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BRIEF COMMUNICATION

Effects of Stress on [^{3}H]Cyclohexyladenosine Binding to Rat Brain Membranes[1,2]

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ANDERSON, S. M., J. R. LEU AND G. J. KANT. *Effects of stress on [^{3}H]cyclohexyladenosine binding to rat brain membranes.* PHARMACOL BIOCHEM BEHAV **26**(4) 829–833, 1987.—The investigation of stress-induced changes in neuronal functioning is important to our understanding of mental disorders, stress-induced psychological impairment, and the emotional reactions of fear and anxiety. Data from previous animal studies have demonstrated various pituitary-adrenal responses to stress and also changes in brain neurotransmitters. We are investigating whether stress-induced neuroendocrine and brain monoamine changes are accompanied by concomitant changes in brain neurotransmitter and/or neuromodulator receptors. We have developed a behavioral paradigm of chronic stress which incorporates sustained stress, continuous performance, and disruption of sleep. Animals which are habituated to press a lever to receive food are trained in an active shock escape task. A matched set of animals housed in identical operant chambers but not exposed to footshock are used as comparative controls. [^{3}H]Cyclohexyladenosine ([^{3}H]CHA) (5–7 nM) binding to A_1 adenosine receptors in hypothalamic membrane preparations from rats stressed for three days was fifteen percent higher than in matched controls. However, no differences in [^{3}H]CHA binding were found in tissue preparations from frontal cortex, hippocampus, or striatum, when comparing stressed and matched control rats. Plasma corticosterone levels were higher in stressed rats than in matched controls.

Stress Receptors Adenosine Receptor modulation Receptor binding

THE body's response to acute intermittent stress is initially advantageous; however, prolonged exposure to stress results in numerous health problems, both physical and mental, leading to deficits in performance, attention, and normal functioning. Some of the behavioral responses to extreme stress include symptoms and functional characteristics of mental disorders and the emotional reactions of fear and anxiety. Accordingly, the investigation of stress-induced changes of neuronal functioning may have an important bearing on the role of stress in the pathophysiology of various psychoses and neuroses. Although much research has been conducted on the response of the pituitary-adrenal axis to stress, much less is known about the relationship between stress and neurotransmitter function, in general, and neurotransmitter receptors, in particular.

A variety of drugs and abnormal concentrations of endogenous substances have been shown to alter receptors for numerous neurotransmitters and neuromodulators [4, 7, 17, 28]. It seems probable then that stress-induced neuroendocrine and brain monoamine changes are accompanied by concomitant changes in brain neurotransmitter and/or neuromodulator receptors. Information on such changes may increase our understanding of the neuropathology of mental illnesses, and also elucidate the stress-induced neurochemical and neurophysiological changes antecedent to a maladaptive stress syndrome.

We are presently investigating stress-induced changes in receptors for endogenous compounds generally regarded as neurotransmitters and also several endogenous neuroactive substances whose role in the central nervous system is less well understood. The putative neurotransmitter/neuromodulator, adenosine, has been shown to play an important

[1]In conducting the research described in this report, the investigator(s) adhere to the *Guide for the Care and Use of Laboratory Animals*, as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

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TABLE 1

SPECIFICALLY BOUND [^{3}H]CYCLOHEXYLADENOSINE KINETIC COMPONENTS

	Higher Affinity "State"		Lower Affinity "State"	
Brain Region	Kd, nM	Bmax, fmol/mg	Kd, nM	Bmax, fmol/mg
Frontal Cortex	0.32 ± 0.08	10.59 ± 1.96	10.00 ± 5.22	14.57 ± 1.55
Hippocampus	0.65 ± 0.23	18.03 ± 8.15	5.37 ± 3.05	30.36 ± 6.50
Striatum	0.80 ± 0.21	18.18 ± 4.80	9.68 ± 5.70	24.04 ± 2.88
Hypothalamus	0.37 ± 0.06	4.83 ± 0.87	7.20 ± 3.12	8.97 ± 0.79

Bmax values are given as fmol per mg tissue (original wet weight). These data are from three separate experiments measuring [^{3}H]CHA (0.045–40 nM) binding to membrane preparations for each brain region using tissue pooled from naive control animals. Values given in the table are maximum likelihood estimates and the standard deviations of those estimates for fitted parameters describing a model of ligand binding to two receptor sites or states. In the generation of parameters of the model giving a least squares-fit solution both mean amount bound and the variance around that mean from three separate experiments was used (for further discussion see [22,53]).

role in the regulation of a variety of physiological processes [3, 13, 42, 49] and has been found in high concentrations in both the peripheral and the central nervous system [9, 30, 42, 48, 55]. A neuromodulatory role for adenosine has been proposed based on the variety of its actions in nervous tissue. Many of these actions tend to decrease the functional activity of neurons either by depressing all firing or by reducing transmitter release [42,49]. Different actions of adenosine are mediated through its interaction with both intracellular sites (P) and extracellular sites (R or A). There are two classes of central extracellular adenosine receptors whose actions have been shown to be mediated through the adenylate cyclase/cAMP system [8, 26, 45, 50, 52]. Stimulatory actions of adenosine on adenylate cyclase occur at micromolar concentrations via A_2 receptors. At A_1 receptors, adenosine in nanomolar concentrations inhibits adenylate cyclase activity [8,56].

Receptors for adenosine have been identified in numerous brain regions by using many different radiolabeled agonists and antagonists of adenosine. Ligand binding to both membrane preparations and slide mounted rat brain tissue sections have been used [5, 46, 58]. The density of A_1 adenosine receptors is greatest in the hippocampus, cortex, striatum, and cerebellum, but they have also been demonstrated in the hypothalamus and brain stem [12, 14, 16, 25, 34, 35, 39, 54]. In this paper we present the results of our examination of A_1 adenosine receptors in the brains of stressed vs. control rats.

METHOD

Animals

Male Sprague-Dawley rats (200–250 g) were purchased from Zivic-Miller and housed individually in a light and temperature-controlled room for at least two weeks prior to use in the behavioral experiment. Lights were on from 0600 to 1800 hr. Prior to use in this experiment food and water were available freely.

Behavioral Paradigm

Rats were placed in operant testing chambers and habituated to lever press for 45 mg food pellets on a FR 1 schedule (fixed ratio 1: one pellet for each lever press). Water was available ad lib and lights were on in the boxes from 0600 to 1800 hr. After three days in the operant chambers, half of the animals were trained to pull a ring (suspended from the ceiling) in response to a shock delivered through the wire grid floor. Following this training these experimental rats were exposed to escape trials for three days. Escape trials were delivered on a variable time schedule with an average intertrial interval of five min (minimum of one min and maximum of nine min between shock series) 24 hours per day. An escape trial series consisted of five seconds of increasing footshock intensity at each of five levels (0.16, 0.32, 0.65, 1.3, 2.6 mA). Eighty percent of the trials could be terminated during the trial by pulling a ceiling chain. Twenty percent of the trials were inescapable. A matched set of animals housed in identical operant chambers but not exposed to footshock were used as comparative controls. All lever presses for food, shock trial escapes, and random chain pulls were recorded using a DEC PDP8 computer. Due to equipment limitations only 3–4 experimental animals and 3–4 matched controls could be tested during each experimental session. Furthermore, rats which did not learn to press the lever for food or to escape the shock were eliminated from the experiment. In order to obtain a reasonable sample size, behavioral, blood hormone, and biochemical data were gathered on three separate occasions in identical test runs which were conducted over a period of several months. The data presented here are values pooled from those three separate runs. Values for body weight, food intake, and plasma hormone levels are means±SEM. Daily fluctuations in plasma hormone levels resulted in large inter-experimental variation among control values, therefore, raw data scores were converted to percent control prior to data analysis.

Biochemical Assays

All animals were killed between 0930 and 1130 hr to minimize the effects of circadian variation. Stressed animals and matched controls were decapitated; trunk blood samples were collected; and brains were quickly removed and dissected into discrete brain regions (frontal cortex, striatum, hippocampus, and hypothalamus). All plasma samples were frozen and stored until a later date at which time they were assayed for corticosterone and prolactin by radioimmunoassay [23,33]. Brain regions were homogenized and washed twice by Polytron and centrifugation at 40,000 × g. Mem-

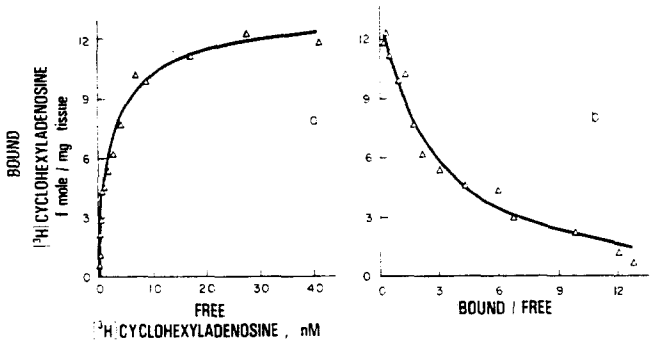

FIG. 1. (a) Binding of 0.045–40.0 nM [³H]CHA to rat hypothalamic membranes shows two nanomolar affinity binding "sites" or "states." (These data are the mean values from three experiments, with each sample measured in triplicate, and non-specific binding defined with 10 μM 2-chloroadenosine.) (b) Eadie-Hofstee plot of data in (a). These data are compatible with a two "site" or "state" receptor model described by tissue parameters: Kd_1=0.37 nM, $Bmax_1$=4.83 fmol/mg tissue; Kd_2=7.20 nM, $Bmax_2$=8.97 fmol/mg tissue.

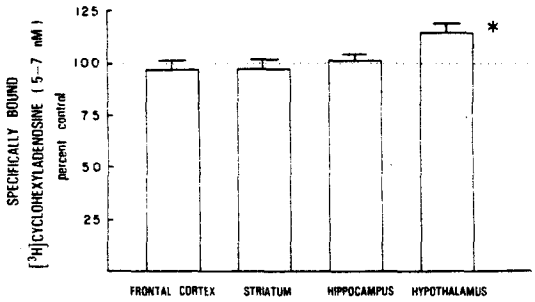

FIG. 2. *Significantly different from control, t-test, $p<0.05$. Alteration of 5–7 nM [³H]cyclohexyladenosine binding to neuronal membranes from specific regions of rat brains from stress animals. Values are mean±SEM of pooled data from three separate experiments (n=9). Control values are 21.24±0.53 fmol/mg tissue to frontal cortex; 24.11±0.75 fmol/mg tissue to striatum; 36.35±1.40 fmol/mg tissue to hippocampus; and 13.17±0.87 fmol/mg tissue to hypothalamus.

branes were incubated with adenosine deaminase (1 unit/20 mg of tissue, Sigma A1155) for 30 min at 37°C, and then pelleted by centrifugation and frozen at −60°C until time of assay. On the day of the receptor binding assay, samples were thawed and resuspended by Polytron and kept on ice as tissue homogenates until time of assay.

A_1 adenosine receptors were measured by the binding of [³H]cyclohexyladenosine (New England Nuclear, s.a. 13.5 Ci/mmol) ([³H]CHA) to membrane tissue homogenates according to a variation of method described previously [5,53]. Membranes from 4 mg of tissue (initial wet weight, approximately 600 μg of protein) were incubated with the tritiated ligand for two hours at 25°C in 50 mM Tris HCl (pH 7.7) buffer. The total assay volume was 2 ml. Specific binding was defined as total binding minus binding in the presence of 10 μM 2-chloroadenosine. Protein determinations were by the method of Lowry *et al.* (1951) [27].

In preliminary assays conducted to characterize [³H]CHA binding to rat brain membranes, brain tissue was pooled from naive control rats which were not exposed to the behavioral tests. Saturation studies were performed by measuring the binding of 0.045–40 nM [³H]CHA to pooled rat brain membranes from those naive control animals. The results were analyzed using nontransformed data from the saturation curves. Single and dual site (or state) receptor models were fitted to the data by using a computer assisted nonlinear least-squares curve fitting technique [22,53]. The binding of [³H]CHA to membranes prepared from the brain regions of individual animals used in the behavioral experiments was measured at 5–7 nM, a concentration less than saturating but at which there is a substantial contribution from both of the A_1 adenosine nanomolar affinity binding sites (or states). Statistical methods used include both parametric and nonparametric procedures and are identified in the Results section.

RESULTS

The kinetic and equilibrium binding properties as indicated in binding profiles in all of the studied brain regions were complex and more consistent with binding to two A_1 adenosine receptor sites (or states) than a single A_1 adenosine receptor site (or state) (Table 1 and Fig. 1). The binding of [³H]CHA (5–7 nM) to brain membranes of the rats from the behavioral experiment prepared from frontal cortex, hippocampus, and striatum did not differ between stressed and control animals (Fig. 2). However, the binding of [³H]CHA (5–7 nM) to hypothalamic membranes from stressed animals was significantly higher than to those membranes from control animals. This increase in [³H]CHA binding to hypothalamic membranes was observed in each of the three experimental runs.

Plasma prolactin did not differ between stressed and control animals (7.92±0.77 ng/ml control vs. 9.30±1.47 ng/ml experimental animals) but plasma corticosterone levels were significantly higher in stressed animals than in matched controls (1.15±0.15 μg/dl control vs. 6.29±3.10 μg/dl experimental animals, $p<0.05$, Mann-Whitney U-Test). Despite equivalent body weight at the initiation of the experiment (309±25 g control vs. 331±24 g experimental animals) food intake during the three day stress period was lower in stressed animals (442±42 pellets for control vs. 284±16 pellets for stressed animals, t-test, $p<0.05$).

DISCUSSION

The results of this study demonstrate increased [³H]CHA binding to hypothalamic membranes from the brains of stressed rats. The lack of change in [³H]CHA binding to membranes from frontal cortex, hippocampus, and striatum suggests that this stress-induced change in A_1 adenosine receptors is regionally specific. These data demonstrating adenosine receptor up regulation accompanying stress are consistent with those from a recently reported pharmacological study in which immobilization stress-induced gastric lesioning was shown to be increased by centrally administered agonists at A_1 adenosine receptors and decreased by A_1 adenosine receptor antagonists [51]. The behavioral model we have developed appears to be a useful paradigm for studying neuroendocrine and neurochemical changes in response to stress [2,24]. In numerous experiments we have found increased levels of plasma corticosterone and im-

paired performance (here indicated by lever pressing for food pellets) in the stressed animals. The lack of difference in plasma prolactin levels in stressed vs. control animals is consistent with evidence for rapid rises in prolactin during acute or initial exposure to stress followed by physiological adaptation [20,21].

Although the action of adenosine on the inhibition of neurotransmitter release has been demonstrated in striatum, cortex, and hippocampus [18, 19, 32, 41], little is known about the physiological role of adenosine in the hypothalamus. Iontophoretic application of adenosine produces depression in neuronal firing [40] and potent adenosine agonists have sedative and anticonvulsant activity [6,47]. The vast majority of studies on the physiological actions of adenosine and its receptors in the CNS have examined the actions of this neuromodulator in the hippocampus and striatum, where the greatest density of adenosine receptors has been found [11, 43, 44]. Recent data on VIP-stimulated release of prolactin and growth hormone by pituitary cells in culture suggest that adenosine may act as an important physiological regulator of hormone release from the anterior pituitary through its interaction with A_1 adenosine receptors associated with adenylate cyclase [1,10]. Although the function of adenosine in the hypothalamus is unknown, the abundance of adenosine-metabolizing enzymes, 5-nucleotidase and adenosine deaminase, [36–38] and adenosine uptake sites [15, 29, 31] suggests an important physiological role for adenosine in the hypothalamus. The localization of adenosine deaminase in specific hypothalamic neurons [36] raises the possibility that adenosine acts as a neurotransmitter within the hypothalamus and/or is released into the portal circulation to directly regulate pituitary hormone secretion.

Further experimentation and clarification is necessary before the physiological significance of the change in adenosine receptors we have seen can be evaluated. Studies are in progress to determine whether this increased [^{3}H]CHA binding is due to changes in affinity for the ligand or in the number of binding sites. Up regulation of adenosine A_1 adenosine receptors in striatum and cortex of REM sleep deprived animals has been reported [57]. Our behavioral paradigm for sustained stress incorporates footshock, continuous performance, decreased food intake, and sleep disruption. Presently, we are measuring A_1 adenosine receptors in brain tissue from animals exposed to these other stressors individually to determine whether the up regulation of A_1 adenosine receptors is associated with stress, in general, or with one specific stressor of our behavioral design.

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