibits response to disruptive or extraneous information.

***Prepulse inhibition***

Another form of modifiable response, PPI, is the normal suppression of the startle reflex when the intense startling stimulus is preceded

30 to 500 msec earlier by a barely detectable prestimulus. In combination with pharma- cological and neurosurgical procedures, the PPI of startle protocol allows the systematic investig atio n o f th e n euro ch emical and neuroanatomical systems that modulate sen- sorimotor gating in rats (Geyer et al., 1990; Swerdlow et al., 1992, 1994). In addition to intramodal PPI, intermodal PPI (when the pre- pulse and pulse stimuli differ in sensory type) can be demonstrated in both experimental ani- mals and humans (e.g., Braff et al., 1992; Kehne et al., 1996).

**Behavioral**

**Neuroscience**

**8.7.9**

Current Protocols in Neuroscience Supplement 3

**Startle Response**

**8.7.10**

Study of the neural substrates of PPI pro- vides insight into possible neural mechanisms responsible for the loss of sensorimotor gating in patients with several neuropsychiatric disor- ders. As a behavioral measure, PPI offers ad- vantages found in measures of the startle reflex, and in addition, PPI measures are particularly useful in predictive models for antipsychotic potency (Geyer et al., 1990; Swerdlow et al.,

1994) and in studies of the neural mechanisms of sensorimotor gating deficits in specific neuropsychiatric disorders.

The inhibitory processes activated by the weak prepulse and the resulting decrement in startle magnitude provide an operational meas- ure of sensorimotor gating, seen as the degree to which a prepulse inhibits a startle reflex. PPI occurs in several sensory modalities and in several species, including mice, rats, and hu- mans. It is not a form of conditioning: it occurs on initial exposure to the prepulse and pulse stimuli, and does not exhibit habituation or extinction. The interval between prepulse and pulse (typically 30 to 500 msec) is too short to evoke conscious voluntary behavioral inhibi- tion. PPI thus appears to reflect the activation of hard-wired centrally mediated behavioral gating.

Deficits in habituation and PPI have been quantified using the startle reflex in patients with different neuropsychiatric disorders. For example, startle habituation is diminished in medicated patients with schizophrenia (Geyer and Braff, 1982; Braff et al., 1992) and in unmedicated schizotypal subjects (Cadenhead et al., 1993). PPI is impaired in patients with disorders characterized phenomenologically by an inability to inhibit intrusive sensory, cog- nitive, or motor information, and characterized anatomically by dysfunction within circuitry linking portions of the limbic system and basal ganglia as in schizophrenic-spectrum disor- ders, obsessive-compulsive disorder, and Hunt- ington’s disease (Braff et al., 1992, 1995; Ca- denhead et al., 1993; Swerdlow et al., 1993).

**Critical Parameters**

Startle magnitudes differ significantly

among strains of laboratory rats (Glowa and Hansen, 1994) and even among groups of the same strain obtained from different providers. Hence, it is particularly important to consider this variable when replicating work from other laboratories and to maintain a consistent line of experimentation within a laboratory.

***Drug effects***

Drug effects on startle variables differ by rat strain. Although these differences are interest- ing and potentially important for exploring ge- netic contributions to startle response vari- ations among different human populations, in most cases they represent confounding vari- ables and should be avoided.

***Stereotaxic ear bars***

Another critical point for startle studies in- volving surgery relates to the use of stereotaxic ear bars for holding the animal firmly in the stereotaxic instrument. If used they must be atraumatic (blunt) ear bars that do not puncture the eardrums.

***Startle apparatus***

Equipment to quantify the startle reflex in rodents may be as simple as a spring and pen recorder, or as complex as a piezoelectric ac- celerometer with a microcomputer interface system for online waveform analyses. Elec- tromyographic (EMG) systems are available for measuring the eyeblink component of star- tle in chair-restrained monkeys, and EMG sys- tems are also available for measures of startle in humans. Different species offer different advantages for specific experimental questions.

The startle reflex can be measured using custom-built equipment or one of the few com- mercially available startle systems. Virtually all current startle monitoring systems are com- puter based. Commercial systems have the ad- vantage of comparability among laboratories and provide greater flexibility and reliability than custom-made systems.

*Housing.* Within the testing apparatus, ani- mals should be housed separately and isolated acoustically to prevent cross-contamination of ultrasonic vocalizations from one animal to the next. This is particularly relevant since startle stimuli themselves elicit ultrasonic vocaliza- tions in rats that can be altered by drug manipu- lations. Startle chambers should also be iso- lated seismically, so that startle responses in one chamber do not influence those in a neighbor- ing chamber. This separation can often be achieved inexpensively by placing each cham- ber atop a separate stack of cinder blocks. Illumination using a low-power source (e.g., a

15-W light) maintains a constant temperature within the chamber. Light sources and level of illumination may be relevant to certain experi- mental designs, particularly where lights are used as a conditioned stimulus. In selecting fans

Supplement 3 Current Protocols in Neuroscience

for adequate enclosure ventilation, noise output from fans should be considered in the experi- mental design: to avoid changes in the acoustic environment, fans must remain on consistently throughout test sessions. The continuous back- ground white noise of 65 dB within each startle chamber not only masks stray sounds but also can have significant nonmonotonic effects on subsequent startle magnitudes (Davis, 1984).

*Animal restraints*. With few exceptions, ani- mals should be minimally restrained for the study of startle responses. Controlling the ani- mal’s location in space reduces potential vari- ance due to differences in perceived stimulus intensities or in the influence of the animal’s movements on the response transducer. Never- theless, to avoid the effects of stress, animals should never be so restrained that they are unable to turn around in the chamber. Restrain- ing devices are made typically of wire mesh or smooth plastic. Wire mesh or rods have the potential disadvantage of detecting both exten- sion and flexion movements, as the animal can hold on to the wire. The more commonly used plastic cylinders are cleaned readily, cannot be gripped by the animal, and tend to place all animals in the same position relative to the stimuli and response equipment.

*Startle recording device.* The startle record- ing device should permit collecting rapid meas- ures of reflex amplitude, typically requiring

1-msec resolution, and should rectify and dig- itize these signals to provide a quantified meas- ure of the startle reflex. Given the high fre- quency and wide dynamic range of the motor response that constitutes startle, it is important to monitor at a rate of 1000 Hz and with a 12-bit analog-to-digital converter. A system that can record the entire waveform of each startle re- sponse is preferable. The type of response transducer varies among systems. Some sys- tems use load cells, with the disadvantage of sensitivity to the weight of the animal and the recording chamber, restricting the available range of response measurement. The bulk of the equipment reported in the literature relies instead on measures of force or acceleration, in keeping with the dynamic nature of the startle response.

*Response measures.* Different researchers measure either the peak response or the inte- grated response during some brief period after the onset of the startle stimulus. For a peak measure the measurement window is less criti- cal, and is often set at 100 or 200 msec (Davis,

1980). For an integrated measure, the window should be set to be just longer than the primary

startle response duration, assuming the re- sponse monitoring system has an appropriately short time-constant. A 100-msec window is suggested for rats (Mansbach et al., 1988) and guinea pigs (Sipes and Geyer, 1996) and a

65-msec window is suggested for mice (Du- lawa and Geyer, 1996). Simultaneous measures of both peaks and integrated responses suggest that the latter are slightly less variable.

*Stimulus control and delivery systems.* It is advisable to select a system that has flexible software control of a variety of different stim- uli. Optimal speakers for eliciting acoustic star- tle in rodents have frequency responses primar- ily within the 5- to 16-kHz range. Most studies use broad-band noise generators for the acous- tic stimuli. Pure-tone stimuli can be used, but are vulnerable to standing waves that can com- plicate their delivery and measurement by mak- ing the sound levels differ markedly from one location to another. Acoustic stimuli (including background noise, prestimuli, and startle-elic- iting stimuli) can be delivered via single or multiple speakers. In most studies a single noise generator and a single speaker provide all the acoustic stimuli for each animal; multiple sound sources may significantly alter behav- ioral response characteristics.

***Nature of stimuli***

As a tactile rather than acoustic stimulus to elicit startle in both rodents and humans, brief puffs of air are directed at the backs of rodents or the necks of humans (Geyer and Braff, 1987). In rodents, even seemingly mild air puffs pro- duce more dramatic startle responses than do the most intense acoustic stimuli. Air puffs are complex stimuli with both acoustic and tactile components, each of which has important and sometimes dissociable effects. Air puffs are delivered via rigid copper or rubber tubing, controlled by an adjustable pressure regulator and gated by a solenoid valve. Adjustment is usually needed to accommodate the delay in delivery of the air puff to the subject, especially if the solenoid and air tank are located in an adjacent room to minimize the acoustic com- ponent of the stimulus. The most critical as- pects of the delivery system are the orifice size in the solenoid and the distance between the delivery tube and the subject. In most systems, a 10- to 20-psi air puff elicits a behavioral response of magnitude similar to that produced by a 120-dB(on the A scale of the dB meter) acoustic stimulus, while air puffs of 40 to 100 psi produce considerably larger responses without harming the animal.

**Behavioral**

**Neuroscience**

**8.7.11**

Current Protocols in Neuroscience Supplement 3

**Startle Response**

**8.7.12**

Both the stimulus delivery and response monitoring systems require periodic calibra- tion. Measure and calibrate sound levels using a sound level meter capable of measuring stim- uli with decibel accuracy in the desired fre- quency ranges. To maximize relevance to hu- man studies, the A weighting scale is typically used. Although most acoustic stimulus delivery systems have some inherent onset spikes, the sound levels reported in the startle literature are almost always those measured with continuous sounds rather than with peak detectors. Stimu- lus calibration requires sustained delivery of loud noises up to 120 dB, and high-quality protective earphones and earplugs are essential to prevent hearing loss in the individual cali- brating these systems. Stimulus characteristics can generally be made uniform with <1% vari- ability across multiple startle chambers.

Also calibrate the sensitivity of the response recording device. Many different devices and techniques have been used to calibrate response sensitivity, most of which produce consistent measures that may not predict the responses of animals. Calibrate load cell systems using static weights which generate response values in grams; such calibration, however, relates only to static measures and shows no relationship to the dynamic changes produced by an animal’s startle response. In the more widely accepted devices with dynamic transducers, a variety of tappers, solenoids, or weight droppers have been tried unsuccessfully. As discussed by Cassella and Davis (1986), reliable calibrations that accurately predict animal responses can be developed by vibrating the test chamber at an appropriate frequency. At least one commer- cially available system has incorporated this approach into a reliable and effective response calibration unit (San Diego Instruments).

The sound and response monitoring equip- ment should be calibrated at regular intervals to ensure consistent performance between chambers across time. With weekly calibration, the monitors will seldom need substantial ad- justments.

**Troubleshooting**

***Little measurable startle reflex***

When the startle stimulus is being presented, observe the animal for behavior that is not detectable by the equipment. Assess hearing damage (e.g., from stereotaxic earbars or postinfection), general debilitation (e.g., ef- fects of drug or surgery) and general ability to exhibit a startle reflex as a response to air puffs.

Confirm that the equipment delivers the star- tling stimuli appropriately. Check recording device sensitivity and recording window times; oscilloscope tracings serve as rapid diagnostic tools.

***Large between-subject variability within a treatment group***

Ensure that rats are not sick or debilitated and are all from same shipment and supplier. Rats of approximately equal weight, age, han- dling and housing are crucial factors in devel- opmental manipulations, as it is impossible to match groups prior to introducing the inde- pendent variable (Geyer et al., 1993).

Occasionally, rats may exhibit very low or very high baseline startle values (e.g., >2 SD above or below group means). Factors that might account for such outliers include audi- tory system pathology of congenital or infec- tious origin, or general health issues (e.g., got hind leg accidently caught in cage lid, a water bottle leaked, or vestibular problems led to “barrel rolling” behavior during test session). While it might be valuable to assess such causes if they become common, generally it is wise to eliminate such outlier subjects from subsequent testing, and thereby greatly reduce variability for critical measures.

Ensure the startle chambers are calibrated and matched for sensitivity and stimulus char- acteristics. Include chamber number as a factor in analyses to assess for systematic differences. Isolate chambers adequately from sonic or seis- mic disturbance.

***Weak or no drug effects despite strong prediction***

Is the rat sex / age / supplier / strain a possible confounding factor?

Did the experimental design allow for com- pletion of testing during the effective time course of drug? Was dose/route of administra- tion/vehicle appropriate? Were there repeated drug exposures leading to a loss of sensitivity? Was the experimental design identical to that in the studies being replicated (e.g., stimulus parameters)?

One precaution is to conduct an additional study varying the interval between drug injec- tion and testing (Geyer and Tapson, 1988).

***Little or no PPI of startle***

*Animal:* Is the rat sex / age / supplier / strain a possible confound? Is the rat sick or debili- tated? Are rats from an appropriate supplier, with adequate handling and housing? Could

Supplement 3 Current Protocols in Neuroscience

subtle hearing loss result in insensitivity to weak (e.g., 2 dB above background) prepulses? If peak reflex latency values are available, they can be a useful control for prepulse detectabil- ity: it is reasonable to assume that the prepulse has been detected if it causes a reduction in peak startle latency (termed latency facilitation) compared to pulse-only trials (Ison and Hoff- man, 1983).

*Equipment/design:* Are stimulus parameters (e.g., prepulse and pulse intensity, background noise, prepulse-pulse interval and configura- tion) appropriate? Is there adequate recording sensitivity/range to detect a reduction in startle magnitude?

In some cases, the intensity of the startle pulse can be an important variable in helping interpret the effects of particular manipulations on PPI. Several manipulations that modify PPI also modify basal startle (pulse-only) magni- tude. Changes in PPI in the context of large increases or decreases in pulse-only startle might be confounded by off-scale “ceiling” or “floor” effects. Thus, it is possible that a ma- nipulation (e.g., strychnine) might increase both pulse-only and prepulse + pulse startle magnitudes to ceiling levels. Because startle magnitude on pulse-only trials might be held artificially under a maximal ceiling, the calcu- lated PPI would be artificially low. One strategy for addressing this potential confound is to include in the test session pulse-only trials that elicit submaximal startle responses (e.g., 100 dB rather than 120 dB), such that the specific manipulation does not elevate pulse-only re- sponse levels to the ceiling.

A second strategy for addressing such po- tential ceiling effects is to compare PPI among treatment subgroups matched for test session levels of pulse-only magnitude. Analogously, potential confounds arise when manipulations significantly reduce startle magnitude; in such cases, floor effects are not so easily addressed by changes in startle stimulus intensity, as when levels above 120 dB do not further increase startle magnitude while causing hearing loss.

**Anticipated Results**

***Startle reactivity***

With a 65-dB background of white noise, startle reflex thresholds begin ∼15 to 25 dB

above background, or 80 to 90 dB total intensity using white-noise pulses. Increasing pulse in- tensities results in increased startle magnitudes, with the maximal acoustic intensities delivered

typically ∼120 dB (50 to 60 dB over back-

ground noise).

***Between-subjects test of startle reactivity***

Factors influencing the degree and rate of startle habituation are startle stimulus intensity, stimulus modality, background noise levels, and intertrial intervals (ITIs). Habituation is most rapid with regular, brief ITIs, and is less rapid with longer, irregular ITIs. Rapid habitu- ation can be achieved, for example, using fixed,

10-sec ITIs, while habituation can be mini- mized using variable ITIs ranging between 15 and 45 sec. In studies where the habituation curve is an important dependent measure, tac- tile (air-puff) stimuli are preferable because the tactile habituation process appears to be more gradual compared to acoustic startle habitu- ation.

***PPI of startle reactivity***

Under conditions where manipulations do not alter levels of pulse-only startle magnitude, percent score and difference score analyses yield comparable information. However, dif- ference score analyses are influenced strongly by individual or group differences in pulse-only startle magnitude. Thus, under conditions that might increase or decrease startle magnitude, percent scores are less affected by changes in baseline response. In extreme cases, a manipu- lation can simultaneously increase PPI differ- ence scores and decrease PPI percent scores simply by virtue of large increases in startle magnitudes (Sipes and Geyer, 1996). Such cases of marked effects on startle reactivity preclude drawing a clear conclusion regarding effects on PPI, and additional studies are needed to eliminate confounding effects on assessment.

In PPI, there are significant differences be- tween the impact of various manipulations on PPI produced by weak (1 to 5 dB over back- ground) versus intense (15 dB above back- ground) prepulses. Such differences are not always intuitively obvious: for example, rats selectively bred for behavioral sensitivity to apomorphine exhibit deficits in PPI produced by weak prepulses (Ellenbroek et al., 1995), while other drug effects are evident with a single intense prepulse.

Expected results for basic startle response and PPI protocols, as well as anticipated effects of drugs and other experimental manipulations on these measures, are reviewed by Geyer et al., 1990; Rigdon, 1990; Rigdon and Viik,

1991; and Swerdlow et al., 1991, 1992, 1994.

**Behavioral**

**Neuroscience**

**8.7.13**

Current Protocols in Neuroscience Supplement 3

**Startle Response**

**8.7.14**

**Time Considerations**

The actual testing of each animal or batch

of animals requires 8 to 35 min, excluding preparation and cleaning time. Variations of these protocols can lead to test sessions lasting up to 60 min; longer sessions can be compli- cated by the influence of prolonged confine- ment, hyperthermia, or asymptotic habituation. Drug pretreatment periods increase the time required. Commercial systems can test up to eight animals at one time. Using four startle chambers, it is feasible for a single experi- menter to complete the simple dose-response experiment described in Basic Protocol 2 over

a two-day period, testing ∼5 to 7 hr per day

(total time, including preparation). Additional

time must be allotted for instrument calibration, definition or programming of test parameters, and animal acclimation and handling (gener- ally 1 week from shipment arrival; see Support Protocol 1), group baseline matching (see Basic Protocol 1), and data analysis.

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Supplement 3 Current Protocols in Neuroscience

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**Behavioral**

**Neuroscience**

**8.7.15**

Current Protocols in Neuroscience Supplement