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THE 3-SECOND AUDITORY CONDITIONED STIMULUS IS A MORE EFFECTIVE STRESSOR THAN THE 20-SECOND AUDITORY CONDITIONED STIMULUS IN MALE RATS

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Abstract—Using fear-conditioning model, we have used a 3-s auditory conditioned stimulus (CS) as a stressor and observed fear and stress responses during a specific experimental period regardless of the presence or absence of the CS. Because the CS was extremely short compared with the experimental period, we observed responses primarily in the absence of the CS. In contrast, most studies in the literature have analyzed responses in the presence of the CS. Therefore, the characteristics of fear and stress responses in the absence of the CS remain to be clarified. To clarify this, we compared the characteristics of fear and stress responses elicited by a 3-s auditory CS with those observed during a 20-s auditory CS. The basolateral complex of the amygdala (BLA), but not the bed nucleus of the stria terminalis (BNST), participated in the fear response elicited by the 3-s CS, whereas both the BLA and BNST were involved in the response observed during the 20-s CS. Additional analyses revealed that the BNST participated in the fear response during the 20-s CS when the CS was paired with a 0.75-mA, but not with a 0.9-mA, foot shock, and to the contextual CS. In addition, the fear response elicited by the 3-s CS was more resistant to extinction than that during the 20-s CS. Finally, the 3-s CS produced more intense freezing and corticosterone secretion than the 20-s CS. On the basis of these characteristics, we conclude that the 3-s auditory CS is a more effective stressor than the 20-s auditory CS. Our findings also suggest that foot shock intensity is an additional determinant in the type of fear response induced by the CS.

Key words: CS duration, foot shock intensity, bed nucleus of the stria terminalis, auditory fear conditioning, corticosterone, sustained fear response.

INTRODUCTION

Pavlovian fear conditioning, or threat conditioning, is an experimental procedure in which a conditioned stimulus (CS) acquires an ability to elicit fear responses in animals that depend on the basolateral complex of the amygdala (BLA) (LeDoux, 2012, 2014). Previous researches that observed the effects of the CS on the acoustic startle reflex have generated the hypothesis that fear responses can be divided into phasic fear response and sustained fear response according to their dependence on the central amygdala (CeA) and bed nucleus of the stria terminalis (BNST) (Walker et al., 2009; Davis et al., 2010). For example, the CeA, but not the BNST, was necessary for the enhancement of the startle reflex in the presence of a 3.7-s visual CS (Walker and Davis, 1997), suggesting that the phasic fear response was elicited. In contrast, the enhancement of the startle reflex during an 8-min auditory CS appeared to be the sustained fear response because the BNST, but not the CeA, was necessary for it (Davis et al., 2010). The nature of the cue is thought to determine the type of fear responses. ''In the laboratory, phasic fear can be measured using a short, discrete cue that is predictably paired with an aversive event'' (Davis et al., 2010), while ''sustained fear is measured using more diffuse cues, or cues associated less predictably with an aversive event'' (Davis et al., 2010).

In addition to the enhancement of the startle reflex, such classification appears applicable to CS-induced freezing behavior. Freezing, however, may be classified only by its dependence on the BNST because the CeA is crucial for the expression of freezing per se (LeDoux et al., 1988; Wilensky et al., 2006). Previous studies found that the BNST was not necessary for freezing during a discrete, predicable cue such as a 20-s (Sullivan et al., 2004), 30-s (Duvarci et al., 2009), or 120-s auditory CS (LeDoux et al., 1988), suggesting that the above responses were the phasic fear responses. In contrast, freezing induced by a more diffuse, less predictable cue such as a contextual CS (Sullivan et al., 2004; Resstel et al., 2008; Duvarci et al., 2009) required a functional BNST, and thus appeared to be the sustained fear response.

We have been analyzing the phenomenon called social buffering in male rats, in which the presence of a same-sex conspecific ameliorates freezing and hypothalamic-pituitary-adrenal axis activation that is

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Abbreviations: BLA, basolateral complex of the amygdala; BNST, bed nucleus of the stria terminalis; CeA, central amygdala; CS, conditioned stimulus; PBS, phosphate-buffered saline.

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elicited by an auditory CS (Kiyokawa et al., 2007, 2009, 2014a,b; Takahashi et al., 2013). We have chosen an auditory CS as a stressor because the characteristics of auditory CS-elicited responses have been analyzed extensively. We observed the responses during a specific experimental period regardless of the presence or absence of the CS. However, because we used a 3-s tone as the CS, which was extremely short compared with the experimental period, we observed the responses primarily in the absence of the auditory CS. In contrast, most studies in the literature observed the responses in the presence of tens of seconds of auditory CS. Therefore, the characteristics of fear and stress responses in the absence of the CS remain to be clarified. In particular, the fear response in the absence of the CS can be the BNST-dependent sustained fear response, rather than the BNST-independent phasic fear response, because the timing of the next CS is unpredictable to the experimental animals.

To clarify this, as well as to seek a suitable stressor for our future social buffering studies, we compared the characteristics of fear and stress responses elicited by the 3-s auditory CS with those observed during one of the most widely used 20-s auditory CS (Sullivan et al., 2004; Lamprecht et al., 2009; Amano et al., 2011). We first assessed the contribution of the BNST and BLA in the fear response, and then evaluated extinction of the fear response. Finally, we directly compared the fear and stress responses induced by the two types of CS.

EXPERIMENTAL PROCEDURES

All experiments were approved by the Animal Care and Use Committee of the Faculty of Agriculture at the University of Tokyo, according to the guidelines adapted from the *Consensus Recommendations on Effective Institutional Animal Care and Use Committees* by the Scientists Center for Animal Welfare.

Experimentally naïve male Wistar rats (aged 7.5–8 weeks) were purchased from Charles River Laboratories Japan (Kanagawa, Japan). They were housed 2–4 animals per cage in a room with a controlled ambient temperature of 24 ± 1 °C and humidity of 45 ± 5% with food and water available *ad libitum*. The animals were maintained under a 12/12-h light/dark cycle (lights on at 0800).

Experiments to assess the contribution of the BNST and BLA to fear responses

The BNST or BLA was bilaterally lesioned with an injection of ibotenic acid as described in our previous study (Kiyokawa et al., 2012). All subjects were anesthetized with sodium pentobarbital (Somnopentyl; Schering-Plough Animal Health, Harefield, UK) and placed in a stereotaxic frame. The cranium was exposed, and a small burr hole was drilled. Injections of 0.08 μL (per site) of ibotenic acid (Wako Pure Chemical Industries, Osaka, Japan) dissolved in phosphate-buffered saline (PBS; 10 μg/μL) were directed at the BNST (AP, −0.2 mm; ML, 1.6 mm; DV, 6.1 mm) or BLA (AP, −2.7; ML, 4.8; DV, 7.5). All injections were administered at a rate of 0.1 μL/min with a Hamilton microsyringe (Hamilton, Reno, NV, USA) connected to a glass micropipette. Sham subjects received PBS injections following the same procedure. Following the completion of each injection, the pipette was left in place for at least 5 min and then slowly withdrawn. The wound was then closed, and the subjects were housed individually. All subjects were handled for 5 min per day for 3 days prior to fear conditioning.

Fear conditioning was performed 5 days after the surgery in an illuminated room between 0900 and 1300. The subjects were placed in an acrylic conditioning box with a metal grid floor (28 × 20 × 27 cm) and received seven repetitions of either a 3-s or 20-s auditory CS (8 kHz, 80 dB) that terminated concurrently with a 0.7-mA foot shock (0.5 s). The inter-trial interval varied randomly between 90 s and 180 s. The subjects were returned to their home cages after fear conditioning.

An auditory fear expression test was performed 24 h after fear conditioning in a dark room illuminated with dim red light. The subjects were placed in an acrylic test box that was constructed of three acrylic walls, one wire mesh wall, and a wire mesh ceiling (25 × 25 × 35 cm) and contained clean bedding. The subjects were then exposed to the CS 5 times at 2-min intervals. Researchers who were blinded to the experimental conditions recorded the duration of freezing (immobile posture with the cessation of skeletal and vibrissae movement except respiration) using a Microsoft Excel-based Visual Basic software that records the duration and number of keyboard keys pressed. Freezing during the 20-s period starting 2 min before the onset of the first CS was defined as baseline freezing and expressed as a ratio to the 20-s period. The duration of freezing during the 20-s period from the onset of each CS was recorded and expressed as the ratio to the 20-s period. The mean ratio of baseline freezing and freezing in the auditory fear expression test was analyzed using Student's *t*-test. Data are expressed as mean ± standard error of the mean. P values < 0.05 were considered significant for all statistical analyses.

After the auditory fear expression test, we verified the lesions using immunohistochemical staining for neuronal nuclei (Kiyokawa et al., 2012). Subjects were deeply anesthetized with sodium pentobarbital and intracardially perfused with 0.9% saline, followed by 4% paraformaldehyde in 0.1 mol/L phosphate buffer. Their brains were removed and immersed overnight in the same fixative before being placed in 30% sucrose/phosphate buffer for cryoprotection. Consecutive coronal sections (50 μm) of the lesion site were collected and incubated with 0.3% H_2O_2 in PBS for 30 min. The sections were then incubated with citric acid buffer (Mitsubishi Chemical Medicine, Tokyo, Japan) for 2 h, followed by incubation with the primary antibody to neuronal nuclei protein (MAB377; Millipore, Billerica, MA, USA) overnight, and then with biotinylated anti-mouse secondary antibody (VECTASTAIN ABC kit, PK-6102; Vector Laboratories, Burlingame, CA, USA) for 2 h. Finally, the sections were processed with the VECTASTAIN ABC kit (PK-6102, Vector Laboratories) and developed using diaminobenzidine solution with nickel intensification. We verified the