Amygdala and bed nucleus of the stria terminalis: differential roles in fear and anxiety measured with the acoustic startle reflex

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SUMMARY

Neural stimuli associated with traumatic events can readily become conditioned so as to reinstate the memory of the original trauma. These conditioned fear responses can last a lifetime and may be especially resistant to extinction. A large amount of data from many different laboratories indicate that the amygdala plays a crucial role in conditioned fear. The amygdala receives information from all sensory modalities and projects to a variety of hypothalamic and brainstem target areas known to be critically involved in specific signs that are used to define fear and anxiety. Electrical stimulation of the amygdala elicits a pattern of behaviours that mimic natural or conditioned states of fear. Lesions of the amygdala block innate or conditioned fear and local infusion of drugs into the amygdala have anxiolytic effects in several behavioural tests. Excitatory amino acid receptors in the amygdala are critical for the acquisition, expression and extinction of conditioned fear.

1. INTRODUCTION

Over the past several years, our laboratory has been studying how a simple reflex, the acoustic startle reflex, can be modified by prior emotional learning. Thus far, most of our work has concentrated on an experimental paradigm called the fear-potentiated startle effect, where the amplitude of the startle reflex can be modified by a state of fear. More recently, however, we have been trying to develop experimental methodologies to measure both fear and anxiety using changes in the acoustic startle reflex. Fear is a natural, adaptive change in an organism elicited by a potentially threatening stimulus, which prepares the organism to cope with the provocation. Fear generally is elicited by a clearly identifiable stimulus and subsides shortly after its offset. Anxiety also is a change in the state of an organism which has many of the same signs and symptoms of fear. However, it may not be clearly associated with a single eliciting stimulus, may last for long periods of time once activated and may lack clear adaptive significance.

2. FEAR-POTENTIATED STARTLE AS A MEASURE OF EXPLICIT CUE CONDITIONING

(a) The fear-potentiated startle test

Although fear is a complex emotion, it can be objectively measured in the laboratory using classical conditioning procedures in which a neutral stimulus, such as a light (conditioned stimulus, CS), is consistently paired with an aversive stimulus, such as a shock (unconditioned stimulus, US). Following a small number of pairings, the light now comes to elicit a constellation of behaviours that are typically used to define a state of fear in animals. These may include a change in heart rate, an increase in blood pressure, pupil dilation, laboured respiration, vocalization, cessation of ongoing behaviour and hyper-responsivity to sensory stimuli.

Our laboratory measures conditioned fear in rats using changes in the amplitude of the acoustic startle response, a short-latency reflex that can be elicited in all mammals (Landis & Hunt 1939; Davis 1984). In our typical paradigm, rats are placed in a chamber specially designed to elicit and measure the amplitude of the acoustic startle reflex and are presented with a small number of startle eliciting stimuli. The average startle amplitude across the last several stimuli (once habituation has occurred so that startle has reached a relatively stable level) is used as a measure of the basal startle amplitude (baseline startle). The next day the rats are returned to the same chamber and presented with light-shock pairings. No startle stimuli are given on this training day. At later times (1–30 days) the rats are returned to the test chamber and presented with acoustic startle stimuli alone, as well as in the presence of the light previously paired with shock. Under these conditions startle amplitude is significantly increased when elicited by the same auditory stimulus in the presence of the light (Brown *et al.* 1951; Davis &

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Astrachan 1978). The difference in startle amplitude elicited in the presence versus the absence of the light, or versus the original baseline level of startle, is used to define the magnitude of conditioned fear (fear-potentiated startle). When startle is elicited at various times during testing, we find that it increases almost immediately after light onset and returns to its baseline level shortly after the light goes off (Davis *et al*. 1989). Hence, fear-potentiated startle is highly time-locked to the presence of the emotionally significant stimulus, making it an example of explicit cue conditioning. Drugs that reduce or increase fear in humans selectively reduce or increase fear-potentiated startle in rats (Davis 1986).

(b) The role of the amygdala in fear-potentiated startle

A great deal of data now indicate that the amygdala is critically involved in explicit cue conditioning (Henke 1980; Gloor *et al*. 1981; Mishkin & Aggleton 1981; Ellis & Kesner 1983; Kapp *et al*. 1984; Sarter & Markowitsch 1985; Liang *et al*. 1986; LeDoux 1987; Davis 1992a,b). The natural pattern of behaviours produced by conditioned fear can be blocked by lesions of the amygdala and produced by electrical stimulation of the amygdala. Anatomical data indicate that the central nucleus of the amygdala projects directly to hypothalamic and brainstem target areas critically involved in specific signs and symptoms of fear. Lesions of the amygdala completely block fear-potentiated startle (Hitchcock & Davis 1986), and low-level electrical stimulation of the amygdala increases startle (Rosen & Davis 1988). Both conditioned fear and electrical stimulation of the amygdala appear to increase startle amplitude by ultimately altering transmission at a particular point along the acoustic startle pathway called the nucleus reticularis pontis caudalis (RPC), which receives a direct monosynaptic connection from the central nucleus of the amygdala (Rosen *et al*. 1991). Finally, local infusion of NMDA antagonists into the amygdala blocks the acquisition but not the expression of conditioned fear (Miserendino *et al*. 1990; Campeau *et al*. 1992), whereas pre-test infusion of non-NMDA receptor antagonists blocks the expression of fear-potentiated startle (Kim *et al*. 1993).

3. FEAR VERSUS ANXIETY

Although a good deal is known about the neural circuitry involved in explicit cue conditioning, much less is known about the closely related, but somewhat different emotion of anxiety. For example, in explicit cue conditioning, a state of fear is clearly elicited by a very specific stimulus that has previously been associated with an aversive event. In fact, a great deal of progress has been made in precisely tracing out the exact pathways to the amygdala that allow an auditory stimulus previously paired with a footshock to produce a state of fear using either freezing (LeDoux 1992) or potentiated startle (Campeau & Davis 1995a,b) as measures of conditioned fear. With anxiety, however, it is often difficult to specify the actual sensory event that

triggers anxiety, or to predict exactly when this change in emotion will subside. Moreover, certain animal tests purported to measure anxiety because of their sensitivity to drugs that reduce anxiety clinically are not always affected by lesions of the amygdala. For example, benzodiazepines have consistently been shown to have anxiolytic effects in the elevated plus maze, yet lesions of the amygdala fail to have an anxiolytic effect in this test (Treit *et al*. 1993). We believe that observations such as these are extremely important because they suggest that different areas of the brain may be involved in different types of aversive emotional states which ultimately may lead to a distinction between brain areas involved in fear versus anxiety. Because anxiety, rather than stimulus-specific fear, is a major problem in many types of psychiatric disorders, identifying separate neural substrates for fear versus anxiety could ultimately lead to more effective anxiety treatments. Because the acoustic startle reflex offers several advantages as a marker for aversive emotional states (Davis 1986), we have been trying to develop procedures that use the acoustic startle response to assess anxiety and brain areas involved in anxiety.

4. LIGHT-ENHANCED STARTLE
(a) The light-enhanced startle effect

Although fear-potentiated startle offers several advantages as an animal model of fear and anxiety, one disadvantage, common to all procedures which rely on conditioning, is that treatment effects cannot unambiguously be attributed to effects on fear vs memory. It is difficult to say, for example, whether a given drug that reduces fear-potentiated startle does so because the drug is anxiolytic or, alternatively, because the drug has a more general effect on memory retrieval. Consequently, it would be valuable to develop a procedure that preserves the benefits of fear-potentiated startle, but which relies on unconditioned stimuli to elicit anxiety.

Previous reports suggest that bright light may be an anxiety-provoking stimulus for rats and mice. For example, open field activity is decreased by high illumination (e.g. McLearn 1960; DeFries *et al*. 1966; Candland & Nagy 1969; Livesey & Egger 1970; Naggy & Glaser 1970; Valle 1970; see also Walsh & Cummins (1976) for a review), and this effect has been attributed, by some, to an activity-suppressing influence of fear (e.g. DeFries *et al*. 1966). An anxiogenic influence of light is also suggested by work from File & Hyde (1978), who have shown that social interactions among rat pairs are significantly reduced in high- versus low-illumination environments, particularly if the environment is an unfamiliar one. This group has also reported that plasma corticosterone concentrations of rats placed for 20 min in a brightly lit and unfamiliar environment are almost twice that of rats placed in a dimly lit unfamiliar environment (File & Peet 1980) and that both the behavioural and physiological effects are blocked by chlordiazepoxide (File & Hyde 1978; File & Peet 1980). The tendency of rodents to spend a greater amount of time in the darkened side of a two-chambered

dark–light box also suggests that light is aversive (Crawley & Goodwin 1980; Crawley 1981) and, consistent with this view, anxiolytic compounds such as diazepam, chlordiazepoxide and buspirone decrease this preference and other indices of anxiety (i.e. suppression of activity, transitions between the two compartments) in this paradigm (Crawley 1981; Costall *et al.* 1989; Onaivi & Martin 1989).

If, as would seem to be the case, high illumination levels are indeed anxiogenic, then illumination might also elevate the amplitude of acoustic startle. In fact, previous reports suggest that the unconditioned effects of light on startle are biphasic, with inhibition being reported at very short light-onset to startle-elicitation intervals (i.e. as short as 40 ms; Ison & Hammond 1971) and facilitation occurring at somewhat longer intervals (i.e. 400 ms; Ison & Hammond 1971) to at least as long as 52 s (Davis *et al.* 1989). Flashing lights also have been reported to 'sensitize' startle responding (Groves & Thompson 1970; Russo & Ison 1979; Wedeking & Carlton 1979), and to do so for as long as the visual stimuli are presented (up to 30 min in Wedeking & Carlton (1979)).

Recently, we have tested the effects of sustained illumination on the startle reflex and the possible role of the amygdala versus the bed nucleus of the stria terminalis on the facilitatory effects of light on the startle reflex (Walker & Davis 1996). Twenty-four rats were randomly divided into three groups of eight rats each. In each group, the effect on startle of either an 8, 70 or 700 footlambert light source was evaluated. Each animal was tested on two separate days. On one day, startle was measured in the dark during phase I and in the light during phase II. On a second day, the light remained off during both phases. These two session types were counterbalanced such that half of the rats in each illumination group began the experiment with a dark–light session type and half the rats began the experiment with a dark–dark session type. For both session types, a difference score (startle amplitude during phase II minus startle amplitude during phase I) was obtained.

Figure 1 shows the mean startle amplitude over successive blocks of three stimuli during phase I, combined across all groups, and during phase II shown separately for the different groups that were tested in phase II in the dark or with 8, 70 or 700 footlamberts of light. In both phases, startle responses habituated over the course of testing. Superimposed on this habituation was a general increase of startle amplitude, which was directly related to light intensity and which remained relatively stable for the duration of the 20 min test. Figure 2 shows these same data in terms of difference scores indicating that light-enhanced startle was directly related to the intensity of the light in phase II with significant increases occurring at the 700 and 70 footlambert intensities, but not with the 8 footlambert light intensity.

These results indicate that high levels of sustained illumination produce an increase in the amplitude of the acoustic startle response, which persists for at least 20 min after placement into the illuminated test chamber. It is possible, however, that the elevation in
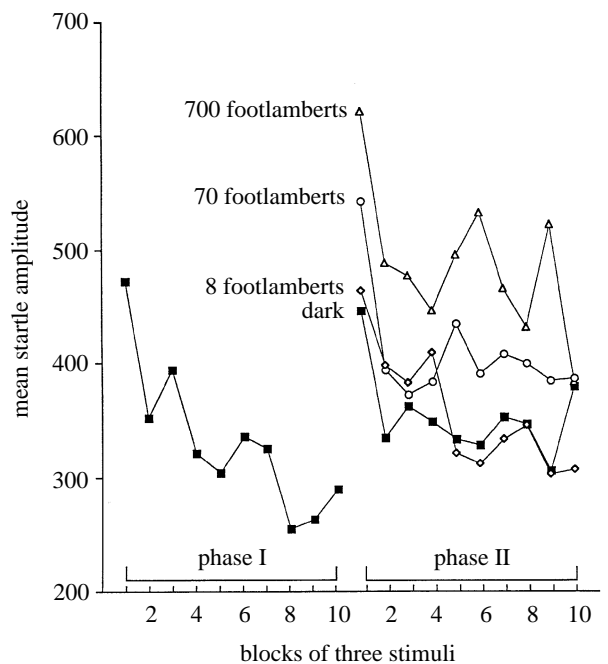
Figure 1. Mean startle amplitude over successive blocks of three stimuli during phase I, combined across all groups, and during phase II shown separately for the different groups that were tested in phase II in the dark or with 8, 70, or 700 footlamberts of light in phase II.
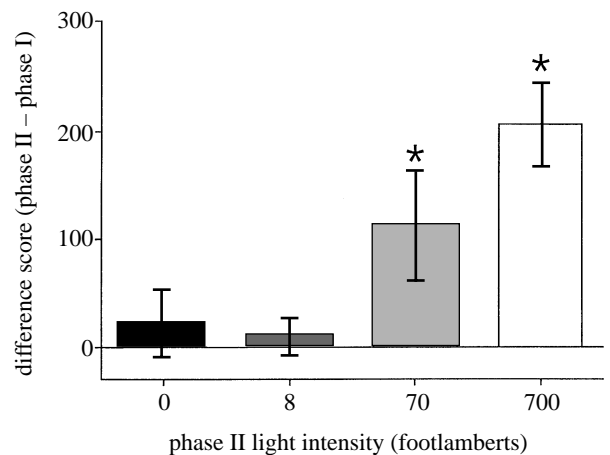

Figure 2. Mean change in startle amplitude from phase I to phase II as a function of the light intensity in phase 2. * = significantly different from dark–dark.

startle was produced simply by the change in illumination (e.g. via dishabituation) rather than the continued presence of the very bright light. If so, then decreasing the level of illumination should also increase startle amplitude. To evaluate this possibility the same animals were retested using the procedure described above, with the exception that rats were tested initially in the presence of the light (phase I) and subsequently in the absence of the light (phase II). The results showed that when illumination levels were decreased from phase I to phase II, the mean amplitude of acoustic startle also decreased. These results could not

be accounted for by dishabituation and confirm that the amplitude of acoustic startle is greater when elicited in an illuminated as opposed to a darkened environment.

(b) *Effects of buspirone on light-enhanced startle*

Treatments that increase the amplitude of acoustic startle are often associated with aversive or anxiogenic properties. For example, cues previously paired with footshock increase the amplitude of acoustic startle (Brown *et al.* 1951), as does footshock itself (Davis 1989), and drugs that produce anxiety in humans increase the amplitude of acoustic startle in rats (cf. Davis 1980, 1990). Perhaps, then, the effects of light shown here reflect an unconditioned anxiogenic effect of high illumination levels. As an initial test of this hypothesis, we assessed the susceptibility of the unconditioned light effect to the compound buspirone, a clinically used anxiolytic which also blocks fear-potentiated startle in rats (Davis *et al.* 1988; Kehne *et al.* 1988; Mansbach & Geyer 1988). Sixteen rats were tested under each of four conditions (dark–light saline, dark–light buspirone, dark–dark saline, and dark–dark buspirone). The ordering of session type and treatment was counterbalanced across animals. During each phase, startle responses were elicited by 30 noise bursts, ten at each of three intensities (90, 95 and 105 dB). Light intensity was set at 700 footlamberts. Immediately prior to phase I, buspirone (5 mg kg^{-1}) or saline (1 ml kg^{-1}) was injected subcutaneously in the neck.

Figure 3 shows that the effect of light was blocked by buspirone. However, consistent with previous results (Eison *et al.* 1986; Kehne *et al.* 1988), buspirone itself increased the amplitude of baseline startle. Although this effect appears to be independent of buspirone's anxiolytic effects on fear-potentiated startle, because buspirone can still block fear-potentiated startle under conditions where it has no effect on baseline startle (Kehne *et al.* 1988), the unusually large effect on baseline startle seen in the present experiment may have prevented measurement of any further increases of startle amplitude by light because of a ceiling effect. To address this possibility, the buspirone data were reanalysed using only those startle responses elicited by the lower intensity noise bursts (i.e. 90 and 95 dB). When these data were compared with the data from saline-treated animals (i.e. using 90, 95 and 105 dB noise bursts), this time across comparable baselines, similar results were obtained (see figure 3).

These data show that the elevation of startle amplitude by light is blocked by buspirone. Response amplitude in the dark was not decreased, but instead showed a moderate increase. As such, the effect of buspirone cannot be attributed to a general suppression of startle amplitude, but reflects, instead, a specific blockade of the light-induced increase. As previously indicated, buspirone is clinically used in the treatment of anxiety and has also been shown to block the effects of conditioned fear on startle (Davis *et al.* 1988; Kehne *et al.* 1988; Mansbach & Geyer 1988). Thus, these results are consistent with the view that the effects of light reflect anxiogenic properties of high illumination levels.
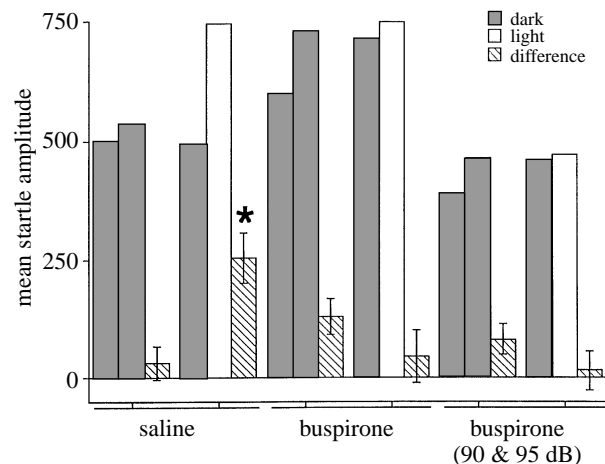
Figure 3. The mean startle amplitude in the dark–dark or dark–light conditions following injection of saline or buspirone. The hatched bars represent the difference between phase I and phase II in either the dark–dark or dark–light conditions. The same data following injection of buspirone are shown only at the 90 and 95 dB test stimuli. * = significantly different from dark–dark.

Recently, we have found that humans show a significant increase of startle amplitude (i.e. of the eyeblink response) in the dark (Grillon *et al.* 1997). The opposite effects of illumination in rats versus humans may be attributable to the fact that rats are nocturnal whereas humans are diurnal. Interestingly, Grillon *et al.* (1997) also report that dark-enhanced startle only occurred in those subjects who rated the experiment as more unpleasant in the dark than in the light, and was correlated with the subjects' self-ratings of how fearful of the dark when they were young. Again, these results are consistent with the view that the effects of light on startle are related to fear or anxiety.

Overall, we believe there are sufficient grounds for attributing the effects of light on startle to increased anxiety and that this paradigm may be a useful model for the study of anxiety in animals. In general, animal models not involving conditioning may afford significant benefits (Lister 1990) and, indeed, several such models have been proposed (e.g. Gardner 1985; Pellow *et al.* 1985; Treit & Fundytus 1989; Hogg & File 1994). When evaluating the effects of various treatments on conditioned fear, for example, it is difficult to distinguish drug effects on anxiety from drug effects on memory. Because light-enhanced startle does not involve conditioning, treatment effects may be more confidently attributed to the former. Also, because tests of conditioned behaviour generally lead to either an increase or decrease of response strength (depending on whether the conditioned stimulus or response is or is not reinforced during test), separate groups of animals are usually required when evaluating multiple treatments to avoid the potential contamination of baseline responding by previous tests and treatments. Because light-enhanced startle is not reinforced and does not markedly habituate across sessions (D. Walker and M. Davis, unpublished observations), this paradigm may be particularly suitable for studies in which repeated testing is desirable.
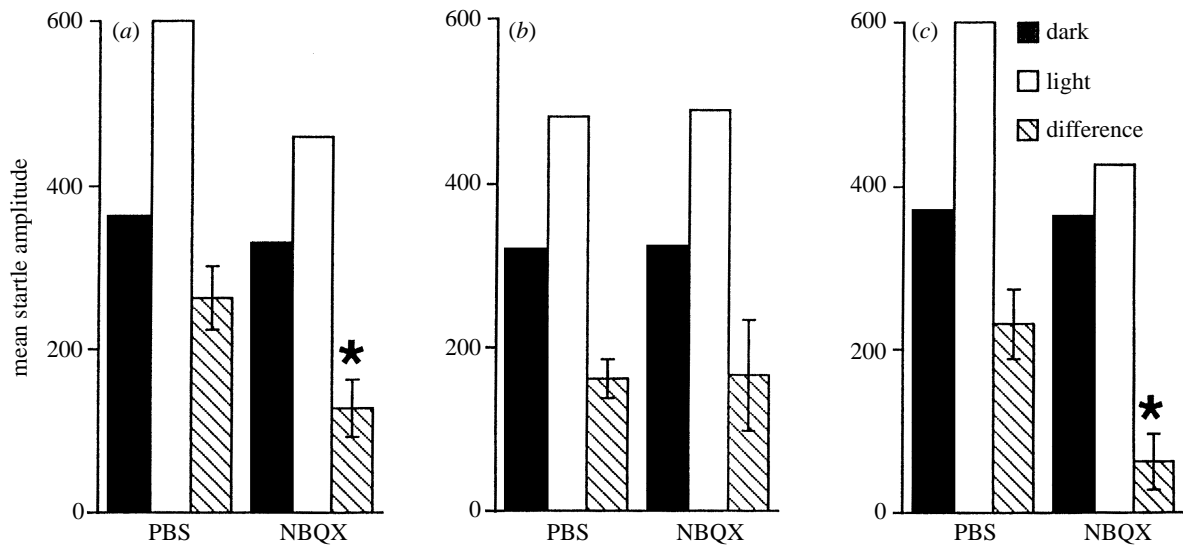
Figure 4. Mean amplitude startle in the dark (phase I)–light (phase II) conditions after local infusion of either phosphate buffered saline (PBS) or the glutamate antagonist NBQX into either the basolateral amygdala (*a*), the central nucleus of the amygdala (*b*) or the bed nucleus of the stria terminalis (*c*). The difference between the two phases defines the amount of light-enhanced startle. * = significantly different from PBS

(*c*) *Effects of glutamate antagonists infused into the bed nucleus of the stria terminalis versus the amygdala on light-enhanced startle*

Because local infusion of glutamate antagonists into the central nucleus of the amygdala completely blocks the expression of fear-potentiated startle (Kim *et al.* 1993), we wondered whether this treatment would also block light-enhanced startle. As a control, we measured the effects of local infusion of glutamate antagonists into the bed nucleus of the stria terminalis. The bed nucleus of the stria terminalis is considered to be part of the so-called extended amygdala, because it is highly similar to the central nucleus of the amygdala in terms of its transmitter content, cell morphology and efferent connections (Alheid *et al.* 1995). However, lesions of the bed nucleus of the stria terminalis fail to block either fear-potentiated startle (Hitchcock & Davis 1991) or conditioned freezing using an explicit cue (LeDoux *et al.* 1988), suggesting that it may not be involved in explicit cue conditioning. On the other hand, several ongoing studies in our laboratory suggested that the bed nucleus of the stria terminalis might be involved in elevations of startle that were more long-lasting than explicit cue conditioning. For example, lesions of the bed nucleus of the stria terminalis blocked long-term sensitization of the startle reflex (Davis *et al.* 1995) or conditioned freezing using the experimental context as the conditioned stimulus rather than an explicit cue (McNish *et al.* 1997). It also blocked the excitatory effect of the peptide corticotropin-releasing hormone on startle (Lee & Davis 1995, and see below).

To evaluate the role of the bed nucleus of the stria terminalis versus the amygdala in light-enhanced startle, animals were implanted with bilateral cannulas in either the bed nucleus of the stria terminalis, the basolateral complex of the amygdala (i.e. the lateral and basolateral nuclei) or the central nucleus of the amygdala. One week later animals were tested for light-enhanced startle using the procedures described above. During phase I, animals were placed in the darkened chamber and presented with startle stimuli over a 20 min period. They were then removed from the chamber, handled and placed back in the brightly illuminated chamber and startled for another 20 min. Prior to being placed into the chamber during phase II, half of the animals were infused with the AMPA/kainate antagonist 6-nitro-7-sulphamoylbenzo(f) quinoxaline-2-3-dione (NBQX; µg/side) and the other half with its vehicle, phosphate buffered saline (PBS). Two days later these procedures were repeated except animals previously infused with NBQX were now infused with PBS and vice versa.

Figure 4 shows the results. Consistent with previous results in non-infused rats, light increased the amplitude of the startle reflex when animals were shifted from the darkened chamber in phase I to the brightly illuminated chamber in phase II after infusion of PBS into each of the three brain structures. Infusion of the glutamate antagonist NBQX into the central nucleus of the amygdala had no effect on light-enhanced startle. On the other hand, infusion of NBQX into either the lateral/basolateral amygdala complex or the bed nucleus of the stria terminalis significantly decreased light-enhanced startle.

These data indicate an important role for both the lateral/basolateral amygdala complex and the bed nucleus of the stria terminalis in light-enhanced startle. It is possible, however, that infusion of NBQX into the bed nucleus of the stria terminalis or the basolateral amygdala caused a depressant effect on startle that simply subtracted from the expected excitatory effect of testing in the brightly illuminated chamber. Previous studies in our laboratory had shown that local infusion of NBQX into the basolateral amygdala did not depress startle (D. Walker and M. Davis, unpublished observations), so that this explanation

could not account for the basolateral amygdala results. To address this issue with regard to the bed nucleus of the stria terminalis, other animals were implanted with cannulas into the bed nucleus of the stria terminalis and then tested for startle in the darkened chamber at the same time after infusion of NBQX that occurred during phase II in the light-enhanced experiment described above. NBQX had no depressant effect on startle when testing took place in the darkened chamber at the same time after infusion when it decreased light-enhanced startle (data not shown). These data strengthen the conclusion that the bed nucleus of the stria terminalis and the basolateral amygdala, which receives visual input and projects to the bed nucleus of the stria terminalis, are critically involved in light-enhanced startle whereas the central nucleus of the amygdala is not.

It is, however, possible that the cannulas in the central nucleus of the amygdala were misplaced and that this accounted for the lack of an effect of inactivation of the central nucleus on light-enhanced startle. Previous studies have shown that local infusion into the central nucleus of the amygdala blocks the expression of fear-potentiated startle (Kim *et al*. 1993). If the central nucleus implants in the present study were located properly, then infusion of NBQX into these animals should also block fear-potentiated startle. To evaluate this, the rats used in the light-enhanced startle experiment were trained and tested for fear-potentiated startle after infusion of NBQX into either the amygdala or bed nucleus of the stria terminalis. During training, animals were placed into a darkened chamber and 5 min later presented with the first of ten light shock pairings using a 3.7 s light which coterminated with a 0.5 s, 0.6 mA footshock. Light shock pairings were presented at an average inter-trial interval of 4 min (range = 35 min). These same procedures were repeated 24 h later creating a total of 20 light shocks pairings. Two days later the animals were placed back in a darkened startle chamber and 5 min later presented with a total of 60 startle stimuli, half of which occurred 3.2 s after the onset of the light and half of which occurred in darkness. Three different noise bursts intensities were used (90, 95 or 105 dB), creating a total of ten occurrences of each of six test trial types.

Figure 5 shows the mean startle amplitude, collapsed over the three test intensities, when startle was elicited in darkness (noise-alone trials: black bars) or 3.2 s after the onset of the light (light-noise trials: white bars). Consistent with previous results (Kim *et al*. 1993), infusion of the glutamate antagonist into the central nucleus of the amygdala completely blocked the expression of fear-potentiated startle. This was also true after an infusion of NBQX into the basolateral nucleus of the amygdala. In contrast, infusion of NBQX into the bed nucleus of the stria terminalis had no effect on fear-potentiated startle. These data indicate, therefore, that the location of the cannulas into the central nucleus of the amygdala was adequate to allow infusion of NBQX to totally block fear-potentiated startle. Hence, the ineffectiveness of NBQX infused into the central nucleus of the amygdala to block light-enhanced

startle cannot be attributed to misplaced cannulas. Moreover, these data show a double dissociation between inactivation of glutamate receptors in the central nucleus of the amygdala versus the bed nucleus of the stria terminalis in relationship to fear-potentiated versus light-enhanced startle.

5. CRH-ENHANCED STARTLE AS A MEASURE OF ANXIETY

In addition to evaluating the role of the amygdala versus the bed nucleus of the stria terminalis in fear-potentiated startle and light-enhanced startle, we have been testing how these same brain areas might be involved in the excitatory effect of the peptide corticotropin-releasing hormone (CRH) on the startle reflex. Intraventricular administration of CRH produces a variety of behavioural and neuroendocrine effects similar to those seen during fear and anxiety, whereas intraventricular administration of the CRH antagonist alpha-helical CRH9-41 block the behavioural and neuroendocrine effects of natural stressors or conditioned fear (Dunn & Berridge 1990). Swerdlow *et al*. (1986) reported that intraventricular administration of CRH increased the acoustic startle reflex and that this effect could be blocked by the benzodiazepine chlordiazepoxide, suggesting that the excitatory effect of CRH on startle reflected an anxiogenic effect of the hormone. We have confirmed and extended this work showing that intraventricular (I.C.V.) infusion of CRH (0.1–1.0 µg) produced a pronounced, dose-dependent enhancement of the acoustic startle reflex in rats (Liang *et al*. 1992*b*).

More recently, we have been attempting to determine the primary receptor site on which CRH acts after intraventricular administration to increase startle amplitude. Three criteria were used to evaluate whether a given structure represented a primary receptor site for CRH-enhanced startle. First, lesions of the structure should block the excitatory effects of CRH on startle. Second, local infusion of CRH into the structure should elevate startle at doses considerably less than those required to increase startle after intraventricular infusion. Third, local infusion of a CRH antagonist directly into the structure should block the excitatory effects of CRH given intraventricularly.

(a) *Effects of lesions of the septal area on CRH-enhanced startle*

The first brain area examined was the septal nucleus. The lateral septal nucleus contains a high density of CRH receptors and is adjacent to the lateral ventricle. Lesions of the lateral septal nucleus are known to increase startle amplitude (Lee *et al*. 1988) and this effect can be prevented by concomitant lesions of the amygdala (Melia *et al*. 1992), suggesting that septal lesions increase startle via disinhibition of the amygdala. Perhaps I.C.V. CRH might functionally inhibit the lateral septum and thereby increase startle via a disinhibition of the amygdala. This hypothesis seemed plausible because previous data had shown that large electrolytic lesions of the amygdala
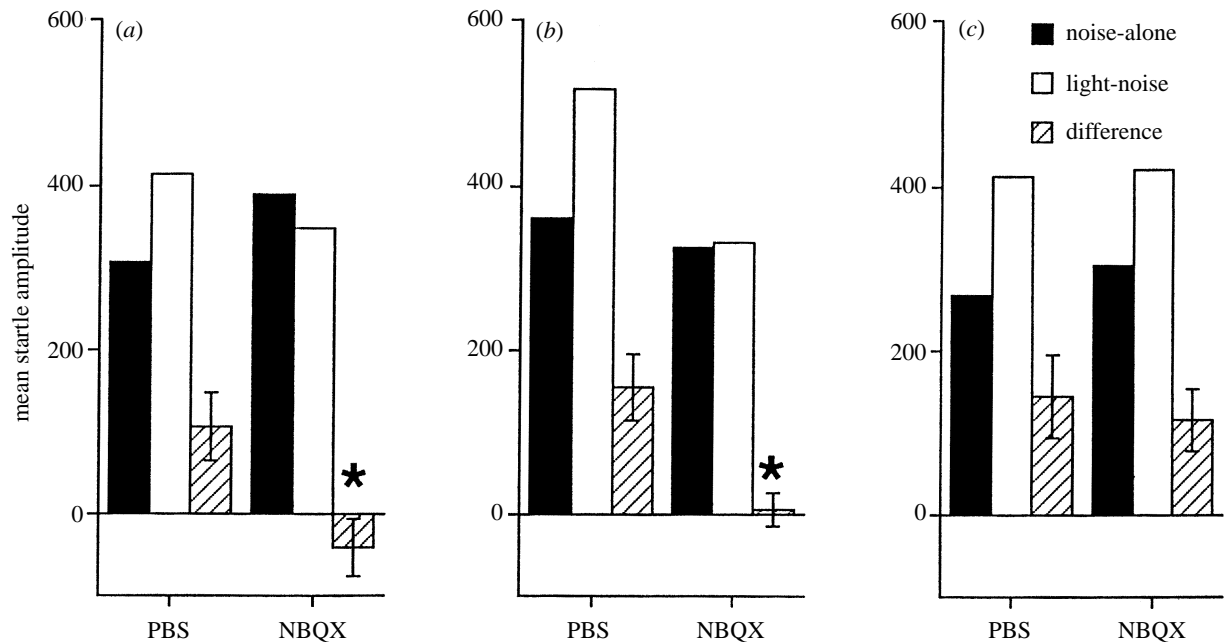
Figure 5. Mean amplitude startle on the noise-alone and light-noise trials after local infusion of either phosphate buffered saline (PBS) or the glutamate antagonist NBQX into either the basolateral amygdala (*a*), the central nucleus of the amygdala (*b*) or the bed nucleus of the stria terminalis (*c*). The difference between the two trial types defines the amount of fear-potentiated startle.

attenuated CRH-enhanced startle even though local infusion of CRH into the amygdala did not significantly elevate startle (Liang *et al*. 1992*a*). Thus the amygdala seemed to be part of the neural circuitry required for CRH to elevate startle, but did not appear to be the primary receptor area where CRH acts.

To test the role of the septal area in CRH-enhanced startle, animals were implanted with intraventricular cannulas and at the same time given electrolytic lesions of either the medial septum, the lateral septum, the medial and lateral septum together or sham lesions. Two weeks later, at a time when the excitatory effects of septal lesions on startle have dissipated (Melia & Davis 1991), all animals were placed in a startle test chamber and given a pretest consisting of presentation of 60 startle stimuli at a 30 s interstimulus interval. The animals were then removed from the chambers and infused intraventricularly with either CRH or its vehicle, artificial cerebrospinal fluid (ACSF). Immediately thereafter they were presented with the first of 240 startle stimuli at a 30 s interstimulus interval, creating a 2 h post infusion test session. Two days later these same procedures were repeated except animals previously infused with CRH were now infused with ACSF and vice versa.

Figure 6 shows the results. Consistent with previous reports, intraventricular infusion of CRH caused a marked elevation of startle that began approximately 20 min after infusion and reached a stable plateau from 60 to 120 min thereafter in the sham lesioned animals. Electrolytic lesions of either the medial septum or the whole septum completely blocked CRH-enhanced startle. On the other hand, counter to expectation, electrolytic lesions of the lateral septum did not block CRH-enhanced startle.

These data suggested an important role for fibres or cell bodies in the medial septum in CRH-enhanced startle. To evaluate the role of cell bodies versus fibres of passage, other animals were given either chemical lesions of the medial septum or sham lesions and then tested for CRH-enhanced startle as described earlier. Figure 7 shows that chemical lesions of the medial septum failed to block CRH-enhanced startle in contrast to the total ablation of CRH-enhanced startle following electrolytic lesions of the same structure. These data indicate that fibres passing through the medial septum are likely to be involved in CRH-enhanced startle whereas cell bodies within the medial nucleus themselves appear not to be involved. Further studies showed that local infusion of CRH into the medial septum also failed to increase startle (data not shown), indicating once again that receptors within the medial septal area do not seem to be involved in CRH-enhanced startle.

(*b*) *Effects of transection of the fimbria on CRH-enhanced startle*

The most prominent fibre tract traversing the medial septum is the fornix, which connects the hippocampus to the bed nucleus of the stria terminalis as well as carrying fibres from the medial septum to the hippocampus itself (Amaral & Witter 1995). Hence, we hypothesized that the blockade of CRH-enhanced startle produced by electrolytic lesions of the medial septum resulted from destruction of the fornix. To test this, other animals were given transections of the fimbria, the fibre bundle outside of the medial septum that forms the fornix, so as to evaluate the role of the fornix without concomitant damage to cell bodies in
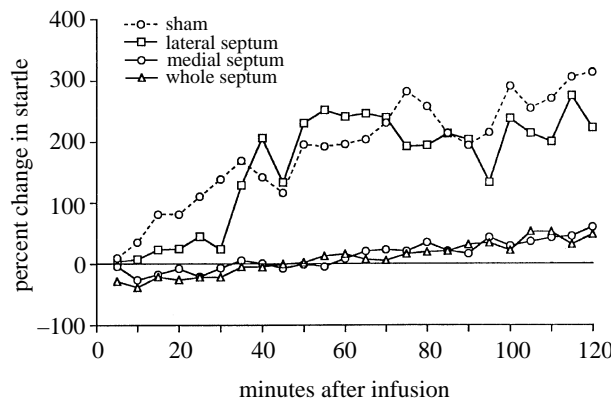
Figure 6. Mean per cent change in startle amplitude, relative to the pre-infusion baseline, after intraventricular infusion of CRH in animals previously given either sham lesions, electrolytic lesions of the lateral septum, electrolytic lesions of the medial septum or electrolytic of the whole septum.
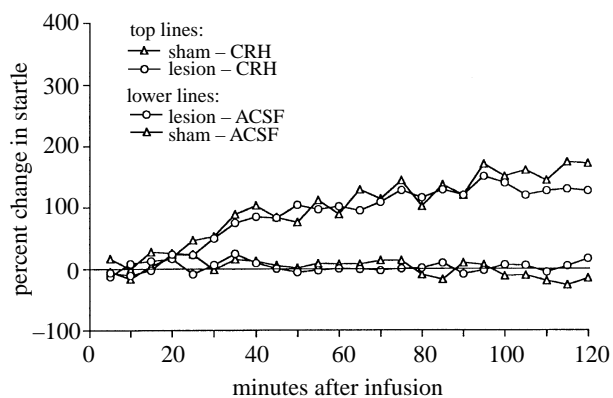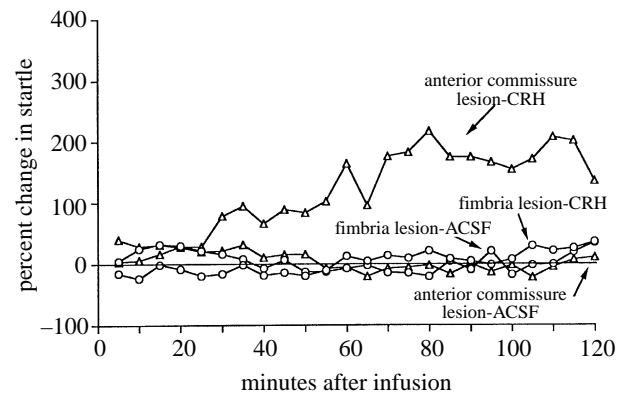

Figure 8. Mean per cent change in startle amplitude, relative to the pre-infusion baseline, after intraventricular infusion of either CRH or artificial CSF (ACSF) in animals previously given either electrolytic lesions of the anterior commissure or transections of the fimbria.

Figure 7. Mean per cent change in startle amplitude, relative to the pre-infusion baseline, after intraventricular infusion of CRH or artificial CSF (ACSF) in animals previously given either sham lesions of the medial septum or chemical lesions of the medial septum.
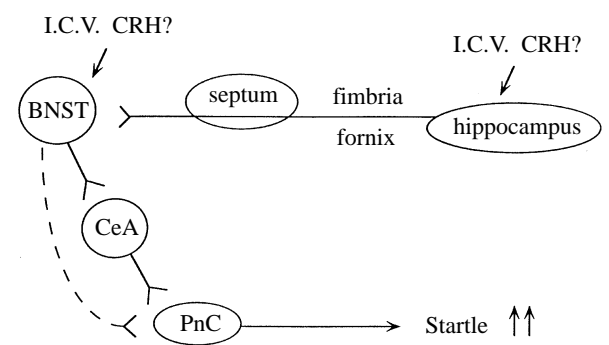

Figure 9. Schematic diagram showing the connections between the hippocampus and the bed nucleus of the stria terminalis via the fimbria fornix, which passes through the septum. Outputs of the bed nucleus of the stria terminalis project to the nucleus reticularis pontis caudalis in the brainstem, critical for startle, either directly or indirectly via the central nucleus of the amygdala.

the medial septum. As a control, other animals sustained lesions of another major fibre bundle, the anterior commissure. Figure 8 shows that transections of the fimbria completely blocked CRH-enhanced startle whereas electrolytic lesions of the anterior commissure had no significant effect. Taken together with the electrolytic lesion data of the medial septum these results strongly implicate the fornix as being critical for the expression of CRH-enhanced startle.

(c) Effects of lesions of the amygdala, hippocampus or bed nucleus of the stria terminalis on CRH-enhanced startle

At the present time it is not clear exactly what role the fornix plays in CRH-enhanced startle. Figure 9 shows a schematic diagram indicating possible connections between limbic structures that may be involved in CRH-enhanced startle. The fornix is known to connect the hippocampus to the bed nucleus of the stria terminalis (Canteras & Swanson 1992; Cullinan et al. 1993;

Amaral & Witter 1995), which in turn projects either directly to the startle pathway (Y. Lee, C. Shi and M. Davis, unpublished observations) or indirectly via the central nucleus of the amygdala (DeOlmos et al. 1985). Hence, the first question was whether chemical lesions of the hippocampus, bed nucleus of the stria terminalis or the amygdala would block the excitatory effect of CRH on startle after intraventricular administration. Figure 10 shows that NMDA induced lesions of the ventral hippocampus or the bed nucleus of the stria terminalis completely blocked CRH-enhanced startle. In contrast, chemical lesions of either the central nucleus of the amygdala or the basolateral/lateral amygdala nuclei had no significant effect on CRH-enhanced startle. However, the lack of an effect of chemical lesions of the amygdala to block CRH-enhanced startle might have resulted from inaccurate or incomplete lesions of these structures. To evaluate this, the same animals were trained for the fear-potentiated startle effect as described previously and then
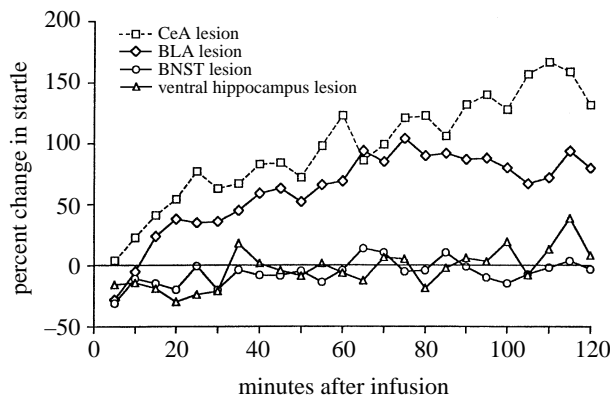
Figure 10. Mean per cent change in startle amplitude, relative to the pre-infusion baseline, after intraventricular infusion of CRH in animals previously given chemical lesions of either the central nucleus of the amygdala (CEA), the basolateral nucleus of the amygdala (BLA), the bed nucleus of the stria terminalis (BNST), or the ventral hippocampus.
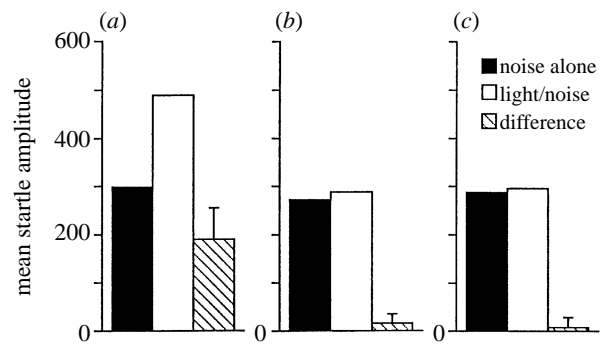

Figure 11. Mean startle amplitude on the noise alone and light-noise trials and the difference between these two trial types in animals previously given NMDA lesions of either (*a*) the bed nucleus of the stria terminalis (BNST), (*b*) the central nucleus of the amygdala (CEA) or (*c*) the basolateral nucleus of the amygdala (BLA).

tested one week later. Figure 11 shows that chemical lesions of either the central or the basolateral amygdala nuclei completely blocked fear-potentiated startle, consistent with prior results (Sananes & Davis 1992; Campeau & Davis 1995*b*). In contrast, chemical lesions of the bed nucleus of the stria terminalis, which completely blocked CRH-enhanced startle, failed to block fear-potentiated startle. Like the difference between light-enhanced startle and fear-potentiated startle, chemical lesions of the bed nucleus of the stria terminalis and amygdala also resulted in a double dissociation in relationship to CRH-enhanced startle.

These data strongly implicate the hippocampus and bed nucleus of the stria terminalis, but not the amygdala, in CRH-enhanced startle. The prior findings that large electrolytic lesions of the central nucleus of the amygdala blocked CRH-enhanced startle is still not resolved by these data. However, our suspicion is that the electrolytic lesions destroyed fibres projecting from the bed nucleus of the stria terminalis to the startle pathway. Further studies using electrolytic lesions in combination with retrograde or anterograde tracing techniques will be required to address this issue.

(d) Effects of local infusion of CRH into the hippocampus or the bed nucleus of the stria terminalis on startle

To further evaluate the role of the hippocampus and the bed nucleus of the stria terminalis in CRH-enhanced startle, other groups of animals were implanted with bilateral cannulas in either the ventral hippocampus or the lateral division of the bed nucleus of the stria terminalis. One week later they were infused with various doses (40, 80 or 160 ng) of CRH or ACSF and tested for startle in the usual way. Local infusion of CRH into the hippocampus failed to increase startle at any of the test doses (data not shown). On the other hand, local infusion of CRH

into the bed nucleus of the stria terminalis caused a dose-dependent elevation in startle that mimicked to some extent the excitatory effect of CRH given intraventricularly (figure 12). However, the excitatory effect of CRH given locally into the bed nucleus of the stria terminalis was never as large in magnitude as that seen after intraventricular administration. Nonetheless, these data suggest that the bed nucleus of the stria terminalis may be at least one of the primary receptor areas critical for CRH-enhanced startle.

(e) Effects of local infusion of the CRH antagonist alpha-helical CRH$_{9-41}$ into the bed nucleus of the stria terminalis on CRH-enhanced startle

To evaluate the role of the bed nucleus of the stria terminalis more fully, other animals were implanted with bilateral cannulas into the bed nucleus of the stria terminalis along with a single cannula into the fourth ventricle. Previous data had shown that both the time-course and magnitude of CRH-enhanced startle was similar when infusions were made in the lateral or fourth ventricle (Liang *et al.* 1992*b*). This arrangement allowed us to test whether local infusion of a CRH antagonist into the bed nucleus of the stria terminalis could block the ability CRH given intraventricularly to elevate startle. Placement of the intraventricular cannula into the fourth ventricle, rather than the lateral ventricle, was necessary because there was not enough space to allow implantation of both an intraventricular cannula and bilateral cannulas into the bed nucleus of the stria terminalis. One week after the surgery, animals were infused with 0.5 µg of CRH intraventricularly 5 min after local infusion into the bed nucleus of the stria terminalis of 1.5 or 3 µg/side of the CRH antagonist alpha-helical CRH$_{9-41}$. Figure 13 shows that intraventricular infusion of CRH using the fourth ventricle produced the usual excitatory effect on startle after infusion of artificial CSF into the bed nucleus of the stria terminalis. However, infusion the CRF antagonist alpha-helical CRH$_{9-41}$ into the bed nucleus of the stria terminalis caused a dose-dependent
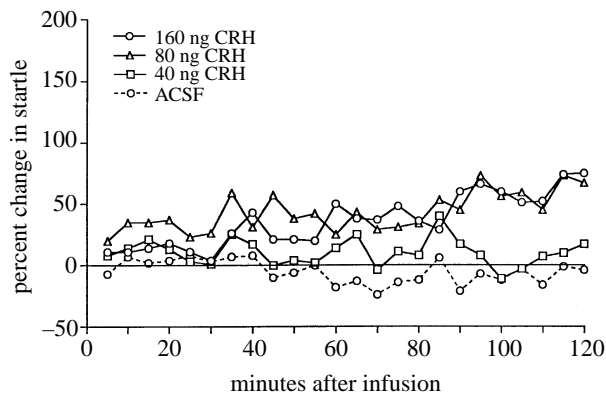
Figure 12. Per cent change in startle amplitude, relative to the pre-infusion baseline, after local infusion into the bed nucleus of the stria terminals of various doses of CRH or artificial CSF (ACSF).
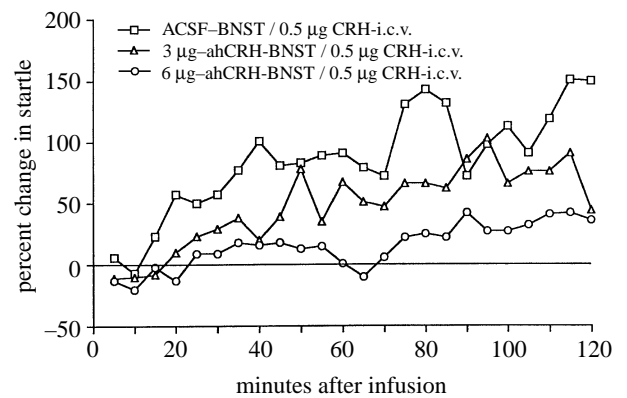

Figure 13. Mean per cent change in startle amplitude, relative to the pre-infusion baseline, after intraventricular infusion of CRH 5 min after local infusion into the bed nucleus of the stria terminals of either artificial CSF (ACSF), 3 µg of the CRH antagonist α-helical CRH, or 6 µg of α-helical CRH.

attenuation of CRH-enhanced startle. These data strongly suggest that CRH receptors in the bed nucleus of the stria terminalis are importantly involved in CRH-enhanced startle. Further studies indicated that the ability of 6 µg of alpha-helical CRH_{9-41} infused locally into the bed nucleus of the stria terminalis to block CRH-enhanced startle could not be attributed to leakage into the ventricular space, because the same dose of alpha-helical CRH_{9-41} given directly intraventricularly had no effect on CRH-enhanced startle. Furthermore, the effect was anatomically specific because 6 µg of alpha-helical CRH_{9-41} infused directly into the central nucleus of the amygdala failed to block CRH-enhanced startle (figure 14).

In summary, these data suggest that the bed nucleus of the stria terminalis may well be a primary receptor site involved in CRH-enhanced startle. Chemical lesions of the bed nucleus of the stria terminalis blocked CRH-enhanced startle. Microinfusion of CRH into the bed nucleus of the stria terminalis mimicked, at least partially, the excitatory effects of CRH on startle given intraventricularly. Finally, local infusion of a CRH antagonist directly into the bed nucleus of the stria terminalis blocked CRH-enhanced startle. In contrast, the amygdala does not seem to be involved in CRH-enhanced startle because (i) chemical lesions of the amygdala fail to block CRH-enhanced startle; (ii) local infusion of CRH into the amygdala does not mimic CRH-enhanced startle; and (iii) local infusion into the amygdala of a CRH antagonist does not block CRH-enhanced startle.

6. DIFFERENTIAL ROLES OF THE AMYGDALA VERSUS THE BED NUCLEUS OF THE STRIA TERMINALIS IN FEAR VERSUS ANXIETY

The series of experiments outlined above shows a clear distinction between the central nucleus of the amygdala and the bed nucleus of the stria terminalis in relation to fear-potentiated startle versus CRH-enhanced and light-enhanced startle. Lesions or chemical inactivation of the central nucleus of the amygdala completely block the expression of

fear-potentiated startle but have no effect whatsoever on either light-enhanced startle or CRH-enhanced startle. Conversely, lesions or chemical inactivation of the bed nucleus of the stria terminalis significantly attenuated either light-enhanced startle or CRH-enhanced startle without having any effect whatsoever on fear-potentiated startle.

At the present time, it is still unclear why these two structures separate so completely in relation to fear-potentiated startle versus light-enhanced and CRH-enhanced startle. It is especially interesting, for example, that the basolateral amygdala appears to be involved in light-enhanced startle, as well as fear-potentiated startle, but not in CRH-enhanced startle. Visual information is known to reach the bed nucleus of the stria terminalis via projections from the peri-rhinal cortex to the basolateral nucleus. Because light-enhanced startle eventually must depend on visual information getting to the bed nucleus of the stria terminalis, the ability of chemical inactivation of the basolateral nucleus of the amygdala to block light-enhanced startle may reflect interruption of visual information passing through the basolateral nucleus of the amygdala to the bed nucleus of the stria terminalis. On the other hand, chemical lesions of the basolateral nucleus of the amygdala did not block CRH-enhanced startle. This may make sense because CRH-enhanced startle would not require visual input to the bed nucleus of the stria terminalis and hence interruption of the visual pathway to the bed nucleus of the stria terminalis would not be expected to block CRH-enhanced startle. It should be emphasized that the very same light is used in both fear-potentiated test and the light-enhanced startle test. The only difference is that in fear-potentiated startle the light is previously paired with a shock and is presented for a brief period of time whereas in light-enhanced startle the light is not paired with a shock and is presented for a relatively long period of time. It is likely that the necessity of the central nucleus of the amygdala in fear-potentiated startle is dependent on prior classical fear conditioning because a great deal of data show that the amygdala is
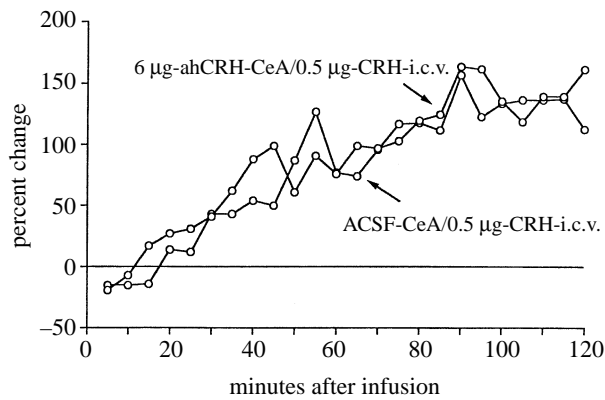
Figure 14. Mean per cent change in startle amplitude, relative to the pre-infusion baseline, after intraventricular infusion of CRH 5 min after local infusion into the central nucleus of the amygdala of either artificial CSF (ACSF) or 6 μg of the CRH antagonist α-helical CRH.
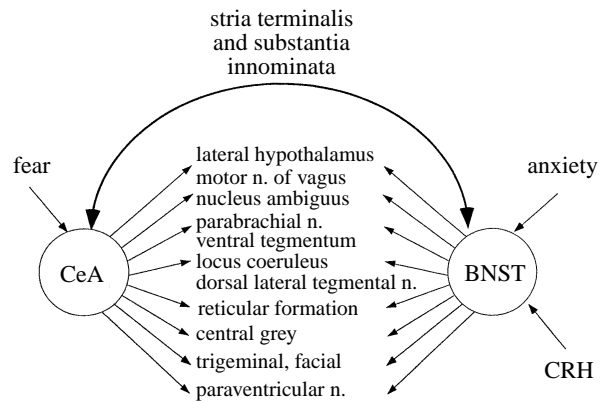

Figure 15. Hypothetical schematic suggesting that the central nucleus of the amygdala (CeA) and the bed nucleus of the stria terminalis (BNST) may be differentially involved in fear versus anxiety, respectively. Both brain areas have highly similar hypothalamic and brainstem targets known to be involved in specific signs and symptoms of fear and anxiety. However, the stress peptide CRH appears to act on receptors in the bed nucleus of the stria terminalis rather than the amygdala, at least in terms of an increase in the startle reflex. Furthermore, the bed nucleus of the stria terminalis seems to be involved in the anxiogenic effects of a very bright light presented for a long period of time but not when that very same light has previously been paired with a shock. Just the opposite is the case for the central nucleus of the amygdala, which is critical for fear conditioning using explicit cues such as a light or tone paired with aversive stimulation (i.e. conditioned fear).

critically involved in both the acquisition and expression of stimulus associations. This does not seem to be the case for the bed nucleus of the stria terminalis because lesions of this structure fail to block changes in behaviour produced by prior aversive conditioning.

It is considerably less clear, however, why sustained activation of the central nucleus of the amygdala via a very bright light source does not seem to be involved in light-enhanced startle. Similarly, the prolonged increase of startle produced by intraventricular administration of CRH also did not seem to involve the amygdala but instead the bed nucleus of the stria terminalis. It is possible, therefore, that in addition to differences between the two structures as they relate to prior classical conditioning, differences in the ability of neural networks in the two areas to respond in a sustained way to sensory activation could also explain the differences between these two areas in fear-potentiated startle versus light-enhanced startle. For example, perhaps the amygdala is especially able to respond to the onset of an aversive stimulus but then rapidly adapts to such activation so as to be prepared for a subsequent presentation of another aversive stimulus. On the other hand, the bed nucleus of the stria terminalis may be arranged in such a way that networks within this nucleus can respond in a much more sustained way to aversive stimulation leading to long-lasting changes in various behavioural responses via projections from the bed nucleus of the stria terminalis to different target areas in the hypothalamus and brainstem. This could also explain why the bed nucleus is more prominently involved in the excitatory effects of CRH on startle compared to the central nucleus of the amygdala. That is, the long-lasting behavioural effects of CRH given intraventricularly would require activation of a structure that could respond in a continuous fashion to receptor occupation by CRH compared to a structure that could only respond in a phasic way. In fact, it might make sense to have separate brain areas respond phasically and tonically to aversive stimulation so as to be able to register both the immediate onset of an aversive experience as well as its prolonged presence,

while maintaining a system such as the amygdala to allow responding in a phasic way to another aversive stimulus. Otherwise, if the 'fear system' were completely saturated, then a subsequent presentation of a threatening stimulus might not be fully processed, severely comprising survival of the organism.

We suggest, therefore, that the bed nucleus of the stria terminalis may be a system that responds to signals more akin to anxiety than those akin to fear, whereas the amygdala is clearly involved in fear and perhaps not as much in anxiety (figure 15). Both these structures have very similar efferent connections to various hypothalamic and brainstem target areas known to be involved in specific signs and symptoms of fear and anxiety (cf. Davis 1992b). Both receive highly processed sensory information from the basolateral nucleus of the amygdala and hence are in a position to respond to emotionally significant stimuli. CRH is known to be released during periods of stress or anxiety, some of which may come from CRH-containing neurons in the amygdala, which project to the bed nucleus of the stria terminalis and act on CRH receptors in the bed nucleus of the stria terminalis. Thus, phasic activation of the amygdala by certain stressors could lead to a long-term activation of the bed nucleus of the stria terminalis via CRH. If so, then compounds that specifically block CRH receptors in the bed nucleus of the stria terminalis might be especially effective in the treatment of anxiety, while leaving the fear response largely intact. Indeed, as our colleague George Heninger once remarked 'One would like to develop drugs to reduce anxiety but not prevent

you from jumping out of the way of the on coming car'. CRH antagonists designed to specifically bind to receptors in the bed nucleus of the stria terminalis might be especially effective in this regard.

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