

(S)-(−)-HA-966, a γ-Hydroxybutyrate-Like Agent, Prevents Enhanced Mesocorticolimbic Dopamine Metabolism and Behavioral Correlates of Restraint Stress, Conditioned Fear and Cocaine Sensitization

BRET A. MORROW, EDWARD J. K. LEE, JANE R. TAYLOR, JOHN D. ELSWORTH, HEATHER E. NYE and ROBERT H. ROTH

Laboratory of Neuropsychopharmacology, Departments of Pharmacology (E.J.K.L., R.H.R.) and Psychiatry (B.A.M., J.R.T., J.D.E., H.E.N., R.H.R.), Yale University School of Medicine, New Haven, Connecticut 06520-8066

Accepted for publication July 15, 1997

ABSTRACT

This report investigates the effect of the negative enantiomer of 1-hydroxy-3-aminopyrrolidone-2 (HA-966) on behavioral and biochemical changes elicited by pharmacological or experimental paradigms which activate mesocorticolimbic dopaminergic neurotransmission. Several paradigms were used, including cocaine sensitization and two stressors: restraint for 30 min and an aversive conditioning model. (S)-(−)-HA-966 (3 and 5 mg/kg i.p.) prevented restraint stress-induced dopamine utilization in both the medial prefrontal cortex and nucleus accumbens, in contrast to the positive enantiomer. Conditioned fear increased dopamine metabolism in both the core and shell subdivisions of the nucleus accumbens, an effect blocked by (S)-(−)-HA-966. The conditioned stress-induced increase in dopamine metabolism in the medial prefrontal cortex was also blocked by (S)-(−)-HA-966. In addition, (S)-(−)-HA-966 suppressed fear-induced behaviors: immobility and defecation. In other studies, (S)-(−)-HA-966 (3 mg/kg i.p.) prevented locomotor sensitization without altering the acute motoric response elicited by cocaine. The highest dose of (S)-(−)-HA-966 (5 mg/kg i.p.) blocked acute cocaine-induced locomotion but resulted in sedation. In addition, the highest dose of (S)-(−)-HA-966 tested suppressed weight gain in control rats, unlike its enantiomer, (R)-(+) HA-966. Because (S)-(−)-HA-966 has been proposed to act at the γ-aminobutyric acid (GABA)B receptor, we examined the ability of (S)-(−) and (R)-(+) HA-966 to displace [3H]-baclofen from cortical membranes to assess GABA B receptor binding. Neither enantiomer significantly altered [3H]-baclofen binding at relevant concentrations, indicating the actions of (S)-(−)-HA-966 reported here are the results of a mechanism apparently independent of the baclofen binding site on the GABA B receptor.

The pharmacological, physiological and behavioral properties of (±)-HA-966 have been widely documented in the past. As a heterocyclic imido five-member ring, (±)-HA-966 appears to be structurally similar to a cyclic form of GABA. Bonta et al. (1971) observed that racemic HA-966 had sedative qualities that mimicked the behavioral properties of γHB. Furthermore, it was found that after the administration of (±)-HA-966, DA levels were increased in the striatum of the rat brain (Bonta et al., 1971) and the spontaneous firing rate of substantia nigra dopaminergic neurons was reduced in a dose-dependent fashion (Nowycky and Roth, 1977; Shepard and Lehmann, 1992), actions similar to those noted with γHB (Roth et al., 1977). In contrast to these findings, Davies and Watkins (1972) discovered that (±)-HA-966 was an excitatory amino acid antagonist, later discovered to act through the glycine modulatory site of the NMDA receptor (Foster and Kemp, 1989).

Only after the enantiomers of (±)-HA-966 were resolved, however, was it possible to attribute the unique characteristics of the racemic compound to its components. Radioligand binding and electrophysiological studies demonstrated that the antagonistic effects of (±)-HA-966 on the glycine/NMDA receptor mainly resided in the (R)-(+) enantiomer, whereas behavioral studies indicated that the sedative/ataxic properties of (±)-HA-966 were primarily due to the (S)-(−) enantiomer. The effects of the racemic compound on nigrostriatal DA neurons seem to reside with the (S)-(−) enantiomer, including increased striatal DA levels (Singh et al., 1990) and

ABBREVIATIONS: GABA, γ-aminobutyric acid; γHB, γ-hydroxybutyric acid; NMDA, N-methyl-D-aspartate; DOPAC, 3,4-dihydroxyphenylacetic acid; DA, dopamine; NAS, nucleus accumbens; mPFC, medial prefrontal cortex; (±)-HA-966, 1-hydroxy-3-aminopyrrolidone-2; 5-HIAA, 5-hydroxyindolacetic acid; 5-HT, serotonin; ANOVA, analysis of variance; HPLC, high-performance liquid chromatography.
inhibition of nigrostriatal DA neuron firing (Shepard and Lehmann, 1992). The simultaneous blockade in spontaneous firing with diminished release and continued synthesis of DA in striatal dopaminergic neurons has been used to explain the increases in striatal DA levels attributed to (−)-HA-966 (Singh et al., 1990).

Increased striatal DA levels, as well as the sedative behavioral properties, were noted to mirror those seen with γHB and γ-butyrolactone (Roth et al., 1977), both of which are structurally similar to (±)-HA-966 (Bonta et al., 1971; Singh et al., 1990). It was postulated that (−)-HA-966 exerted its effects through the GABAB receptor based on the resemblance of HA-966 to the cyclic form of GABA and because γHB has been proposed to elicit its actions through the GABAB receptor (Engberg and Nissbrandt, 1993). In support of this, the GABAB antagonist CGP 35348 was found to attenuate the increase in DA synthesis by γ-butyrolactone, HA-966 and the GABAB agonist (−)-baclofen (Waldmeier, 1991). The binding site by which γHB affects GABAB receptors is not clear, although a highly specific membrane γHB binding site, without affinity for GABA or GABAergic agonists, has been identified (Benavise et al., 1982; Mair et al., 1983). These data suggest that (S)-(−)-HA-966 may act by affecting GABAB-ergic neurotransmission.

Shepard et al. (1995) proposed that both enantiomers of HA-966 may mediate DA firing pattern through a common mechanism related to GABAB. Both enantiomers are able to dose-dependently inhibit nigral DA neuronal firing, and in each case, this inhibition can be reversed by a GABAB antagonist, CGP-35348. However, (R)(+)-HA-966 was found to be ~10-fold less potent than the negative enantiomer. In addition, both enantiomers, at different doses, display a normalization of firing pattern of midbrain DA neurons, resulting in an increase in the regularity of cell firing without changing the overall firing rate in chloral hydrate anesthetized rats (Shepard et al., 1995). Again, a 10-fold difference in potency was noted between the enantiomers. The normalization of firing pattern was not mimicked by the administration of a competitive NMDA receptor antagonist, NPC-12626, indicating that this effect is probably not due to blockade of the NMDA receptor complex (McMillen et al., 1992). Previously, we proposed that the normalization in firing pattern seen by Shepard et al. may be the mechanism by which (R)-(−)-HA-966 prevents stress-induced changes in DA metabolism without disrupting basal DA turnover (Morrow et al., 1993). In addition, it seemed feasible that this hypothesis could be extended to explain the ability of (R)(+)-HA-966 to prevent cocaine-induced locomotor sensitization (Morrow et al., 1995a, 1995b). If the normalization of DA neuron firing is necessary for these actions, the negative enantiomer of HA may provide efficacy in these models but at lower doses.

**Methods**

**Restraint stress protocol.** Male Sprague-Dawley rats initially weighing ~250 g were used in these experiments. For restraint stress experiments, rats were injected with saline or (S)-(−)-HA-966 (1.3 or 5 mg/kg i.p.; Research Biochemicals, Natick, MA), returned to the home cage for 20 min, moved to a separate room and placed into a wire restraint apparatus for 30 min. The apparatus restrained without immobilizing the rat. In addition, special care was taken to avoid pinching or crushing the animal during the restraint period. Control rats remained in the home cages in a separate room. At the end of the experiment, rats were rapidly decapitated, the brains were removed and the following regions dissected as previously described: mPFC, NAS and striatum (Horger et al., 1995).

**Fear conditioning.** Aversive conditioning was performed in test cages as previously described (Morrow et al., 1995b). The test cages were Plexiglas and stainless steel (24 × 30 × 27 cm) with a grid floor wired for footshock. To minimize external interference, the test cages were located within a sound-attenuated chamber illuminated by a red light. A white noise generator provided a constant background noise, and the cages were cleaned and dried before each session with 70% ethanol to minimize olfactory cues. A PC controlled the 2.8-kHz tone along with triggering the shock generators (BRS/LVE) that delivered the foot shock. This tone did not startle naive rats. The intensity of the foot shock was calibrated to 0.4 mA (Morrow et al., 1995b).

Habituation, aversive conditioning and test sessions were given on 3 consecutive days. On day 1, the habituation day, rats were placed into the test cages and left for 30 min. No tones or foot shocks were given on day 1. On day 2, the aversive conditioning day, rats were treated with (S)-(−)-HA-966, 5 mg/kg or saline; returned to the home cage for 20 min and then placed into the test cages and given 10 tones with or without a paired footshock over 30 min. The intervals between tones were randomly selected by the computer to be between 1 and 4 min. The tones were 5 sec long and paired with the 0.5-sec-long foot shocks so that the tone and foot shock terminated together. Thus, conditioned rats received a total of 10 × 0.5 sec of mild foot shock. The response of the rats to the foot shock itself was typical of that to mild foot shock: initial startled and eventual immobility. No rat attempted escape during the administration of foot shock, and no vocalization was noted. On day 3, the test day, rats were returned to the test cages and given 10 computer-controlled, random tones without foot shock over 30 min. Immediately after the completion of the test session, rats were killed by decapitation, and the following brain regions were rapidly dissected on a 1°C stage: mPFC, NAS core, NAS shell, striatum and VTA (Horger et al., 1995). Care was taken to avoid the anterior portion of the NAS, which is predominately the shell subdivision. The tissue was stored at ~70°C until assayed for monoamines. Rats were in one of four groups: saline/nonstress, (S)-(−)-HA-966/nonstressed, saline/conditioned and (S)-(−)-HA-966/conditioned groups.

The videotape of the rat’s behavior on the test day was evaluated for the duration of immobility associated with the presentation of the tone. A 1-min interval that included the 5-sec tone and 55 sec after the tone was selected for analysis and scored by an experimenter blinded to each subject’s treatment. Immobility was defined as no visible movement except those necessary for respiration. Occasionally, nonstressed, control rats would fall asleep during the last three tones; those data were excluded from analysis. Sleeping was noted only in nonstressed rats and was not associated with the tone but was usually preceded by grooming and other preparatory behaviors. The number of fecal boli left after each session was also counted.

**Behavioral sensitization to cocaine.** Thirty-two naïve, male Sprague-Dawley rats (~250 g) were tested for locomotor sensitization to repeated cocaine administration, as previously described (Morrow et al., 1995a). Locomotor testing was performed in cages identical to the housing cages that were contained within a sound-attenuated chamber illuminated by a red light and equipped with a white noise generator. Locomotor activity was monitored by an automated 16-photocell array (Omnitech Digiscan Micro-Monitor, Columbus, OH) set to count photocell beam interjections per 10-min interval. The cages were cleaned with 70% ethanol before each testing session, and fresh bedding was used for each rat. On the first day of the experiment, rats were injected with (S)-(−)-HA-966 (3 or 5 mg/kg i.p.; Research Biochemicals) or saline and placed into the test cages. After 30 min of monitoring locomotion, the rats were injected 2

2 B. A. Morrow, unpublished observations.
with cocaine HCl (15 mg/kg i.p.; Sigma Chemical, St. Louis, MO) or saline, immediately returned to the test cage and monitored for an additional 60 min. Locomotion during this 60-min period after the administration of cocaine was used for analysis. This procedure was repeated for 5 consecutive days, with the sole exception that on the second and fourth days, the rats were treated in the home cage. Seven days after the end of the chronic treatment, rats were placed into the test cages; after 30 min, all rats were injected with cocaine (15 mg/kg i.p.), and locomotor activity was monitored for 60 min.

**GABA<sub>B</sub> binding assay.** The ability of either (S)-(−) or (R)-(−)-HA-966 to displace [3H]-(−)-baclofen from a rat tissue preparation was tested in a protocol based on Hill and Bowery (1981) and Mochizuki et al. (1989) with minor modifications. The cerebral cortex from normal rats was dissected, weighed and stored at −70°C. These frozen tissue samples were homogenized in 10 volumes of 0.32 M ice-cold sucrose using a glass homogenizer and Teflon pestle. The preparation was separated by centrifugation at 1000 × g for 10 min at 4°C, and the resulting supernatant was centrifuged at 35,000 g for 20 min at 4°C. The P2 pellet was resuspended in 10 volumes of water at 4°C and centrifuged at 8000 × g for 20 min at 4°C. Finally, the supernatant and buffy layer on the pellet were removed and centrifuged at 35,000 × g for 20 min at 4°C. The final pellet was collected and frozen at −70°C as the crude mitochondrial fraction enriched in synaptic membranes. For the binding assay, the tissue fraction was thawed on ice and suspended in 10 volumes of 50 mM Tris(HCl)/2.5 mM CaCl<sub>2</sub> buffer, pH 7.4, and centrifuged at 35,000 × g for 10 min at 4°C. This wash was repeated two further times. After the final wash, the pellet was resuspended in the Tris(HCl)/CaCl<sub>2</sub> buffer and 250 µl, 0.4 mg of protein, was removed for the assay. (butyl-4-3H)-(−)-Baclofen (New England Nuclear Research Products, Boston, MA; 32 Ci/mmol), 20 nM/250 µl, and various concentrations of racemic baclofen, GABA and (S)-(−) or (R)-(−)-HA-966 were added in a 100-µl volume and allowed to incubate for 20 min at room temperature. Nonspecific binding was determined by the addition of GABA, 100 µM/µl, instead of test compounds. The bound and free [3H]-(−)-baclofen was separated by filtration through Whatman GF/B paper, presoaked in 0.1% bovine serum albumin, using a Brandel Cell Harvester. Filters were washed three times with ice-cold buffer, air dried and counted after adding scintillation fluid. Total counts were corrected for nonspecific binding, and IC<sub>50</sub> values were calculated from triplicate determination in two separate experiments for each ligand tested, except (R)-(−)-HA-966, for which only one experiment was performed.

**Biochemical and statistical analyses.** DA and DOPAC values were determined from the tissue samples using HPLC-EC, as described by Elesworth et al. (1989) and Morrow et al. (1995b). Briefly, frozen brain regions were sonicated in ice-cold 0.1 N perchloric acid, and the precipitated protein was removed by centrifugation at 4°C. The supernate was assayed for dihydroxybenzylamine (an internal standard for catecholamines), DOPAC, DA, 5-HIAA and 5-HT by HPLC-EC. Because of the low levels of DOPAC and DA in the cortex, the mPFC samples were prepared as described above, and an aliquot was removed to determine the levels of DOPAC and DA. The aliquot was brought to pH 8.4 with 3 M Tris buffer and extracted onto acid-prepared alumina (Sigma Chemical), and the catechols were eluted from the alumina with 0.1 M oxalic acid. The concentrated sample containing the internal standard, DOPAC and DA was quantified using the HPLC setup specified above. Protein content of the sample was estimated from pellet using the folin-phenol method. DOPAC and DA were quantified against external standards after correcting for any loss using the internal standard dihydroxybenzylamine. The concentration of the transmitter or metabolite was divided by the amount of protein in the sample and metabolic activity, or utilization, was calculated by dividing the metabolite value by the parent value (DOPAC/DA).

ANOVA with Duncan’s range test or Student’s t test were used for analysis of single timepoint data. In all cases, a value of P < .05 was considered significant. Multiple timepoint data from the behavioral sensitization to cocaine and conditioned fear experiments were analyzed by repeated measures ANOVA using Duncan’s range test to determine group differences. Where significant effects were observed, the individual timepoints of repeated measures data were examined using one-way ANOVA with Duncan’s range test to determine specific differences.

**Results**

(S)-(−)-HA-966 and restraint stress. The administration of (S)-(−)-HA-966 (1, 3 or 5 mg/kg i.p.), 50 min before killing, did not alter basal DA utilization (DOPAC/DA) in the mPFC (fig. 1, top) or striatum (data not shown) of nonstressed animals. In addition, treatment with (S)-(−)-HA-966 (1, 3 or 5 mg/kg i.p.) did not alter the level of DA, itself, in the mPFC, NAS or striatum (data not shown). The levels of DOPAC and DA, respectively, in the saline control rats were as follows: mPFC, 0.129 ± 0.029 and 0.673 ± 0.142, and NAS, 13.39 ± 2.25 and 92.54 ± 10.91 nmg/mg protein. Basal DA metabolism in the NAS in nonstressed rats was increased by the highest dose of (S)-(−)-HA-966 tested, 5 mg/kg, but not by lower doses. After 30 min of restraint, DA metabolism was elevated in both the mPFC and NAS of saline-treated rats [fig. 1, PFC: F(1,20) = 4.83, P < .01; NAS: F(1,20) = 2.69, P < .05]. No change in the DOPAC/DN ratio was observed in the striatum (data not shown). However, (S)-(−)-HA-966 blocked the stress-induced increase in DA metabolism in the mPFC in a dose-dependent manner. The highest dose tested completely blocked the stress-induced DA metabolism in the mPFC, whereas (S)-(−)-HA-966 at 3 mg/kg only blunted this stress response; the stress-induced increase in rats treated with 3 mg/kg (S)-(−)-HA-966 was significantly different from the saline/stress group (P < .05) as well as the (S)-(−)-HA-966, 3 mg/kg nonstressed control (P < .05). At 1 mg/kg, (S)-(−)-HA-966 failed to alter the stress-induced DA metabolism in the mPFC. This relationship between the dose of (S)-(−)-HA-966 and the stress-induced response in DA metabolism was not observed in the NAS. The highest dose of (S)-(−)-HA-966 tested blocked the stress-related increase in DA utilization in the NAS and shifted the base-line DA metabolism in the nonstressed controls. Lower doses of (S)-(−)-HA-966 were without effect on the stress-activated DA metabolism in the NAS.

**Effect of (S)-(−)-HA-966 on aversive conditioning.** Several significant effects were noted during the extinction period in previously conditioned rats treated with (S)-(−)-HA-966 with regard to treatment [treatment: F(3,20) = 10.75, P < .005] and the interaction of treatment and time [F(27,234) = 2.01, P > .05] but not with time [F(9,234) = 60, P < .05]. As expected, prior conditioning, tone plus foot shock, resulted in evidence of fear responses in rats during the extinction phase, when the subjects were presented with the tone by itself. This was indicated by an increase in immobility during the 1-min period after the tones for the 30-min test period (fig. 2, top, P < .05). On the test day, the saline and (S)-(−)-HA-966 control rats remained mobile during and after exposure to the tone (fig. 2, top). (S)-(−)-HA-966 conditioned rats were significantly less immobile than the saline/conditioned rats at several time points, indicating a less fearful reaction to the conditioned tone (fig. 2, top, P < .05).

In addition, fear-induced immobility in the (S)-(−)-HA-966 conditioned rats was not different from the nonconditioned,
Based on these results, we conclude that the $(S)-(-)-HA-966$ blunted, but did not block, fear-induced immobility. Furthermore, fear conditioning increased defecation $[F(3,28) = 4.56, P < .05]$. Prior administration of $(S)-(-)-HA-966$ did not alter defecation in nonshocked control rats and blocked fear-induced defecation in conditioned rats (fig. 2, bottom, $P > .05$).

As expected, treatment with $(S)-(-)-HA-966$ (5 mg/kg i.p.) did not alter the level of DA, itself, in the mPFC, NAS core or shell or the striatum (data not shown). The levels of DOPAC and DA, respectively, in the saline control rats were as follows: mPFC, $0.111 \pm 0.005$ and $0.664 \pm 0.042$; NAS core, $26.09 \pm 1.41$ and $149.8 \pm 9.6$; and NAS shell, $19.43 \pm 1.45$ and $139.0 \pm 8.1$ ng/mg protein. Fear conditioning increased DA metabolism (DOPAC/DA) in the mPFC and the NAS shell, as expected $[F(3,28) = 6.42, P < .005$, NAS shell: $F_{(3,28)} = 3.11, P < .05]$.

In contrast to published studies on other stressors, 30 min of a continuous conditioned stimulus (CS) or foot shock (FSC) increased DA turnover in the mPFC and NAS shell, but failed to further increase DA turnover in the striatum.

**Fig. 1.** The effect of $(S)-(-)-HA-966$ on basal and restraint stress-induced DA metabolism (DOPAC/DA). Rats were treated with $(S)-(-)-HA-966$, 1, 3 or 5 mg/kg i.p., or saline and, after 20 min, placed into a wire restraint apparatus or left in the home cage for 30 min (hatched and open bars, respectively). Top, in the mPFC, $(S)-(-)-HA-966$ dose-dependently blocked the stress-induced increase in DA metabolism ($n = 5–11$ for each group). Bottom, in the NAS, restraint stress increased DA metabolism in the NAS of rats treated with saline and $(S)-(-)-HA-966$, 1 and 3 mg/kg. The highest dose of $(S)-(-)-HA-966$ tested, 5 mg/kg, increased DA metabolism in the nonstressed control group ($P < .05$). Stress failed to increase DA turnover further in rats treated with 5 mg/kg $(S)-(-)-HA-966$ ($n = 6–14$ for each group). $+, P < .05$ vs. the appropriate nonstressed control.

**Fig. 2.** The effect of treatment with $(S)-(-)-HA-966$ fear conditioning. Rats were treated with $(S)-(-)-HA-966$ [$(S)-(-)-HA$] or saline, placed into the test chamber and given 10 tones plus foot shock (Conditioned) or 10 tones alone (Control). The next day, rats were returned to the chamber and exposed to 10 tones alone. Top, fear-induced immobility in control and conditioned rats treated with saline or $(S)-(-)-HA-966$. During this extinction test, no foot shocks were delivered. Treatment with $(S)-(-)-HA-966$ on the conditioning day significantly reduced fear-induced immobility ($n = 8$ for each group). Bottom, effect of $(S)-(-)-HA-966$ on fear-induced defecation. The number of fecal boli remaining in the cage after the test session was significantly increased in saline-treated, conditioned rats. $(S)-(-)-HA-966$ treatment reduced the effect of fear conditioning, so defecation did not differ between the conditioned and control groups treated with $(S)-(-)-HA-966$ ($n = 8$ for each group). $+ and +, P < .05$ vs. the saline control and saline conditioned groups, respectively.
tioned stress increased DA turnover in the NAS core \(F_{(3,26)} = 3.13, P < .05\), in addition to the NAS shell. Treatment with \((S)(-)-HA-966\) (5 mg/kg i.p.) on the conditioning day prevented the stress-induced increase on the extinction day in DA utilization in all regions activated by stress: mPFC, NAS shell and NAS core. \((S)(-)-HA-966\) did not significantly alter DA metabolism in any region tested of the nonstress control rats. Nevertheless, a tendency for an increase in DA metabolism was noted in both the NAS core and shell, which failed to reach significance (fig. 4). Neither conditioning nor \((S)(-)-HA-966\) had any effect on DA metabolism in the striatum (data not shown). Elevated 5-HT metabolism was noted only in the mPFC in response to this aversive conditioning paradigm [fig. 3, bottom: \(F_{(3,26)} = 4.72, P < .01\)]. Prior treatment with \((S)(-)-HA-966\) did not significantly alter basal or fear-induced activation of 5-HT metabolism. These data indicate that \((S)(-)-HA-966\) given during the conditioning was able to prevent fear-induced increase in DA, but not 5-HT, metabolism in each of the regions activated.

**Effect of \((S)(-)-HA-966\) on baseline and cocaine-induced locomotion.** Several significant differences were noted between \((S)(-)-HA-966\)- and saline-treated rats during the acquisition of cocaine sensitization [treatment: \(F_{(5,28)} = 18.77, P < .0001\], time: \(F_{(2,56)} = 5.20, P < .01\], and interaction: \(F_{(10,56)} = 3.49, P < .01\]. In saline controls, the highest dose of \((S)(-)-HA-966\) tested, 5 mg/kg, significantly dimin-
ished locomotion compared with the saline/saline controls (fig. 5, P < .05). A closer examination of the individual days indicated that (S)-(−)-HA-966, 5 mg/kg, significantly reduced novelty-induced locomotion in saline controls on the first day only (fig. 5, day 1, P < .05) but not on the subsequent days, (day 3, P > .05 and day 5, P > .05), indicating that some adaptation to the motoric effects of (S)-(−)-HA-966 had occurred. The lower dose of (S)-(−)-HA-966, 3 mg/kg did not affect baseline locomotion in saline controls on any day monitored: days 1, 3 and 5 (fig. 5, P > .05).

The repeated administration of cocaine resulted in an increasing locomotor response (fig. 5, P < .05). An examination of each day separately indicated that cocaine significantly increased locomotion in saline-treated rats every day tested [day 1: F(5,28) = 4.39, P < .005; day 3: F(5,28) = 7.52, P < .0005; day 5: F(5,28) = 18.60, P < .0001]. Cocaine-induced locomotion only significantly increased on days 1 and 3 for rats treated with (S)-(−)-HA-966, 3 mg/kg. Cocaine administration failed to significantly increase locomotor behavior in rats treated with (S)-(−)-HA-966, 5 mg/kg on any day tested (P > .05). After 7 days, during which no cocaine or (S)-(−)-HA-966 was administered, rats were administered an acute cocaine challenge, and several differences were noted [fig. 6, treatment: F(5,28) = 2.93, P < .05, time: F(5,25) = 52.12, P < .0001 and interaction: F(25,140) = 3.86, P < .0001]. Rats preexposed to cocaine had an augmented locomotor response to the challenge dose of cocaine compared with the saline preexposed controls (fig. 6, P < .05). Coadministration of (S)-(−)-HA-966, at 3 or 5 mg/kg, with the chronic cocaine exposure prevented the behavioral locomotor sensitization to a subsequent challenge dose of cocaine on the test day (fig. 6, P < .05). This indicates that concurrent exposure of (S)-(−)-HA-966 during chronic cocaine administration can prevent the development of behavioral sensitization to cocaine.

As expected, rats exposed to cocaine failed to gain weight as rapidly as the saline controls during the chronic phase of this experiment (data not shown). Interestingly, rats given repeated doses of (S)-(−)-HA-966, 5 mg/kg without cocaine, also failed to gain weight as rapidly as the saline controls over the 5-day period [table 1, days 1–5, F(3,22) = 5.51, P < .01]. This effect was primarily due to the suppression of weight gain during the initial period, days 1 to 3 [F(3,22) = 5.24, P < .01], but not during the later period, days 3 to 5 [F(3,22) = .64, P > .05]. The lower dose of (S)-(−)-HA-966, 3 mg/kg, as well as the positive enantiomer, (R)-(−)-HA-966, 15 mg/kg, did not alter weight gain during repeated exposure (P > .05). The suppression of weight gain did not continue after the drugs were discontinued (data not shown). The effect of weight gain does not appear to be related to short-acting locomotor effects of (S)-(−)-HA-966. The administration of (S)-(−)-HA-966 occurred 7 to 10 hr before the beginning of the dark cycle, when the rats would do most of their feeding.

**Effect of the enantiomers of HA-966 on GABA<sub>B</sub> binding.** Neither (S)-(−) nor (R)-(−)-HA-966 displaced [3H]-baclofen from the cortical tissue preparations at relevant concentrations (table 2). Racemic baclofen and GABA displaced [3H]-baclofen at approximately the expected concentrations. The results of this study indicate that it is unlikely that the actions of (S)-(−)-HA-966 are mediated directly through the baclofen binding site of the GABA<sub>B</sub> receptor.

![Fig. 5.](https://jpet.aspetjournals.org/) The effect of chronic exposure to (S)-(−)-HA-966 on baseline and cocaine-induced locomotion in rats. Rats were given (S)-(−)-HA-966, 3 or 5 mg/kg i.p., or saline, placed into a locomotor testing apparatus for 30 min, given saline or cocaine, 15 mg/kg i.p., and immediately returned to the chamber for 60 min. Total locomotor activity, as measured by a photocell system, in this 60-min period was used for analysis. This protocol was repeated for a total of 5 consecutive days with the exception that on days 2 and 4, rats were left in the home cage and not monitored for locomotor activity. The highest dose of (S)-(−)-HA-966 tested significantly lowered locomotor activity on day 1 compared with saline and (S)-(−)-HA-966 3 mg/kg in rats not receiving cocaine. As expected, cocaine administration significantly increased locomotion in control rats. (S)-(−)-HA-966, 5 mg/kg, prevented the cocaine-induced increase in locomotion on all 3 days tested. The lower dose of (S)-(−)-HA-966, 3 mg/kg, prevented cocaine-induced locomotion on day 5 only. * and +, P < .05 vs. the saline control and the same treatment, noncocaine control, respectively (n = 5–7 for each group).
Minutes

Fig. 6. The effect of a cocaine challenge on rats previously exposed to cocaine and (S)-(-)-HA-966. Rats were treated for 5 consecutive days with (S)-(-)-HA-966 and cocaine as described for figure 5. After 7 drug-free days, rats were returned to the test chamber for 30 min; cocaine, 15 mg/kg, was administered at time 0; and locomotor activity was monitored for an additional 60 min. Enhanced cocaine-induced motor activity, or locomotor sensitization, was observed in rats previously exposed to cocaine (sal/coc) compared with the saline controls (sal/sal). Prior administration of (S)-(-)-HA-966, 5 or 3 mg/kg, with cocaine [(S)-HA-5/coc and (S)-HA-3/coc, respectively] prevented locomotor sensitization to cocaine. * P < .05 vs. the same treatment control (n = 5–7 for each group).

TABLE 1

Weight gain during repeated HA-966 treatment

Rats were treated for 5 continuous days with saline, (R)-(+)-HA-966 or (S)-(-)-HA-966. Rats were weighed immediately before administration of drug, and the weight gain over the select period was obtained by simple subtraction. No cocaine was administered to any of the animals during the chronic treatment period indicated above. Rats treated with the highest dose of (S)-(-)-HA-966 gained weight slower during the 5-day period compared with the saline controls and those treated with the lower dose of (S)-(-)-HA-966 and (R)-(+)-HA-966 [F(5.22) = 5.51, P < .05]. This effect was due to the initial effect of the (S)-(-)-HA-966, (5 mg/kg/day) on weight gain between days 1 and 3 [F(3,22) = 5.24, P < .05]. No difference in weight gain between the groups was noted between days 3 and 5 [F(5.22) = 6.4, P > .05].

<table>
<thead>
<tr>
<th>Days 1–3</th>
<th>Days 3–5</th>
<th>Days 1–5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>14.7 ± 2.1</td>
<td>12.2 ± 2.0</td>
</tr>
<tr>
<td>(S)-(-)-HA-966</td>
<td>7.6 ± 0.4*</td>
<td>13 ± 1.8</td>
</tr>
<tr>
<td>5 mg/kg/day</td>
<td>15.8 ± 1.9</td>
<td>16.3 ± 2.5</td>
</tr>
<tr>
<td>(R)-(+)-HA-966</td>
<td>15 ± 1.6</td>
<td>12.8 ± 1.6</td>
</tr>
</tbody>
</table>

* P < .05 vs. the saline control.

Discussion

(±)-HA-966 has been available for >35 years and has been clinically tested for the treatment of extrapyramidal motor disorders. Now that the distinct enantiomers have been resolved, the means by which (±)-HA-966 achieves its range of actions can be clarified. This report demonstrates that (S)-(-)-HA-966 can block stress-induced activation of behavior, stress-induced increases in dopaminergic systems and locomotor sensitization to cocaine. However, the site of action of (S)-(-)-HA-966 remains uncertain, as it does not appear, as suggested, to be the baclofen binding site of the GABA_B receptor. The actions of the positive enantiomer, although similar, which are generally attributed to the NMDA/glycine receptor complex, show several differences compared with (S)-(-)-HA-966 (Morrow et al., 1993). These data do not support the hypothesis that normalizing DA cell firing rates in anesthetized rats is a likely means by which both enantiomers produce behavioral effects in conscious rats.

Comparison with the effects of (R)-(+)-HA-966. One goal of these studies was to contrast the effects of (S)-(-)-HA-966 with its enantiomer, (R)-(+)-HA-966. The results of this current study and work by others are summarized in table 3. Shepard et al. (1995) proposed that the two enantiomers share a common mechanism of action, possibly through GABA_B receptors. Based on this finding and current investigation in this laboratory, we proposed that (R)-(+)-HA-966 could block stress-induced changes in DA neurotransmission by normalizing mesocortical DA neuronal firing rates (Morrow et al., 1993). As the (S)-(-) enantiomer was shown to be more potent at this effect than the (R)-(+)-HA-966 (Shepard et al., 1995), we tested (S)-(-)-HA-966 in several paradigms in which the efficacy of the (R)-(+) enantiomer

TABLE 2

IC_{50} values for (R)-(+) and (S)-(−)-HA-966 displacement of [3H](-)-baclofen

A crude mitochondrial fraction enriched in synaptic membranes was prepared from rat cerebral cortex tissue. The values shown are the averages of duplicate experiments, except (R)-(+) HA-966, which was assayed only once.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>IC_{50} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA</td>
<td>0.068</td>
</tr>
<tr>
<td>(-)-Baclofen</td>
<td>0.650</td>
</tr>
<tr>
<td>(R)-(+) HA-966</td>
<td>350</td>
</tr>
<tr>
<td>(S)-(−)-HA-966</td>
<td>200</td>
</tr>
</tbody>
</table>

TABLE 3

A comparison of the biochemical and behavioral effects of the enantiomers of HA-966

<table>
<thead>
<tr>
<th>Paradigm</th>
<th>(R)-(+)</th>
<th>(S)-(−)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normalization of DA cell firing</td>
<td>10 mg/kg*</td>
<td>1 mg/kg*</td>
</tr>
<tr>
<td>(ID_{50})</td>
<td>57.8 mg/kg*</td>
<td>5.7 mg/kg*</td>
</tr>
<tr>
<td>Sedation</td>
<td>&gt;100 mg/kg^b</td>
<td>10 mg/kg^b</td>
</tr>
<tr>
<td>Mice</td>
<td>&gt;30 mg/kg^c</td>
<td>5 mg/kg</td>
</tr>
<tr>
<td>Rats</td>
<td>15 mg/kg^c</td>
<td>3 mg/kg</td>
</tr>
<tr>
<td>Blocking cocaine sensitization</td>
<td>15 mg/kg^c</td>
<td>5 mg/kg</td>
</tr>
<tr>
<td>Blocking stress-induced in-</td>
<td>15 mg/kg^c</td>
<td>5 mg/kg</td>
</tr>
<tr>
<td>crease in DA metabolism</td>
<td>15 mg/kg^c</td>
<td>5 mg/kg</td>
</tr>
<tr>
<td>mPFC</td>
<td>15 mg/kg*</td>
<td>5 mg/kg</td>
</tr>
<tr>
<td>NAS</td>
<td>No (15 mg/kg)</td>
<td>Yes (5 mg/kg)</td>
</tr>
<tr>
<td>Suppression of weight gain</td>
<td>[3H]Baclofen</td>
<td>350</td>
</tr>
<tr>
<td>(-)-Baclofen</td>
<td>4.1*</td>
<td>&gt;900*</td>
</tr>
<tr>
<td>(S)-(−)-GABA</td>
<td>12.5*</td>
<td>339^b</td>
</tr>
</tbody>
</table>

The data presented here represent minimum effective doses, unless otherwise stated, condensed from this (uncited) and the following studies: * Shepard et al., 1995; ^b Singh et al., 1990; ^c Hutson et al., 1991; ^d Morrow et al., 1995a; ^e Morrow et al., 1993; and ^f Pullan et al., 1990. Studies of the two enantiomers were selected to match in methods and laboratory with the exception of those examination sedation. Sedation studies of the HA-966 enantiomers were performed in mice and rats in rotarod and horizontal locomotor apparatus, respectively. Electrophysiological studies were performed on chloral hydrate anesthetized rats using i.v. administration of drugs. The concentrations of [3H]GABA were as follows ^f 20 nM and ^b 50 nM.
omer was established. Several similarities between the two enantiomers were that (1) both were noted to have anxiolytic-like actions, (2) both were able to prevent locomotor sensitization to cocaine and (3) both were able to block stress-induced mesocortical DA utilization. Nevertheless, several differences were noted between these two compounds. First, unlike (S)-(-)-HA-966, (R)-(+)HA-966 selectively prevented stress-induced activation of the mPFC but not the NAS (Morrow et al., 1993; Goldstein et al., 1994). This selectivity of (R)-(+)HA-966 is likely due to actions on the glycine/NMDA receptor at the level of the DA cell bodies in the VTA (Morrow et al., 1993). The selective blockade of stress-induced DA metabolism in the mPFC and not the NAS was also noted with a low dose of the noncompetitive NMDA antagonist MK-801 (Morrow et al., 1993), supporting the NMDA receptor complex as the sight of action of (R)-(+)HA-966. Second, a suppression of weight gain was noted with (S)-(-), but not (R)-(+), HA-966 during chronic administration. This effect was not observed after cessation of (S)-(-)-HA-966 and does not appear to be related to the sedation observed with (S)-(-)-HA-966 because no food was available during the experiment. In addition, the dose of (S)-(-)-HA-966 was given 7 to 10 hr before nighttime, the active cycle of the rat, when most of the feeding is done. Finally, with regard to sedation, a clear difference was observed: in the (S)-(-) enantiomer, unlike (R)-(+)HA-966, the sedative dose and the dose effective at preventing stress-induced DA changes were similar. In this report, a significant decrease in spontaneous locomotor activity was noted with (S)-(-)-HA-966 at 5 mg/kg but not 3 mg/kg. In contrast, the positive enantiomer of HA-966 at 100 mg/kg did not disrupt locomotor activity in mice (Bristow et al., 1993; Hutson et al., 1991). These data indicate that the effective anxiolytic dose of (R)-(+)HA-966 (15 mg/kg) is much lower than the sedating dose, whereas with regard to (S)-(-)-HA-966, the sedating and anxiolytic doses are much more similar. In addition, the dose of the (S)-(-) and (R)-(+) enantiomers required to block the spontaneous firing of midbrain DA neurons was similar to the sedative dose. In the case of (R)-(+), but not (S)-(-), HA-966, this effective dose was much greater than the dose required to block stress-induced changes to DA metabolism (Shepard et al., 1993, 1995). It is curious that both enantiomers have similar, but distinct, actions on the mesocorticolimbic DA system and related behaviors through, most likely, different neurotransmitter systems.

Possible involvement of GABA<sub>B</sub> and DA neurons. The mechanism of action of (S)-(-)-HA-966 is not clear, although pharmacological evidence at hand suggests that the GABA<sub>B</sub> receptor may be involved. The strongest support for a GABA<sub>B</sub>-related mechanism for (S)-(-)-HA-966 comes from studies using the GABA<sub>B</sub> receptor antagonist CGP 35348. Shepard et al. (1993) noted that CGP 35348 could completely antagonize the suppression of DA neuronal firing induced by (S)-(-)-HA-966. The effects of racemic HA-966, baclofen and γ-butyrolactone on DA synthesis could also be suppressed with CGP 35348 (Waldmeier, 1991). Second, the actions of racemic HA-966, as well as (S)-(-)-HA-966, have been associated with the sedative γHB. (S)-(-)-HA-966 and the γHB both suppress the spontaneous firing rate of midbrain DA neurons (Nowycky and Roth, 1977; Roth et al., 1973; Shepard and Lehmann, 1992) and have been shown to have sedative effects at higher doses (Singh et al., 1990; Tunnicliff, 1992, a review). In addition, (S)-(-)-HA-966, γHB and the GABA<sub>B</sub> agonist baclofen cause an initial increase in DA accumulation, which is believed to be the result of the cessation of impulse flow in DA neurons and the activation of DA synthesis (Nowycky and Roth, 1977; Waldmeier, 1990). However, in this report, we did not see any significant displacement of [3H]-baclofen by either enantiomer of HA-966, indicating that the actions of (S)-(-)-HA-966 are apparently distinct from the baclofen-binding site on the GABA<sub>B</sub> receptor. Taking into account the positive pharmacological studies using CGP 35348 and this current negative binding study, two possibilities for the actions of (S)-(-)-HA-966 seem likely. First, a distinct subtype of the GABA receptors has been observed with poor sensitivity to phaclofen but high sensitivity to CGP-35348 (Bonanno and Raiteri, 1993). (S)-(-)-HA-966 might bind to this GABA receptor subtype and thus have GABAergic actions without displacing baclofen. Second, because of the similarities in pharmacological action between (S)-(-)-HA-966 and γHB, another possibility is that (S)-(-)-HA-966 acts on the proposed γHB transmitter system (Benavisea et al., 1982; Doherty et al., 1978). Previous radioligand binding studies have indicated that γHB may be acting through highly specific membrane binding sites that do not have affinity for GABA or GABAergic agonists (Benavisea et al., 1982; Maitre et al., 1983). The exact nature of the γHB binding site is not clear, but it appears to be functionally linked to GABA<sub>B</sub> activity (Broxterman et al., 1981; Engberg and Nissbrandt, 1993; Waldmeier, 1991). The possibility that (S)-(-)-HA-966 acts at a baclofen-insensitive site of the GABA<sub>B</sub> receptor or at the γHB binding site warrants further investigation.

(S)-(-)-HA-966 altered components of locomotor activation. We used doses of (S)-(-)-HA-966 well below those previously reported to cause sedation (Singh et al., 1990). Nevertheless, unlike lower doses, 5 mg/kg (S)-(-)-HA-966 disrupted base-line and cocaine-stimulated locomotion. An apparent tolerance developed to this effect by the third exposure. In addition, on the first day of cocaine exposure, (S)-(-)-HA-966 blunted the cocaine-induced locomotor activity at the higher dose tested. Neither effect was noted with the lower dose of (S)-(-)-HA-966.

Repeated exposure to cocaine has been demonstrated to cause an enhanced sensitivity to the locomotor effects of a subsequent challenge dose of cocaine, an effect termed behavioral sensitization (Downs and Eddy, 1932; Post and Contel, 1983). Although this observation is thought to involve enhanced dopaminergic transmission, a precise mechanism has not been established (see Wise and Leeb, 1993, for review). Drugs acting at the GABA<sub>B</sub> receptor, as well as other neurotransmitter systems, have been noted to prevent behavioral sensitization (Kalivas et al., 1988; Kalivas and Stewart, 1991). However, these studies used local application of baclofen into the VTA, which blocked the motoric effects of acute cocaine, in addition to locomotor sensitization (Kalivas et al., 1988; Kalivas and Stewart, 1991). This current study demonstrates that 3 mg/kg (S)-(-)-HA-966, unlike baclofen in previous studies, prevents behavioral sensitization to cocaine without disrupting the acute motoric effects of cocaine.

Role of (S)-(-)-HA-966 in behavioral and biochemical stress activation. Interestingly, the doses of (S)-(-)-HA-966 that blocked psychomotor stimulant-induced behavioral sensitization also affected stress-induced increases in DA
metabolism. Several other compounds have been demonstrated to have both actions, including \((R)-(+)-\text{HA-966} \) (Morrow et al., 1993, 1995a, 1995b) and MK-801 (Karler et al., 1990; Morrow et al., 1993; Wolf and Jeziorski, 1993). The highest dose tested, 5 mg/kg, of \((S)-(-)-\text{HA-966}\) prevented behavioral and biochemical indices of stress activation. Lower doses of \((S)-(-)-\text{HA-966}\) blunted the stress activation of DA. However, the highest dose of \((S)-(-)-\text{HA-966}\) was not able to block the stress-induced increase in 5-HT metabolism in the conditioned fear paradigm. The \((R)-(+)-\text{enantiomer also failed to alter stress-induced changes in 5-HT metabolism (Goldstein et al., 1994). The significance of the resistance of the stress response of 5-HT neurons to drug demonstrated to have anxiolytic-like activity is not clear.

Fear conditioning activated DA metabolism in both the core and shell subdivisions of the NAS, in contrast with several published studies that noted a selective activation in dopaminergic activity after foot shock in the NAS shell only (Deutch and Cameron, 1992; Kalivas and Duffy, 1995). These previous studies using a similar intensity stressor but differ from this current study by (1) duration of stressor and (2) type of stressor. First, we used a longer duration stressor than previous studies. It is quite possible that the activation of the subdivisions of the NAS may simply be temporally distinct, so the NAS shell activates earlier than the NAS core. A similar situation was observed in comparing the restraint stress-induced DA metabolism in the whole NAS and the mPFC: the onset of activation of the mesocortical DA system was more rapid than the mesolimbic, 10 vs. 20 min, respectively (Roth et al., 1988). By extending the duration of stress in this study, we observe an activation of both the core and shell of the NAS. Neuroanatomical studies have associated the NAS shell and core with the limbic system and striatum, respectively (Cools et al., 1993; Deutch and Cameron, 1992; Heimer et al., 1991; Zahm, 1991; Zahm and Heimer, 1990). This report notes that the activation of the DA projections to both the NAS core and shell can be achieved without a relatively intense stressor and without any activation of the striatum, indicating that the dopaminergic neurons projecting to the NAS core activate differently than the nigrostriatal DA neurons and more similar to those DA neurons projecting to the NAS shell. Second, the type of stressor (i.e., psychological vs. physical) may be important in determining the response of the core and shell subunits of the NAS. It is possible that the use of foot shock stress immediately before killing the rat activates neuronal pain pathways that may suppress the activation of the NAS core. This is avoided with the use of conditioned fear because no painful foot shock is given on the test day.

Conclusion. We report anxiolytic-like actions of the \((S)-(-)-\text{enantiomer of HA-966}. These actions are similar to those observed with the \((R)-(+)-\text{enantiomer, a weak partial agonist for the glycine/NMDA receptor complex, yet have several clear points of distinction that seem to indicate a distinct mechanism of action. Although the mechanism of action of \((S)-(-)-\text{HA-966} is not clear, it is likely that it acts through the GABAergic neurons, possibly at a \(\gamma\text{H}B\) binding site or a baclofen-insensitive site of the GABA\(_B\) receptor. This compound may represent a novel class of potential anxiolytic agents with \(\gamma\text{H}B\)-like actions.

Acknowledgments

\((S)-(-)-\text{HA-966} was provided by Research Biochem as chemicals for the Chemical Synthesis Program of the National Institute of Mental Health, Contract N01-MH30003.

References


McMillen, B. D., Williams, H. L., Lehmied, H. and Sheppard, P. D.: On central


