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RAPID COMMUNICATION

Fear-Potentiated Startle Elevates Catecholamine Levels in the Dorsomedial Hypothalamus of Rats

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SHEKHAR, A., J. S. KATNER, W. P. RUSCHE, T. J. SAJDYK AND J. R. SIMON. *Fear-potentiated startle elevates catecholamine levels in the dorsomedial hypothalamus of rats.* PHARMACOL BIOCHEM BEHAV **48**(2) 525–529, 1994.—The norepinephrine (NE), dopamine (DA), and serotonin (5-HT) systems are thought to be important in the development of anxiety and stress. The dorsomedial hypothalamus (DMH) of rats has been implicated in the regulation of physiological and behavioral responses associated with fear and anxiety. In order to elucidate the interactions between the monoamine systems and the DMH, we studied the effects of subjecting rats to the fear-potentiated startle test, a commonly used test of anxiety in rats, on the NE, DA, and 5-HT levels in the DMH. Rats in the potentiated startle test, but not those exposed to just foot shocks or acoustic startle, showed significantly higher levels of NE and DA in the DMH compared to cage controls. In contrast, foot shocks significantly elevated the 5-HT levels in the DMH.

Anxiety Norepinephrine Dopamine Serotonin Conditioned fear

ANXIETY disorders are the most common psychiatric disorders, affecting as many as 10% of the population in the United States. While the neural substrates that lead to chronic anxiety are still poorly understood, several animal models have been proposed to study anxiety responses. The fear-potentiated startle test is one such model that has been extensively studied (4). The potentiated startle has a number of advantages, such as a short training period, lack of any shock during testing, and the test being nonoperant in nature as well as empirically validated for a number of human anxiolytic and anxiogenic drugs. The basic neural pathways involved in this "startle circuit" have been delineated, and the amygdala appears to be a major forebrain site that regulates the potentiated startle response (10).

We have studied another area of the forebrain, the dorsomedial hypothalamus (DMH), which also appears to be an important site of regulation of anxiety responses in rats. Blocking γ-aminobutyric acid ($GABA_A$) receptor function in the DMHs of rats elicits increases in heart rate, respiration, and blood pressure (6,20), as well as behavioral effects such as increased locomotor activity (21); increased "fear" responses in a Sidman avoidance schedule (22); and increased "anxiety" in the conflict (23), elevated plus-maze (20), and social interaction (Shekhar and Katner, unpublished) tests. Similar physiological responses have been elicited by injecting excitatory amino acid receptor agonists into the DMH (7).

A number of other neuroanatomical sites and neurotransmitter systems have also been implicated in the generation of anxiety responses. Several studies have suggested that activation of the locus ceruleus and increased norepinephrine (NE) release is important in the generation of anxiety responses (3,17). The ascending serotonin (5-HT) terminals from the raphe nuclei are considered to be another essential system in the generation of anxiety responses (15). Stimulation of the

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raphe nuclei has been reported to elicit behavioral signs of stress in rats (8). Both the locus ceruleus and the raphe nuclei have extensive projections to the DMH (24). Activation of the mesolimbic dopamine (DA) pathways during stress and classical conditioning are also well documented (9,19,26).

Although a number of neuroanatomical regions have been suggested as being important in regulating anxiety in rats, the specific interactions between these diverse CNS regions are still unclear. It has been demonstrated that anxiety and stress increase the activity of the locus ceruleus (1) as well as increase NE turnover (27) and NE release (26) in a number of forebrain sites including the hypothalamus. Similarly, there is increased DA release in the limbic areas during stress (19). Therefore, to further understand the role of the ascending monoaminergic projections to the DMH in anxiety and stress responses, we studied the changes in the monoamine levels in the DMHs of rats elicited by the fear-potentiated startle response.

METHODS

Experiments were conducted on male Sprague-Dawley rats (275–325 g) housed in individual cages in a temperature-controlled (72°F) room with a 12-h light/dark cycle and given ad lib food and water. The training and testing protocol for the potentiated startle test was as follows. The startle apparatus (San Diego Instruments) consisted of a pressure-sensitive startle chamber with floor grids to deliver foot shocks. This chamber was placed in a sound-attenuated box equipped with a light and sound source. The apparatus was controlled by a desktop computer. All startle responses, measured as arbitrary units of force generated by the animal's whole body contraction, were fed directly into the computer and stored on disks. The startle response recorded for each trial was an average of the force generated during the 100-ms interval following the stimulus. The rats were given two training sessions separated by 24 h. Each training session consisted of 15 1.0-mA foot shocks (unconditioned stimulus) paired with a light (25 W; conditioned stimulus). After 10 min of acclimatization to the chamber with a background white noise of 85 dB, the light was turned on in the chamber 3200 ms prior to the shock and remained on during the 500-ms foot shock. This 3700-ms sequence was repeated at 20-s intervals 15 times during each training session. The rats were placed in the potentiated startle test 48 h after the last training session. During testing, the startle response was elicited by an auditory stimulus (110 dB

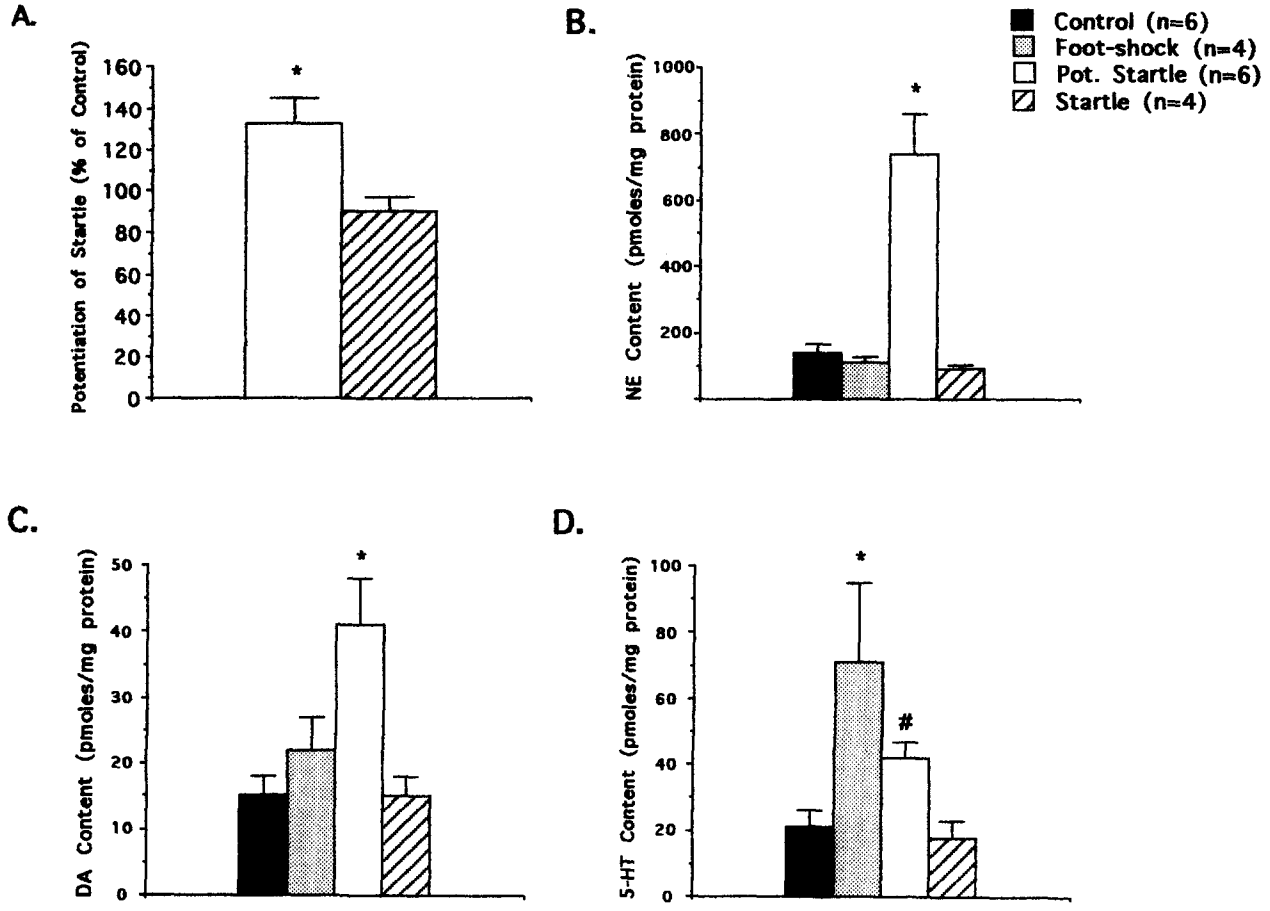

FIG. 1. Summary of the behavioral responses and the monoamine levels in the dorsomedial hypothalamus (DMH) of rats. All data are presented as means ± SEMs. (A) Potentiation of startle by the presence of light (conditioned stimulus) in the trained (light previously associated with foot shocks; potentiated startle) and the untrained (startle) group of rats. Total norepinephrine (NE) (B), dopamine (DA) (C), and serotonin (5-HT) (D) content in the DMHs of the rats in the four experimental conditions of cage controls (Control), two sessions of 10 foot shocks only (Foot-shock), startle testing without training (Startle), and startle testing after conditioned fear training (Pot. Startle). The left and right DMHs were separately assayed and averaged for each rat (n = number of rats). *Significantly different from controls. #Significantly different from startle by analysis of variance coupled with Newman-Keuls test, $p < 0.05$.

white noise over a background noise of 85 dB) in the presence (light–noise, potentiated startle) and absence (noise only, startle) of light. Twenty each of potentiated startle and startle trials were given in a random manner separated by 30-s intervals. The mean startle responses in the potentiated startle and startle trials were obtained, and the difference between them was taken as a measure of anxiety.

The experimental protocol was designed to obtain four groups of rats, consisting of cage controls, rats trained and tested in the potentiated startle test, rats given foot shock only, and rats subjected to acoustic startle only. This design was followed to provide adequate controls to see what effects the fear potentiation of startle would have on the monoamine levels in the DMH when compared to untouched controls, acoustic startle, or foot shocks alone. The four groups of rats were subjected to the following different experimental conditions. Group 1 (control, $n = 6$) was comprised of untrained, control rats that were kept in their home cages for a week. Group 2 (potentiated startle, $n = 6$) had rats that were trained and tested in the potentiated startle paradigm as described above. The rats in group 3 (foot shock, $n = 4$) were subjected only to foot shocks in the two training sessions (i.e., they underwent the training sessions with the light turned off). They were not tested in the potentiated startle paradigm before being sacrificed. The last group of rats (startle, $n = 4$) were placed in the training chamber for 15 min on two days (training program was turned off) and were tested 48 h later in the potentiated startle paradigm without any training.

An additional group of three rats was subjected to two training sessions where they were exposed to 15 unpaired, random shocks and light cues. They were tested in the potentiated startle paradigm described above 48 h after the last training session.

Animals were sacrificed 24 h after completing their respective behavioral experiments. Their brains were removed and immediately placed in a brain slicer (Zivic-Miller, 1-mm sections) and frozen on dry ice. The brain section containing the DMH [6 mm from the occipital pole according to the atlas of Paxinos and Watson (14)] was placed on a dry ice platform. A square area (1 × 1 mm) of tissue immediately adjacent to the dorsal half of the third ventricle (area corresponding to the DMH) and the 1-mm-square area lateral to it (the lateral hypothalamus, LH) were microdissected, weighed, and stored in a −70°C freezer until assayed. The measurement of tissue monoamine levels was conducted using high-performance liquid chromatography (HPLC). Tissue samples were sonicated in 200 μl of 0.5-M perchloric acid and centrifuged in a Fisher Scientific model 235C centrifuge for 5 min, and the supernatant was injected onto the HPLC column. The HPLC consisted of an ISCO Model 2350 pump, an EG&G Princeton Applied Research Model 400 EC detector, a Hewlett Packard HP3396 Series II integrator, and a 3-μm ODS hypersil column (4.6 × 150 mm; Keystone Scientific, Bellefonte, PA). The mobile phase (1800 ml of 0.08-M sodium phosphate buffer, pH 3.1; 150 ml acetonitrile; 300 mg/l 1-octanesulfonic acid; and 800 μl of 0.1-M ethylenediaminetetraacetic acid [EDTA]) was pumped at 1 ml/min, and the monoamines were detected electrochemically using a glassy carbon electrode set at a potential of 0.7 V. The centrifuged pellet was dissolved in 0.1-M NaOH and the protein content of the tissue was measured (13). The total tissue contents of NE, DA, and 5-HT were calculated and expressed as pmol/mg of protein.

RESULTS

The enhancement of the startle responses in the potentiated startle and the acoustic startle groups by the presence of light in the chamber are summarized in Fig. 1A. As expected, only the potentiated startle group showed a significant enhancement of the acoustic startle response when the light was on in the test chamber. The acoustic startle group of rats that had not acquired the conditioned fear response showed no potentiation of startle.

The results of the tissue levels of NE, DA, and 5-HT in the DMHs of rats subjected to the different behavioral conditions are summarized in Fig. 1B–D. The NE contents in the DMHs in the foot shock and startle groups were not significantly different from the control animals. In contrast, the rats subjected to the potentiated startle had a dramatic, three- to four-fold increase in the NE content in their DMHs (Fig. 1B).

The DA levels in the DMHs of the four groups of rats also showed a similar pattern of changes (Fig. 1C). The DA contents in the DMHs of foot shock and acoustic startle groups were not significantly different from the control rats, while the DMHs of rats in the potentiated startle group had a significant increase in their DA content (Fig. 1C).

The changes in the 5-HT content in the DMHs of the four groups of rats are summarized in Fig. 1D, and the pattern of change in 5-HT levels is quite different from NE and DA levels. Only the foot shock group had tissue 5-HT levels in the DMH significantly higher than the control rats. The potentiated startle group showed a significant increase in the 5-HT levels compared to the acoustic startle group but not compared to the control group. The 5-HT content in the DMHs of the acoustic startle group did not significantly differ from control 5-HT levels.

The rats that were exposed to unpaired, random shocks and light cues did not show significant elevation of either NE (125.4 ± 32.0 pmol/mg of protein, $n = 3$) or DA (9.1 ± 2.0 pmol/mg of protein, $n = 3$) in the DMH.

The NE and DA levels in the LHs of the control and potentiated startle groups of rats are summarized in Table 1. There were no significant changes in the NE and DA levels in the LHs of the potentiated startle group compared to the controls. Serotonin levels were lower than the detectable limit in the LHs in both the control and potentiated startle groups.

TABLE 1

THE TOTAL TISSUE LEVELS (mean ± SEM) OF NOREPINEPHRINE (NE) AND DOPAMINE (DA) IN THE LATERAL HYPOTHALAMUS (LH) OF RATS SUBJECTED TO DIFFERENT BEHAVIORAL CONDITIONS

Behavioral Condition	NE Levels (pmol/mg protein)	DA Levels (pmol/mg protein)
Control ($n = 4$)	63 ± 11	77 ± 14
Potentiated startle ($n = 3$)	49 ± 13	34 ± 16

The left and right DMHs were separately assayed and the results were averaged for each rat (n = number of rats).

DISCUSSION

The data presented above clearly indicate some dramatic changes in the monoamine levels in the DMH 24 h after the rats were subjected to different behavioral experiments. In the potentiated startle test the rats are exposed to a conditioned fear paradigm without employing any painful foot shocks during the test. Rats placed in this test show a significant increase in both the NE and DA levels in the DMH. Being startled by an acoustic stimulus or receiving painful foot shocks by themselves do not elicit these neurochemical changes. Rats

that received random, unpaired shocks and light cues during training also failed to show the elevation in catecholamine levels in the DMH. It appears that the conditioned fear response selectively increases catecholamine levels.

In contrast to the catecholamines, the increases in 5-HT levels seem to be associated with the experience of the painful foot shock. The foot shock group showed a significant increase in the 5-HT level compared to controls. The rats in the potentiated startle group also received foot shocks during the training but did not show a significant increase in 5-HT levels compared to controls. However, the 5-HT levels in the potentiated startle group were significantly different from those in the startle group. The startle group is perhaps a more appropriate comparison group for the potentiated startle rats. Thus, both groups of rats that received foot shocks may in fact be exhibiting an increase in 5-HT levels compared to their appropriate comparison groups.

While the increases in the catecholamines in the DMH are seen in the fear-potentiated startle test, other explanations for these effects are possible. One possibility is that rats responding in the fear-potentiated startle show greater motor response during the startle test compared to the untrained or sham-trained animals. This enhanced motor response and not the fear could be resulting in these neurochemical changes in the DMH. Further, neurochemical measures were done at one time point of 24 h after testing in the startle paradigm. A stronger case for associating these neurochemical changes with anxiety can be made if the time course of the catecholamine increases parallels the development and extinction of the fear-potentiated startle response.

In view of the fact that only monoamine content was determined in the present study, it is difficult to speculate whether there is an actual increase in the synthesis or a decrease in the release of these monoamines. Therefore, further studies are needed to clarify the functional significance of these neurochemical changes. However, there is evidence that uncontrollable stress (and not controllable stress) specifically increases the activity of the locus ceruleus and the release of NE in its projection areas, including the hypothalamus (25,27). These changes in NE were blocked by pretreatment with the anxiolytic drug diazepam (11). The fear-potentiated startle paradigm is a model of anxiety that utilizes a conditioned fear response to an uncontrollable stressor. A selective increase in the catecholamines in the DMHs of rats reported here is consistent with these studies. Further, stimulation of the locus ceruleus is known to enhance memory (5) and has been associated with the development of traumatic memories (3). Therefore, the fear-potentiated startle test, which involves learning associated with aversive foot shocks, could activate the ascending NE fibers.

There is a variety of evidence suggesting a connection between the hypothalamus and the ascending brain stem monoamine projections (2). The autonomic responses elicited by stimulation of the locus ceruleus can be modified by hypothalamic influences (16), and hypothalamic defensive responses elicit overall changes in brain NE levels (18). Similarly, 5-HT projections to the forebrain centers have been implicated in the CNS responses to stimuli such as stress, fear, and pain (15). The amygdala, the forebrain site that is most notably associated with the potentiated startle response (4), has clear projections to the hypothalamus (2,12). The results of the present study support the hypothesis that the DMH and its monoamine afferents may be involved in the development of conditioned fear and "anxiety" in rats.

In summary, this study reports that, following the potentiated startle test, dramatic neurochemical changes take place in the DMH, an area in the CNS that has been previously implicated in the regulation of experimental anxiety in rats.

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