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The NMDA Glycine Site Antagonist (+)-HA-966 Selectively Regulates Conditioned Stress-induced Metabolic Activation of the Mesoprefrontal Cortical Dopamine but Not Serotonin Systems: A Behavioral, Neuroendocrine, and Neurochemical Study in the Rat

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Animals confronting threatening stimuli respond with a coordinated set of autonomic, neuroendocrine, neurochemical, and behavioral responses that constitute the stress response. The role of the NMDA receptor and its glycine modulatory site was investigated in a rat conditioned stress model. Behavioral, neuroendocrine, and neurochemical analyses were conducted. Regional dopamine (DA) and serotonin (5-HT) utilization was assessed by postmortem tissue measurements of metabolite-to-parent neurotransmitter ratios. Rats were conditioned to fear a tone previously paired with footshock. The following day, rats were systemically administered saline or the NMDA glycine site antagonist (+)-HA-966 before exposure to thirty minutes of conditioned stress. Conditioned stress resulted in a selective increase in medial prefrontal cortical DA and 5-HT utilization, elevation in serum corticosterone, and freezing behavior in control animals. The conditioned stress-induced increase in DA utilization in control animals was also detected in the lateral prefrontal cortex and nucleus accumbens, whereas DA utilization was not affected in the perirhinal or cingulate cortices, lateral-basolateral amygdaloid complex, anterior ventromedial caudatoputamen, or posterior dorsolateral caudatoputamen. Pretreatment with (+)-HA-966 at 15 mg/kg completely abolished the conditioned stress-induced increase in DA utilization in the medial and lateral prefrontal cortices. This effect was regionally specific since (+)-HA-966 pretreatment did not block increased DA utilization in the nucleus accumbens. This effect was also neurochemically specific since the stress-induced increase in 5-HT utilization in the medial prefrontal cortex was not affected by (+)-HA-966 pretreatment. Pretreatment with (+)-HA-966 did not affect stress-induced serum corticosterone elevation but did attenuate the freezing response. Control experiments demonstrated that (+)-HA-966 pretreatment did not (1) induce sedation, (2) interfere with habituation to a novel environment, (3) alter basal DA, 5-HT, or serum corticosterone levels, or (4) block acquisition of aversive memories. These data suggest that the NMDA receptor complex and associated glycine modulatory site may play an important role in the afferent control of the mesoprefrontal cortical DA system during conditioned stress. The relevance of these findings to schizophrenia and human anxiety disorders such as post-traumatic stress disorder are discussed.

[Key words: dopamine, 5-HT, NMDA, glycine site, (+)-HA-966, prefrontal cortex, freezing, ultrasonic vocalization, corticosterone, conditioned fear, stress, schizophrenia, post-traumatic stress disorder]

Animals confronting threatening situations react in complex, highly coordinated patterns termed defense reactions or the stress response. This response functions in the natural environment to maximize survival in the face of predatory threat (Fanselow, 1984) and includes increased vigilance, enhanced reactivity to environmental stimuli, increased autonomic reactivity, alterations in cardiovascular functioning, and activation of neuroendocrine responses. In the rat, behavioral reactions to stress also include cessation of ongoing behavior, characteristic changes in respiration, freezing, and ultrasonic vocalization (Sewell, 1967; Blanchard and Blanchard, 1969; Bouton and Bolles, 1980; Fokkema et al., 1986). Many of the behavioral, neuroendocrine, autonomic, and neurochemical changes associated with the stress response have been evolutionarily conserved and are found in most mammals, including humans. Psychophysiological similarities in the stress response across mammalian species may reflect common neurobiological processes underlying the emotional states of fear in animals and anxiety in humans (Charney et al., 1993).

Exposure to threatening stimuli is also known to activate monoaminergic systems in the CNS (Rubin et al., 1970; Maas et al., 1971; Thierry et al., 1976; Deutch and Roth, 1990; Inoue et al., 1993). Acute inescapable footshock in rodents results in increased dopamine (DA) utilization in the medial prefrontal cortex (m-PFC) as measured by *ex vivo* tissue analysis (Thierry it al., 1976; Herman et al., 1982; Roth et al. 1988) and *in vivo* microdialysis (Abercrombie et al., 1989; Sorg and Kalivas, 1993). In the rat, unconditioned stress, such as spatial proximity to other rats exposed to footshock (Kaneyuki et al., 1991), or conditioned stress (Herman et al., 1982; Deutch et al., 1985) such as exposure to an environmental context previously paired with

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footshock, are known to robustly activate the mesoprefrontal cortical DA system. More substantial or prolonged exposure to stress results in recruitment of DA systems that innervate regions such as the nucleus accumbens septi and striatum (Roth et al., 1988).

The effect of stress on the 5-HT system has been less rigorously studied. Footshock was first shown to elevate 5-HT turnover in whole brain and to activate 5-HT metabolism in cortex (Bliss et al., 1968; Thierry et al., 1968). Since then a variety of unconditioned stressors have been shown to increase 5-HT utilization in the frontal cortex of rats and mice (Adell et al., 1988; Dunn, 1988; Miyauchi et al., 1988; Pei et al., 1990; Dunn and Welch, 1991; Heinsbroek et al., 1991). In addition, reexposure to a context previously paired with intense footshock has recently been shown to increase 5-HT metabolism in the m-PFC (Inoue et al., 1993).

Control of the mesoprefrontal cortical DA system is complex and involves interaction between a variety of neuroactive peptides and monoamines, as well as excitatory and inhibitory amino acids (Deutch and Roth, 1990; Kalivas, 1993). Recent studies have demonstrated that excitatory amino acids may be involved in the selective metabolic activation of subsets of DA neurons within the ventral tegmental area (VTA) that project to the m-PFC (Kalivas et al., 1989). Indeed, receptors for excitatory amino acids are found in the VTA (Monaghan and Cotman, 1985).

The NMDA receptor complex is an excitatory amino acid ligand-gated ion channel that is selectively activated by NMDA and regulated at several pharmacologically distinct sites (Collingridge and Lester, 1989). One of these sites is a high-affinity, strychnine-insensitive glycine binding site that is found in many regions of the mammalian brain (Bristow et al., 1986). Competitive antagonists of the strychnine-insensitive glycine site that cross the blood–brain barrier have become available, making possible *in vivo* pharmacological manipulation of the NMDA receptor through this regulatory site. One such high-affinity, selective antagonist at the NMDA receptor glycine site is (+)-HA-966, the recently resolved enantiomer of the drug (+)-3-amino-1-hydroxy-2-pyrrolidinone (Singh et al., 1990). In the rat, systemic administration of this compound has been shown to normalize DA neuron firing (McMillen et al., 1992; Shepard and Lehmann, 1992). Previous work in this laboratory has also demonstrated that systemic and intra-VTA administration of (+)-HA-966 blocks physical restraint-induced increases in DA utilization in the m-PFC of the rat (Morrow et al., 1993).

In the present study, a nonsedating systemic dose of (+)-HA-966 was tested for its effects on the behavioral, neuroendocrine, and neurochemical responses induced in the rat by conditioned psychological stress. We examined its effects on dopaminergic and serotonergic responses in a variety of cortical, limbic, and striatal terminal regions. Since the NMDA receptor complex has been implicated in memory processing (e.g., Morris et al., 1986; Miserendino et al., 1990), we also tested the effect of pretraining administration of this compound on acquisition of the behavioral, neuroendocrine, and neurochemical responses to conditioned stress.

Materials and Methods

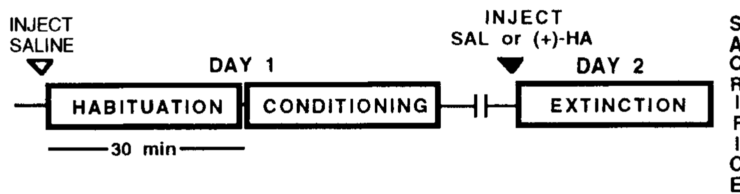
Animals and housing conditions. All procedures met guidelines and protocols approved by the Yale Animal Care and Use Committee. Male albino Sprague–Dawley rats (Camm, Wayne, NJ) weighing 300–400 gm were used. All rats were individually caged and acclimated in this laboratory's animal colony room for at least 2 weeks prior to experimentation. Rats were maintained on a 12:12 hr light-dark schedule with lights on at 1500. Food and water were available ad libitum. Contact with human personnel was limited to a single attendant.

Apparatus. Testing was conducted in the dark in a modified standard Plexiglas and aluminum conditioning chamber isolated in a sound attenuation cubicle. In order to avoid contamination of the test chamber with olfactory cues, all surfaces of the chamber were extensively washed with 70% ethanol and fan dried after each animal exposure. An infrared television camera (Sanyo Electronics) and infrared illuminator were positioned over the test cage and connected to a standard television–video recording system located in a monitoring station in the next room. This setup allowed remote monitoring and recording of test subjects in complete darkness. The top of the testing chamber was constructed of transparent Plexiglas into that was inserted a miniature narrow-bandwidth ultrasound microphone (Panasonic, Tokyo, Japan) with a center frequency of 21 kHz, the same frequency utilized by adult rats for distress vocalization (Sewell, 1967). The signal from the ultrasound microphone was fed to an amplifier in the monitoring room where it was filtered, digitized (sampling frequency, 15 Hz), and analyzed by a computer programmed to measure number, duration, and amplitude of the ultrasonic calls. Shock intensity was measured by a digital oscilloscope running in the differential mode with a 1 kΩ load in series with a 100 kΩ resistor bridge across adjacent cage bars. Current was defined as the root mean square voltage across the 1 kΩ resistor (mA = 0.707 × 0.5 × peak-to-peak voltage). Shock intensity was set at 0.40 mA. Shock duration was 0.5 sec.

Drugs. (+)-HA-966 was provided by Research Biochemicals, Inc. (Natick, MA) under the National Institute of Mental Health Funded Synthesis Program. Solutions of (+)-HA-966 were freshly prepared before use with sterile normal saline.

Preliminary test of drug-Induced sedation. Previous studies in this laboratory using (+)-HA-966 at an intraperitoneal dose of 15 mg/kg suggested that this dose does not produce sedation in rats (L. Goldstein, unpublished observations). This dose was then more closely examined for sedative effects. Rats were pretreated with either saline (1 ml/kg, i.p.) or (+)-HA-966 (15 mg/kg at 1 ml/kg, i.p.) 15 min before introduction into a novel, dark, sound-attenuating test chamber equipped with an eight cage computer-assisted activity monitoring system (Omnitech Digiscan, Columbus, OH). Test subjects were allowed to freely explore the novel chambers for two consecutive 30 min periods. Ambulation was continuously monitored by infrared photobeam interruption and analyzed by computer. Cumulative locomotor activity was compiled into two successive 30 min test intervals.

Conditioning and testing procedures. All studies were conducted in the dark during the active phase of the rat diurnal activity cycle, beginning 1–6 hr after dark onset. The rationale for testing in the dark was to approximate the psychophysiological conditions when these nocturnal animals are most likely to encounter threatening stimuli under natural conditions. The experimental model used in this study is schematically presented in Figure 1. To test the effect of (+)-HA-966 pretreatment on the expression of conditioned stress, all subjects were injected with saline (1 ml/kg, i.p.) 15 min prior to testing on day 1, and returned to their home cages (Fig. 1*A*). Test animals were then introduced into the dark test chamber and allowed to explore freely the cage for thirty minutes to facilitate habituation to the chamber. During the last 5 min of this habituation period, three 5 sec 56 dB white noise tones were presented by computer. Immediately following, a 30 min conditioning period was initiated. During conditioning, the animals were randomly presented with ten 5 sec white noise tones (conditioned stimulus, CS) that coterminated with a 0.5 sec 0.4 mA footshock (unconditioned stimulus, US). At the conclusion of the conditioning period, the subjects were returned to their home cages. Unstressed, nonshocked control animals were treated as above except that these animals did not receive footshock (US) during the conditioning period. The next day, subjects in the conditioned stress groups were injected with either saline (1 ml/kg, i.p.) (SAL-CS group), or (+)-HA-966 (15 mg/kg at 1 ml/kg, i.p.) (HA-CS group) 15 min before behavioral testing. Animals in the nonshocked control group (SAL-NS group) were injected with saline (1 ml/kg, i.p.) 15 min prior to behavioral testing. The animals were then reintroduced into the testing chamber. During this extinction trial, animals in all groups were randomly presented with ten 56 dB white noise tones without footshock. To test the effects of (+)-HA-966 on the acquisition of conditioned stress, rats were tested as described above, but



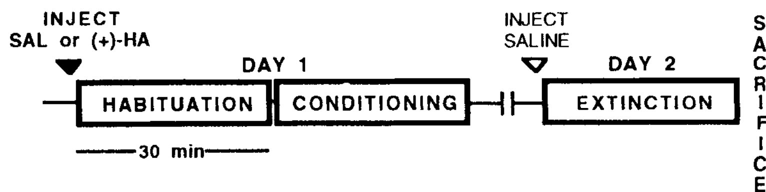


Figure 1. Experimental models. *A* shows conditioned stress model used to test the effect of systemic administration of (+)-HA-966 (15 mg/kg, i.p.) on the expression phase of conditioned stress. Injection of (+)-HA-966 or saline is given 15 min before beginning the extinction period on day 2. *B* shows conditioned stress model used to test the effect of systemic administration of (+)-HA-966 (15 mg/kg, i.p.) on the acquisition phase of conditioned stress. Pretraining injection of (+)-HA-966 or saline is given 15 min before beginning the habituation period on day 1.

were given either saline or (+)-HA-966 15 min before training on day 1. All animals were given saline before testing on day 2 (Fig. 1*B*).

At the conclusion of the testing period on day 2, the animals were rapidly decapitated. Anesthesia prior to death was not used due to possible interference with neurochemical and neuroendocrine measurements. Trunk blood was collected for determination of serum corticosterone levels and brain tissue was obtained for neurochemical analyses as described below.

A separate group of animals was used for determination of basal neurochemical and serum corticosterone levels. These animals were injected with either saline (1 ml/kg, i.p.) or (+)-HA-966 (15 mg/kg, i.p.) and returned to their home cages in the colony facility for 45 min. This time period corresponds to the time from injection to death in animals tested in the behavioral studies. After this 45 min period, animals were removed from their home cages and rapidly decapitated as described above.

Behavioral measurements. Behavioral data (freezing, grooming, crossings, and rears) were remotely coded by an observer using an electronic pushbutton/toggle switch assembly connected to a computer by an analog-to-digital converter. Ultrasonic vocalizations were electronically monitored as detailed above and compiled by computer. The following criteria had to be met to qualify as an ultrasonic vocalization: (1) remote behavioral observation of the animal confirmed deep, prolonged respiration at approximately 0.5 Hz preceded by short, shallow rapid panting at approximately 3–4 Hz (Frysztak and Neafsey, 1991); (2) call duration was greater than 20 msec but no longer than 3 sec as visualized on a digital oscilloscope; and (3) call tracings appeared on both the oscilloscope and computer monitor screen. Use of these criteria prevented the recording of spurious signals such as those due to animals sniffing or scratching the aluminum sidewalls. Freezing behavior was strictly defined as no visible movement of the animal, including vibrissae, except that related to respiration. This behavior has been used as an index of fear in the rat (Fanselow, 1980; Conti et al., 1990). Freezing was coded by throwing a toggle switch while the animal was engaged in this behavior. This signal was continuously recorded and compiled by computer as the percentage of time the animal froze during each of ten 3 min intervals comprising the 30 min test period. Crossings were coded each time the base of an animal's tail crossed a grid drawn on the video screen and were compiled as the number of such events occurring per 3 min interval. Rears were counted when an animal simultaneously raised both forepaws off the cage bars and was not otherwise engaged in grooming behavior. Rears were coded and likewise compiled as the number occurring per 3 min interval. Animals were initially coded by an observer not blind to the experimental conditions. Video records of 11 randomly selected animals were recoded by an observer blind to the experimental conditions. Statistical analysis showed a high correlation between results generated by unblind and blind behavioral observations (crossings, $r = 0.98$, $p < 0.0001$; rearings, $r = 0.98$, $p < 0.0001$; freezing, $r = 0.99$, $p < 0.0001$). Therefore, the initial behavioral results were used for statistical analysis.

Brain dissection and neurochemical measurements. At the conclusion of behavioral testing animals were rapidly decapitated. The brain was quickly removed from the calvarium, immersed in chilled normal saline (−2.5°C), and transferred ventral side up to an ice-chilled stainless steel brain mold with precision milled transverse slots. Coronal slices (2 mm) were rapidly made by insertion of chilled razor blades. The slices were then transferred to a frozen plastic dissection platform filled with a gel coolant that maintained the platform temperature at approximately 1.0°C. Dissection and punch sites are schematically depicted in Figure 2. Tissue biogenic amines were isolated according to a modification of the procedure of Reinhard and Roth (1982). Briefly, frozen tissue was sonicated in 0.4 ml of 0.1 M perchloric acid containing 8 ng of 3,4-dihydroxybenzylamine and 24 ng *N*-methyl-serotonin as internal standards. Samples were sonicated in plastic Eppendorf tubes immersed in a saline and ice slurry to prevent thermal decomposition of the monoamines and their metabolites. Forty microliters of solution was removed for protein measurement by the method of Lowry et al. (1951). The remaining solution was centrifuged under refrigeration for 20 min. Sample supernatants were collected and mixed with 25 μl of 3.0 M Tris base (pH 11.0), resulting in a solution buffered to pH 8.6. This solution was then passed over minicolumns packed with alumina (Aldrich Chemical Co., Milwaukee, WI). The effluents, containing 5-HT and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), were immediately stored at −70°C until analysis. The alumina minicolumns with adsorbed catechols were then centrifuged, washed with distilled water, and eluted with 150 μl of 0.1 M oxalic acid. The eluates containing DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) were stored at −70°C until analysis. The samples were assayed for monoamines using reversed-phase high-performance liquid chromatography (HPLC) coupled with amperometric electrochemical detection. Separate systems were chromatographically optimized for measurement of DA and DOPAC and for 5-HT and 5-HIAA. Sample aliquots of 50 μl were injected onto an HPLC system consisting of (1) a 10 cm × 2.1 mm narrow-bore column packed in our laboratory with 3 μm C-18 packing for DA and DOPAC, or an Altech C-18 Microsorb column (Alltech Associates, Deerfield, IL) for 5-HT and 5-HIAA, and (2) a glassy carbon electrode assembly maintained at +0.70 V (vs Ag/AgCl reference electrode) coupled with an LC-3 electrochemical detector (Bioanalytical Systems, West Lafayette, IN). The systems were optimized to increase the signal-to-noise ratio by placing a low-pass RC filter on the recording device. The mobile phase for determination of DA and its metabolite DOPAC consisted of 2–8% methanol in 0.1 M monobasic sodium phosphate buffer, 0.2 mM octanesulfonic acid, and 0.1 mM EDTA, with pH optimized between 2.7 and 3.2 for acid metabolite detection. The mobile phase for determination of 5-HT and its metabolite 5-HIAA consisted of 0.02 M sodium acetate, 10% (v/v) methanol, 0.3 mM EDTA, and 7.2 mM

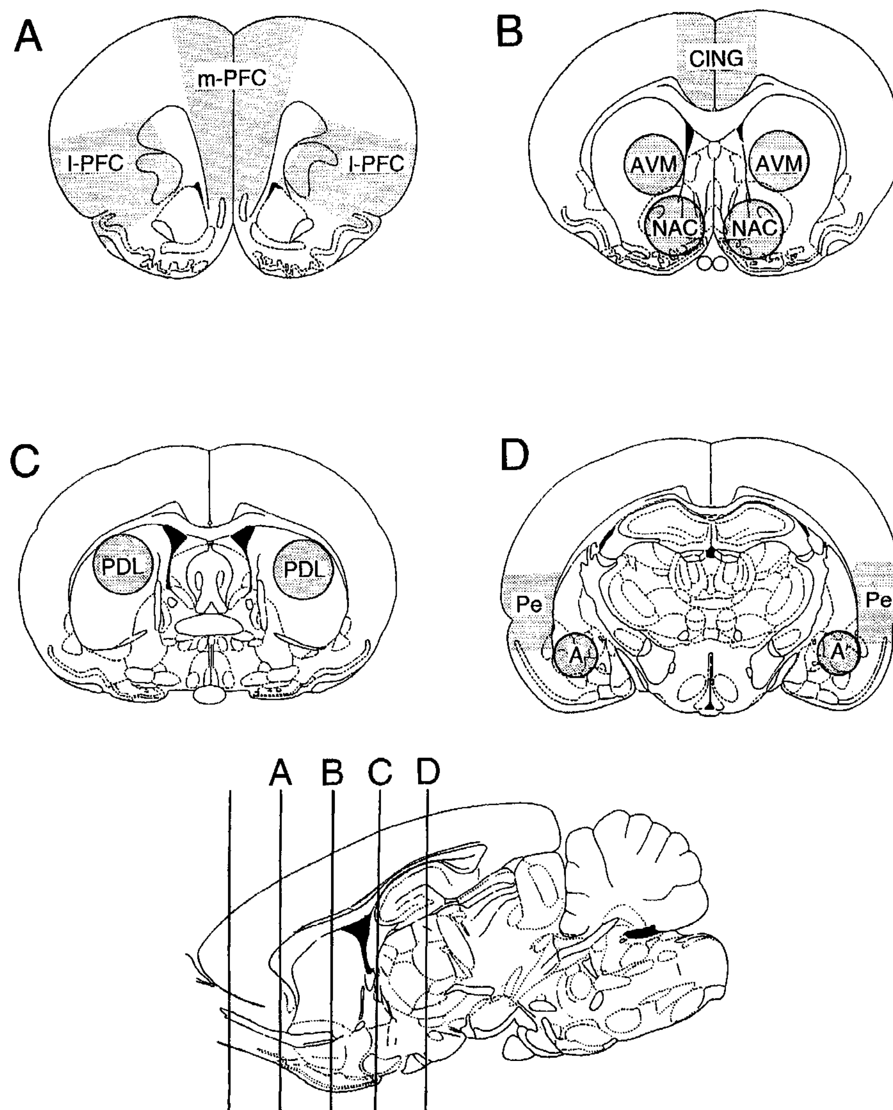


Figure 2. Dissection and punch diagrams indicating areas of interest analyzed for neurochemical responses to conditioned stress. *Bottom panel* shows a sagittal section of the rat brain indicating approximate levels at which slices were taken. For convenience of illustration only a single sagittal plane is represented. *Letters above lines* represent slices that are shown as coronal sections with the same letter designation in the top four panels: *m-PFC*, medial prefrontal cortex; *l-PFC*, lateral prefrontal cortex; *CING*, posterior cingulate cortex; *NAC*, nucleus accumbens septi; *AVM*, anterior ventromedial caudatoputamen; *PDL*, posterior dorsolateral caudatoputamen; *Pe*, perirhinal cortex; *A*, amygdaloid complex.

triethylamine, at pH 3.90. Quantitation of biogenic amines and their respective metabolites was achieved by measuring peak heights and comparing them to internal standard values. DA and 5-HT utilization was assessed by calculating the ratio of the acid metabolite to the parent neurotransmitter. The DOPAC:DA and 5-HIAA:5-HT ratios correlate with the net metabolic flux within the tissue sample at the time of death. Some brain regions from some animals were excluded from analysis due to technical difficulties with individual dissections or sample preparation.

Neuroendocrine measurement. Serum corticosterone was measured by a magnetic radioimmunoassay developed in this laboratory. Trunk blood was collected at decapitation in glass tubes and immediately placed on ice prior to centrifugation. Serum was collected and stored at −70°C until it was assayed. Serum samples (2 μl) were diluted in phosphate-buffered saline with gelatin. The radioimmunoassay used a polyclonal rabbit anti-corticosterone primary antibody (kindly provided by Dr. G. Niswinder, Colorado State University), commercially purchased ^{125}I-corticosterone (ICN Biomedicals, Inc., Costa Mesa, CA), and a custom-prepared polyclonal goat anti-rabbit IgG secondary antibody conjugated to paramagnetic particles (Corning Diagnostics, Corning, NY). Incubation with the primary antibody was conducted overnight under refrigeration. Incubation with the secondary antibody was conducted at room temperature for 1 hr. Bound versus unbound fractions were then separated by placing the test tubes in the presence of a magnetic field. This drew the antibody complexes to the side walls of the tubes. Free labeled corticosterone and antibody were then withdrawn from the test tubes using vigorous suction. Corticosterone levels were determined using a standard gamma counter. Serum counts corrected for nonspecific binding were compared to those obtained on a standard curve of varying known concentrations of corticosterone. Using this procedure, corticosterone levels of 10 ng/ml in 2 μl of serum could be routinely measured.

Statistics. Data were analyzed by analysis of variance for significant differences among all means. The Student-Newman-Keuls post hoc multiple comparison procedure was used for comparison among means where appropriate. Temporal analyses of behavioral data were conducted with a repeated measures analysis of variance model. These analyses were conducted using the SUPERANOVA computer program (Abacus Concepts, Inc., Berkeley, CA). The two-tailed Fisher Exact Probability Test was used for statistical analysis of the effects of treatment on the number of animals vocalizing per group. Data are expressed as means ± standard error of the mean (SEM). Statistical significance was set at $p < 0.05$.

Results

Test for drug-induced sedation

No difference in locomotor activity was observed when animals were injected with either saline or (+)-HA-966 (15 mg/kg, i.p.) 45 min before introduction into a novel environment (Fig. 3). In addition, the initial burst of ambulatory activity in response

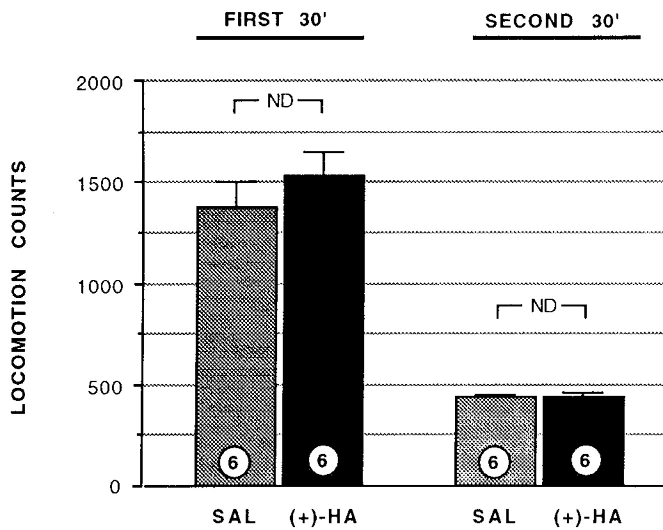


Figure 3. Systemic administration of (+)-HA-966 at 15 mg/kg is nonsedating. Intraperitoneal administration of (+)-HA-966 at 15 mg/kg 15 min before subjects were introduced into a novel dark chamber for 1 hr does not affect novelty-induced locomotor activity. Control animals were injected with saline (*SAL*). Locomotion was assessed by photobeam interruptions in an activity chamber. The test session was divided into two consecutive 30 min periods. No significant difference was noted between the saline and (+)-HA-966 pretreated groups. However, a significant effect of time was observed ($p < 0.0001$). *Numbers in circles* at base of histogram bars indicate number of rats in each group.

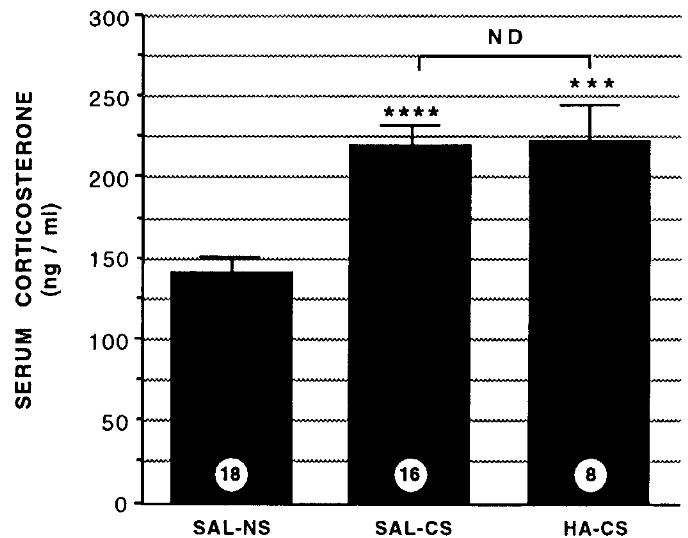


Figure 4. Effect of conditioned stress and (+)-HA-966 pretreatment on serum corticosterone levels in the rat. Rats were pretreated with either saline (1 ml/kg, i.p.) or (+)-HA-966 (15 mg/kg, i.p.) 15 min before beginning the extinction period on day 2. Animals were then tested as described in Materials and Methods for 30 min. Data are presented as means (±SEM). *Numbers in circles* at base of histogram bars indicate number of rats in each group. **** and ***, significant differences compared to the SAL-NS group control ($p < 0.0001$ and $p < 0.005$, respectively). *ND*, no significant difference between the SAL-CS and HA-CS groups.

to a novel environment was similar in both saline and (+)-HA-966 pretreated groups. A significant effect of time in the novel environment was detected [$F(1,20) = 139.9$, $p < 0.0001$]. No interaction between treatment group and time was detected, indicating a similar pattern of habituation to the test chamber in both the saline and (+)-HA-966 pretreated groups.

Effect of (+)-HA-966 on basal serum corticosterone and neurochemical measures

Dopaminergic and serotonergic neurochemistry and serum corticosterone values in untreated naive rats are shown in Table 1. Note that these rats did not receive injections.

To test whether (+)-HA-966 had effects on basal DA and 5-HT utilization or serum corticosterone concentration, rats were injected with either saline (1 ml/kg, i.p.) or (+)-HA-966, (15 mg/kg, i.p.) and returned to their home cages for 45 min before death. This 45 min period corresponds to the time from pretreatment injection to death in the conditioned stress protocol. No difference was detected between saline and (+)-HA-966 pretreated rats in DA or 5-HT utilization (Table 2) as measured by the ratio of major metabolite-to-parent neurotransmitter levels in any of the regions tested. Nor was there a difference in basal corticosterone between these groups.

Effect (+)-HA-966 on neuroendocrine and behavioral indices of conditioned stress

A significant effect of treatment on serum corticosterone was observed [$F(2,39) = 13.83$, $p < 0.0001$] (Fig. 4). Post hoc analysis demonstrated a significant difference in serum corticosterone between shocked and nonshocked saline control groups (SAL-CS, 219.9 ± 12.1 ng/ml; SAL-NS, 140.6 ± 10.2 ng/ml) ($p < 0.0001$). This elevation in serum corticosterone in response to conditioned stress was not attenuated by pretreatment with (+)-HA-966 (HA-CS, 222.5 + 21.5 ng/ml).

Table 1. Basal regional concentrations of DA, 5-HT, and their acid metabolites (DOPAC and 5-HIAA) in the naive rat

Region (*n*)	DOPAC (ng/mg prot)	DA (ng/mg prot)	DOPAC:DA	5-HIAA (ng/mg prot)	5-HT (ng/mg prot)	5-HIAA:5-HT
m-PFC (4)	0.11 ± 0.01	0.87 ± 0.08	0.126 ± 0.005	24.09 ± 1.03	76.04 ± 22.84	0.246 ± 0.030
NAC (4)	17.40 ± 5.40	132.56 ± 41.68	0.133 ± 0.005	12.60 ± 3.73	28.77 ± 10.52	0.466 ± 0.047
AVM (6)	44.03 ± 7.80	509.83 ± 107.11	0.089 ± 0.006	56.89 ± 14.14	109.20 ± 36.05	0.573 ± 0.051
PDL (6)	40.90 ± 6.55	495.08 ± 50.23	0.081 ± 0.005			
LBL (4)	4.36 ± 0.41	49.55 ± 3.78	0.088 ± 0.006			
PR-CX (4)	0.27 ± 0.05	2.33 ± 0.62	0.129 ± 0.020			
CING (4)	0.33 ± 0.05	3.29 ± 1.70	0.158 ± 0.042			

Neurochemistry values in naive animals are expressed as mean concentration (±SEM) in selected cortical and noncortical regions. Animals were removed from their home cages and rapidly killed. m-PFC, medial prefrontal cortex; NAC, nucleus accumbens septi; AVM, anterior ventromedial caudatoputamen; PDL, posterior dorsolateral caudatoputamen; LBL, lateral-basolateral amygdala; PR-CX, perirhinal cortex; CING, posterior cingulate cortex. DOPAC, 3,4-dihydroxyphenylacetic acid; DA, dopamine; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin. Number of naive rats in each group is indicated in parentheses following region abbreviations.

Table 2. Effect of pretreatment with saline or (+)-HA-966 on basal neurochemistry and serum corticosterone

	Saline pretreatment	(+)-HA-966 pretreatment
Neurochemistry		
m-PFC		
DOPAC:DA	0.175 ± 0.022 (7)	0.194 ± 0.018 (7)[ND]
5-HIAA:5-HT	0.291 ± 0.014 (7)	0.340 ± 0.034 (7)[ND]
l-PFC		
DOPAC:DA	0.312 ± 0.038 (5)	0.303 ± 0.028 (6)[ND]
5-HIAA:5-HT	0.300 ± 0.031 (7)	0.320 ± 0.028 (6)[ND]
NAC		
DOPAC:DA	0.146 ± 0.008 (7)	0.130 ± 0.130 (6)[ND]
5-HIAA:5-HT	0.530 ± 0.068 (7)	0.572 ± 0.070 (6)[ND]
AVM		
DOPAC:DA	0.109 ± 0.109 (7)	0.116 ± 0.116 (7)[ND]
Serum corticosterone (ng/ml)	119.5 ± 10.2 (7)	114.7 ± 9.7 (7)[ND]

Systemic pretreatment with (+)-HA-966 at 15 mg/kg does not alter basal dopamine or serotonin utilization or change basal serum corticosterone levels in control rats. Animals were injected with either saline (1 ml/kg, i.p.) or (+)-HA-966 (15 mg/kg, i.p.) and returned to their home cage in the colony facility for 45 min. This time period corresponds to time from pretreatment injection to death in the conditioned stress protocol. m-PFC, medial prefrontal cortex; l-PFC, lateral prefrontal cortex; NAC, nucleus accumbens septi; AVM, anterior ventromedial caudatoputamen. Numbers in parentheses indicate number of rats in each group.

[ND] No significant difference between saline and (+)-HA-966 pretreatments.

A significant effect of treatment was also detected for total freezing behavior [$F(2,37) = 104.24$, $p < 0.0001$] (Fig. 5*A*). Animals in the SAL-CS group froze more than those in the SAL-NS control group ($p < 0.0001$). The SAL-NS group froze 0.99 (±0.61)% of the extinction trial while the SAL-CS group froze 72.57 (±4.32)% of the time. Pretreatment with (+)-HA-966 (HA-CS) significantly reduced the percentage of time spent freezing during the extinction trial to 37.49 (±6.50)%, versus the SAL-CS group ($p < 0.0001$). Thus, pretreatment with (+)-HA-966 resulted in a 48.3% decrease in conditioned stress-induced freezing.

An ANOVA with repeated measures also revealed a significant interaction between treatment and time ($p < 0.0001$) (Fig. 6*A*). In the saline pretreated groups, shock on the training day markedly elevated the freezing curve during the extinction trial on the following day ($p < 0.0001$). Pretreatment with (+)-HA-966 (HA-CS) resulted in a downward shift in the temporal course of freezing during the extinction trial when compared to the saline pretreated conditioned stress group (SAL-CS) ($p < 0.0001$). In the HA-CS group, freezing was reduced during all 3 min intervals during the extinction trial, except the first. Unlike the SAL-CS group, the HA-CS group was less likely to freeze between tones. During the last 9 min of the testing period, the HA-CS group froze only 8.4 ± 3.5% of the time while the SAL-CS group froze 56.6 ± 5.9% of the time.

Combined horizontal and vertical locomotion (crossings plus rears) was also influenced by treatment [$F(2,37) = 59.29$, $p < 0.0001$] (Fig. 5*B*). A decrease in locomotion in the SAL-CS group compared to the SAL-NS group was noted ($p < 0.0001$). Mean locomotion scores for the SAL-CS and SAL-NS groups were 50.87 (±6.20) and 317.06 (±25.27), respectively. The mean locomotion score for the HA-CS group was 142.89 (±14.10), a 180.9% increase over the SAL-CS group ($p < 0.01$). In contrast, note that (+)-HA-966 pretreatment did not increase spontaneous locomotion in a novel, non-noxious environment (compare Fig. 3).

An ANOVA with repeated measures also revealed a significant interaction between treatment and time for locomotion ($p < 0.0001$). Note the upward shift in the tail of the locomotion curve, which was most evident during the last 9 min of the extinction trial (Fig. 6*B*).

Effects of conditioned stress and (+)-HA-966 on regional DA utilization

The results of analyses of DA utilization in cortical, limbic, and striatal regions in response to conditioned stress after injection of either saline or (+)-HA-966 are presented in Figure 7. All data are derived from neurochemical analysis of post mortem brain tissue collected at death immediately following the extinction trial on day 2. DA utilization is expressed as the ratio of the dopamine metabolite DOPAC to the parentage neurotransmitter, DA (i.e., DOPAC:DA). Basal neurochemistry values for naive rats are shown in Table 1.

The only cortical areas that showed a significant effect of treatment on DA utilization were the medial and lateral prefrontal cortices (m-PFC and l-PFC, respectively). In the m-PFC, an effect of treatment on DA utilization was observed [$F(2,39) = 40.53$, $p < 0.0001$]. The SAL-CS group showed a 96.1% increase in the DOPAC:DA ratio versus the SAL-NS group ($p < 0.0001$). Mean DOPAC:DA ratios in the m-PFC for the SAL-NS and SAL-CS groups were 0.153 (±0.013) and 0.300 (±0.014), respectively. This increase in the DOPAC:DA ratio was exclusively due to an increase in the tissue concentration of DOPAC, a pattern noted in the other regions showing increased DOPAC:DA ratios in response to conditioned stress. Significantly, this increase in m-PFC DA utilization in the SAL-CS group was completely blocked by pretreatment with (+)-HA-966 ($p < 0.0001$). Mean DOPAC:DA ratios in the m-PFC for the SAL-CS versus HA-CS groups were 0.300 (±0.014) and 0.160 (±0.013), respectively. There was no difference between the conditioned stress group that had been pretreated with (+)-HA-966 (HA-CS) and the nonstressed saline pretreated control group

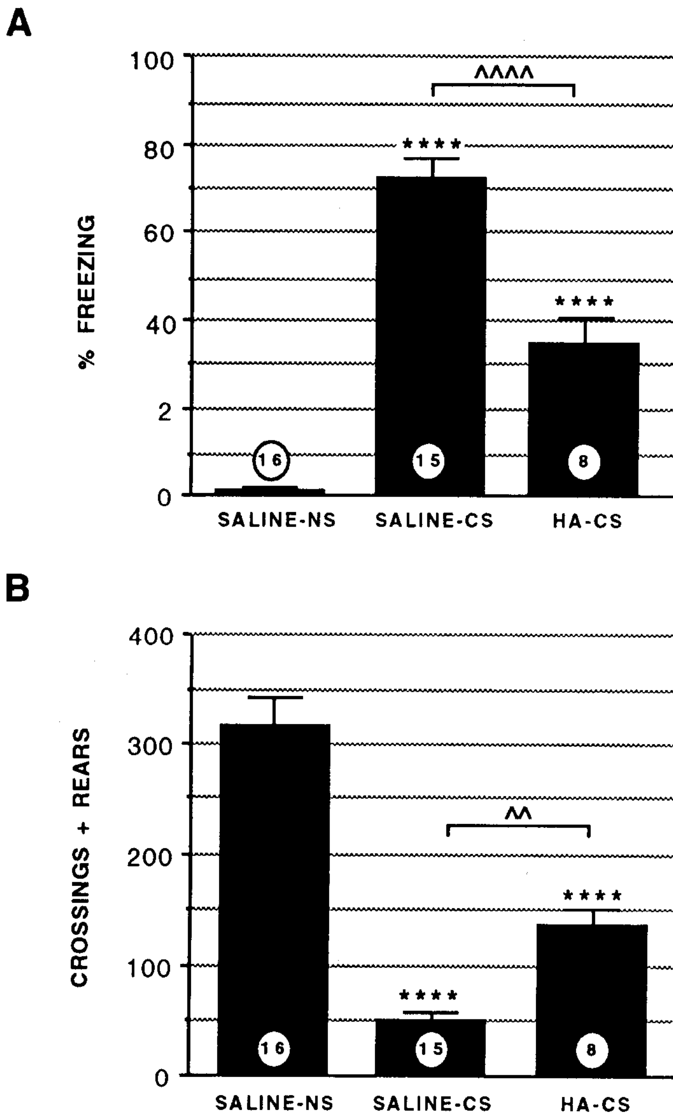


Figure 5. Effect of (+)-HA-966 administration on freezing and locomotor behavior during conditioned stress. Rats were pretreated with saline (1 ml/kg, i.p.) or (+)-HA-966 (15 mg/kg, i.p.) 15 min before beginning the extinction period on day 2. *A* shows effect on freezing behavior. *B* shows effect on locomotor behavior (crossings and rears). Percentage freezing represents the percentage of total time (in seconds) animals were engaged in this behavior during the 30 min extinction trial (1800 sec). Crossings + rears indicates number of times the animals crossed hatchings in cage plus the number of rears during the 30 min extinction trial. Data are presented as means (±SEM) for the 30 min period. *Numbers in circles* at base of histogram bars indicate number of rats in each group. ****, significant difference versus the SAL-NS control group ($p < 0.0001$). ^^^^, significant difference between the HA-CS and SAL-CS groups ($p < 0.0001$). ^^, significant difference between the HA-CS and SAL-CS groups ($p < 0.01$).

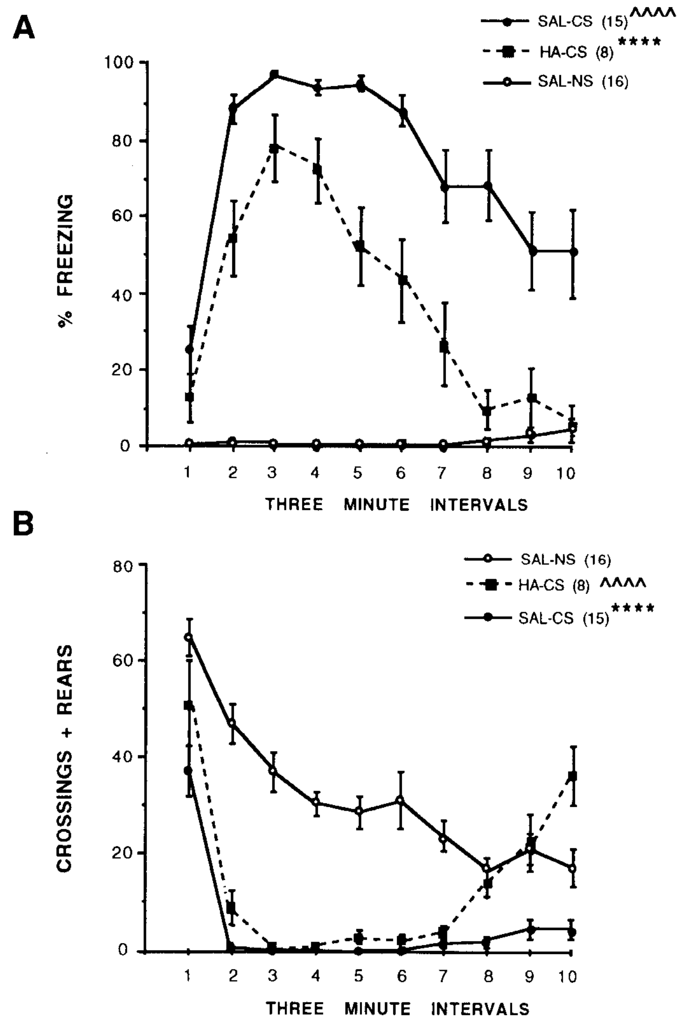


Figure 6. Temporal effect of (+)-HA-966 administration on freezing and locomotor behavior during conditioned stress. Rats were pretreated with saline (1 ml/kg, i.p.) or (+)-HA-966 (15 mg/kg, i.p.) 15 min before beginning the extinction period on day 2. *A* shows effect on freezing behavior. *B* shows effect on locomotor behavior (crossings and rears). Percentage freezing represents the percentage of total time (in seconds) animals were engaged in this behavior during each successive 3 min test interval (180 sec). Crossings + rears indicates number of times the animals crossed hatchings in cage plus the number of rears per 3 min test interval. Data are presented as means (±SEM) for successive 3 min intervals. *Numbers in circles* at base of histogram bars indicate number of rats in each group. ****, significant difference between SAL-NS and SAL-CS groups ($p < 0.0001$). ^^^^, significant difference between SAL-CS and HA-CS groups ($p < 0.0001$).

(SAL-NS), suggesting that (+)-HA-966 prevented the regional increase in the DOPAC:DA ratio normally induced by stress.

In the l-PFC, there was also an effect of treatment on DA utilization [$F(2,19) = 7.13$, $p < 0.005$]. The SAL-CS group DOPAC:DA ratio nearly doubled in comparison to the SAL-NS group ($p < 0.01$). Mean DOPAC:DA ratios in the l-PFC for the SAL-NS and SAL-CS groups were 0.183 (±0.023) and 0.359 (±0.046), respectively. This increase in l-PFC DA utilization in the SAL-CS group was completely blocked by pretreatment with (+)-HA-966 ($p < 0.05$). Mean DOPAC:DA ratios for the SAL-CS versus HA-CS groups were 0.359 (±0.046) and 0.215 (±0.024), respectively. No difference in l-PFC DA utilization between the conditioned stress group pretreated with (+)-HA-966 (HA-CS) and the nonstressed saline pretreated control group (SAL-NS) was observed, suggesting that (+)-HA-966 prevented the regional increase in the DOPAC:DA ratio normally induced by stress. Note that these effects of (+)-HA-966 on stress-induced increases in DA utilization in the m-PFC and l-PFC were not due to (+)-HA-966 lowering basal DA utilization (see Table 2).

DA utilization in the two other cortical regions, the perirhinal and cingulate cortices, was not increased by exposure to con-

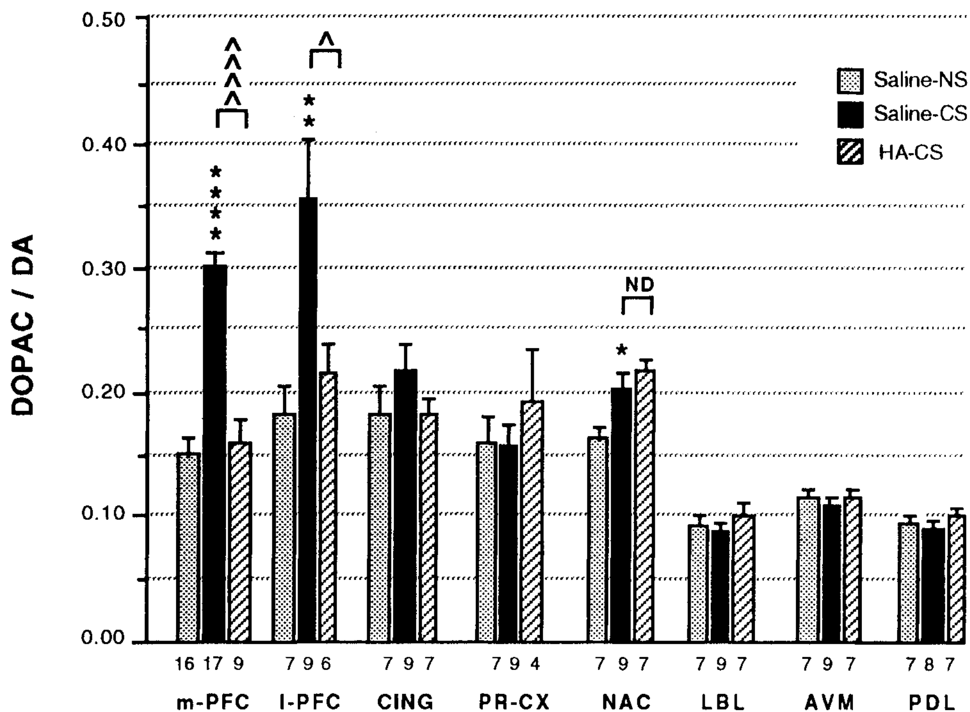


Figure 7. Effect of saline versus (+)-HA-966 pretreatment on dopamine utilization in cortical, limbic, and striatal terminal regions in response to conditioned stress. (+)-HA-966 or saline was administered by intraperitoneal injection at doses of 15 mg/kg and 1 ml/kg, respectively, 15 min prior to the extinction trial on day 2. Control animals were subjected to the same training procedures as the conditioned stress groups except that the control group did not receive footshock during the conditioning period. Control animals were also injected with saline (1 ml/kg, i.p.) 15 min prior to the extinction trial on day 2. Data are expressed as means (±SEM). *m-PFC*, medial prefrontal cortex; *l-PFC*, lateral prefrontal cortex; *CING*, posterior cingulate cortex; *PR-CX*, perirhinal cortex; *NAC*, nucleus accumbens septi; *LBL*, lateral-basolateral nuclei of the amygdala; *AVM*, anterior ventromedial caudatoputamen; *PDL*, posterior dorsolateral caudatoputamen. *Numbers below histogram bars* indicate number of rats in each group. ****, significant difference between the SAL-NS and SAL-CS groups ($p < 0.0001$). ^^^^, significant difference between the SAL-CS and HA-CS groups ($p < 0.0001$). **, significant difference between the SAL-NS and SAL-CS groups ($p < 0.01$). ^, significant difference between the SAL-CS and HA-CS groups ($p < 0.05$). *, significant difference between the SAL-CS and SAL-NS groups ($p < 0.05$). ND, no significant difference between the SAL-CS and HA-CS groups.

ditioned stress. Nor was there a significant effect of pretreatment with (+)-HA-966 in these two cortical regions.

In the NAC, a modest effect of treatment on DA utilization was detected [$F(2,20) = 5.01$, $p < 0.05$]. The SAL-CS group showed a 22.9% increase in the DOPAC:DA ratio versus the SAL-NS group ($p < 0.05$). Mean DOPAC:DA ratios in the NAC for the SAL-NS and SAL-CS groups were 0.166 (±0.011) and 0.204 (±0.013), respectively.

In contrast to the effect seen in the m-PFC and l-PFC, this increase in NAC DA utilization in the SAL-CS group was not blocked by pretreatment with (+)-HA-966. Mean DOPAC:DA ratios in the NAC for the SAL-CS versus HA-CS groups were 0.204 (±0.013) and 0.222 (±0.012), respectively. A separate control experiment demonstrated that (+)-HA-966 administration did not alter the basal DOPAC:DA ratio in this region (see Table 2). In contrast to the effect of stress noted in the NAC, the LBL showed no effect of treatment on DA utilization [$F(2,20) = 0.21$, $p > 0.05$]. Mean DOPAC:DA ratios in the LBL for the SAL-NS and SAL-CS groups were 0.106 (±0.010) and 0.112 (±0.011), respectively. Pretreatment with (+)-HA-966 before conditioned stress did not affect DA utilization in this region. Mean DOPAC:DA ratios in the LBL for the SAL-CS versus HA-CS groups were 0.204 (±0.013) and 0.222 (±0.012), respectively.

Finally, we examined the effect of conditioned stress on DA utilization in two striatal regions, the anterior ventromedial caudatoputamen (AVM) and the posterior dorsolateral caudatoputamen (PDL). In neither of these two areas was DA utilization affected by treatment. Nor did (+)HA-966 affect DA utilization during conditioned stress. In the AVM, mean DOPAC:DA values for the SAL-NS, SAL-CS, and HA-CS groups were 0.116 (±0.006), 0.110 (±0.008), and 0.117 (±0.004), respectively. In the PDL, mean DOPAC:DA values for the SAL-NS, SAL-CS, and HA-CS groups were 0.096 (±0.006), 0.091 (±0.005), and 0.103 (±0.007), respectively.

Effects of conditioned stress and (+)-HA-966 on regional 5-HT utilization

Results of the effects of conditioned stress and (+)-HA-966 pretreatment on 5-HT utilization are presented in Figure 8. Analyses were conducted on the same animals and tissue samples as those used for the DA studies detailed above. All data are derived from neurochemical analysis of brain tissue collected at death immediately following the extinction trial on day 2. 5-HT utilization is expressed as the ratio of the 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA) to the parent neurotransmitter, 5-HT (i.e., 5-HIAA:5-HT). Basal neurochemistry values for naive rats are shown in Table 1.

In the m-PFC, an effect of treatment on 5-HT utilization was noted [$F(2,39) = 10.29$, $p < 0.001$]. The SAL-CS group showed a 40.6% increase in the 5-HIAA:5-HT ratio versus the SAL-NS group ($p < 0.01$). Mean 5-HIAA:5-HT ratios in the m-PFC for

Table 3. Effect of saline or (+)-HA-966 pretreatment 15 min prior to day 1 conditioning

	Pretraining injection day 1	
	Saline	(+)-HA-966
Day 1 responses		
Ultrasonic callers	12/17	4/9ND
% calls/rat	297.9 ± 74.4 (17)	178.0 ± 77.9[ND] (9)
% freeze	53.4 ± 5.3 (17)	45.0 ± 9.8[ND] (9)
Day 2 responses		
% Freeze	72.6 ± 4.3 (15)	48.1 ± 8.9* (9)
Serum corticosterone (ng/ml)	219.9 ± 12.1 (15)	222.4 ± 27.0[ND] (9)
DOPAC:DA	0.300 ± 0.014 (17)	0.238 ± 0.011** (9)

Data show effect of pretreatment with (+)-HA-966 on acquisition of conditioned stress. Saline or (+)-HA-966 was administered 15 min prior to the conditioning period on day 1. Data are presented as the mean ± SEM with the number of test subjects in parentheses below. Ultrasonic caller data is presented as the ratio of callers to all animals in the treated group. The day 1 responses represent data collected during the conditioning period on day 1. The day 2 responses represent data collected from the same rats during the extinction period on day 2. Note that all rats received saline injections before testing on day 2.

* Significant difference ($p < 0.05$).

** Significant difference ($p < 0.01$).

ND No significant difference.

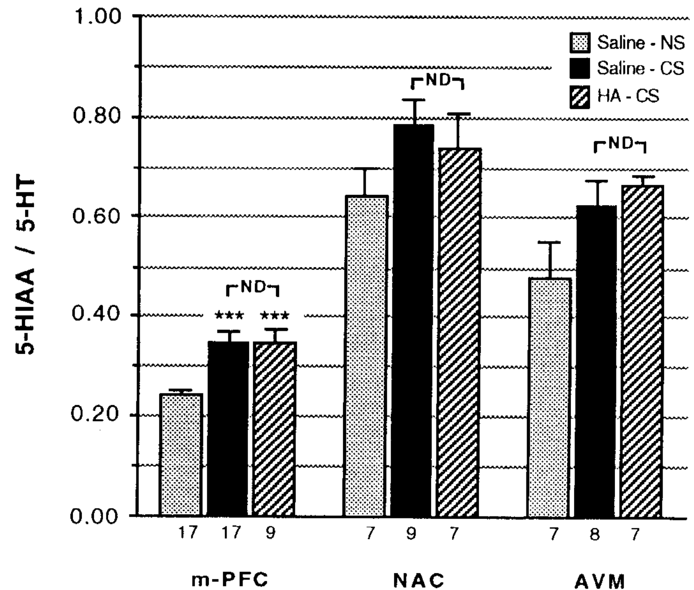


Figure 8. Effect of saline versus (+)-HA-966 pretreatment on 5-HT utilization in the medial prefrontal cortex (*m-PFC*), nucleus accumbens (*NAC*), and anterior ventromedial caudatoputamen (*AVM*) in response to conditioned stress. Saline or (+)-HA-966 were administered systemically at intraperitoneal doses of 1 ml/kg or 15 mg/kg, respectively, 15 min prior to the extinction trial on Day 2. *Numbers below histogram bars* indicate number of rats in each group. ***, significant difference versus the SAL-NS control group ($p < 0.001$). ND, no significant difference between the SAL-CS and HA-CS groups.

the SAL-NS and SAL-CS groups were 0.246 (±0.012) and 0.346 (±0.025), respectively. The increase in the ratio of 5-HIAA to 5-HT in the m-PFC reflects the composite effects of a decrease in tissue 5-HT and increase in tissue 5-HIAA. In contrast to findings in the m-PFC DA system, the conditioned stress-induced increase in 5-HT utilization in the m-PFC was not blocked by pretreatment with (+)-HA-966. Mean 5-HIAA:5-HT ratios for the SAL-CS group (0.346 ± 0.025) were indistinguishable from the HA-CS group (0.368 ± 0.018).

5-HT utilization was also analyzed in the NAC and the AVM. No effect of treatment on 5-HIAA:5-HT ratios was noted in either region. In the NAC, mean ratios of 5-HIAA:5-HT for the SAL-NS, SAL-CS, and HA-CS groups were 0.582 (±0.077), 0.786 (±0.051), and 0.744 (±0.069), respectively. In the AVM, mean ratios of 5-HIAA:5-HT for the SAL-NS, SAL-CS, and HA-CS groups were 0.481 (±0.074), 0.627 (±0.048), and 0.669 (±0.021).

Effect of pretraining administration of (+)-HA-966 on behavioral, neuroendocrine, and neurochemical indices of conditioned stress

Since the NMDA receptor complex has been implicated in memory processing, we tested the effect of pretraining administration of (+)-HA-966 on acquisition of the conditioned behavioral, neuroendocrine, and neurochemical responses to conditioned stress (Table 3). Administration of (+)-HA-966 prior to training on day 1 and administration of saline prior to testing on day 2 resulted in attenuation of freezing to the conditioned stimulus ($p < 0.05$). The mean percentage of time engaged in freezing during the extinction trial for saline and (+)-HA-966 pretreated animals was 72.57% (±4.31) and 48.11% (±8.86), respectively. Nevertheless, it should be noted that the (+)-HA-966 pretreated group did exhibit substantial freezing on day 2, indicating that these animals had learned to fear the conditioned stimulus on the previous day. This is consistent with the observation that serum corticosterone values in the saline and (+)-HA-966 pretreated groups were indistinguishable on day 2. An attenuation in DA utilization in the m-PFC was also observed on day 2 when (+)-HA-966 was administered before training on day 1 ($p < 0.01$).

Discussion

The present study indicates that pretreatment with (+)-HA-966, an antagonist at the glycine modulatory site of the NMDA receptor complex (Singh et al., 1990), blocks the increase in DA utilization in both the medial and lateral prefrontal cortices in response to conditioned stress. This effect is regionally selective, in that the conditioned stress-induced increase in DA utilization in the nucleus accumbens was not blocked by pretreatment with (+)-HA-966. The effect is neurochemically specific since the conditioned stress-induced increase in 5-HT utilization in the m-PFC was not blocked by pretreatment with (+)-HA-966. The effect is also state dependent in that (+)-HA-966 caused no changes in basal DA or 5-HT utilization or in serum corticosterone in control rats. This lack of effect on basal neurochemistry stands in contrast to the dose-dependent increases in DA release and metabolism induced by the noncompetitive NMDA receptor antagonist dizocilpine (MK-801; Loscher et al., 1991).

Glycine may be an important endogenous modulator of the NMDA receptor channel complex (Kemp and Leeson, 1993). This hypothesis is supported by *in vitro* studies that have shown that the response of the NMDA receptor to agonist stimulation is absolutely dependent on the presence of glycine (Kleckner and Dingledine, 1988) and that glycine mediates its actions by

binding to the NMDA receptor complex (Johnson and Ascher, 1992). Glycine is found in relatively high (micromolar) concentrations in brain extracellular fluid (M. During, personal communication). These data in concert with the present results suggest that the glycine modulatory site of the NMDA receptor complex may play a physiologically significant role in the selective regulation of the mesoprefrontal cortical DA system response to conditioned stress. Furthermore, these data also suggest that endogenous ligands at this site, such as the amino acid glycine and the antagonist kynurenic acid, may be physiological modulators of mesoprefrontal dopaminergic activity.

Effect of (+)-HA-966 on behavioral and neuroendocrine responses

In addition to having effects on prefrontal cortical DA utilization, we observed that (+)-HA-966 pretreatment reduced freezing and increased locomotion in the face of conditioned stress. This was manifested as a downward shift in the freezing extinction curve and in early reemergence of locomotion (Fig. 6). Pretreatment with (+)-HA-966 attenuated, but did not completely block the behavioral stress response. This finding corroborates and extends recent work by Dunn et al. (1992) suggesting that (+)-HA-966 has anxiolytic behavioral properties.

However, pretreatment with (+)-HA-966 prior to conditioned stress did not reduce the stress-induced elevation in serum corticosterone. This is not surprising since (+)-HA-966 pretreated animals still exhibited a substantial, albeit reduced, behavioral stress response. In addition, it should be emphasized that even very large doses of classic anxiolytic agents, such as the benzodiazepine agonist diazepam may only partially suppress the rat adrenocortical response to psychological stress (e.g., Kaneyuki et al., 1991).

Differential responses of dopaminergic and serotonergic systems to conditioned stress

Unconditioned stress, such as spatial proximity to other rats exposed to footshock (Kaneyuki et al., 1991) or exposure to the urine of stressed conspecifics (Goldstein et al., unpublished observations), as well as conditioned stress such as exposure to an environmental context previously paired with footshock (Herman et al., 1982; Deutch et al., 1985), have been shown to increase DA utilization in the m-PFC. Results from the present experiments are in agreement with these previous studies. The present experiments also demonstrate that conditioned stress increases DA utilization in the l-PFC, but not in the cingulate or perirhinal cortices. This suggests that the different subsets of DA neurons innervating these cortical areas (Lindvall and Bjorklund, 1984) may have different afferent regulatory mechanisms.

A previous study has reported high basal DA utilization in the l-PFC (Jones et al., 1986), a finding corroborated in the present experiments. In one of few other reports examining this region, Herman et al. (1982) observed increased DA utilization in response to footshock, but not in response to contextual cues associated with footshock. The present study, however, demonstrates that increased DA utilization in this region does occur in response to conditioned aversive cues. Furthermore, this increase was completely blocked by pretreatment with (+)-HA-966. The discrepancy between these studies may be accounted for by differences in conditioning protocols. In our experiments, a randomly presented discrete conditioned stimulus (e.g., a sub-startle-threshold tone) was presented in an overlapping manner with the unconditioned stimulus (e.g., footshock). No such discrete conditioned stimulus was used in the Herman study. We have previously shown in unpublished work that the behavioral, neurohumoral, and neurochemical indices of conditioned stress in our model are under stimulus (i.e., tone) control, suggesting that the presence of this discrete conditioned stimulus may have increased the degree of perceived threat and contributed to the effects on l-PFC DA utilization seen in the present study. Also, in contrast to the previous study (Herman et al., 1982), the present experiments were conducted during the dark phase of the rat diurnal activity cycle. It is also possible that tissue dissections of the l-PFC may have differed between studies.

In addition to increasing DA utilization in the prefrontal cortices, conditioned stress was shown to modestly but reliably activate the mesoaccumbens DA system. Exposure to mildly stressful stimuli is known to activate the medial prefrontal cortical DA system selectively whereas more profound or prolonged stimulation from footshock or physical restraint results in recruitment of the mesoaccumbens and finally the mesostriatal DA systems (Roth et al., 1988; Deutch and Roth, 1990). The fact that the mesoaccumbens DA system was activated suggests that the conditioned stress model used in the present study is at least moderately stressful for these animals. It also demonstrates that an increase in DA utilization in the nucleus accumbens is not singularly associated with an increase in locomotion. In addition, dissection of the nucleus accumbens into its core and shell subdivisions reveals that DA utilization in the limbic system–related shell region occurs at levels of stress that do not activate the core (Deutch and Cameron, 1992). The accumbens dissection used in the present study included both core and shell regions, and was similar to that used in previous studies from this laboratory (Roth et al., 1988; Deutch and Roth, 1990) in which the m-PFC was selectively activated by mild stress. Thus, the fact that the mesoaccumbens DA system was only modestly activated by conditioned stress in the present study might reflect a tissue dilution artifact of mesoaccumbens DA activation in the shell subdivision.

Though the effect of various stressors on DA utilization in the m-PFC has been known for some time (reviewed by Deutch and Roth, 1990), much less is known about 5-HT utilization in response to stress. The literature on this biogenic amine system and stress is contradictory. In a recent review, Jacobs (1992) concluded that presumptive serotonergic neurons in the raphe nuclei of cats change their firing rate and pattern in response to conditions eliciting arousal, but do not further change their firing frequency or pattern in response to physical stress. Kaneyuki et al. (1991) observed that while exposure of rats to unconditioned psychological stress increased medial prefrontal cortical DA utilization, increased 5-HT utilization was not seen. However, others have demonstrated increases in 5-HT utilization in response to unconditioned physical stress in whole brain (Thierry et al., 1968) and in cortex (Bliss et al., 1968; Adell et al., 1988; Dunn, 1988; Miyauchi et al., 1988; Pei et al., 1990; Heinsbroek et al., 1991). In addition, Inoue and coworkers (1993) observed a selective increase in medial prefrontal cortical 5-HIAA levels in rats reexposed to a context previously paired with intense footshock. This latter study is consistent with our finding that exposure to conditioned stress induces a regionally specific increase in 5-HT utilization in the m-PFC.

We have consistently found this selective increase in medial prefrontal cortical 5-HT utilization in response to conditioned stress to be less substantial, but as reliable as the selective in-

crease in DA utilization in this region under the same experimental conditions. Differences in results between studies reported in the literature are most likely due to a combination of factors including differences in stress models, duration and intensity of stress exposure, neurochemical endpoints, and means of endpoint measurement. Clearly, further studies of the relationship between regional 5-HT utilization and exposure to conditioned stress are needed.

Effect of (+)-HA-966 on conditioned stress-induced increases in mesoprefrontal cortical DA and 5-HT utilization

The increase in mesoprefrontal cortical DA utilization in response to conditioned stress may be related to alterations in DA neuron firing patterns. DA neurons in the VTA that innervate the prefrontal cortex are known to fire in two different patterns: an irregular mode characterized by spikes frequencies of 3–9 Hz, and a bursting mode in which spikes occur in groups with short interspike intervals (Bunney et al., 1973; Wang, 1981; Grace and Bunney, 1983, 1984). In anesthetized animals, DA neuron bursting is correlated with dramatic increases in DA release in terminal regions. For example, Bean and Roth (1991) have shown that electrical stimulation of the median forebrain bundle in a bursting pattern results in increased DA release in the m-PFC. In addition, a recent study has demonstrated that pressure ejection of NMDA into the VTA results in burst firing in presumptive DA neurons and enhancement of DA release in the NAC (Suaud-Chagny et al., 1992). These data suggest that the increase in mesoprefrontal cortical DA utilization induced by conditioned stress may be due in part to increased bursting of DA neurons in the VTA with a concomitant increase in DA release and metabolic utilization in stress-activated terminal regions.

That the NMDA receptor complex may be involved in the regulation of DA neurons at the cell body region is supported by several other lines of evidence. NMDA sensitive glutamate binding has been shown to be present in the VTA (Monaghan and Cotman, 1985) and excitatory amino acid afferents to the VTA arise from the cortex (Carter, 1982; Nieoullon and Dustidier, 1983; Christie et al., 1985). Indeed, Sesack and Pickel (1992) have elegantly demonstrated direct monosynaptic input from prefrontal cortical efferents to dopaminergic neurons in the VTA. A recent study has shown that this prefrontal cortical projection to the VTA may be physiologically relevant in that glutamate injection into this cortical region selectively increased burst firing of DA neurons in the VTA and enhanced the release of DA in the NAC, whereas prefrontal cortical injection of the local anesthetic lidocaine resulted in the opposite effects on burst firing and DA release (Murase et al., 1993).

Electrophysiological studies have also shown that NMDA exerts a powerful excitatory effect on midbrain DA neurons in both *in vitro* and *in vivo* preparations (Seutin et al., 1990; Mercuri et al., 1992; Chergui et al., 1993). However, electrophysiological investigations to date have not determined whether these findings extend to the DA neurons in the A10 VTA region that project to the prefrontal cortex. A recent study demonstrated that A9 DA neurons in an *in vitro* slice preparation change their firing pattern from a regular to bursting mode in response to bath application of NMDA (Johnson et al., 1992). This effect was observed in the presence of the spider venom apamin, a potent antagonist of calcium-dependent potassium channels that is itself known to induce bursting in these neurons (Shepard and Bunney, 1988). An *in vivo* study in the rat has demonstrated that iontophoretic or pressure injection of the specific NMDA antagonist AP-5 into the A9/A10 DA cell body region regularized cell firing by reducing bursting, whereas injection of NMDA-induced bursting (Chergui et al., 1993). Recent data obtained by Shepard and colleagues demonstrate that modulation of the NMDA receptor at the glycine site exerts a profound effect on A9 DA neuron firing (McMillen et al., 1992; Shepard and Lehmann, 1992). These investigators showed that (+)-HA-966 normalizes the dopaminergic neuron firing pattern such that the variability of intervals between spikes is dramatically reduced.

Kalivas and colleagues (1989) have shown that infusion of NMDA into the VTA selectively increases DA metabolites in the m-PFC. Likewise, direct injection of glutamate into the VTA increases DA metabolites in the m-PFC, NAC, and the VTA, while coadministration of the NMDA receptor antagonist CPP selectively blocks the effects of glutamate on m-PFC DA utilization. Similarly, administration of CPP into the VTA prevents footshock-induced increases in DA utilization in the m-PFC. A recent finding from this laboratory has demonstrated that intra-VTA infusion of (+)-HA-966 prevents physical restraint-induced increases in m-PFC DA utilization (Morrow et al., 1993). In light of these findings, we hypothesize that (+)-HA-966 blocks the increase in mesoprefrontal cortical DA utilization in response to conditioned stress by inhibiting excitatory amino acid–induced excitation of DA cells in the VTA.

Another possible explanation for the ability of (+)-HA-966 to block increased DA utilization in the prefrontal cortices during stress invokes interaction of this compound with excitatory amino acid receptors that may regulate DA release at neuronal terminals. A presynaptic excitatory amino acid regulatory mechanism is well established for DA terminals in the striatum (Roberts and Anderson, 1979; Chesselet, 1990). In fact, Crawford and Roberts (1989) demonstrated that a racemic mixture of HA-966 antagonized NMDA-induced enhancement of ^{3}H-DA release from rat striatal slices. This antagonism was reversed by inclusion of glycine in the bathing solution. A similar, though speculative, mechanism operating at the DA terminals in the cortex could explain the present results. Even though excitatory amino acid presynaptic regulation of DA release in the m-PFC has not been demonstrated, Moghaddam et al. (1993) have shown that glutamate and aspartate levels in the m-PFC increase in response to restraint and swim stress.

In contrast to its effect on DA utilization in the prefrontal cortices during conditioned stress, (+)-HA-966 does not block the conditioned stress-induced increase in medial prefrontal cortical 5-HT utilization. This finding clearly points to the neurochemical specificity of the effects of (+)-HA-966. Furthermore, these data indicate that the afferent control of the activation of the mesoprefrontal cortical serotonergic system probably does not involve glycine-modulated NMDA receptor mediated mechanisms.

Effect of (+)-HA-966 on acquisition of conditioned aversive memory

The NMDA receptor complex has been implicated in memory processing events (e.g., Morris et al., 1986; Miserendino et al., 1990). A recent report indicates that the NMDA glycine site may also be involved in memory processing since the NMDA glycine site agonist D-cycloserine appears to facilitate retention of an avoidance task in mice (Flood et al., 1992). These studies have suggested that (+)-HA-966 might impair memory. However, in the present study, rats pretreated with (+)-HA-966 prior

to conditioning demonstrated elevations in serum corticosterone, freezing, and m-PFC DA utilization in response to the conditioned stimuli, demonstrating that the animals had indeed learned to associate the tone with footshock. This finding indicates that (+)-HA-966, at a dose of 15 mg/kg, does not block aversive memory formation under these experimental conditions.

Nevertheless, at least two components of the stress response, freezing and increased m-PFC DA utilization, were attenuated under this regimen indicating that (1) the perception of and/or reaction to the unconditioned stimulus was blunted at the time of the conditioning, consistent with the hypothesis that (+)-HA-966 exhibits anxiolytic properties (Dunn et al, 1992), and/or (2) the conditioned aversive memories were rendered less stable during memory acquisition or consolidation, consistent with this compound's known ability to alter NMDA receptor function at the glycine modulatory site. In addition, we cannot rule out the possibility that the effects of preconditioning administration of (+)-HA-966 on behavioral and neurochemical indices measured the following day may reflect prolonged pharmacokinetic clearance of (+)-HA-966 or the persistence of bioactive metabolites. However, the finding that pretraining treatment with this compound alters behavioral and neurochemical expression of conditioned stress without blocking formation of aversive memories suggests that (+)-HA-966 administered at this dose may differentially affect processes that are influenced by NMDA receptor systems. Further work is necessary to clarify these issues.

Clinical relevance and possible therapeutic potential of (+)-HA-966

Both *in vitro* and *in vivo* studies have demonstrated that DA and 5-HT influence cortical electrophysiological activity (Bunney and Aghajanian, 1976; Mantz et al., 1988, 1990; Sesack and Bunney, 1989; Gellman and Aghajanian, 1993). Thus, catecholamines may contribute to cognition, possibly by enhancing signal processing in the cortex (Servan-Schreiber et al., 1991). Studies in the rat have demonstrated cognitive impairment following destruction of the A10 DA neurons that project to the m-PFC (Simon et al., 1980). Cortical DA depletion in the dorsolateral prefrontal cortex of rhesus monkeys is associated with cognitive deficits that are reversed by administration of the DA precursor L-dopa or the DA agonist apomorphine (Brozoski, 1979). In addition, direct injection of a DA antagonist into the prefrontal cortex of rhesus monkeys induces specific cognitive deficits related to working memory (Sawaguchi and Goldman-Rakic, 1991).

One of the hallmarks of schizophrenia is alteration in formal thought processes and cognition. Though the neuropathology of this disorder is unclear, cortical dysfunction in this disorder has been related to dopaminergic dysregulation (Weinberger et al., 1986; Davis et al., 1992). In addition, certain symptoms of this disorder are thought to be induced or exacerbated by stress (Zubin and Spring, 1977; Brown and Birley, 1968; Bebbington et al., 1993). The fact that (+)-HA-966 blocks the conditioned stress-induced activation of the m-PFC DA system at a non-sedating dose without altering basal dopaminergic metabolic activity suggests the possibility that this drug may be a novel pharmacological agent for the treatment of schizophrenia.

Post-traumatic stress disorder (PTSD) is a psychiatric disorder caused by exposure to overwhelmingly stressful circumstances. Inescapable footshock and conditioned fear in animals have been suggested as models of this anxiety disorder (van der Kolk et al., 1985; Charney et al., 1993). In PTSD, cues associated with an initial trauma later become capable of eliciting a psychological and physiological reaction similar to that induced by the initial trauma. Thus, the finding that (+)-HA-966 appears to hasten the rate of extinction of defensive responses to conditioned stressful stimuli suggests that this drug may also be useful in the treatment of PTSD. Furthermore, the finding that (+)-HA-966 does not block acquisition of aversive memories, but mitigates the neurochemical and behavioral responses to these memories, suggests that administration of this drug may be relatively free of unwanted cognitive side effects.

Further electrophysiological, neurochemical, and behavioral studies are clearly necessary to elucidate the means by which (+)-HA-966 modulates stress activation of the mesoprefrontal cortical DA system. This future work may help clarify the possible contribution of the mesoprefrontal cortical DA system and the NMDA receptor complex to the pathophysiology and therapeutic management of stress-induced or stress-exacerbated neuropsychiatric disorders.

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