Neuroscience 169 (2010) 1689–1704

CONDITIONED TURNING BEHAVIOR: A PAVLOVIAN FEAR RESPONSE EXPRESSED DURING THE POST-ENCOUNTER PERIOD FOLLOWING AVERSIVE STIMULATION

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Abstract—Rats were trained to fear an auditory conditioned stimulus (CS) by pairing it with a mild electric shock (the unconditioned stimulus, or US) delivered to one eyelid. After training, the CS elicited two different conditioned fear responses from rats: a passive freezing response, and an active turning response. The balance between these two modes of conditioned responding depended upon the rat's recent history of encounters with the US. If rats had not recently encountered the US, then they responded to the CS by freezing. But after recently encountering the US, rats exhibited CS-evoked turning responses that were always directed away from the trained eyelid, even if the US had recently been delivered to the opposite (untrained) eyelid. This post-encounter turning behavior was not observed in rats that had been trained with unpaired presentations of the CS and US, indicating that even though CS-evoked turning was selectively expressed after recent encounters with the US, it was nonetheless a conditioned Pavlovian fear response that depended upon a learned association between the CS and US. Further supporting this conclusion, pharmacological inactivation experiments showed that expression of both freezing and turning behaviors depended upon lateralized circuits in the amygdala and periaqueductal gray (PAG) that are known to support expression of Pavlovian fear responses. These findings indicate that even though the ability of a CS to elicit Pavlovian fear responses depend upon the long-term history of CS–US pairings, the mode of conditioned responding (freezing versus turning in the present experiments) can be modulated by short-term factors, such as the recent history of US encounters. We discuss neural mechanisms that might mediate such short-term transitions between different modes of defensive responding, and consider how dysregulation of such mechanisms might contribute to clinical anxiety disorders.

Key words: amygdala, midbrain, periaqueductal gray, lateralization, predatory imminence.

Most animals (including humans) are endowed with an innate repertoire of defensive responses for coping with threats to their survival. Defensive responses change as threat levels increase, and can thus be organized along a spectrum referred to as the "predatory imminence continuum" (Blanchard and Blanchard, 1969a,b; Bolles, 1970; Fanselow and Lester, 1988; Mobbs et al., 2007, 2009). In rats, low levels of threat are characterized by engagement in non-defensive behaviors, such as exploration or goal-seeking. At intermediate threat levels (referred to as "circa-strike"), the rat begins to perceive danger and engage in behaviors such as freezing to avoid detection by potential predators, or emitting warning calls to notify conspecifics of a possible threat. The highest threat levels (referred to as "post-encounter") occur after the rat has suffered injury or come under attack by a predator, triggering responses such as distress calls, fleeing from danger, or fighting back against the predator if no escape is possible.

Much of what is currently known about neural circuits mediating defensive responses has been learned from rodent studies of Pavlovian fear conditioning, in which rats (or mice) are trained to fear a neutral conditioned stimulus (CS) by pairing it with an aversive unconditioned stimulus (US) (for review see Davis, 1992; LeDoux, 2000). In such studies, conditioned fear is typically assessed by presenting a CS to previously trained subjects that recently have not encountered the US, while they are in a baseline state of low predatory imminence (e.g. freely exploring their environment, or engaged in a task such as licking or bar-pressing). Under these testing conditions, the CS can elicit circa-strike defenses—such as freezing or startle potentiation—which are measured to index the level of conditioned fear. An underlying assumption of such experiments is that expression of the measured responses is monotonically related to the intensity of conditioned fear (i.e. more responding indicates more fear). However, this monotonicity assumption may not always be valid, because if fear intensity exceeds the threshold for triggering post-encounter defensive strategies, then decreases in the expression of circa-strike behaviors (like freezing or startle) may reflect greater—not lesser—fear of the CS (see Blanchard and Blanchard, 1969a,b; Bolles, 1970; Davis and Astrachan, 1978; Fanselow and Lester, 1988). Consequently, the range of conditioned fear intensities that can be accurately indexed by Pavlovian circa-strike behaviors is constrained to remain below the threshold for expression of post-encounter defenses. This is an unfortunate limitation, because rodent fear conditioning has been widely adopted as an animal model for investigating the neurobiological basis of clinical anxiety disorders (Davis and Whalen, 2001; Rau et al., 2005; Davis et al., 2006; Milad et al., 2006; Miller and McEwen, 2006; Rauch et al., 2006). But some anxiety symptoms in human patients—such as panic attacks—may involve activation of post-encounter response

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Abbreviations: CS, conditioned stimulus; dPAG, dorsal PAG; mPFC, medial prefrontal cortex; PAG, periaqueductal gray; UNP, unpaired; US, unconditioned stimulus; vPAG, ventral PAG.

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doi:10.1016/j.neuroscience.2010.06.046

systems (see Craske, 1999). Standard rodent models of fear conditioning may not recruit these post-encounter response systems, since they are based upon methods that favor the expression of circa-strike behaviors.

We have previously conducted fear conditioning experiments using a paradigm in which rats are given an auditory CS paired with a unilateral shock US delivered to one eyelid (Moita et al., 2003, 2004; Blair et al., 2005a,b; Tarpley et al., 2009; Johansen et al., 2010). During these experiments, we have observed that in addition to CS-evoked freezing behavior, well-trained rats also tend to exhibit another distinctive response to the CS: turning in circles away from the eyelid where shock is anticipated. Here, we conducted a formal investigation of this novel turning response. We report that, like freezing, CS-evoked turning behavior is a Pavlovian response which depends upon lateralized circuits in the amygdala and periaqueductal gray (PAG) that mediate acquisition and expression of conditioned fear (Fanselow, 1991; Bandler and Depaulis, 1991; Davis, 1992; Maren, 2005; LeDoux, 2000). But unlike freezing, the turning response is expressed selectively after recent encounters with the US, and not at other times. These results suggest that conditioned turning responses may be expressed selectively when the intensity of conditioned fear exceeds the threshold for triggering post-encounter defenses, which does not occur unless the US has recently been encountered. Based on these findings, we propose that conditioned turning responses may provide a useful behavioral index for investigating clinically relevant questions concerning neural substrates that mediate post-encounter defensive strategies.

EXPERIMENTAL PROCEDURES

All experimental procedures were approved by the UCLA Animal Research Committee and were conducted in accordance with USA federal guidelines for animal research.

Subjects and surgery

Male Long-Evans rats weighing 350–400 g were ordered from a commercial breeder (Charles River Laboratories, Hollister, CA, USA) and housed singly upon arrival. After at least five days of acclimatization, they were reduced to 85% of *ad libitum* weight through limited daily feeding. Under deep isoflourane anesthesia, all but two rats (see below) were implanted with a pair of very thin insulated stainless steel wires (75 μm diameter) threaded into the skin of each eyelid for delivering the mild periorbital shock US. Rats in the experimental groups were implanted with a pair of 26 gauge microinjector guide cannulae (Plastics One, Roanoake, VA, USA) targeted bilaterally in the lateral nucleus of the amygdala (3.0 mm posterior, 5.3 mm lateral and 8.0 mm ventral to bregma) or PAG (7.8 mm posterior, 0.75 mm lateral and 5.8 mm ventral to bregma). All implants were secured in place with bone cement and anchoring screws. At the conclusion of the surgery, rats were removed from the stereotaxic frame and observed until they fully emerged from anesthesia, then returned to their home cages and allowed to recover for at least 5 days prior to begin experiments. Two rats (one implanted in the amygdala, the other in PAG) were not implanted with periorbital stimulus wires, and were not removed from the stereotaxic frame at the end of the surgery, but instead were given intracranial infusions (0.4 μl a rate of 0.25 μl/min) of fluorescent muscimol (tagged with Bodipy® TMR-X fluorophore, Invitrogen product #M2400), dissolved at 0.25 mg/mL in sterile 0.9% saline vehicle (this was the same volume, concentration, and rate used for infusions of non-fluorescent muscimol in behavioral experiments, see below). 30 min after the infusion was completed, rats were removed from the stereotaxic frame, euthanized with an i.p. overdose of pentobarbital (100 mg/kg), and transcardially perfused with formalin so that brain tissue could be prepared for histological analysis of muscimol diffusion (results shown in Fig. 3C).

Fear conditioning experiments

After recovery from surgery, rats were pre-exposed for 5 days (15 min/day) to the experimental platform before any fear conditioning sessions were conducted. Throughout pre-exposure and fear conditioning sessions, rats constantly foraged on a 70×70 cm^2 platform for 20 mg purified food pellets (Bioserv, Frenchtown, NJ, USA) dropped from an overhead dispenser at ~30 s intervals, to provide a baseline of motor activity against which stimulus-evoked freezing, movement, and turning behavior could be measured. The CS for fear conditioning was a train of 70 dB white noise pips, each lasting 250 ms, delivered at 1 Hz for 20 s through an overhead speaker. The US was a train of very brief 2.0 mA shock pulses, each lasting 2.0 ms, delivered to the skin above one eyelid at a rate of 6.66 Hz for 2 s. During CS–US pairing trials, the first shock pulse was always delivered 300 ms after the offset of the final (20th) CS pip. The interval between CS onset of successive trials was uniformly random between 180 and 240 s for all testing and training trials. Rats implanted with amygdala cannulae were trained drug-free for 7 days prior to their first intracranial infusion, whereas rats implanted with PAG cannulae were trained drug-free for 4 days prior to their first infusion.

Rats in the unpaired control group were trained with explicitly unpaired presentations of the CS and US, by delivering the US exactly halfway between CS onset of successive trials (which were separated by a uniformly random interval of 180 and 240 s, as in paired training). In studies of Pavlovian conditioning, it is usually preferable to randomize the order of CS and US alone trials in the unpaired controls. But here, the conditioned response under study (CS-evoked turning) was strongly modulated by the recency of US delivery. This made it necessary to preserve the alternating order of CS and US presentations in both the paired and unpaired groups, because presenting several CS alone trials in a row to unpaired rats (which would sometimes occur with a randomized trial order) could diminish the CS-evoked turning response by increasing the separation between the CS and the most recent US, and thus reduce conditioned responding by mechanisms unrelated to associative learning. Explicitly unpaired presentations of the CS and US—as we have used here—can cause the CS to acquire properties of a conditioned inhibitor (Rescorla, 1969), and this potential confound is addressed in the Results section.

Behavioral scoring

The rat's moment-to-moment position on the platform was sampled at 30 Hz by an overhead video tracking system (Neuralynx Corporation, Bozeman, MT, USA), which monitored the location of three light-emitting diodes (red, blue, green) attached to the animal's headstage for automated scoring of freezing, movement, and turning behavior using software developed in our laboratory. The algorithm for scoring freezing behavior has been described elsewhere (Moita et al., 2003, 2004). The algorithm for scoring movement and turning behavior first performed one iteration of smoothing (5-point adjacent averaging) upon the position data for each of the three colored LED's. The center point of the three LEDs was obtained by averaging their x and y-coordinates, and the displacement distance of this center position between each successive video frame gave the rat's linear movement speed. The angles of the axes passing through each pair of tracking