

Timing of the Acoustic Startle Response in Mice: Habituation and Dishabituation as a Function of the Interstimulus Interval

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The hypothesis that the standard acoustic startle response (ASR) paradigm contains the elements of interval timing was tested. Acoustic startle stimuli were presented at a 10-s interstimulus interval (ISI) for 100 trials leading to habituation of the ASR. The ISI was then changed to either a shorter (5-s) or a longer (15-s) duration using a between-subjects design. Dishabituation of the ASR was used to measure the degree of temporal generalization for the interval-timing process. The ASR showed dishabituation at both shorter and longer ISI values on the first trial following the change in ISI. The dishabituation resulting from the change in ISI was temporary and the ASR rapidly returned to levels of response habituation showing rate sensitivity to the frequency of stimulus presentation. Interval timing may be a standard feature of this habituation paradigm, it serves to anticipate the time of occurrence of the subsequent stimulus and to prepare the startle response, and provides a computational dimension lacking in the habituation process per se.

The acoustic startle response (ASR) is a protective behavioral reaction consisting of muscle contractions of the eyelid, the neck, and the extremities that is elicited by sudden, loud acoustic stimuli. The ASR has been shown to be mediated by a simple subcortical pathway located in the ponto-medullary brainstem that consists of three synaptic stations in the central nervous system (e.g., Davis et al., 1982; Li et al., 2001). The central element is the caudal pontine reticular nucleus (PNC) that receives direct input from the auditory system and directly projects to spinal and cranial motor neurons. Despite this simple underlying pathway, the ASR is not a reflex like all-or-none response, but can be modulated by a variety of experimental changes in the perceptual or emotional state of the organism (e.g., Leaton & Cranney, 1990). Many modulatory influences affect the primary startle circuit at the level of the PNC. Examples of modulations leading to a decrease of the ASR are habituation and prepulse inhibition. Habituation is the decrease of the ASR during repetitive stimulation. It is assumed to be an intrinsic process occurring within the primary startle pathway. Prepulse inhibition (PPI) is the decrease of startle amplitude by a prepulse presented shortly before the startle stimulus that does not elicit a startle response itself. The prepulse activates an inhibiting loop that suppresses the

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processing of succeeding sensory inputs. A pharmacologically induced deficit of PPI has been proposed as an animal model for schizophrenia and Huntington's disease (e.g., Braff et al., 1992). Examples of modulations leading to an increase of the ASR are sensitization and fear conditioning. Sensitization is the enhancement of the ASR following a strong stimulus such as footshocks or strong startle stimuli. In the fear-potentiated startle paradigm the animals are trained to associate a neutral stimulus with an aversive stimulus. After a few pairings, the conditioned stimulus induces a state of fear that can be measured by a potentiation of the ASR. Footshock sensitization and fear conditioning are mediated by direct and indirect projections from the amygdala to the PNC (e.g., Fendt & Fanselow, 1999; Hitchcock & Davis, 1991; Young & Leaton, 1994, 1996). This broad spectrum of modulations together with the methodological advantage that all experimental procedures can be carried out automatically by a computer, makes the ASR a valuable tool for the study of general principles of the plasticity of sensorimotor information processing.

Startle can be elicited in a variety of animal species including humans (e.g., Schicatano & Blumenthal, 1995, 1998). By far most of the studies on the neurobiology of the ASR have been done in rats. During the last few years, an increasing number of various specific knockout and transgenic mice have been developed. The combination of this powerful new tool together with the ASR opens up fascinating new possibilities for the elucidation of the neural mechanisms of behavioral plasticity (e.g., Dirks et al., 2001; Dulawa & Geyer, 2000; Geyer, 1999; Ralph et al., 2001). As a first step, it is mandatory to characterize startle behavior in healthy mice. Then the phenotype of a mutant mouse can be attributed to the effect of the targeted gene. Recently, researchers have been characterizing the ASR of different inbred and outbred mouse strains (e.g., Plappert & Pilz, 2001). These studies contribute to a phenotypic description of the different strains that may serve as the basis for future genetic studies. In general, research has shown enormous mouse strain differences concerning ASR magnitude, course of ASR amplitude during stimulation, amount of footshock and acoustic sensitization and amount of prepulse inhibition or prepulse augmentation, respectively. There has also been use of genetically altered mice with defined neuronal deficits to elucidate the neural mechanisms of learning and memory. For example, although wild-type mice and mice with a targeted disruption of the alpha and delta isoforms of the transcription factor cAMP response element binding protein (CREB) had similar startle amplitudes and magnitudes of prepulse inhibition of startle the CREB-deficient mice failed to show fear-potentiated startle. These results suggest that CREB-activated transcription plays a selective role in the formation of long-term memory as wild-type mice are able to demonstrate fear-potentiated startle for up to 45 days after training (e.g., Falls et al., 2000). Other research has used habituation of the ASR as a "control condition", i.e., studies of social recognition memory have shown that mice lacking in the oxytocin gene show specific impairments in the memory of odors of conspecifics, but no deficit in the habituation to acoustical stimuli, thus showing general learning processes to be intact (e.g., Fergusen et al., 2000).

Our goal in this paper is to disentangle habituation of the ASR from interval timing. Interval timing is the ability to estimate durations in the seconds-to-minutes range. Animals use this stopwatch-like behavior in everyday life (e.g., Hinton & Meck, 1997). It allows them to estimate the intervals separating events and to calculate their rates of occurrence. The ability to estimate durations has been sug-

gested to be evolutionarily essential, for example, in anticipating attacks and escaping predators (e.g., Gallistel, 1990).

The phenomenon of interval timing can be also expressed in experimental settings. For example, in one common interval-timing procedure called the peak-interval procedure (Meck et al., 1987), animals are given an auditory or visual signal to time and receive food when they make a correct response after the criterion signal duration has passed. After 20-30 daily training sessions, the animal's mean response rate gradually increases toward the time of the reinforcement and then declines afterwards in a fairly symmetrical fashion. The peak-interval procedure has been used to study interval timing in various species including rats, mice, pigeons, starlings, fish, and humans (e.g., Brunner et al., 2001; Meck, 2001; Paule et al., 1999; Sasaki et al., 2002).

Recently, neuropsychological and pharmacological studies in humans and other animals have suggested that interval timing may be related to dopamine regulation in frontal-striatal circuits (e.g., Gibbon et al., 1997; Malapani et al., 1998; Matell & Meck, 2000; Meck, 1996; Meck & Benson, 2001). Subjects perceive signal durations as being proportionally shorter with the administration of dopamine antagonists or a lesion of the substantia nigra and proportionally longer with the administration of dopamine agonists. These results suggest that the speed of the "internal clock" used to make temporal judgments is modified by the effective levels of dopamine (e.g., Meck, 1983, 1986, 1996). Unfortunately, problems with psychophysical procedures that rely on operant responding can occur when motivational or motor-control factors are adversely affected by the drug or lesion under study. In addition, many of the standard interval-timing tasks require substantial training in order to evaluate the temporal control of behavior and may not be practical for the rapid screening of the large numbers of animals often required for genetic analysis.

In the present study, we describe an alternative to the traditional interval-timing procedures which consists of measuring the ASR. The amplitude of the ASR is markedly decreased when stimuli are repeatedly presented at a constant inter-stimulus interval (ISI), thus showing habituation (Davis, 1970, 1974, 1984). Davis (1970) also included a fixed vs. variable ISI comparison which showed that habituation is slowed with a variable ISI, thus suggesting that the habituation of the ASR is sensitive to the predictability of the startle stimulus. One way in which the ASR could be used to investigate interval timing is to present the startle stimulus at a regular ISI. Once habituation to the stimulus is observed, the same startle stimulus with a different ISI can be introduced and the degree of dishabituation observed as a function of temporal generalization from the baseline ISI to the test ISIs. Such a result would suggest that two learning processes are involved in this task: one is the process of habituation to the startle stimulus and its frequency of occurrence (e.g., Staddon & Higa, 1996), and the other is the active timing of the ISI, that is, the comparison of the current time with the expected time of stimulus presentation (e.g., Gibbon, 1977).

Method

Subjects

A total of 30 mice served as subjects. In order to provide generality to our behavioral procedures two strains of wild-type mice were used. Twelve of these mice were male, wild-type

C57/129 mice, 8 to 12 weeks of age, obtained from our breeding colony of parental (+/-) dopamine transporter knockout mice at Duke University (see Gainetdinov et al., 1999; Giros et al., 1996). The remaining eighteen mice were male, black C57 mice 8 to 12 weeks of age obtained from Jackson Laboratories (Maine, U.S.A.). Mice were housed in groups of 3-4 and food and water were available ad libitum in the home cage. The vivarium was kept on a 12:12 h light:dark cycle with lights on at 09:00 h. Behavioral testing procedures took place during the light phase of the animal's light:dark cycle.

Apparatus

The acoustic startle response (ASR) was measured using four identical startle chambers and associated software controlled by an IBM-compatible PC (MED-ASR-PRO, MED-Associates Inc., Vermont, U.S.A.). For the measurement of the ASR mice were placed in a round acrylic animal holder (ENV-263A, MED-Associates).

Procedure

In order to determine the appropriate stimulus intensities to use for our experiments with different strains of mice, a preliminary study was conducted prior to the current experiment. Eight mice were placed individually in the acrylic cylinder and after a 10-min acclimation period with exposure to a 65 dB background white noise, 40-ms pulses of white noise at five different tone intensities (80, 90, 100, 110, and 120 dB) were presented every 60 s. Each of the five stimulus intensities was presented once in a randomized order in a block of five trials. Ten of these blocks were presented for a total of 50 trials in a session. Based on these "input/output" functions, an optimum habituation intensity for the white noise stimulus was defined as the midpoint between the minimum startle and the maximum startle response as determined by a standard regression algorithm. A mean optimum intensity value of 100 dB was obtained from these preliminary studies and there were no significant strain differences observed in this measure for the mice used in our studies, $p > 0.05$. Consequently, the 100 dB startle stimulus was used for all of the mice in the experiments reported below. For additional details on obtaining input/output functions relating the acoustic input (SPL of the startle stimuli) and the behavioral output (ASR amplitudes) in wild-type and mutant mice see Plappert et al. (2001).

Mice were placed in the acrylic cylinder located in a dark, sound-attenuated, and ventilated startle chamber for a 10-min acclimation period with exposure to a 65-dB background noise, which continued throughout the session. Mice readily adapted to being placed in the holder and no strain differences were observed. An acoustic startle stimulus consisting of a 40-ms pulse of white noise presented at 100 dB could also be presented. Using random assignment, the mice from each genotype were evenly divided into two groups. The mice in Group 1 were presented with a continuous sequence of 150 presentations of the startle stimulus. The first 100 startle stimuli were presented with a 10-s ISI, immediately followed by 50 trials with a 5-s ISI (10 s→5 s ISI Group). The mice in Group 2 were also presented with a continuous sequence of 150 startle stimuli. The first 100 startle stimuli were presented with a 10-s ISI, immediately followed by 50 trials with a 15-s ISI (10 s→15 s ISI Group).

The latency to startle is defined by the MED-ASR-PRO software as the time between the onset of the startle stimulus and the onset of the startle response. An amplifier boosts input from the load cells of the response platform before being sent as an analog signal that is digitized by the MED Associates hardware. The desired amplitude range is calibrated prior to the experiment by using a calibrated input utility and a pressure generating calibrator that simulates an animal's response. The resolution of each platform response is calibrated to the same output criterion (± 250 response units) so as to insure consistent amplitude measures between mice. Startle waveforms with latencies to onset that were less than 10 ms or more than 50 ms were not included in the present analyses. This screening resulted in the exclusion of approximately 10-15 % of the trials. Visual inspection of the waveforms that did not meet this criterion showed that these responses lacked a positive wave with no peak response times occurring between 45 and 60 ms. Waveforms that did not meet criterion are similar to those observed in the null trials, without the startle stimulus, and are correlated with the mouse's independent movement in the Plexiglas tube.

In order to reduce outliers in our graphical representations the median ASR was calculated for each mouse as a function of blocks of 10 trials. The percent maximum ASR for each mouse

was then calculated by dividing the median for each block's ASR by the median ASR amplitude for the first block of trials. For statistical comparisons, however, the initial level of responding was calculated by taking the mean ASR amplitude during the first 10 trials. In addition, a baseline level of habituation to the startle stimulus was calculated by taking the mean ASR amplitude for the last 30 trials before the ISI change and normalizing it by the initial level of response. This baseline level of habituation was then compared to the normalized ASR for the first trial following the ISI change. All statistical tests were evaluated at a significance level of 0.05.

Results

The mean ASR amplitudes as a function of blocks of trials at the 10-s ISI (100 trials) followed by the transition to a 5-s ISI (50 trials) for Group 1 or to a 15-s ISI (50 trials) for Group 2 are shown as a percent of the initial level of responding during the first 10 trials in Figure 1. A comparison between the initial levels of responding and the baseline levels of habituation prior to the change in ISI revealed a significant repeated-measure effect, $F(1,26) = 40.8$, but no reliable effects of mouse type, ISI condition, or any of the interactions.

No statistically significant differences were observed in either the amplitude or the response pattern during baseline training for the different genetic backgrounds, $F_s < 1$. A significant increase in the ASR immediately after the ISI change was observed in both groups as shown by a significant repeated-measure effect of baseline vs. test conditions, $F(1,26) = 8.99$, but no significant interaction between the ISI condition and the repeated measure, $F < 1$ or the mouse type x ISI condition x repeated measure interaction, $F < 1$. The increases in ASR following the change in ISI were temporary and usually disappeared within the first 10 trials following the ISI change as shown in Figure 1. Comparisons between the habituation baseline collected from the last 30 trials before the ISI change and the first trial after the change are shown in Figure 2.

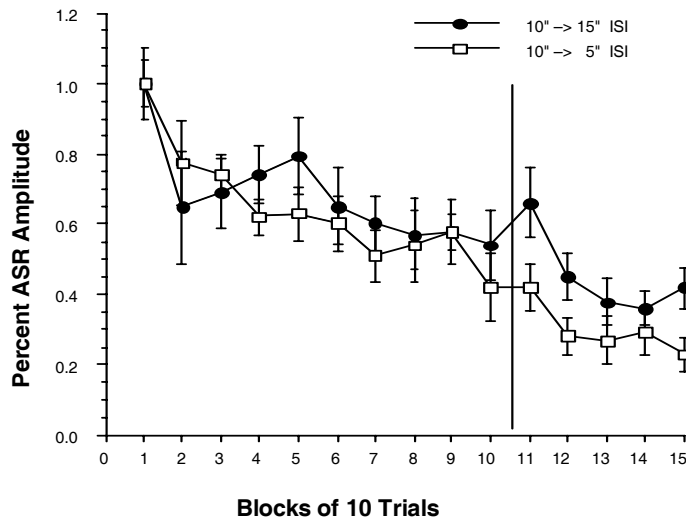


Figure 1. Mean percent acoustic startle response (ASR) amplitude (Mean \pm SEM) as a function of blocks of 10 trials. During baseline training startle stimuli were presented for 100 trials with a 10-s interstimulus interval (ISI). Baseline training was immediately followed by 50 test trials with either a 5-s ISI (Group 1 = 10 s \rightarrow 5 s) or a 15-s ISI (Group 2 = 10 s \rightarrow 15 s). The vertical line indicates the change in ISI. Data for the two mouse types are collapsed because there were no significant differences in performance.

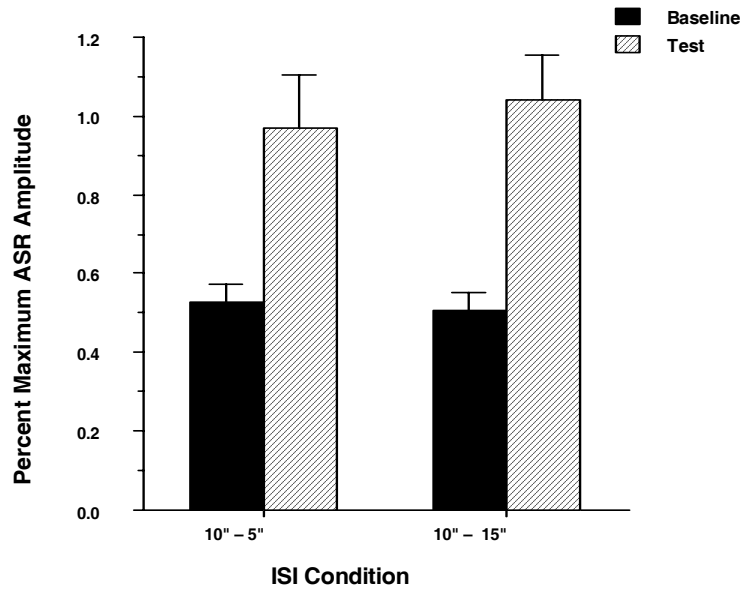


Figure 2. Mean percent acoustic startle response (ASR) amplitude (Mean \pm SEM) for the last 3 blocks of trials prior to the change in the interstimulus interval (ISI) and the first trial following the change in ISI for mice in Group 1(10s \rightarrow 5s) and Group 2 (10 s \rightarrow 15 s) treatments. Data for the two mouse types are collapsed because there were no significant differences in performance.

It is important to note that the value of the new ISI differentially affected the final levels of habituation. ISI shifts from 10 s to 5 s produced higher levels of habituation than those observed for ISI shifts from 10 s to 15 s , $F(1, 26) = 12.34$.

Discussion

Previous studies have demonstrated that through rest and recovery, the effects of habituation training can be reversed; the animal gradually comes to respond again to the habituating stimulus (e.g., Fantino & Logan, 1979). In addition, as shown in the present report, a different and more immediate reversal of habituation can be brought about by the single presentation of a novel stimulus. If following the initial response decrement the subject is immediately exposed to the single presentation of a solitary novel stimulus, subsequent responding to the habituating stimulus will be quickly reinstated. That is, interpolated novel stimulation immediately restores the ability of the habituating stimulus to evoke the once prominent response. This immediate reversal of habituation produced by novel stimulation is termed dishabituation. The critical feature of the interpolated stimulus is its novelty. Typically, when habituating and novel stimuli represent the same stimulus modality, dishabituation is produced by a change in the intensity (higher or lower) of the stimulus or the spatial location of the stimulus (e.g., Thompson & Spencer, 1966). The current data are the first to suggest that the temporal spacing of stimuli may be an important factor in determining the novelty of a stimulus and the subsequent observation of dishabituation (for a more rigorous definition of dishabituation see Tighe & Leaton, 1976).

Because many investigators have made a distinction between independent

short-term and long-term habituation processes (e.g., Leaton et al., 1985) it should be noted that only the short-term habituation process is implicated by our results. In particular, our findings that habituation is sensitive to the temporal expectation of the stimulus, i.e., the ASR to the anticipated stimulus is reduced in amplitude while the ASR to the surprising stimulus is increased in amplitude, fit in nicely with Wagner's (1976, 1978, 1979) short-term memory theory of habituation. The present results are especially intriguing given the well established relationship between ASR amplitude and the ISI (e.g., Davis, 1970) which our data reverse on the first trial of the ISI change. It has also been shown in rats that startle stimuli expected because of context or because they are preceded by a discrete stimulus induce higher, rather than lower, response amplitudes (e.g., Leaton & Cranney, 1990). Thus, in these instances it is the "unexpected" stimulus that produces the smaller ASR. Finally, habituation of the ASR in rats has been shown to be insensitive to changes in context (Jordan et al., 2000) and is unaffected by modifications in the frequency (pitch) of the startle-eliciting stimulus, if intensity is controlled (e.g., Jordan & Poore, 1998; Leaton et al., 1985). Taken together, these findings suggest that an internally generated stimulus such as duration may have a distinctively different effect on the habituation process than externally presented stimuli. If these results can be shown to apply across a variety of species (e.g., rats and mice) it will be important to determine how the processing of the temporal interval connects to the neural circuits involved in the ASR. If sensitivity to the temporal expectation of the stimulus can only be demonstrated in mice it will be important to determine whether or not peripheral effects could account for the observed differences. For example, given the relative size discrepancy between the mouse and the rat, differences in the post-stimulus and/or anticipatory posture that affect the ASR may not be too surprising (R. Leaton, personal communication.).

A classic finding in the literature is that habituation occurs faster with shorter ISIs, but shows greater maintenance with longer ISIs (Davis, 1970). This phenomenon is referred to as the rate sensitivity property of habituation and it has been demonstrated using a variety of stimuli (e.g., pure tones, visual cues, and tactile stimuli) in rats, pigeons, *C. elegans*, and *Aplysia* (e.g., Broster & Rankin, 1994; Carew et al., 1972; Davis, 1970; Staddon & Higa, 1996). Here we show that this hallmark of habituation can be observed in mice using white-noise as the startle stimulus. In addition, we show that there is evidence for the anticipation of the startle stimulus as predicted by scalar expectancy theory (e.g., Gibbon, 1977; Gibbon et al., 1984). Memory for the interval separating individual startle stimuli was revealed by showing increases in ASR amplitude immediately after a change in the ISI. Consequently, our experiment suggests that there are at least two types of learning involved in this task; one is habituation to the stimulus characteristics of the white noise and its relationship to the background (e.g., Davis, 1974) and another is the anticipation of the time of occurrence of the startle stimulus—referred to here as interval timing.

As indicated above, previous studies have shown that a variety of species have the ability to time arbitrary intervals in the seconds-to-minutes range (e.g., Paule et al., 1999). In many of these experiments, peak-interval timing procedures have been used in which subjects are trained to make a response at a certain time in order to receive feedback (e.g., food reward). By showing their maximum response rate around the time that they expect feedback to occur, subjects commonly produce

a Gaussian-shaped response function. The symmetry of this response function on an arithmetic time scale and the observation that the standard deviation of the function grows proportional to the mean of the function have been depicted as hallmarks of interval-timing behavior. In order to formally characterize the shape of the temporal generalization functions obtained from our startle response procedure, future experiments will need to include a broader range of ISI values (see Church & Gibbon, 1982; Church et al., 1991). This could be done either as a within- or between-subjects design. One problem, however, is that the test trials required to evaluate the degree of temporal generalization are dependent upon the level of habituation to the baseline ISI and the novelty of the dishabituating stimulus. Intermixing training trial ISIs with test trial ISIs is a thorny problem as noted by Davis (1974). Additional work will have to be conducted in order to clarify the appropriate experimental design for obtaining more extensive temporal generalization functions. Our assumptions, however, are that the ASR amplitude is at its nadir at the habituated ISI and that the ASR amplitude systematically varies with the size of the deviation from the training ISI in a manner consistent with the properties of the internal clock and scalar expectancy theory (e.g., Church, 1984; Gibbon et al., 1984).

In summary, after a shift in the baseline ISI we observed the rate-sensitivity property of habituation, i.e., increased levels of habituation with shorter ISIs and decreased levels of habituation with longer ISIs (e.g., Fantino & Logan, 1979). In addition, immediately after the ISI change, the startle response increased dramatically (dishabituation) but quickly rehabilitated to the levels appropriate to the new ISI. Taken together, these results suggest a dissociation of the anticipatory timing and rate sensitivity mechanisms of habituation. An interval timing interpretation of these results would predict that habituation would be slowed if one were to use a variable ISI, thereby interfering with the anticipatory timing of the stimulus. Indeed, Davis (1970) provides evidence for this, at least following a modest number of habituation trials. Consequently, temporal generalization of the ASR may serve as a useful alternative to traditional peak-interval timing procedures in terms of its potential for the separation of clock, memory, motivational, and motor components of the task. In addition, temporal generalization of the ASR appears sufficiently sensitive to serve as a rapid screening assay for interval-timing deficits in transgenic and knockout mice.

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