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13. ABSTRACT *(Maximum 200 words)*

Cerebral noradrenergic systems and their interactions with corticotropin-releasing factor (CRF) in stress-related responses were studied. 1. Microdialysis studies indicated that hypotension and footshock increased release of hypothalamic and cortical norepinephrine (NE). 2. A new voltammetric probe was designed to provide voltammetric data that a hypotensive agent, sodium nitroprusside, increases cortical NE secretion. 3. CRF infused into the locus coeruleus (LC) but not into surrounding structures, such as the parabrachial nucleus, increased secretion of cortical NE, supporting the notion that CRF affects the activity of LC-NE neurons. 4. Peripheral administration of interleukin-1 (IL-1) known to activate cerebral noradrenergic systems increased extracellular NE in the hypothalamus, and this activation was implicated in the induction of Fos protein in the hypothalamic paraventricular nucleus. 5. Chlordiazepoxide, administered systemically or locally decreased the secretion of NE induced by footshock and CRF. 6. Activation of noradrenergic systems by idazoxan or treatment with propranolol attenuated footshock-induced freezing and ultrasonic vocalization. Idazoxan did not alter the acquisition of conditioned fear, but it depressed the expression of that behavior. 7. 6-Hydroxydopamine lesions of the dorsal noradrenergic bundle did not consistently alter stress-induced behavioral patterns.

14. SUBJECT TERMS

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CEREBRAL NEUROCHEMICAL MECHANISMS IN STRESS AND ANXIETY

Final Technical Report - February 1, 1993 - June 30, 1996

I. INTRODUCTION

Stress and anxiety have a major impact on human performance. Our limited understanding of anxiety disorders suggests that a variety of different central nervous system (CNS) mechanisms may be involved. Stressful stimuli are associated with activation of the hypothalamic-pituitary-adrenal (HPA) axis which many have used to define a stress and concomitants activation of catecholaminergic system (the sympathetic nervous system, the adrenal medulla, and cerebral catecholaminergic systems). These systems are regarded to have complementary actions, but the relationships between them are poorly understood. In previous work, we and others have demonstrated that intracerebral injections of corticotropin-releasing factor (CRF) can mimic many of the endocrine, physiological, neurochemical and behavioral responses observed in stress (Dunn and Berridge, 1990). However, the CNS noradrenergic system is also known to be activated in stress. Our previous studies have implicated noradrenergic activity in the stress-related changes in different behavioral paradigms. The pharmacological analyses of these stress-related changes suggested that they were mediated primarily by noradrenergic neurons activating CRF-containing neurons. This sequential activation of noradrenergic and CRF systems rationalizes the coactivation of these two systems which may have complementary functions in the brain during stress and, possibly, influence each other reciprocally.

The experiments were concerned with the cerebral mechanisms involved in stress. The focus was on the HPA axis and locus coeruleus noradrenergic system (LC-NE) and its interaction with CRF. Effects of several different kinds of stressors (physical, psychogenic, immune) were examined in laboratory rats.

II. EXPERIMENTS AND RESULTS

A. Neurochemical responses

1. Sodium nitroprusside infusions activate cortical and hypothalamic noradrenergic systems in rats.

To demonstrate the sensitivity, and therefore the suitability, of *in vivo* microdialysis for studies of cerebral norepinephrine (NE) release in stress, the responses to acute hypotension (a hemodynamic stressor) induced by intravenous infusion of sodium nitroprusside (NP) was examined. NP treatment is known from electrophysiological data to activate the locus coeruleus noradrenergic neurons (LC-NE). In anaesthetized rats, iv infusion of NP caused a rapid decrease in blood pressure which lasted for the duration of the infusion but rapidly reversed when the infusion was terminated. It also enhanced the apparent release of NE in two LC projection fields, the medial prefrontal cortex (PFM) and medial hypothalamus. The increase in extracellular NE in the hypothalamus (67%) was greater than that in the PFM (41%). The increase in extracellular NE in both regions had returned to baseline in the first microdialysate sample after cessation of NP infusion. It is concluded that microdialysis is sensitive enough to monitor physiologically induced changes in the activity of the cerebral NE system. Also, activation of the LC results in a neurochemical response in prefrontal cortex. The observed

activation of the cortical NE system reflects activation of the LC-NE neurons and may be responsible for the behavioral responses in stress. It is believed that the hypothalamic NE system is activated as a part of a larger system responsible for the regulation of blood pressure, and its activation may reflect an autonomic component of the organismic response to stress.

The data were presented at the 1993 Annual Meeting of the Society for Neuroscience (Smagin, Swiergiel, Brown, and Dunn, 1993) and published in *Neuroscience Research Communications* (Smagin, Swiergiel, and Dunn, 1994b).

2. Cortical catecholamine secretion following intravenous nitroprusside infusion: studies with *in vivo* voltammetry.

Experiments like those described above but using *in vivo* voltammetry were performed. Using *in vivo* voltammetry, a real-time method of estimating changes in extracellular concentration of catecholamines, enables monitoring the time relationship between the NP-induced changes in blood pressure and the activity of the cortical noradrenergic system.

As in our previous experiments, iv administration of NP rapidly decreased blood pressure, but this rapidly reversed when the infusion was terminated. After a delay of between about 2 and 8 minutes (mean 5 min), there was an increase in extracellular concentrations of a NE-like substance. Presumed cortical release of NE lasted for several minutes but had almost returned to baseline by the time the NP infusion was terminated at 15 minutes. In many cases, the first peak was followed by a second one, usually of smaller amplitude but more prolonged than the

first one. There was no clear response to the cessation of infusion of NP. The initial response is comparable to the previously reported electrophysiological response of LC-NE neurons to NP. These results suggest that activation of the NE-LC neurons by NP results in a delayed synaptic release of NE in the cerebral cortex which rapidly attenuates. Local administration of idazoxan produced voltammograms showing peaks similar to the first peak, suggesting that the recorded oxidation current was related to release of NE (at least for the first peak). The voltammetric experiments confirm the results of the microdialysis studies and suggest that NP activates the noradrenergic system for a rather short period of time. However, the delayed voltammetric peaks may not be attributable to NE.

The present results suggest that reduction of blood pressure by nitroprusside infusion results in a delayed noradrenergic response of cortical synaptic terminals; an initial rapid secretion of NE followed by a smaller and longer lasting release of NE or some other electroactive compound The system may function so that the cortex is cognizant of changes in peripheral physiology.

A manuscript has been submitted to *Brain Research Bulletin* ((Swiergiel, Palamarchouk, and Dunn, 1997a), copy attached).

3. *In vivo* voltammetric measurements of exogenous NE clearance in medial prefrontal cortex of DSP-4-treated rats.

DSP-4 is a potent and selective neurotoxin of central noradrenergic neurons. In rodents, a single systemic injection of DSP-4 produces long-lasting reductions in NE concentrations and NE uptake capacity in brain.

The reductions in NE and the loss of NE uptake sites are far more extensive in regions innervated by NE axons of the locus coeruleus than in regions supplied by non-coerulear noradrenergic cells. The aim of the experiments was to estimate *in vivo* contribution of synaptic uptake to the decrease in NE concentration in the prefrontal cortex of rats.

Adult Sprague-Dawley rats were injected with DSP-4 (50 mg/kg, ip). 72 hours later they were anesthetized with urethane and carbon fiber electrodes attached to micropipettes implanted into the prefrontal cortex. Local applications of NE (500 μM) were performed by pressure ejection of 10 to 200 nl of NE solution (doses of 0.5-10 pmol of NE). Rapid chronoamperometric measurements of the exogenous NE oxidation current were performed. The rate of clearance of NE from vicinity of the carbon electrode is reflected by an exponential decline in oxidation current. Three major phenomena determine the rate of clearance of locally applied exogenous NE in brain: synaptic uptake, catabolism and diffusion.

The results showed that the rate of clearance of exogenously applied NE was substantially slower in DSP-4-treated animals than in the control ones. The mean rate constant describing the clearance of exogenous NE from the medial prefrontal cortex was 14.9 ± 1.5 sec^{-1} in intact and 8.5 ± 1.4 sec^{-1} in DSP-4 treated rats ($p < 0.01$) It is concluded that synaptic uptake of NE is largely dependent on intact axonal terminals. The decreased uptake delays return of the NE level to a baseline extracellular concentration.

The data were presented at the 1995 Annual Meeting of the Society for Neuroscience ((Swiergiel, Palmartchouck, and Dunn, 1995), copy attached).

4. A new design of carbon fiber microelectrode for *in vivo* voltammetry using fused silica.

In the course of the above experiments a new construction method for carbon fiber voltammetric microelectrodes was developed. Electrodes (fused silica, FS type) were constructed from stainless steel and fused silica tubing ensheathed carbon fiber. Electrochemical tests were carried out to compare these electrodes with commercially available glass-sealed, IVEC-5 electrodes.

Electrodes of both types displayed similar descending baseline curves and calculated coefficients of stabilization (the tangent of baseline during a stable period). There were no significant differences in sensitivity between the two designs of electrodes to NE and dopamine (DA). All tested electrodes showed linear characteristics of current dependent on concentration of NE and DA with a correlation coefficient greater than 0.995. FS electrodes have been suitable for electrochemical measurements (*in vivo* voltammetry) and have similar or better characteristics than commercially available glass carbon fiber micro-electrodes. FS electrodes can easily be made in the laboratory and do not require any special equipment (such as a micropipette puller) or skills. An additional piece of fused silica can be glued to the electrode for micro-injections. The electrodes are very robust, easy to handle and can be mounted on the standard arm of a stereotaxic frame. The electrodes can be made very long to reach the deepest parts of the brains of large animals.

The method has been accepted for publication in the *Journal of Neuroscience Methods* ((Swiergiel, Palamarchouk, and Dunn, 1997b).

5. Corticotropin-releasing factor administered into the locus coeruleus but not the parabrachial nucleus stimulates norepinephrine release in the prefrontal cortex.

Several studies have indicated that intracerebroventricular application of CRF activated noradrenergic neurons in the brainstem LC (Valentino, Foote, and Aston-Jones, 1983) and NE metabolism in several brain regions ((Dunn and Berridge, 1987; Lavicky and Dunn, 1993). To assess whether CRF has direct effects on LC noradrenergic neurons, CRF was infused into the LC and concentrations of NE and its metabolites were measured in microdialysates collected from the medial prefrontal cortex (PFM). Infusion of 100 ng of CRF into the LC significantly increased dialysate concentrations of NE and of its catabolite, MHPG, in the ipsilateral PFM, whereas no significant changes were observed following infusion of artificial cerebrospinal fluid). No response was observed when the infusions of CRF occurred outside of the LC, including infusions in the parabrachial nucleus. Although CRF administered into the LC slightly increased dialysate concentrations of NE in the contralateral PFM, this effect was not statistically significant. The effect of CRF injected into the LC on dialysate NE was prevented by combination with a tenfold excess of the CRF antagonist, alpha-helical CRF_{9-41}, indicating some specificity in the response. These results are consistent with anatomical and electrophysiological evidence suggesting that CRF may directly activate noradrenergic neurons in or close to the LC.

The results were presented at the 1994 Annual Meeting of the Society for Neuroscience (Smagin, Swiergiel, Guerin, and Dunn, 1994) and The First World Congress on Stress and have been published in *Brain Research Bulletin* (Smagin, Swiergiel, and Dunn, 1994a) (copies attached).

6. Peripheral administration of interleukin-1 increases extracellular concentrations of norepinephrine in rat hypothalamus: comparison with plasma corticosterone.

Independent work in our laboratory has shown that interleukin-1 (IL-1) is a potent activator of cerebral NE systems (Dunn, 1988). The noradrenergic activation is much more pronounced for the hypothalamus (innervated by brain stem nuclei A1 and A2), than other forebrain regions (innervated by A6 (LC)). Therefore, we have used IL-1 as a tool to activate cerebral NE systems. A remarkable parallel occurs between the behaviors observed in sickness and those elicited by peripheral administration of IL-1. Administration of IL-1 activates the hypothalamo-pituitary-adrenocortical (HPA) axis and NE metabolism in the hypothalamus and may be considered as a model of an immune stressor. Because changes in cerebral noradrenergic activity may be important in the IL-1-induced behavioral responses in stress, the release of hypothalamic NE using *in vivo* microdialysis following intravenous (iv) or intraperitoneal (ip) injection of human IL-1β was studied. The results indicate that extracellular concentrations of NE in the hypothalamus increased following iv and ip administration of IL-1β. The elevation was more rapid following iv IL-1β, and reached a peak at 1 hour, whereas the slower and smaller increase following ip IL-1β did not reach a peak until 2 hours. The results were similar in both anesthetized and unanesthetized rats. These changes paralleled increases in plasma concentrations of corticosterone in freely moving unanesthetized rats. The results demonstrated that the cerebral noradrenergic system is activated by an immune factor and are consistent with the possibility that a central noradrenergic mechanism

mediates the activation of the HPA axis by peripherally administered IL-1β.

The results were published in *Psychoneuroendocrinology* (Smagin, Swiergiel, and Dunn, 1996) (copy attached).

7. The role of cerebral noradrenergic system in the Fos response to interleukin-1.

Peripheral administration of IL-1 has been shown to activate induction of Fos protein in the brain, but the mechanism is not known. Because cerebral noradrenergic system have been implicated in Fos induction and we have shown that IL-1 potently activates cerebral NE in the hypothalamus, the IL-1-induced appearance of Fos in mice pretreated with the noradrenergic neurotoxin, 6-hydroxydopamine (6-OHDA) was studied. It was observed that IL-1 induced Fos protein appearance in several regions of the brain, but most markedly in the paraventricular nucleus of the hypothalamus (PVN). This response was substantially reduced in animals pretreated with 6-OHDA. The results further implicate central noradrenergic system in the activation of the HPA axis, because the PVN contains the cell bodies of CRF-containing neurons concerned with the activation of the HPA axis.

The data were presented at the 1995 Annual Meeting of the Society for Neuroscience (Dunn, Swiergiel, and Stone, 1995) and have now been published in *Brain Research Bulletin* (Dunn, Swiergiel, and Stone, 1996) (copies attached).

8. Effects of repeated footshock on overflow of hypothalamic norepinephrine in freely moving rats.

Footshock augments the release of catecholamines in the hypothalamus. The study examined the effects of repeated footshock on release of hypothalamic NE. Rats were subjected to two 20-minute sessions of footshock (60 x 0.1-0.2 mA) separated by 100 min and the NE concentrations were measured in microdialysates collected over subsequent 20 min periods. After the first footshock session the concentration of NE in the dialysate samples was augmented by 64% ($p < 0.01$) in comparison with baseline. After the second footshock session the increase reached 313% of baseline ($p < 0.05$) of the baseline preceding the first stress session, and 251% ($p < 0.05$) of the value immediately preceding the second footshock session. It is concluded that the NE response to footshock may be potentiated by a previous period of footshock. (Figure 1 attached).

9. Effects of chlordiazepoxide on stress-induced overflow of cerebral norepinephrine.

Synaptic release of cerebral catecholamines is increased during stress. Benzodiazepines, such as chlordiazepoxide (CDP), are the most commonly used anxiolytic drugs. The effects of the benzodiazepines on stress-related release of catecholamines have been controversial. The effect of CDP pretreatment on the footshock-induced release of cerebral NE was examined. Freely moving rats were implanted with microdialysis probes in the medial hypothalamus and the medial prefrontal cortex (PFM). Footshock (60 x 0.1-0.2 mA shocks in 20 min) significantly increased microdialysate concentrations of NE in the first sample collected after

initiation of footshock. In the hypothalamus, dialysate NE was augmented 50% above baseline, and in the PFM by 143%. CDP administration (5 mg/kg ip) had no statistically significant effects on the basal dialysate concentrations of NE, although there was a tendency towards a reduction. CDP administered one sample before the footshock attenuated the increased dialysate concentrations of NE in footshock animals. These results suggested that footshock increased the synaptic release of NE in the cortex and hypothalamus, and that the response could be attenuated by CDP. The experimental design used could not distinguish whether CDP altered the input to LC noradrenergic neurons, or whether the benzodiazepines exerted a direct effect on the noradrenergic neurons. Interestingly, when CDP was included in the solution used to perfuse the microdialysis probe, 1-5 mM decreased the footshock-induced increase in NE concentrations, suggesting the possibility of a local action of CDP at the noradrenergic terminals.

The results were presented at the 1994 Society for Neuroscience Meeting (Swiergiel, Wei, Li, and Dunn, 1994) (copy attached).

10. Chlordiazepoxide attenuates overflow of extracellular NE in prefrontal cortex caused by CRF infused into LC.

In an earlier experiment, it was found that in anaesthetized rats administration of CRF into LC increased the concentration of NE in microdialysates collected from the prefrontal cortex. Those results were replicated in the experiment in which the effect of a benzodiazepine on the noradrenergic response was tested. Preliminary results indicated that the response could be attenuated by pretreatment with peripherally administered chlordiazepoxide (CDP, 5 mg/kg ip). (Figure 2 attached).

B. BEHAVIORAL RESPONSES IN STRESS

Several behavioral studies using different animal models of anxiety were used.

11. Effects of lesions of the dorsal noradrenergic ascending bundle (DNAB) on shock-induced freezing and vocalization in rats.

NE is known to play a role in fear and anxiety. The main evidence came from the effects of yohimbine that acts mainly at α_2-adrenoreceptors. Yohimbine causes anxiety in humans (Charney et al., 1983). Furthermore, several lines of physiological and pharmacological evidence led to the proposal that the locus coeruleus, the main noradrenergic nucleus in the brain stem, is a mediator of anxiety and its behavioral manifestations (Redmond, 1979). Adult rats received bilateral infusions of 6-OHDA into the dorsal noradrenergic ascending bundle (DNAB) that resulted in almost complete depletions of cortical and hippocampal NE. The following behavioral patterns were observed after the lesions: 1. air-puff-induced freezing and ultrasonic vocalization (non painful); 2. electric footshock-induced freezing and vocalization (painful); 3. conditioned freezing and vocalization (non-painful); 4. exploratory behavior in elevated plus-maze; 5 withdrawal behavior and exploration in an open field provided with a refuge. The above behavioral patterns are sensitive to a variety of anxiolytic and anxiogenic compounds that may interact with the brain noradrenergic system. However, no significant effects of the DNAB lesions on the observed behaviors were found. Ultrasonic vocalization was potentiated in the DNAB rats but not to

an extent that would suggest any significant role of the ascending NE system in this response. The study is to be repeated.

Results were presented at the 1993 Annual Meeting of the Society for Neuroscience (Swiergiel and Dunn, 1993) (copy attached).

12. Effects of idazoxan and chlordiazepoxide on stress-induced freezing and ultrasonic vocalization.

The effects of idazoxan (1.25, 2.5 and 5 mg/kg) and CDP (2.5 and 5 mg/kg) on stress-induced freezing and ultrasonic vocalization were studied. Idazoxan is a potent and relatively specific α_2-adrenergic antagonist. It increases the activity of the noradrenergic system by accelerating the discharge rate of the LC-NE neurons and augmenting synaptic release of NE. There is widely accepted neuroanatomical, neurochemical, neurophysiological and behavioral evidence suggesting that the development of anxiety or fear is related, in part, to increased brain NE activity. In several experiments the hypothesis that increased noradrenergic activity contributes to the display of fear in several animal models of anxiety was tested. Preliminary results indicated that activation of NE system by idazoxan resulted in almost complete inhibition of stress-induced vocalization, and relatively small changes in stress-induced freezing. Although unexpected, the results agreed with previous experiments in which it had been demonstrated that depletion of the dorsal noradrenergic system with a neurotoxin DSP-4 dramatically enhanced stress-induced vocalization (Swiergiel, unpublished). The experiments are to be completed.

13. Effect of pretreatment with idazoxan on acquisition of footshock-induced freezing.

Several experiments were performed to determine whether pretreatment with idazoxan affects acquisition of a freezing response i.e. display of conditioned freezing on the next day. On the first day, the rats were given idazoxan (1.25 or 2.5 mg/kg) and tested for footshock-induced freezing and vocalization. On the next day, display of conditioned freezing and vocalization was observed.

The results showed that an injection of idazoxan ten minutes before footshock did not change the latency to freeze or the total freezing time upon subsequent re-exposure to the same stressful environment. Thus, idazoxan does not affect (impair) acquisition of footshock-induced conditioned behavior. The experiments are to be completed.

14. Effect of idazoxan on expression of footshock-induced conditioned freezing.

As a sequel to the above study, it was decided to determine whether pretreatment with idazoxan affect the display of conditioned freezing. On the first day, rats were subjected to behavioral tests for footshock-induced freezing and vocalization. On the next day, the rats were given idazoxan (1.25 and 2.5 mg/kg) and the display of conditioned freezing and vocalization was observed.

The results showed that peripheral administration of idazoxan ten minutes before re-exposure to the stressful environment caused a significant decrease in total freezing time. Thus, idazoxan depressed previously conditioned footshock-induced freezing. In both experiments idazoxan did not alter nociception as determined in a hot-plate test.

15. Effects of propranolol on fear responses in rats.

Propranolol has been reported to have anxiolytic properties in several animal models of anxiety (Gorman and Dunn, 1993). The effects of peripheral administration of the β-adrenergic antagonist, propranolol (1 and 2 mg/kg L-propranolol, i.p) on footshock-induced behavioral patterns in adult male rats were studied. Propranolol significantly decreased shock-induced freezing and vocalization. Neither grooming nor the number of fecal boli were significantly affected. The results further implicated β-adrenergic receptors in mediating behavioral responses in stress. They support earlier studies that indicate the ability of propranolol to reverse anxiety-related behavioral responses. The experiments are to be completed.

III. CONCLUSIONS

The experimental results obtained suggest that activation of brain noradrenergic systems can be quantified using *in vivo* microdialysis and *in vivo* voltammetry. There appears to be differential activation of the cortical and hypothalamic noradrenergic systems by different stressors; footshock activates hypothalamic and cortical projections approximately equally, whereas hemodynamic and "immune" stressors (such as interleukin-1) appear to have a more selective activation of the hypothalamic projections. This suggests an obvious distinction between the forebrain projections from the locus coeruleus (A6), as compared to the ventral forebrain projections from brainstem nuclei A1 and A2.

Infusion of corticotropin-releasing factor (CRF) locally into the locus coeruleus activates cortical NE release. This may indicate a functional CRF input to the LC from collaterals of CRF-containing cells in

the hypothalamic paraventricular nucleus (PVN). This pathway may provide a means by which activation of the HPA axis (normally initiated in the PVN) is communicated to the cerebral cortex.

It has frequently been speculated that activation of the LC-NE neurons, which would result in cortical release of NE and desynchronization of the electroencephalogram, may be responsible for the behavioral responses in stress. The results reported here suggest an involvement of noradrenergic systems in the behavioral responses, but have not provided sufficient data to either prove or disprove the notion that cerebral noradrenergic systems are critical mediators of the observed behavioral responses in stress. We consider it important that experiments using well established behavioral models of anxiety be performed in animals with partial or complete noradrenergic lesions and with specific adrenergic antagonists to resolve the issue. It may well be that cotransmitters (e.g., neuropeptides, such as galanin, neuropeptide Y, etc.) that may be colocalized in noradrenergic neurons, play a significant role, and that a full elucidation of the role of the noradrenergic neurons in stress-related behaviors, will require understanding of the role of these peptides and their interactions with NE.

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