temic administration or intra-amygdala infusions of D-cycloserine as assessed with fear-potentiated startle in rats. *Journal of Neuroscience, 22,* 2343–2351.

Neural Systems Involved in Fear and Anxiety Measured With Fear-Potentiated Startle

Michael Davis
Emory University

A good deal is now known about the neural circuitry involved in how conditioned fear can augment a simple reflex (fear-potentiated startle). This involves visual or auditory as well as shock pathways that project via the thalamus and perirhinal or insular cortex to the basolateral amygdala (BLA). The BLA projects to the central (CeA) and medial (MeA) nuclei of the amygdala, which project indirectly to a particular part of the acoustic startle pathway in the brainstem. N-*methyl-D-aspartate (NMDA) receptors, as well as various intracellular cascades in the amygdala, are critical for fear learning, which is then mediated by glutamate acting in the CeA. Less predictable stimuli, such as a long-duration bright light or a fearful context, activate the BLA, which projects to the bed nucleus of the stria terminalis (BNST), which projects to the startle pathway much as the CeA does. The anxiogenic peptide corticotropin-releasing hormone increases startle by acting directly in the BNST. CeA-mediated behaviors may represent stimulus-specific fear, whereas BNST-mediated behaviors are more akin to anxiety. NMDA receptors are also involved in extinction of conditioned fear, and both extinction in rats and exposure-based psychotherapy in humans are facilitated by an NMDA-partial agonist called D-cycloserine.*

Keywords: amygdala, anxiety, extinction, fear, startle

Editor's Note
Michael Davis received the Award for Distinguished Scientific Contributions. Award winners are invited to deliver an award address at the APA's annual convention. A version of this award address was delivered at the 114th annual meeting, held August 10–13, 2006, in New Orleans, Louisiana. Articles based on award addresses are reviewed, but they differ from unsolicited articles in that they are expressions of the winners' reflections on their work and their views of the field.

Over the last 25 years, a great deal of progress has been made in delineating the neural pathways and the cellular and molecular mechanisms involved in fear, anxiety, and extinction of fear. The two most widely used measures of conditioned fear are freezing and fear-potentiated startle (Fendt & Fanselow, 1999), and the systematic study of these behaviors by a host of investigators has rapidly led to a detailed understanding of the neural pathways and the cellular and molecular mechanisms of both the acquisition and the expression of conditioned fear. The purpose of this article is to describe the fear-potentiated startle test and how it has been used in our laboratory[1] to understand the anatomical and cellular basis of fear, anxiety, and extinction.

The Fear-Potentiated Startle Effect

Brown, Kalish, and Farber (1951) demonstrated that the amplitude of the acoustic startle reflex in the rat can be augmented by presenting the eliciting auditory startle stimulus in the presence of a cue (e.g., a light) that has previously been paired with a shock. This phenomenon has been termed the *fear-potentiated startle effect* and has been replicated using either an auditory or a visual conditioned stimulus (CS) when startle is elicited by either a loud sound or an air puff (Davis, 1986). In this paradigm, we typically use a 3.7-s light that coterminates with a 0.5-s 0.4-mA shock. This is called the *training session*. At some later time (i.e., hours to months), the rat is placed in a cage specially designed to measure the amplitude of the startle reflex elicited by a burst of noise at the time when the shock was presented in training (e.g., 3.2 s after onset of the light [light–noise test trial]) or in darkness (noise-alone trial). Conditioned fear is operationally defined by elevated startle amplitude in the presence versus the absence of the cue previously paired with a shock (fear-potentiated startle). Thus, the CS does not elicit startle. Furthermore, the startle-eliciting stimulus is never paired with a shock; instead, the CS is paired with a shock, and startle is elicited by another stimulus either in the presence or absence of the CS. Facilitation of a simple reflex is used to assay the hypothetical state of fear, which would be expected to facilitate reflexes. Fear-potentiated startle only occurs following paired versus unpaired or "random" presentations of the CS and the shock, which indicates that it is a valid measure of classical conditioning (Davis & Astrachan, 1978). Discriminations between visual and auditory conditioned stimuli (Davis, Hitchcock, & Rosen, 1987) or between auditory

[1] In many places in this review, I have decided to use the first person when describing work that has been done in my laboratory over the last 35 years. Of course the work was generally not done directly by me but by my very dedicated technicians, students, postdocs, and faculty colleagues. By writing in the first person, I hope to convey my thanks to all of them. When I say *we*, *our*, or *us*, I mean the people listed in the references relevant to that paragraph.

cues or visual cues that differ in duration (Davis, Schlesinger, & Sorenson, 1989; Siegel, 1967) have also been demonstrated with potentiated startle. Odors are especially good conditioned stimuli for fear-potentiated startle: Reliable conditioning can be found after only a single pairing of an odor with footshock (Paschall & Davis, 2002). Increased startle in the presence of the CS still occurs very reliably at least one month after original training, making it appropriate for the study of long-term memory as well (Campeau, Liang, & Davis, 1990). Fear-potentiated startle can also be measured in mice (Falls, Carlson, Turner, & Willott, 1997) and rhesus monkeys (Winslow, Parr, & Davis, 2002).

Fear-Potentiated Startle in Humans

Fear-potentiated startle can be seen in humans using several different ways of eliciting fear. In humans, the eyeblink component of startle is the most easily measured and the most reliable, because although it habituates with repeated presentation of startle stimuli, it typically reaches a nonzero asymptote, so both excitatory and inhibitory effects can be measured. One way to potentiate startle in humans is via conditioning, using procedures that closely parallel those in rats. For example, Christian Grillon and I (Grillon & Davis, 1997) presented undergraduates with a light consistently paired with a shock (paired group), a light explicitly unpaired with a shock (unpaired group), or a light that served as a signal to push a button as soon as a second light came on (reaction time group). Potentiated startle was measured in the same session and then again one week later. Fear-potentiated startle was seen only in the paired group in both sessions. This indicates that fear-potentiated startle was not simply a function of heightened arousal following shock presentation or instructions to perform in a reaction time experiment, consistent with earlier work (Hamm, Start, & Vaitl, 1990). It is interesting to note that these earlier studies showed that arousal associated either with shock or with a reaction time experiment increased the galvanic skin response, a measure of activation of the sympathetic nervous system. Thus, changes in startle reflected a change in valence (threat vs. safe) and arousal, whereas the galvanic skin response did not differentiate between valence and arousal. Finally, in Session 2, there was a pronounced increase in startle amplitude at the beginning of the session in the unpaired group but not in the paired group, indicative of context conditioning, a result predicted by contemporary learning theory (Rescorla & Wagner, 1972).

Another way to potentiate startle is simply to tell people that when a certain colored light comes on, they might get a shock (Grillon, Ameli, Woods, Merikangas, & Davis, 1991). Thus, even though they have never actually received a shock, just the anticipation of this possibility, which is rated to be very fearful, is enough to increase startle magnitude in humans. Finally, startle elicited in the presence of pictures of scary scenes, such as a snake or dog ready to attack, is potentiated compared with when it is elicited in the presence of neutral pictures (e.g., of baskets or cans; Lang, Bradley, & Cuthbert, 1990). In contrast, startle is actually inhibited when it is elicited in the presence of pleasant pictures, such a babies or sexy scenes, whereas the galvanic skin response is increased in the presence of both scary and pleasant scenes. Once again, therefore, startle is sensitive to valence but not simply arousal (Lang et al., 1990).

Neural Pathways Involved in Fear-Potentiated Startle

One of the major advantages of the fear-potentiated startle test is that the hypothetical state of fear is inferred from an increase in a simple reflex. Moreover, because the acoustic startle reflex has such a short latency (e.g., 8 ms measured electromyographically in the hind leg, 5 ms in the neck; Cassella, Harty, & Davis, 1986), it must be mediated by a simple neural pathway. We now believe that the primary acoustic startle reflex pathway involves three central synapses: (a) auditory nerves fibers to cochlear root neurons, (b) cochlear root neuron axons to cells in the nucleus reticularis pontis caudalis, and (c) pontis caudalis axons to motor neurons in the facial motor nucleus (pinna reflex) or spinal cord (whole body startle; see Figure 1).

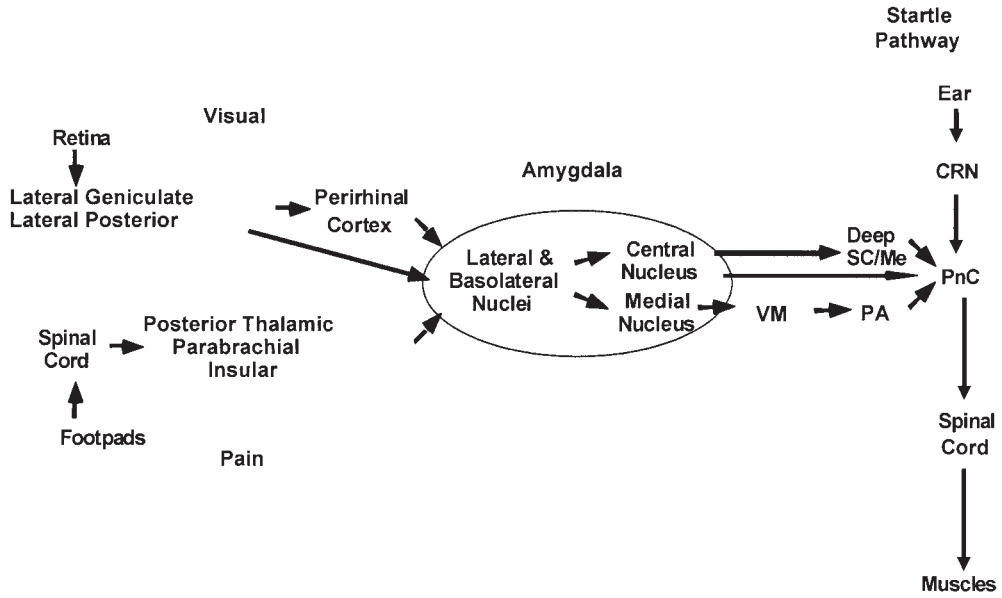
Cochlear Root Neurons

In rats, there are a small group (about 20 on each side) of very large cells (35 μm in diameter) embedded in the cochlear nerve in rodents, called *cochlear root neurons* (CRN). These neurons receive direct input from the spiral ganglion cells in the cochlea, making them the first acoustic neurons in the central nervous system (Lopez, Merchan, Bajo, & Saldana, 1993). They send exceedingly thick axons (sometimes as wide as 7 μm) through the trapezoid body, at the very base of the brain, to the contralateral side, to an area just medial and ventral to the lateral lemniscus, and they continue on up to the deep layers of the superior colliculus. However, they give off thick axon collaterals that terminate directly in the pontis caudalis (PnC; Lopez et al., 1993), exactly at the level known to be critical for the acoustic startle reflex (cf. Lee, Lopez, Meloni, & Davis, 1996). Bilateral, chemical lesions of the CRN essentially eliminate acoustic startle in rats (Lee et al., 1996). Although damage to the auditory root, where the CRN reside, has not been fully ruled out, other tests have indicated that these animals could clearly orient to auditory stimuli (e.g., suppression of licking) and had normal compound action potentials recorded from the cochlear nucleus (Lee et al., 1996).

Nucleus Reticularis PnC

Very discrete *N*-methyl-D-aspartate (NMDA)-induced lesions of cell bodies in the PnC have completely eliminated

Figure 1
Schematic Diagram of Parallel Visual Pathways and Parallel Shock Pathways to the Amygdala, Projections From the Basolateral Amygdala to the Central and Medial Nuclei of the Amygdala, and Parallel Outputs From the Central and Medial Nuclei to the Startle Pathway



Note. VM = ventromedial hypothalamus; Deep SC/Me = deep white layers of the superior colliculus/deep mesencephalic reticular nucleus; PA = periaqueductal gray; CRN = cochlear root neurons; PnC = pontis caudalis.

startle. Local infusion of the NMDA antagonist DL-2-amino-5-phosphonopentanoic acid (AP5) into the PnC reduced startle by 80%–90% (Miserendino & Davis, 1993). Moreover, comparably low doses of the non-NMDA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) also depressed startle after local infusion into the PnC (Miserendino & Davis, 1993). Single-pulse electrical stimulation of the PnC elicited startle responses with a latency of about 5 ms recorded in the hindleg, compared with about 8 ms when elicited acoustically (Davis, Gendelman, Tischler, & Gendelman, 1982).

Facial and Spinal Motor Neurons

In rats, the pinna component of the startle reflex consists of a rapid backward movement of the pinna that covers and protects the ear, and the pinna reflex shows many of the features of whole-body startle (Cassella & Davis, 1986). The motor neurons that innervate the relevant pinna muscles are located in the dorsolateral division of the facial motor nucleus, to which the PnC has direct projections. Startle stimuli elicit action potentials in facial motor nucleus neurons with a latency of 5 ms (Cassella & Davis, 1987) prior to movement of the pinna muscles, and local infusion of the alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/kainate antagonist CNQX into the facial motor nucleus eliminated the click-elicited pinna reflex on the ipsilateral but not the contralateral side (Meloni & Davis, 1990).

Motor neurons in the lumbar spinal cord innervate muscles in the hind leg that provide the major extension–flexion component of startle in rodents (Davis, 1984). When startle is measured by electromyography (EMG) in the hind leg, two distinct components can be measured: a short-latency component (~8 ms) and a slightly longer latency component (~15 ms). Infusion in the space between the spinal cord and the membranes that surround the spinal cord (intrathecal infusion) of the AMPA/kainate antagonist CNQX in the vicinity of the lumbar motor neurons eliminated the short-latency component but not the longer latency component, whereas infusion of the NMDA antagonist AP5 had just the opposite effect (Boulis, Kehne, Miserendino, & Davis, 1990). Infusion of both compounds together totally eliminated the EMG component of startle in the hind leg. This suggests that the acoustic startle reflex involves motor neurons in the spinal cord that are activated by release of glutamate acting on both non-NMDA and NMDA receptors. Intrathecal administration of cyclic AMP (cAMP) or cAMP analogues markedly facilitate acoustic startle amplitude (Boulis & Davis, 1990; Kehne, Astrachan, Astrachan, Tallman, & Davis, 1986), probably by increasing the amount of glutamate released from the terminals of neurons in the PnC activated by the startle stimulus. Intra-

thecal administration of the glycine receptor antagonist strychnine markedly increases startle amplitude (Kehne, Gallager, & Davis, 1981), as do norepinephrine (Davis, Astrachan, Kehne, Commissaris, & Gallager, 1984) and serotonin agonists (Davis, Astrachan, Gendelman, & Gendelman, 1980; Davis et al., 1984), which are known to facilitate the response of motor neurons to glutamate.

Fear-Potentiated Startle Measured Electromyographically

Having delineated what we believe is the primary acoustic startle pathway, we hoped to use this information to deduce where fear ultimately alters neural transmission so as to increase acoustic startle amplitude. Because startle can be measured with a latency of only 8 ms, the light should potentiate this 8-ms response. Typically, however, startle is not measured electromyographically, but instead, it is measured as a movement of a cage over a relatively long interval after onset of the startle-eliciting stimulus (e.g., 200 ms). Hence, it is possible that the visual CS does not actually alter the very short-latency startle response but, instead, might facilitate transmission in other auditory systems, which could produce cage movements at longer latencies. If so, this might mean that the visual CS would not actually alter transmission along the short-latency pathway outlined in Figure 1. However, if the light did increase the very short short-latency startle reflex, we would have to conclude that it alters transmission at some point in the short-latency pathway. In fact, we found that a light previously paired with a footshock markedly potentiated the short-latency startle response (5 ms) measured electromyographically in the neck muscles (Cassella et al., 1986), indicating that the visual CS must ultimately alter neural transmission somewhere along the short-latency pathway outlined in Figure 1. Moreover, potentiated startle never resulted in additional longer latency EMG activity, indicating that longer latency startle pathways were not recruited during a state of fear.

The Point in the Startle Pathway Where Fear Modulates Transmission

Having demonstrated that fear facilitates transmission in this very short-latency pathway, our next task was to try to deduce where a light previously paired with a shock ultimately modulates transmission in this short-latency pathway. We had previously shown that startle could be elicited with single electrical pulses at various points along the startle pathway, with progressively shorter latencies as the electrode was moved from the cochlear root axons to the reticulospinal axons connecting the PnC with spinal motor neurons (Davis, Gendelman, et al., 1982), and we had used this method to deduce where habituation and sensitization occurred within the startle pathway (Davis, Parisi, Gendelman, Tischler, & Kehne, 1982). This approach assumes that electrically elicited startle will be potentiated in the presence of a light previously paired with shock if the modulation occurs at neural sites prior to the point in the pathway where startle is elicited but that it will not be potentiated by electrical stimulus in parts of the startle pathway downstream of the point of modulation. Using this strategy, Keith Berg and I (Berg & Davis, 1985) found that startle elicited electrically from CRN axons adjacent to the ventral cochlear nucleus, or farther along the base of the brain en route to the PnC, was facilitated by the light, whereas startle elicited in the PnC or the reticulospinal tract was not, even though acoustically elicited startle was increased in all cases. Systemic administration of diazepam (Valium), which reduces fear and anxiety in people, selectively decreased fear-potentiated startle elicited electrically from points afferent to the PnC, indicating that elicitation of startle in this way could pick up the anxiolytic effect of diazepam (Berg & Davis, 1984). These data suggested that the light ultimately alters transmission in the PnC.

Projections to the PnC

Having determined that the PnC was the probable site where fear ultimately altered transmission to increase startle amplitude, our next question was this: What parts of the brain project to the part of the PnC critical for startle, and are these projections critical for fear potentiated startle? Over the course of several years, we have come to believe there are three parallel pathways, each of which may play a part in fear-potentiated startle (see Figure 1).

Direct Projections From the CeA

Local infusion of the retrograde tracer Fluoro-Gold (hydroxystilbamidine; Fluorochrome, Denver, CO) into the part of the PnC critical for startle resulted in labeling of neurons in the medial division of the CeA (Rosen, Hitchcock, Sananes, Miserendino, & Davis, 1991). This was an exciting finding, because earlier work in several laboratories had implicated the CeA in conditioned fear using several different measures. Local infusion into the CeA of an anterograde tracer confirmed this connection and was used to delineate the course of the pathway from the CeA to the PnC. Electrolytic lesions at various points along this pathway blocked fear-potentiated startle but had no effect on baseline startle amplitude (Hitchcock & Davis, 1991). In contrast, electrolytic lesions of outputs of the CeA to the bed nucleus of the stria terminalis (BNST) had no effect on fear-potentiated startle, consistent with earlier work (LeDoux, Iwata, Cicchetti, & Reis, 1988).

Indirect Projections From the CeA via the Deep Mesencephalic Reticular Formation

Although these results with electrolytic lesions were consistent with the idea that this direct projection mediates fear-potentiated startle, it was still possible that synaptic,

rather than direct, projections might be involved. In fact, injection of a retrograde tracer into the PnC showed that several nuclei along this direct pathway contained neurons that also projected directly to the PnC. One of the most prominent of these was in the mesencephalic reticular formation and deep layers of the superior colliculus (deep SC/DpMe). The amygdala sends heavy, broad projections to this part of the rostral midbrain (Rosen et al., 1991), which, in turn, projects to the PnC (Meloni & Davis, 1999), and the rostral midbrain had been proposed to be a relay between the amygdala and the PnC in fear-potentiated startle (Yeomans & Pollard, 1993).

Consistent with this hypothesis, inactivation of the deep SC/DpMe with muscimol (Meloni & Davis, 1999) or the AMPA/kainate glutamatergic receptor antagonist 2,3-dihydroxy-6-nitro-7-sulphamoylbenzo(F)-quinoxaline (NBQX; Zhao & Davis, 2004) blocked the expression but not the acquisition of fear-potentiated startle if infused into the deep SC/DpMe. In contrast, infusion of the same doses of NBQX—either 1 mm lateral into the lateral mesencephalic reticular formation or 1 mm medial into the dorsal–lateral periaqueductal gray—had no effect. None of the infusions altered the baseline startle response.

Indirect Projections From the MeA via the Ventromedial Hypothalamus (VMH) and the Ventral Periaqueductal Gray (PAG)

The MeA and its outputs to the VMH and the PAG have been implicated in defensive behavior in cats (Adamec, 1994), and we found that blockade of AMPA/kainate glutamate receptors in the MeA blocked conditioned fear elicited not only by an odor but also by a light previously paired with footshock (Walker, Paschall, & Davis, 2005).

In a series of studies, we have found that local infusion into the MeA of either morphine (Davis, Yang, Shi, & Zhao, 2004) or Substance P, antagonists (Zhao, Yang, & Davis, 2004) blocked the expression of fear-potentiated startle, without any effect on baseline startle amplitude. The MeA sends heavy projections to the VMH, and local infusion of either NBQX or morphine or Substance P antagonists into this region also totally blocked fear-potentiated startle, without any effect on baseline startle amplitude. Although the VMH does not project directly to the PnC, it does project to the PAG, which in turn projects to the PnC. Recall, however, that local infusion of NBQX into the PAG did not block the expression of fear-potentiated startle. Nonetheless, local infusion of either morphine or Substance P antagonists into the PAG did block fear-potentiated startle. This suggests that fear-potentiated startle may be mediated or importantly modulated by release of Substance P in the MeA, the VMH, and the PAG. The effect of morphine at each of these areas might be attributable to its ability to decrease the release of Substance P by acting on terminal mu opioid autoreceptors.

Thus, there appear to be three parallel routes whereby the amygdala can modulate startle during a state of conditioned fear: (a) a direct pathway from the CeA to the PnC; (b) an indirect pathway from the CeA to the deep SC/DpMe to the PnC, where glutamate acting on AMPA/kainate receptors seems to be critical, and (c) an indirect pathway from the MeA to the VMH to the PAG to the PnC, where Substance P receptors seem to be critical (see Figure 1).

The Role of the Amygdala in Fear

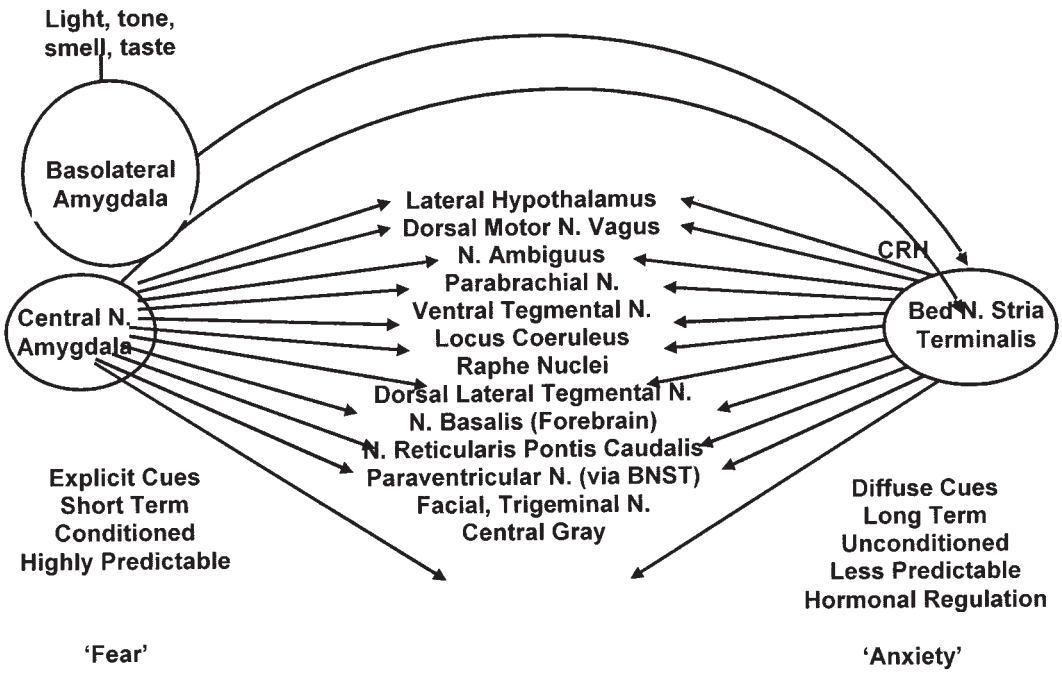
I have just provided a detailed description for how fear modulates a simple reflex in terms of the neural circuitry involved in the reflex and the way in which the amygdala connects to the reflex pathway. However, this is just one example of many showing that outputs of the CeA to the hypothalamus and brainstem are involved in many of the specific signs of fear, as illustrated in Figure 2. However, this is only the "output" side of the story. One still needs to explain how sensory stimuli, including footshocks, activate the amygdala and how pairing sensory stimuli with footshock can produce a "memory" that can last for a very long time. The amygdala receives input from numerous areas of the brain, many of which are critical for fear conditioning.

Shock Inputs

During fear conditioning, foot shock information is transmitted to the amygdala via parallel pathways that include the posterior intralaminar nuclei in the thalamus and the parietal insular cortex. Besides receiving acoustic inputs from the inferior colliculus, the posterior intralaminar nuclei also receive somatic pain inputs from the spinal cord and, in turn, project to the amygdala, particularly the lateral amygdaloid nucleus (cf. Shi & Davis, 1999). However, pretraining lesions of the posterior intralaminar nuclei alone did not prevent the acquisition of fear conditioning (Campeau & Davis, 1995b; Romanski & LeDoux, 1992), indicating that an additional pathway or pathways must contribute footshock information to the amygdala.

The caudal part of insular cortex, the so-called *parietal insula*, receives convergent inputs from somatosensory cortices, ventroposterior and posterior thalamic nuclei, posterior intralaminar nuclei, and the midbrain parabrachial nucleus (cf. Shi & Davis, 1999). Further, this portion of the insular cortex is probably a primary source in providing cortical somatosensory information to the amygdala. Both the parietal insular cortex and the posterior intralaminar nuclei of thalamus in turn project to the lateral, basolateral, basomedial, and central nuclei of the amygdala. Combined lesions of both parietal insular cortex and posterior intralaminar nuclei of the thalamus were necessary to interrupt transmission of footshock information to the amygdala and, thus, block the acquisition of fear-potentiated startle

Figure 2
Schematic Diagram of the Outputs of the Central Nucleus or the Lateral Division of the Bed Nucleus of the Stria Terminalis (BNST) to Various Target Structures and Possible Functions of These Connections



Note. N. = nucleus; CRH = corticotropin-releasing hormone.

(Shi & Davis, 1999). It is important to note, however, that these lesions did not block the expression of fear-potentiated startle once conditioning had taken place, as one would expect if these pathways were involved in fear acquisition. These combined lesions also reduced the degree to which rats reacted to footshock, as do lesions of the amygdala (Hitchcock, Sananes, & Davis, 1989). This may explain why chemical lesions in some of these thalamic nuclei failed to block fear conditioning, because these lesions also failed to alter shock reactivity (Brunzell & Kim, 2001), and we found that when chemically induced lesions of the posterior intralaminar nuclei reduced shock reactivity, they also blocked acquisition of fear-potentiated startle (Shi & Davis, 2000).

Auditory Inputs

A great deal of work has been done using auditory cues to study the role of the amygdala in fear conditioning, as exemplified by the elegant work in Joseph LeDoux's laboratory. Auditory inputs from modality-specific areas of thalamus and cortex exclusively or primarily target the dorsolateral and ventrolateral divisions of the lateral amygdaloid nucleus (cf. Romanski, Clugnet, Bordi, & LeDoux, 1993) and both electrolytic and excitotoxic posttraining lesions of the lateral nucleus of the amygdala, sparing a large number of other neurons in the basolateral amygdala, disrupted fear-potentiated startle to both auditory and visual conditioned stimuli (Campeau & Davis, 1995a), consistent with earlier work using auditory cues, pretraining lesions, and freezing as the measure of fear (LeDoux, Cicchetti, Xagoraris, & Romanski, 1990). Complete electrolytic or excitotoxic lesions of the entire auditory thalamus specifically disrupted fear-potentiated startle to an auditory but not a visual CS, whether the lesions were made before or after conditioning (Campeau & Davis, 1995b), consistent with earlier work using pretraining lesions and freezing (LeDoux, Sakaguchi, Iwata, & Reis, 1986; LeDoux, Sakaguchi, & Reis, 1984).

Although it has been argued that the direct projection from the thalamus to the amygdala is critical for conditioned fear to an auditory stimulus (Romanski & LeDoux, 1992), this conclusion is based on results of experiments in which lesions of a given pathway are made prior to fear conditioning. When lesions are made after fear conditioning, it is found that the subcortical pathway probably is not normally used but, instead, can take over if the thalamocortical pathway is disrupted. The cortical pathway I am referring to is not the primary auditory cortex but, rather, a secondary multisensory cortex called the *perirhinal cortex*. These conclusions are based on the following observations.

Lesions of the ventral and dorsal divisions of the medial geniculate body, giving rise to the main thalamocortico–amygdala pathway, significantly disrupted fear-potentiated startle to an auditory but not a visual CS (Campeau & Davis, 1995b). In contrast, animals with posterior thalamic lesions, which project directly to the lateral nucleus of the amygdala, actually had higher levels of fear-potentiated startle, especially to an auditory CS. However, the subcortical pathway can be recruited to mediate fear-potentiated startle to an auditory CS when animals sustaining ventral and dorsal medial geniculate body lesions were retrained (Campeau & Davis, 1995b). This could explain why lesions of the cortical pathway made prior to fear conditioning, such as those done in the LeDoux lab, would not disrupt conditioned fear, because under these circumstances the subcortical pathway would have "taken over."

Neither pre- nor posttraining auditory cortex ablations, mostly restricted to the primary auditory area, had reliable effects on fear-potentiated startle (Campeau & Davis, 1995b). In contrast, posttraining lesions to the secondary auditory and perirhinal cortices completely blocked fear-potentiated startle to both auditory and visual conditioned stimuli, but importantly, pretraining lesions did not reliably affect fear-potentiated startle to either CS. The posttraining deficits were observed only after the lesions included most of the rostral–caudal extent of the perirhinal area, which also receives visual input. These results are consistent with the findings of LeDoux's lab—showing that pretraining perirhinal-area lesions do not reliably disrupt conditioned fear response to an auditory stimulus—as well as those of our lab (Rosen et al., 1992) showing that posttraining lesions of perirhinal cortex disrupt fear-potentiated startle to a visual stimulus. Thus, by using posttraining lesions, we concluded that the pathway going from the auditory thalamus to the perirhinal cortex to the lateral nucleus of the amygdala is normally used in fear-potentiated startle when an auditory CS is used. The difference between this conclusion and earlier ones reflects the use of pre- versus posttraining lesions and was confirmed by later work in LeDoux's lab using posttraining lesions of the perirhinal cortex (Corodimas & LeDoux, 1995). Finally, although the neocortex does not seem to be involved in these situations using single-cue conditioning, it probably is necessary when more complex discriminations are involved.

Visual Inputs

As with the auditory system, the use of posttraining lesions has led us to conclude that subcortical projections from the visual thalamus (e.g., the lateral posterior nucleus) to the amygdala are not normally used in fear-potentiated startle using a visual CS. Instead, we believe that projections from the lateral posterior nucleus of the thalamus to the perirhinal cortex and then into the amygdala are the ones normally used. Lesions or chemical inactivation of superficial layers of superior colliculus, which receive massive retinal input, do not disrupt the expression of fear-potentiated startle to a visual CS (Meloni & Davis, 1999; Tischler & Davis, 1983). The lateral posterior nucleus of the thalamus also receives direct projections from the retina and sends heavy projections to area TE2 and the dorsal perirhinal cortex and moderate projections to the lateral amygdaloid nucleus (Shi & Davis, 2001). However, posttraining lesions restricted to the lateral posterior nucleus did not block the expression of fear conditioning using a visual CS. Posttraining lesions of neither the dorsal lateral geniculate nucleus (Shi & Davis, 2001), which receives retinal inputs, nor the visual cortex (including V1 and V2) prevented the expression of conditioned fear responses using a visual CS (Falls & Davis, 1994; LeDoux, Romanski, & Xagoraris, 1989; Rosen et al., 1992; Tischler & Davis, 1983).

However, both TE2 and the perirhinal cortex receive visual inputs from lateral posterior nucleus (Shi & Davis, 2001) and visual cortices and, in turn, project to the amygdala, and combined lesions of both dorsal lateral geniculate nucleus and lateral posterior nucleus, which would cut off both thalamic and cortical routes to Te2 and the perirhinal cortex, totally blocked the expression of conditioned fear using a visual CS. Local infusion of the glutamate antagonist NBQX had the same effect, suggesting that the lesion effect did not result from damage to fibers of passage. Thus, we concluded that visual input carried by projections from dorsal lateral geniculate nucleus and lateral posterior nucleus via connections through Te2 and the perirhinal cortex to the amygdala are normally used in conditioned fear using a visual CS.

Olfactory Inputs

Olfactory receptors in the nose send axons to the olfactory bulb, which then projects directly to the corticomedial nucleus of the amygdala as well as to the piriform cortex, which projects to the BLA (lateral, basolateral, and basal nuclei) as well as the perirhinal cortex. Inactivation of the MeA with NBQX blocks the expression of fear-potentiated startle to olfactory cues, but infusion of NMDA antagonists fails to block the acquisition of fear-potentiated startle, suggesting that the MeA is more on the output than on the input side in terms of conditioned fear using olfactory cues. However, NMDA antagonists infused into the BLA block the acquisition of conditioned fear using olfactory conditioned stimuli (Walker et al., 2005), and chemical lesions of the perirhinal cortex block fear conditioning using olfactory cues measured with freezing (Herzog & Otto, 1997). Once again, the more indirect pathway from the olfactory bulb to the piriform cortex to perirhinal cortex to the BLA is probably the route necessary for conditioned fear using olfactory cues.

The Role of Glutamate Receptors in the Amygdala in Fear-Potentiated Startle

Local infusion into the BLA of the NMDA antagonist AP5 blocked the acquisition but not the expression of fear-potentiated startle using visual (Miserendino, Sananes, Melia, & Davis, 1990), auditory (Campeau, Miserendino, & Davis, 1992) or olfactory (Walker et al., 2005) cues as conditioned stimuli. It is important to note that the same doses did not disrupt the ability of the CS to potentiate startle when infused prior to testing. Because the amygdala is essential for the expression of fear-potentiated startle (Campeau & Davis, 1995a; Hitchcock & Davis, 1987; Kim, Campeau, Falls, & Davis, 1993; Sananes & Davis, 1992; Walker & Davis, 1997b), these findings indicate that the effects of NMDA receptor blockade on fear learning cannot be attributed to a general disruption of amygdala activity or to a more specific disruption of the ability of rats to process the CS. More recent findings showing a disruption of fear expression by intra-amygdala AP5 (cf. Lee, Choi, Brown, & Kim, 2001) probably result from actions on a particular subtype of the NMDA receptor, the NR2A subtype, because infusion of ifenprodil, another NMDA antagonist that acts at the NR2B but not the NR2A subtype, blocked acquisition of conditioned freezing without having any effect on its expression (Rodrigues, Schafe, & LeDoux, 2001).

It could still be argued that AP5-induced learning impairments are attributable to a disruption of processing the footshock. However, Gewirtz and Davis (1997) reported that intra-amygdala AP5 infusions blocked second-order fear conditioning—a procedure in which a previously trained CS substitutes for shock as the aversive reinforcing stimulus. Furthermore, in the same rats, AP5 did not disrupt expression of fear-potentiated startle to the first-order auditory CS, arguing strongly that AP5 disrupted the association between light and noise rather than preventing amygdala activation by the noise stimulus that was used as the reinforcement in second-order conditioning.

Involvement of AMPA Receptors in the BLA and CeA in Fear Learning

Pretraining infusions of NBQX into either the BLA or the CeA significantly disrupted fear learning, suggesting that both areas play a role in conditioning (Walker & Davis, 2000). It is unlikely that the effects in the CeA were due to diffusion to the BLA, because in an earlier study using the same dose, infusion volume, infusion rate, and stereotaxic coordinates, we were able to demonstrate differential effects of infusions into the BLA versus the CeA on light-enhanced startle (Walker & Davis, 1997b)—an anxiety paradigm in which sustained exposure to bright light elevates startle amplitude (see below; Walker & Davis, 1997a). Our results, and those of others (cf. Samson & Pare, 2005), are consistent with the idea that both areas participate in fear learning and with recent evidence that long-term potentiation can occur in the CeA (Samson & Pare, 2005).

Intracellular Events Involved in Fear-Potentiated Startle

Broad-Based Survey of Gene Changes in Amygdala Following Fear Conditioning

We examined 21 genes known to be involved in neural plasticity on the basis of their induction with kainic-acid-induced seizures (Ressler, Paschall, Zhao, & Davis, 2002). We found that a substantial number of these genes were transcriptionally regulated in the amygdala, as well as in several other brain areas, from about 30 min to 6 hr after fear conditioning, depending on the gene. These messenger RNA (mRNA) changes occurred only when the conditioned and unconditioned stimuli were paired, not when they were unpaired or the unconditioned stimulus was presented alone. These results suggest that fear-memory consolidation occurs within a broad neural circuit that includes, but is not limited to, the amygdala. It is associated with early and late changes in gene expression of a variety of transcription factors, cytoskeletal proteins, adhesion molecules, and receptor stabilization molecules that together may contribute to the neural plasticity underlying long-term memory in mammals.

Gephyrin

Although several genes were up-regulated in the amygdala following fear conditioning, mRNA that codes for a protein called *gephyrin*, involved in the clustering of gamma-aminobutyric acid (GABA) and glycine receptors, was down-regulated (Ressler et al., 2002). There also was a decrease in gephyrin protein as well as in the surface expression of $GABA_A$ receptors in the BLA after fear conditioning (Chhatwal, Myers, Ressler, & Davis, 2005). Because a decrease in gephyrin would be expected to decrease GABA transmission, this suggests that fear conditioning leads to a period of increased excitability in the amygdala for several hours. This is interesting, because it is difficult to establish long-term potentiation in amygdala brain slices unless GABA antagonists are added. Although this seems unphysiological, these results with gephyrin suggest that fear conditioning down-regulates $GABA_A$ in the amygdala, perhaps to allow long-term potentiation to take place, which may be important for consolidation of long-term memory.

BDNF

The growth factor BDNF has been implicated in learning and memory in hippocampally dependent tasks (cf. Rattiner, Davis, & Ressler, 2005), and we found that BDNF mRNA was elevated in the BLA 2 hr following fear conditioning (Rattiner, Davis, French, & Ressler, 2004). Dominant-negative inhibition of TrkB (the receptor that binds

BDNF) within the amygdala impaired fear-potentiated startle without disrupting baseline amygdala function. These results strongly suggest a requirement for TrkB signaling in the acquisition and consolidation of fear memory.

cAMP Response-Element Binding (CREB) Protein

Because cAMP CREB protein has been implicated repeatedly in learning and memory, we used viral vector gene transfer to up-regulate CREB to see if it would facilitate fear conditioning using suboptimal parameters (massed, as opposed to spaced, training trials). We found a dramatic, pairing-specific increase in the magnitude of fear-potentiated startle associated with increased CREB protein during training, but not testing, in the BLA following local infusion of herpes simplex virus CREB in the amygdala (Josselyn et al., 2001). We have now replicated this using a totally different paradigm—namely, social defeat in hamsters (Jasnow, Shi, Israel, Davis, & Huhman, 2005)—which is an ethologically relevant, amygdala-dependent form of long-term fear conditioning in this species.

The Role of the BNST in Anxiety

The BLA projects to a variety of brain areas that are involved in fear and anxiety. Two structures are of particular interest—the CeA and the BNST. As shown above, the CeA is critical for the expression and probably the acquisition of conditioned fear. Recall, however, that lesions of the BNST did not block fear-potentiated startle or conditioned freezing. However, the lateral BNST and CeA are anatomically, neurochemically, cytoarchitectonically, and embryologically related (cf. Alheid, deOlmos, & Beltramino, 1995), and the BNST has the same downstream projections as the CeA (see Figure 2). Hence, we wondered how it might be involved in fear and anxiety.

Corticotropin-Releasing Hormone (CRH)-Enhanced Startle

Infusions of CRH into the lateral cerebral ventricle markedly increase the amplitude of the acoustic startle response in rats, and this effect was blocked by the anxiolytic chlordiazepoxide (Swerdlow, Geyer, Vale, & Koob, 1986). CRH-enhanced startle did not occur with intrathecal infusion and was not disrupted by lesions of the paraventricular nucleus of the hypothalamus (Liang, Melia, Campeau, et al., 1992), indicating mediation by CRH receptors in the brain that did not involve activation of the hypothalamic–pituitary–adrenal axis. Lee and Davis (1997a, 1997b) found that excitotoxic lesions of the BNST—but not the septum, hippocampus, BLA, or CeA—completely blocked CRH-enhanced startle, as did intra-BNST infusions of the CRH antagonist, α-helical CRH (α-hCRH). Infusions of CRH directly into the BNST increased startle amplitude at doses much lower than those that were required with intracerebroventricular administration (80 vs. 1,000 ng). Neither BNST lesions nor intra-BNST α-hCRH infusions disrupted fear-potentiated startle. Moreover, local infusion of CRH into the CeA failed to increase startle amplitude (Liang, Melia, Campeau, et al., 1992), and infusion of α-hCRH there failed to block CRH-enhanced startle.

Light-Enhanced Startle

Walker and Davis (1997a) described a new animal model of anxiety, termed *light-enhanced startle*, in which startle amplitude is increased when rats are exposed to bright light for 20 min. Like CRH-enhanced startle, light-enhanced startle was dependent on the BNST and not on the CeA (Walker & Davis, 1997b). Thus, local infusion of the AMPA receptor antagonist NBQX into the BNST, but not the CeA, blocked light-enhanced startle. The amygdala was still involved, however, because local infusion of NBQX into the BLA did block light-enhanced startle, probably because visual information is transmitted to the BNST through the BLA. Thus, as with the CRH experiments described above, these experiments demonstrated a double dissociation between the roles of the BNST and the CeA in startle increases produced by fear-inducing or anxiogenic stimuli.

Long-Term Sensitization of the Acoustic Startle Response by Repeated Footshock

To examine whether the inability of BNST lesions to block fear-potentiated startle was related to the strength of conditioning, we used a procedure in which acquisition of fear-potentiated startle can be measured by giving a few training and test trials each day (Kim & Davis, 1993; Gewirtz, McNish, & Davis, 1998). Even at early time points, when fear-potentiated startle was relatively weak, sham- and BNST-lesioned rats showed comparable levels of fear-potentiated startle. Unexpectedly, BNST lesions did influence one aspect of performance. In shocked but not in nonshocked control rats, baseline startle amplitude (i.e., startle amplitude to the 10 noise bursts delivered at the beginning of each test session) grew steadily over the course of training. The increase did not appear to reflect contextual fear conditioning but seemed, instead, to reflect a long-term sensitization to startle stimuli produced by repeated footshock administration, and it was absent in BNST-lesioned rats.

What Does the BNST Do? A Provisional Hypothesis Based on Results From Fear Conditioning and Acoustic Startle Studies

Fear-potentiated startle to a specific cue is a highly predictable situation that involves prior conditioning and uses a rather short cue that reliably predicts an aversive event. Fear-potentiated startle develops very rapidly once the light comes on and dissipates very quickly once the light goes off (Davis et al., 1989). In contrast, light-enhanced startle is a situation in which the animal is exposed to a potentially dangerous situation that is less predictable, does not

depend on any obvious conditioning, and involves a long period of anticipation that something bad might happen. To try to explain why manipulations of the CeA affect fear-potentiated startle and not light-enhanced startle, and why manipulations of the BNST affect light-enhanced startle and not fear-potentiated startle, we suggested two alternatives. One hypothesis was that the CeA mediates conditioned fear responses, whereas the BNST mediates unconditioned fear responses. Support for this idea came from the finding that increased startle in the presence of the smell of fox feces, presumably an unconditioned fear stimulus, was blocked by inactivation of the BNST but not the amygdala (Fendt, Endres, & Apfelbach, 2003). More recently, however, it was reported that posttraining lesions of the BNST blocked the expression of context conditioning measured with freezing (Sullivan et al., 2004), which clearly is a conditioned response to a context previously paired with shock, a result not consistent with the idea that the BNST is only involved in unconditioned fear. Our second hypothesis was that maybe the CeA mediates fear reactions activated by relatively short stimuli in highly predictable situations, whereas the BNST mediates fear responses to relatively long cues under conditions in which the perceived danger is not highly predictable and requires a sustained state of defensive preparedness. We now believe that this second alternative is the right conclusion.

In the light-enhanced startle paradigm, we found the light had to be on for a least 5 min to see maximal light-enhanced startle. At shorter intervals (i.e., 60 s), the excitatory effect was weak, and at very brief intervals (i.e., 3.2 s), the effect of light was often inhibitory (Davis et al., 1989). When the light is turned off, after a 20-min on time, startle does not abruptly return to baseline but remains elevated for sometime thereafter (de Jongh, Groenink, van der Gugten, & Olivier, 2002; Walker & Davis, 1996). Thus, light-enhanced startle requires a long-duration stimulus, and the effect of this stimulus far outlasts the period when the light is actually on. It also is an inherently unpredictable situation in which the animal may feel "at risk" without knowing exactly when something might happen and how bad it might be, much as all U.S. citizens felt after 9/11. In fact, in humans, we have found that startle is increased in the dark (Grillon, Pellowski, Merikangas, & Davis, 1997) and that this effect is much larger in patients with posttraumatic stress disorder (Grillon, Morgan, Davis, & Southwick, 1998). When we asked these patients how they felt when the light went out, they often reported that they felt like they were back in their bunker, anticipating a mortar attack, but not knowing when this would happen.

CRH-enhanced startle may be similarly characterized. CRH-enhanced startle appears to be a slow-onset (20 min) and slow-offset (several hours) effect, at least with intracerebroventricular administration (Lee & Davis, 1997a; Liang, Melia, Miserendino, et al., 1992). It is not clear whether the protracted time course and slow decay of CRH-enhanced startle reflect response characteristics of the BNST itself, the time required for CRH to occupy and then dissociate from CRH receptors, or emergent properties of the neural circuitry within which the BNST is embedded. For example, Koob (1999) suggested that CRH-responsive neurons in the BNST and elsewhere, once activated by emotional stressors, excite brainstem noradrenergic nuclei, which then feed back to CRH-responsive neurons to stimulate further CRH release.

The effect on startle of repeated footshock also fits the pattern. In Gewirtz et al. (1998), the effect developed gradually over many days and persisted for at least 24 hr (i.e., the interval between the final shock on the preceding training day and the baseline test on the following day).

Overall then, the data presently available argue for the existence of two phenomenologically and anatomically dissociable response systems, each capable of mediating increases in the amplitude of the acoustic startle response (see Figure 2). One, which includes the CeA as an integral component, can be characterized as a rapid response system that mediates short-term responses to specific threat cues (i.e., stimulus-specific fear responses). The other, which includes as an integral component the BNST, can be characterized as a sluggish response system that, once activated, continues to influence behavior long after the initiating stimulus has been terminated. We refer to the first response—a stimulus-specific, short-lasting type of response—as *fear*; we refer to the second—a more sustained type of response—as *anxiety*. Moreover, these two different systems show perfect additivity, consistent with independent, parallel systems that elevate startle (Walker & Davis, 2002). Finally, many other laboratories are finding that the BNST plays a more general role in stress, depression, and anxiety using many different experimental paradigms, including drug craving and withdrawal (cf. Walker, Toufexis, & Davis, 2003).

Extinction of Fear-Potentiated Startle

If, following fear-potentiated startle to a visual stimulus, the light is presented over and over again without shock, there will be a significant decrease in the magnitude of fear-potentiated startle as a direct function of the number of presentations of the light in the absence of shock (Walker, Ressler, Lu, & Davis, 2002). This procedure is known as *extinction training*, and the theoretical process that accounts for this decrease in conditioned fear is known as *extinction*. Behavioral observations indicate that extinction is a form of learning in its own right, rather than an "unlearning" or forgetting of previous learning (for a review, see Myers & Davis, 2002). We found that local infusion in the BLA of the NMDA antagonist AP5 completely blocked the development of extinction when animals were tested the next day drug free (Falls, Miserendino, & Davis, 1992). This impairment could not be attributed to an effect on NMDA receptors outside the amygdala, to damage to

the amygdala, or to an impairment of sensory transmission during extinction training, and it has been confirmed in several laboratories. Blocking NMDA receptors after extinction training also blocks extinction, suggesting that NMDA receptors are important for the consolidation of extinction (Santini, Muller, & Quirk, 2001).

In light of these findings, the question arose as to whether it would be possible to enhance extinction by enhancing the functioning of the NMDA receptor. It is known that a compound called D-cycloserine (DCS) binds to the NMDA receptor and makes it work better. Thus, we predicted that giving DCS prior to extinction training would enhance extinction. DCS given either systemically or directly into the amygdala prior to extinction training dose-dependently enhanced extinction in rats exposed to lights in the absence of shock, but it did not do so in control rats that did not receive extinction training when testing occurred 24 hr later in the absence of the drug (Walker et al., 2002), an effect now replicated with freezing to a tone (Ledgerwood, Richardson, & Cranney, 2003). Ledgerwood et al. (2003) also found that DCS could still facilitate extinction when given up to about 3 hr after extinction training, a finding consistent with the idea that DCS facilitates consolidation of extinction.

Clinical Implications

Because treatments for PTSD and other anxiety disorders typically involve a process similar to extinction, we tested whether DCS would enhance exposure-based psychotherapy in people suffering from an inordinate fear of heights in a double-blind placebo-controlled study. The exposure therapy used a virtual reality situation developed by Barbara Rothbaum and colleagues in which patients rode in a virtual glass elevator to progressively higher floors (Ressler et al., 2004). This situation is very frightening to patients just entering treatment, but it becomes considerably more tolerable with increasing exposure to the virtual environment, typically over six to eight sessions. Thirty patients were rated for their initial fear of heights and divided into three groups that had comparable levels of fear and were similar on other variables (e.g., age, sex), and then they received only two exposure sessions, purposely suboptimal to detect improvement. Single doses of placebo or DCS (50 or 500 mg) were taken 2–4 hr prior to each of the two sessions of virtual reality exposure therapy. Exposure therapy combined with DCS resulted in significantly larger reductions of acrophobia symptoms on all main outcome measures than did the same amount of exposure in combination with placebo. Compared with subjects receiving the placebo, subjects receiving DCS had significantly more improvement within the virtual environment both one week and three months after treatment. They also showed significantly greater decreases in posttreatment skin-conductance fluctuations and greater improvement on general measures of real-world acrophobia symptoms and number of self-exposures to real-world heights. Because of these promising results, DCS is now being tested in combination with psychotherapy all over the world for all the major anxiety disorders.

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Conflict of interest notice: Michael Davis has submitted a patent for the use of D-cycloserine for the specific enhancement of learning during psychotherapy and is entitled to royalties from Therapade in the event this invention is commercialized. The terms of these arrangements have been reviewed and approved by Emory University in accordance with their conflict of interest policies.

Correspondence concerning this article should be addressed to Michael Davis, Yerkes Primate Center and the Center for Behavioral Neuroscience, Emory University, 954 Gatewood Drive, Atlanta, GA 30329. E-mail: mdavis4@emory.edu

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