Brief Communication

AX+ / BX– discrimination learning in the fear-potentiated startle paradigm in monkeys

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Individuals with anxiety disorders often do not respond to safety signals and hence continue to be afraid and anxious. Consequently, it is important to develop paradigms in animals that can directly study brain systems involved in learning about, and responding to, safety signals. We previously developed a discrimination procedure in rats of the form AX+/BX–, where cues A and X presented together are paired with an aversive stimulus and cues B and X presented together predict the absence of an aversive stimulus. The present experiment adapted this procedure to the fear-potentiated startle paradigm in rhesus monkeys.

Excessive fear and anxiety, along with an inability to overcome these emotions, are defining characteristics of many psychiatric disorders such as phobias, panic disorder, and post-traumatic stress disorder. Animal models of fear conditioning and fear inhibition provide useful tools for the study of these phenomena. We have recently developed whole-body acoustic startle applications in non-human primates, including conditioned fear (Winslow et al. 2002, 2007). Further, we have begun to compare neural processes associated with fear conditioning using identical procedures between rats and non-human primates.

Fear-potentiated startle is defined by the relative increase in the amplitude of the acoustic startle reflex when elicited in the presence of a conditioned stimulus (CS) previously paired with an aversive stimulus (unconditioned stimulus [US]) (Brown et al. 1951; Davis and Astrachan 1978). In the laboratory, inhibition of fear traditionally has been studied with the use of two conditioning paradigms: extinction and conditioned inhibition (Myers and Davis 2002).

In the typical conditioned inhibition paradigm, one stimulus (A) is paired with an aversive US when presented in isolation (A+) and is not paired with the US when presented in compound with a second stimulus, B (AB–). After training on this A+/AB– discrimination, A elicits a fear response, and AB elicits less of a fear response. It can be demonstrated that the reduction of fear to AB is due to inhibitory fear learning to B that counteracts the fear that otherwise would be elicited by A (Rescorla 1969).

Although the typical conditioned inhibition paradigm has been widely used in rodents it has certain limitations that make it especially problematic in humans. Thus, A+, AB– can be solved not by considering A and B as separate elements, but instead by viewing stimulus A as one cue and stimulus AB as a second cue. Such a “configural strategy” easily leads to fear to A and less fear to AB. However, inhibition does not develop to B as shown by transfer tests. Thus, B does not inhibit fear when put in compound with another cue, C, that also was paired with an aversive event. As it turns out, humans more typically use a “configural strategy” where they treat A as one cue and AB together as

a different cue and thus do not show transfer when B is put in compound with C. Because of this, the typical conditioned inhibition paradigm has not proven to be an effective way to study fear inhibition in humans (e.g., Grillon and Ameli 2001).

Myers and Davis (2004) recently developed a discrimination procedure in rats that allows for independent evaluation of excitation and inhibition of fear. The procedure was referred to as a conditional discrimination and abbreviated as AX+/BX–. A becomes excitatory with training as the subject learns that A and X presented together predict the US. B becomes inhibitory in that B presented with X predicts “safety” from the US. In a critical subsequent transfer test trial, presentation of A and B together (AB) results in a reduced fear response compared with the response to A. We have also now found this to be true in humans using procedures that reduce configural learning, and we see transfer of inhibition on AB test trials (Jovanovic et al. 2005), in contrast to prior failures to see transfer in humans using a conditioned inhibition paradigm (Grillon and Ameli 2001).

Because it was not obvious whether rhesus monkeys would behave more like rats or humans in terms of treating cues as separate elements vs. using a configural strategy, our goal in the present study was to design a non-human primate fear conditioning paradigm based on the AX+/BX– paradigm that can assess fear-potentiation and fear-inhibition in the same test session under conditions in which they are relatively independent, using the fear-potentiated startle test.

Twelve male rhesus monkeys (average age of 48 mo) participated in these experiments at the outset; 11 subjects were born and mother-reared at the Yerkes National Primate Research Center Field Station and transferred to the Yerkes Main Station and pair-housed at weaning. The remaining monkey was peer-reared in the Yerkes Nursery Facility and pair-housed at 1 yr of age. Caging and husbandry followed NIH guidelines for enhanced environmental enrichment to promote primate psychological well-being.

The testing apparatus and general procedures related to training and measurement of whole-body startle are described in detail elsewhere (Antoniadis et al. 2007, Winslow et al. 2007). Briefly, conditioning was performed in a custom-built startle testing chamber. Three to-be-conditioned stimuli (A, B, X) were ei-

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ther (1) visual: produced by halogen house lights mounted in the chamber ceiling; (2) tactile: airflow generated by a quiet computer fan mounted overhead (Evercool, <24 dB, ~8 cfm at source at 5000 rpm) and channeled through a plastic tube (5 cm diameter) terminating ~10 cm above the head of each subject; or (3) auditory, 80-dB, 210-Hz computer-generated tone emitted from speakers located in the chamber walls. Each stimulus was 4.0 sec in duration, and for training these cues were assigned differently as A, B, or X for each animal and balanced across subjects. The unconditioned stimulus (US) was a 500-msec air pulse (100 psi at source, approx. 400 cfm at nozzle) delivered through stainless steel nozzles directed at the face of the subject from opposite sides and ends of the chamber, ~10 cm from the monkey.

Fear-potentiated startle training of AX+ and BX– progressed in three stages. During the first phase, subjects were trained to associate cue A with presentation of the air-burst US (A+) in four pairings randomly presented during each of three repeated 50-min sessions separated by at least 72 h (Winslow et al. 2007). A+ trials were scheduled such that one occurred at the beginning and one at the end of each session. The remaining two pairings were scheduled during inter-trial intervals within the session. Interposed between A+ trials there were six blocks of six startle trials. Each startle trial represented one of three stimulus intensities (95, 105, or 115 dB) in the presence or absence of A. When A came on, the startle stimulus was presented 3.5 sec after A onset (i.e., at the time when the air burst occurred on A+ training trials). Thus, on any given trial, the monkey could not predict, when A came on, whether an air burst or a startle stimulus would occur. The A cue was either a visual, tactile, or auditory stimulus that was randomly assigned to a subject such that each CS type was conditioned in four animals.

During the next phase, a second conditioned stimulus, B, was added to the sessions such that B was never paired with the air burst US (B–), but A continued to be paired four times (A+) within each session according to the schedule described previously. Also as before, three sessions were scheduled at least 72 h apart and included six blocks of six startle trials. Each trial represented one of two stimulus intensities (105 or 115 dB) in the presence or absence of the A, B, or no CS. When the A or B cue came on, the startle stimulus was presented 3.5 sec after cue onset (i.e., at the time when the air burst occurred on A+ training trials). The order of stimulus presentation was systematically varied within blocks and across sessions. A+ continued to be represented by the same stimulus type assigned to each subject in the initial A+ training phase. The B cue was systematically varied such that all possible combinations of A+, B– cue types were represented in each of two animals.

Prior to the third phase (see below), two monkeys were removed from the study due to veterinary concerns related to social housing. During this phase, an X stimulus for each subject was presented simultaneously with either A or B throughout these training sessions to yield compound stimuli AX+ and BX–. The X cue was included for three reasons. First, Myers and Davis (2004) found that prior experience with compound cues reduced the probability that reduced fear on the critical AB transfer test would occur because the AB compound cue was novel. In other words, prior experience with compound cues reduced external inhibition. Secondly, because A and B are never presented in compound prior to test, this reduces the probability that the AB compound will be viewed as a single cue. Third, because B is never put in compound with A prior to test, this reduces the amount of second-order fear conditioning that can accrue to B, which would interfere with learning that B is a safety signal.

As before, three sessions were scheduled at least 72 h apart and were composed of six blocks of six startle trials. Each trial represented one of two stimulus intensities (105 or 115 dB) in the

presence or absence of the AX, BX, or no CS. When the AX or BX came on, the startle stimulus was presented 3.5 sec after stimuli onset. The order of stimulus presentation was systematically varied within blocks and across sessions. AX+ continued to be represented by the same A stimulus type assigned to each subject in the A+ training phase. BX– continued to be represented by the same B stimulus type assigned to each subject in the A+, B– training phase.

Animals were tested within 24 h after the last AX+/BX– training session in a single session to examine the potential inhibitory effects of B on A. The testing session started with an initial AX–US pairing, and then no further CS–US pairings were scheduled. Startle was then elicited in the presence of AX, BX, A, B, X, and no CS, as well as a newly formed AB compound stimulus. Each trial was represented by one of two stimulus intensities (105 or 115 dB) in the presence or absence of the six test cues or two “no CS” to yield a total of 14 trials. During the testing session, the order of stimulus presentation was systematically varied.

Fear-potentiated startle was computed for each animal by computing “percent fear potentiated startle” as [(mean startle amplitude on CS test trials – mean startle amplitude on noise burst alone (no CS) test trials)/mean startle amplitude on noise burst alone test trials] $\times$ 100 (Walker and Davis 2002). Percent fear-potentiated startle values were typically not normally distributed and were thus examined using a Wilcoxon matched pairs test for dependent samples.

During the first phase, we confirmed that neither individual nor compound to-be-conditioned stimuli significantly altered acoustic startle response relative to unsigned startle stimuli prior to conditioning.

We also determined that subjects developed elevated startle levels in the presence of each of the individual CS types when paired with the air burst (overall mean percent potentiated startle = 289 ± 53.4). Inspection of individual performance revealed that every subject had greater startle amplitude in the presence of the conditioned stimulus. Paired comparisons of averaged percent potentiated startle values found significant fear potentiated startle in each session but no difference in the magnitude of potentiation between sessions.

In the second phase, subjects also discriminated between cues occasionally paired (A+) and cues never paired (B–) with air bursts in repeated training sessions. Paired comparisons of averaged percent potentiated startle values to A and B within each session detected significant differences after 3 d of training ($z = 2.57$, $P < 0.012$, Wilcoxon matched pairs).

In the third phase, subjects also discriminated between compound cues occasionally paired (AX+) and cues never paired (BX–) with air bursts in repeated training sessions. Paired comparisons of averaged percent potentiated startle to AX and BX within each session detected significant differences after three days of training ($z = 2.60$, $P < 0.009$, Wilcoxon matched pairs). For comparison with the first exposure to the compound AB stimulus, we also examined paired comparisons of percent potentiated startle to AX and BX during the first block of trials within each session and likewise detected significant differences after three days of training ($z = 2.0$, $P < 0.04$, Wilcoxon matched pairs; Fig. 1).

On the AB test day, Wilcoxon matched pairs comparison of percent potentiated startle associated with each stimulus (Fig. 2) revealed that AX was significantly greater than BX ($z = 2.19$, $P < 0.03$), AB ($z = 1.98$, $P < 0.047$), and B ($z = 2.80$, $P < 0.005$) but not X. No significant differences were detected in comparisons between percent potentiated startle associated with AB, X, and BX or between A and AX. Notably, startle in the presence of the new compound AB was significantly less than in the presence of A on the first AB test trial. That is, subjects immediately trans-

Figure 1. Mean (± 1 SEM) percent change in startle amplitude during the first block of trials in each of three AX+/BX- training sessions. Data demonstrate discrimination between the compound stimulus occasionally paired with an aversive stimulus (AX+) and BX-, a compound stimulus which was never paired with an aversive stimulus. As previously, discrimination is defined as significantly elevated percent change in startle amplitude in the presence of AX+ vs. BX- relative to unsignaled startle. Asterisks represent $P < 0.05$, Wilcoxon matched pairs test comparing AX+ to BX- within block 1 of each session.

ferred “safety” on the first AB test trial, consistent with the assertion that the decrement in startle to AB relative to A was accounted for by the inhibitory properties of B and not an effect of rapid learning once the animal learned that cue AB was non-reinforced.

Figure 2. Mean (± 1 SEM) percent change in startle amplitude during the first block of trials of the AB Test session. Startle amplitudes were elicited in the presence of compound stimuli AX, BX, individual stimuli A, B, and X as well as a new compound stimulus AB. Data demonstrate the persistence of previously conditioned discriminations between AX and BX as well as A, B, and X. Data also demonstrate that startle in the presence of the new compound stimulus AB was significantly reduced compared to AX or A. Asterisks represent $P < 0.05$ for Wilcoxon matched pairs test for averaged percent change values computed for each subject in the presence of AX+ vs. each of the other stimuli BX, AB, A, B, and X within block 1 of the AB test session. Also depicted for comparison in inset are t -test contrasts between raw unsignaled startle amplitudes (no CS) and values elicited in the presence of each of the CS- or CS+ stimuli. Bold text represents $P < 0.05$ for paired t -tests within the first block of trials.

We present a new method of measuring inhibition of fear in non-human primates adapted from a conditional AX+/BX- discrimination procedure developed in rats and humans (Myers et al. 2004; Jovanovic et al. 2005). Using cues from different stimulus modalities (tactile, auditory, and visual randomly assigned as A, B, or X), we paired cues A and X in simultaneous compound with a burst of air to the head (AX+), whereas cues B and X signaled that no burst would occur (BX-). We found that subjects startled more in the presence of AX and A than in the presence of BX, B, X, or, most importantly, AB.

This represents the first study to demonstrate transfer in a conditioned inhibition experiment with fear-potentiated startle in non-human primates under conditions in which second-order conditioning, configural learning, and external inhibition are minimized. However, it needs to be acknowledged that we cannot yet fully rule out the possibility that the marked inhibition of fear to A we saw on the first AB test trial was not, at least partially, due to external inhibition, whereby putting a cue in compound with a conditioned stimulus can reduce a conditioned response to that stimulus (Pavlov 1927). To test this, we would have had to evaluate whether another cue (e.g., C) would inhibit fear to A on an AC test trial. As mentioned earlier, prior work in rats found that experience with compound cues (e.g., AX, BX) reduced external inhibition (Myers and Davis 2004). Furthermore, in both rats (Toufexis et al. 2007) and humans (Jovanovic et al. 2005) we found no inhibition on AC test trials when there was prior exposure to all cues. In the current design, the monkeys had considerable prior exposure to all cues during the various phases of the study, so we are quite confident that we were measuring conditioned inhibition. Nonetheless, further work needs to explicitly test how much of the inhibition we observed on AB test trials resulted from conditioned vs. external inhibition.

In contrast to extinction, the AX+/BX- paradigm permits an independent analysis of fear potentiation and fear inhibition in the same subject in the same session and thus should provide a unique opportunity to look at neural process associated with the modulation of fear, such as cortical regulation of the amygdala. We are currently setting up to use pictures as cues in monkeys to develop a procedure in which we can carry out repeated AX+/BX- training and testing using different sets of pictures each time to allow within-subject comparisons of cortical inactivation or pharmacological treatments on both the acquisition and expression of inhibitory learning.

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