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# Responses of rat substantia nigra dopamine-containing neurones to (−)-HA-966 *in vitro*

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**1** Extracellular single unit recording techniques were used to compare the effects of (-)-3-amino-1-hydroxypyrrolidin-2-one ((−)-HA-966) and (±)-baclofen on the activity of dopamine-containing neurones in 300 $\mu$m slices of rat substantia nigra. Electrophysiological data were compared with the outcome of *in vitro* binding experiments designed to assess the affinity of (−)-HA-966 for $\gamma$-aminobutyric acid ($GABA_B$) receptors.

**2** Bath application of (−)-HA-966 produced a concentration-dependent inhibition of dopaminergic neuronal firing ($EC_{50}$ = 444.0 $\mu$M; 95% confidence interval: 277.6 $\mu$M – 710.1 $\mu$M, $n$ = 27) which was fully reversible upon washout from the recording chamber. Although similar effects were observed in response to (±)-baclofen, the direct-acting $GABA_B$ receptor agonist proved to be considerably more potent than (−)-HA-966 ($EC_{50}$ = 0.54 $\mu$M; 95% confidence interval: 0.44 $\mu$M – 0.66 $\mu$M, $n$ = 29) *in vitro*.

**3** Low concentrations of chloral hydrate (10 $\mu$M) were without effect on the basal firing rate of nigral dopaminergic neurones but significantly increased the inhibitory effects produced by concomitant application of (−)-HA-966.

**4** The inhibitory effects of (−)-HA-966 were completely reversed in the presence of the $GABA_B$ receptor antagonists, CGP-35348 (100 $\mu$M) and 2-hydroxysaclofen (500 $\mu$M). Bath application of CGP-35348 alone increased basal firing rate. However, the magnitude of the excitation (9.2 ± 0.3%) was not sufficient to account for the ability of the antagonist to reverse fully the inhibitory effects of (−)-HA-966.

**5** (−)-HA-966 (0.1 – 1.0 mM) produced a concentration-dependent displacement of [$^3$H]-GABA from synaptic membranes in the presence of isoguvacine (40 $\mu$M). However, the affinity of the drug for $GABA_B$ binding sites was significantly less than that of GABA (0.0005 potency ratio) and showed no apparent stereoselectivity.

**6** These results indicate that while (−)-HA-966 appears to act as a direct $GABA_B$ receptor agonist *in vitro*, its affinity for this receptor site is substantially less than that of GABA or baclofen and unlikely to account for the depressant actions of this drug which occur at levels approximately ten fold lower *in vivo*.

**Keywords:** Dopamine; substantia nigra; (-)-3-amino-1-hydroxypyrrolidin-2-one; $GABA_B$; HA-966; baclofen; CGP-35348; 2-hydroxysaclofen; $\gamma$-hydroxybutyrate

## Introduction

3-Amino-1-hydroxypyrrolidin-2-one (HA-966) is representative of an unusual group of centrally-acting muscle relaxant/antispasmodic drugs which, in addition to sharing a number of behavioural and neurochemical effects, are capable of selectively disrupting the spontaneous electrical activity of mesencephalic dopamine-containing neurones *in vivo* (Nowycky & Roth, 1977; Shepard & Lehmann, 1992; McMillen *et al*., 1992). Used extensively as pharmacological tools in neurochemical studies of dopamine synthesis and release (Roth, 1987), the mechanisms underlying the unique electrophysiological effects of these compounds have remained elusive. As a chiral molecule, HA-966 exists in enantiomeric forms, each with its own distinct pharmacological profile (Singh *et al*., 1990). (+)-HA-966 exhibits anxiolytic and neuroprotective efficacy (Vartanian & Taylor, 1991; Dunn *et al*., 1992; Anthony & Nevins, 1993; Morrow *et al*., 1993; Goldstein *et al*., 1994) which has been attributed to its ability to antagonize the glycine allosteric site on the N-methyl-D-aspartate (NMDA) receptor (Henderson *et al*., 1990; Singh *et al*., 1990). (−)-HA-966 has negligible affinity for the NMDA receptor complex (Singh *et al*., 1990). However, it possesses marked anticonvulsant and sedative effects (Singh *et al*., 1990; Vartanian & Taylor, 1991) and is considerably more potent in inhibiting the spontaneous activity of mesencephalic dopaminergic neurons *in vivo* than the (+)enantiomer (Shepard *et al*., 1995).

Differences in potency notwithstanding, the electrophysiological effects of (−) and (+)-HA-966 on substantia nigra (SN) dopamine cells are qualitatively indistinguishable (Shepard *et al*., 1995). In addition to inhibiting neuronal firing rate, both drugs suppress bursting activity and evoke a pacemaker-like firing pattern rarely observed *in vivo* (Shepard & Lehmann, 1992; McMillen *et al*., 1992). Previous data indicating that these effects occur independently of an interaction with NMDA receptors (McMillen *et al*., 1992) have raised the possibility that some of the behavioural effects of (+)-HA-966, particularly those observed in response to high doses of the drug, result from an interaction at the as yet unidentified (−)-HA-966 binding site (Shepard *et al*., 1995).

Although the mechanisms underlying the effects of (−)-HA-966 on dopaminergic neurones remains to be established, several lines of evidence point to the possible involvement of $\gamma$-aminobutyric acid ($GABA_B$) receptors. For example, many of the neurochemical, electrophysiological and behavioural effects of (−)-HA-966 are shared by the putative GABA mi-

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metic, γ-hydroxybutyrate, as well as the prototypical $GABA_B$ receptor agonist, baclofen (Waldmeier, 1991; Engberg & Nissbrandt, 1993; Engberg *et al*., 1993). It has also recently been shown that the inhibitory effects of (+)- and (−)-HA-966 on substantia nigra (SN) dopaminergic neurones *in vivo* can be reversed by systemic administration of the $GABA_B$ antagonist, 3-aminopropane-diethoxymethylphosphonic acid (CGP-35348) (Olpe *et al*., 1990; Shepard *et al*., 1995). Although these findings could be interpreted as suggesting direct involvement of GABAergic mechanisms, preliminary studies have indicated that (−)-HA-966 possesses negligible affinity for $GABA_B$ binding sites (Waldmeier, 1991). In an effort to resolve these seemingly paradoxical findings, single unit recording techniques were used to compare the potency of (±)-baclofen and (−)-HA-966 on the activity of nigral dopaminergic neurones in brain slices. The competitive $GABA_B$ antagonists CGP-35348 and 2-hydroxysaclofen were also tested for their ability to reverse (−)-HA-966-induced alterations in neuronal activity. Electrophysiological data were compared with the outcome of *in vitro* binding studies designed to assess the affinity of (−)-HA-966 for $GABA_B$ receptors. Our results indicate that while (−)-HA-966 appears to act as a direct $GABA_B$ agonist *in vitro*, its potency is substantially less than that of (±)-baclofen and may not fully account for the ability of the drug to suppress dopamine cell firing *in vivo*.

## Methods

### *Single unit recording experiments*

Male, Sprague-Dawley rats weighing 100–200 g at the time of experiment were anaesthetized with chloral hydrate (400 mg $kg^{-1}$, i.p.) and decapitated. The brain was excised and immersed in an ice-cold saline solution (ACSF) of the following composition (in mM): NaCl 124, KCl 4.0, $NaH_2PO_4$ 1.25, $MgSO_4$ 1.2, $NaHCO_3$ 26, $CaCl_2$ 2.5, ascorbate 0.15 and glucose 11. A block of tissue containing the SN was prepared over ice and placed on the stage of a manual tissue chopper (Stoelting). Coronal slices (300 μm thickness) were made throughout the anterior-posterior extent of the SN and transferred to a holding dish filled with chilled ACSF. Two slices containing the SN (bilateral) were immediately transferred to the stage of an interface perfusion chamber. Tissue was maintained at 34–36°C in a humidified oxygen environment and continuously superfused (1.5 ml $min^{-1}$) with oxygenated ACSF equilibrated with 95% $O_2$ and 5% $CO_2$ to maintain a pH of 7.35. Slices were maintained in the recording/perfusion chamber for 2 h before the start of the recording studies.

Extracellular single unit activity was recorded from SN neurones by microelectrodes prepared from glass capillary tubing. Electrodes were filled with 2 M NaCl and the tips broken back to achieve an *in vitro* impedance of 2.5–3.5 MΩ. Electrodes were positioned visually within the pars compacta of the SN by use of a dissecting microscope and micromanipulator equipped with a stepping motor. Electrode potentials were amplified, filtered (sampling bandwidth 0.1–4 kHz) and continuously monitored with a digital oscilloscope and audio amplifier. Pars compacta neurones were identified as dopaminergic on the basis of their unique waveform characteristics and firing properties (Grace & Onn, 1989; Yung *et al*., 1991). Action potentials from individual dopaminergic neurones were observed and recorded for a minimum of 5 min to establish basal firing characteristics. Subsequently, each cell was tested with one or more of the following drugs: (−)-HA-966 (50 μM–1 mM), (±)-baclofen (0.25–1.5 μM), chloral hydrate (10 μM), CGP-35348 (100 μM) or 2-hydroxysaclofen (500 μM). Drugs were applied to the perfusion chamber by use of a stopcock assembly that permitted substitution of the normal media with ACSF containing a fixed concentration of the test compound(s). Drugs were applied to the chamber until a stable response was achieved (see Figure 1) and were removed by dilution with control ACSF.

Data acquisition and analysis were conducted by use of an integrated software package for electophysiology (RISI, Symbolic Logic, Dallas, TX). Cumulative rate histograms were compiled in real-time from the output of a window discriminator and displayed with a 10 s bin width. Average firing rates were computed from each rate histogram immediately before and during peak drug responses. The data are presented in the text as mean ± s.e.mean. Concentration-response curves were compiled from groups of cells exposed to a single concentration of (±)-baclofen or (−)-HA-966. Only one cell was tested per slice. Drug-induced alterations in firing rate were expressed as a percentage of the pre-drug (control) firing rate and plotted as a log function of the concentration administered. $EC_{50}$ values, representing the concentration required to inhibit neuronal firing by 50%, were computed by use of a four-parameter logistic equation (Inplot, Graphpad Software, San Diego, CA). Omnibus testing of the overall differences between treatment groups was accomplished by analysis of variance (ANOVA) or in the case of paired comparisons, with a paired *t* test. Post-hoc comparisons between treatment groups were conducted by the Bonferroni t-test method. Statistical analysis was performed with the SigmaStat (Jandel Scientific, San Rafael, CA) and Instat2 (Graphpad Software, San Diego, CA) software packages.

### *[$^3$H]-GABA$_B$ binding*

Crude synaptic membranes were prepared from whole brain as previously described (Bowery *et al*., 1983; Facklam & Bowery, 1993). Briefly, tissue homogenates were prepared in ice-cold 0.32 M sucrose and centrifuged for 10 min at 1,600 *g*. The $P_2$ fraction, containing an enriched mixture of synaptic membranes, was collected by centrifugation of the supernatant layer at 31,000 *g* for 20 min. The resulting pellet was lysed by dispersal in distilled water and recollected by centrifugation at 12,000 *g* for 20 min. The resulting supernatant together with the upper layer (buffy coat) of the pellet were recentrifuged at 48,000 *g* for 20 min, washed twice in distilled water and stored at −20°C for a minimum of 18 h.

Before use in the binding assay, isolated membrane preparations were subjected to an extensive washing procedure to remove endogenous GABA and other potential contaminants. Frozen membranes were thawed at room temperature and dispersed in ice-cold distilled water. Following centrifugation (13,000 *g* for 10 min), the pellet was re-washed in distilled water with incubation periods of 15 min between consecutive spins. Subsequently, membranes were suspended in 50 mM Tris-HCl (pH 7.4) containing 2.5 mM $CaCl_2$, incubated for 15 min and centrifuged at 13,000 *g* for 10 min at 4°C. Following a final wash in modified Tris buffer, membrane pellets were suspended in 100 μl of Tris-HCl and incubated for 10 min at room temperature in the presence of 40 μM isoguvacine HCl, 2.5 mM $CaCl_2$, 5 nM [$^3$H]-GABA and either GABA (0.01–0.1 mM) or (−)-HA-966 (0.1–1.0 mM). Non-specific binding was evaluated by addition of 100 μM (-)-baclofen. The assay was terminated by centrifugation (7,500 *g* × 3 min at 4°C) and repeated washing in ice-cold distilled water. Pellets were solubilized overnight in Soluene-350 and transferred to liquid scintillation vials containing 0.4 ml of 0.2 M HCl. Tritium content was determined by liquid scintillation spectrometry. Non-specific binding was subtracted from the total amount of radioactivity to yield the amount of specific ligand binding.

### *Drugs*

(−)-HA-966 was obtained from Tocris Cookson (Bristol, U.K.). (±)-Baclofen and 2-hydroxysaclofen were purchased from Research Biochemicals International (Natick, MA, U.S.A.). CGP-35348 and (−)-baclofen were generously supplied as a gift by Ciba-Geigy (Basel, Switzerland). [$^3$H]-GABA (95 Ci $mmol^{-1}$) was purchased from Amersham (U.K.).

## Results

Stable extracellular recordings were obtained from a total of 76 neurones in the pars compacta of the SN. All cells exhibited electrophysiological characteristics identical to those previously ascribed to neurochemically-identified dopaminergic neurones including: (1) spontaneous, long duration (>2 ms), triphasic action potentials; (2) uniform firing rates ranging from 2.0–5.1 Hz (mean = 3.3±0.1 Hz) and (3) a pacemaker firing pattern (Grace & Onn, 1989; Yung *et al*., 1991).

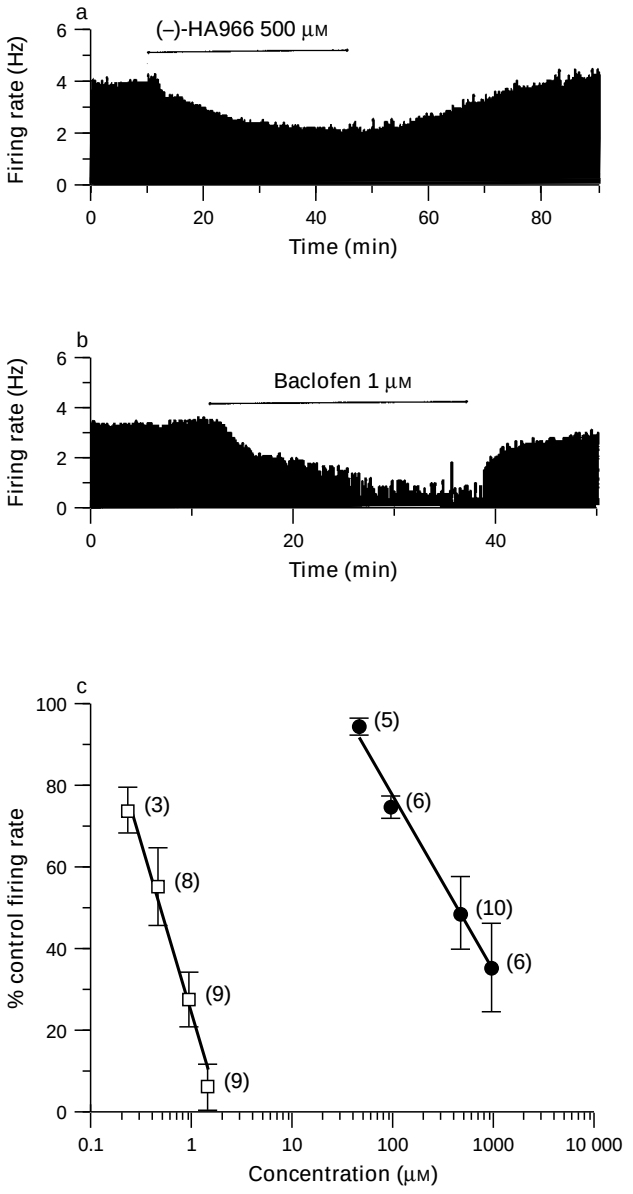


**Figure 1** Inhibitory effects of (−)-HA-966 and baclofen on the firing rate of SN dopaminergic neurones *in vitro*. (a and b) Cumulative rate histograms illustrating the typical effects of bath applied (a) (−)-HA-966 (500 μM) and (b) baclofen (1 μM) on the spontaneous firing rate of nigral dopaminergic neurones. Horizontal line denotes the period during which drugs were applied. Note gradual onset of the inhibitory effects of both compounds. (c) Log linear concentration-response curves comparing the potency of (−)-HA-966 (●) and baclofen (□). Data represent the average response of groups of cells (numbers in parentheses denote sample size) tested with a single concentration of each drug; vertical lines show s.e.mean. Regression lines were drawn to a least-squares fit of the data. No significant differences existed between the pre-drug firing rates of the cells comprising the individual treatment groups (1-way ANOVA $F_{7,48}=0.73$, $P=0.65$).

### *Effects of (−)-HA-966 on dopamine cell activity*

Bath application of (−)-HA-966 (0.05–1.0 mM) inhibited the firing rate of all cells tested (Figure 1). Although statistically significant (mean difference = 0.16 Hz, paired $t=3.14$; $P=0.035$), the magnitude of inhibitory effects produced by the lowest concentration of the drug (50 μM) never exceeded 15% of the control firing rate. Higher concentrations produced a proportionately greater inhibition of activity. However, only 2 of 16 cells tested were completely inhibited by the drug. The transient increase in firing rate that frequently precedes onset of the inhibitory effects of (−)-HA-966 *in vivo* (Shepard & Lehmann, 1992; Shepard *et al*., 1995) was never observed *in vitro*. Although fully reversible upon washout from the chamber, individual cells were slow to respond to bath application of (−)-HA-966. Maximal inhibitory effects of the drug were typically attained after 20 to 30 min of continuous perfusion with comparable intervals required to reverse fully these effects (Figure 1a). Consequently, concentration-response data were compiled from the peak response of individual neurones tested with a single concentration of (−)-HA-966 and only one cell was tested per slice. Least squares analysis of the data indicated that the concentration of (−)-HA-966 required to inhibit dopamine cell activity by 50% ($EC_{50}$) averaged 444.0 μM (95% confidence intervals: 277.6 μM–710.1 μM, $n=27$, Figure 1c).

### *Effects of (±)-baclofen on dopamine cell activity*

In order to provide a basis for comparison, an additional group of experiments was conducted to assess the sensitivity of nigral dopamine neurones to the inhibitory effects of the prototypical $GABA_B$ agonist, (±)-baclofen. As illustrated in Figure 1c, bath application of (±)-baclofen produced a concentration-dependent reduction in firing rate ($EC_{50}=0.54$ μM, 95% confidence intervals: 0.44 μM–0.66 μM, $n=29$). Of nine cells tested with the highest concentration of (±)-baclofen (1.5 μM), seven (78%) were silenced by the drug. The inhibitory effects of (±)-baclofen, like those of (−)-HA-966, were slow to develop, usually requiring more than 20 min to attain a steady-state value. However, the inhibition produced by (±)-baclofen was immediately reversed upon its washout from the chamber (Figure 1b).

### *Effects of chloral hydrate on the inhibitory actions of (−)-HA-966*

Previous studies have shown that the response of SN dopaminergic neurones to γ-hydroxybutyrate is influenced by certain anaesthetics (Diana *et al*., 1991). These findings, together with our own results indicating that the inhibitory effects of (−)-HA-966 *in vivo* are attenuated when ketamine is substituted for chloral hydrate as an anaesthetic (Shepard *et al*., 1993), prompted us to examine the effects of the latter compound on the inhibitory effects of (−)-HA-966 *in vitro*. In these experiments, the average response of ten SN dopamine cells tested with 500 μM (−)-HA-966 was compared to a separate group of cells ($n=9$) tested with an identical concentration of the drug applied in the presence of 10 μM chloral hydrate. Average basal firing rates exhibited by the two treatment groups were not significantly different. Bath application of the anaesthetic had no effect on neuronal firing rate (Figure 2b). The magnitude of the inhibition produced by (−)-HA-966 in the presence of chloral hydrate (65% inhibition; Figure 2b) was only slightly greater than that observed in its absence (48% inhibition; Figure 2a). However, these differences were marginally significant (2-way repeated measures ANOVA, $F_{1,17}=4.4$, $P=0.5$).

### *CGP-35348 and 2-hydroxysaclofen reverse the inhibitory effects of (−)-HA-966*

The potential involvement of $GABA_B$ receptors in mediating the inhibitory effects of (−)-HA-966 was assessed by use of the

selective antagonists, CGP-35348 (100 μM) and 2-hydroxysaclofen (500 μM). Addition of CGP-35348 (Figure 3a, d) or 2-hydroxysaclofen (Figure 3b, e) to the bathing solution completely reversed the inhibitory effects of (−)-HA-966 (500 μM). Notably, in contrast to the characteristically slow washout of (−)-HA-966, reversal of the rate-decreasing effects of the drug by both antagonists was complete within five minutes following their introduction into the chamber (Figure 3a-b). Bath application of CGP-35348 alone increased (9.2 ± 0.3%) the basal firing rate of SN dopaminergic neurones (Figure 3c; mean difference = −0.28 Hz, Paired $t = 3.67$, $P = 0.04$).

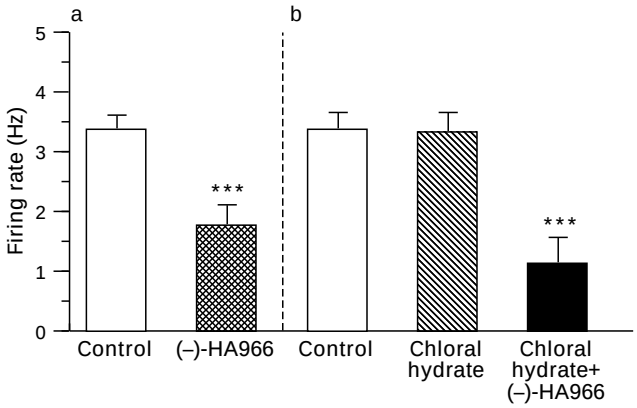


**Figure 2** Bar graph illustrating the effects of chloral hydrate on the inhibition produced by (−)-HA-966. (a) Average response (mean ± s.e.mean) of 10 cells to 500 μM (−)-HA-966. Data were obtained from the same group of cells as that used to compile the concentration-response curve in Figure 1. (b) Response of a second group of cells ($n = 9$) to chloral hydrate (10 μM) in the presence (solid column) and absence (hatched column) of (−)-HA-966 (500 μM). Note that addition of chloral hydrate potentiated the inhibitory effects of (−)-HA-966 (2-way repeated measures ANOVA, $F_{1,17} = 4.4$, $P = 0.05$). Asterisks denote a significant difference from control firing rate (Bonferroni $t$ test, $P < 0.001$).

### *Effect of (−)-HA-966 on [$^3$H]-GABA$_B$ binding*

In order to assess directly the affinity of (−)-HA-966 for $GABA_B$ receptors, the ability of the drug to displace [$^3$H]-GABA from synaptic membranes was determined under conditions in which $GABA_A$ receptors were blocked by isoguvacine. As illustrated in Figure 4, the extent of displacement was concentration-dependent and proportional to the inhibitory effects produced by the drug. However, (−)-HA-966 was significantly less potent than GABA (relative affinity = 0.0005 GABA) and exhibited no apparent stereoselectivity (data not shown).

## Discussion

Previous studies have confirmed the ability of (−)-HA-966 to modify both the firing rate and discharge pattern of nigrostriatal dopamine-containing neurones in the chloral hydrate anaesthetized rat (Shepard & Lehmann, 1992; Shepard *et al*., 1995). In the present series of experiments, we have extended these findings by demonstrating that the inhibitory effects of

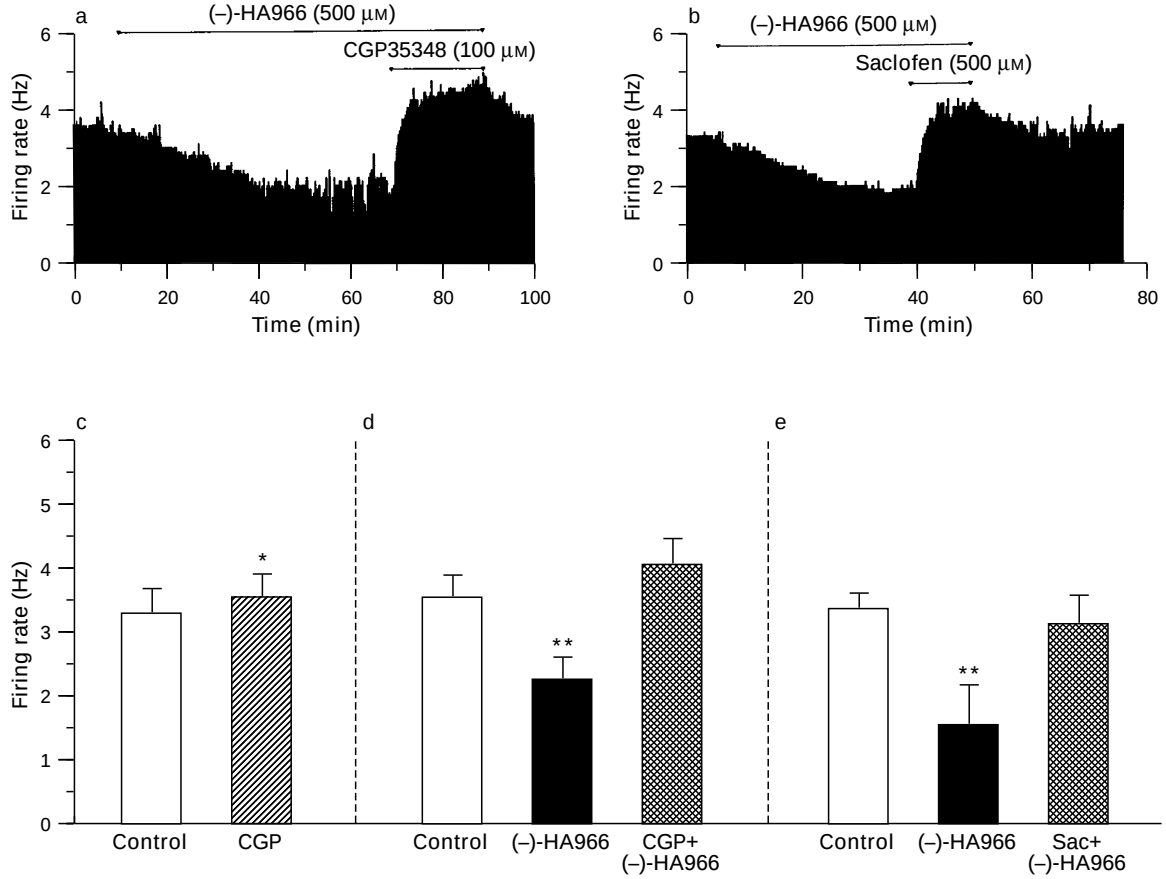


**Figure 3** Effects of CGP-35348 and 2-hydroxysaclofen on the inhibitory actions of (−)-HA-966. (a and b) Rate histograms illustrating the characteristic effects of bath applied (a) CGP-35348 (100 μM) and (b) 2-hydroxysaclofen (500 μM) on the inhibitory response produced by concomitant application of (−)-HA-966 (500 μM). Horizontal bars denote the duration of drug application. (c–e) The effects of CGP-35348 on dopamine cell activity (c) and the effects of (d) CGP-35348 and (e) 2-hydroxysaclofen on (−)-HA-966-induced inhibition of neuronal activity. Asterisks denote a significant difference from control values (*$P < 0.05$; **$P < 0.01$).

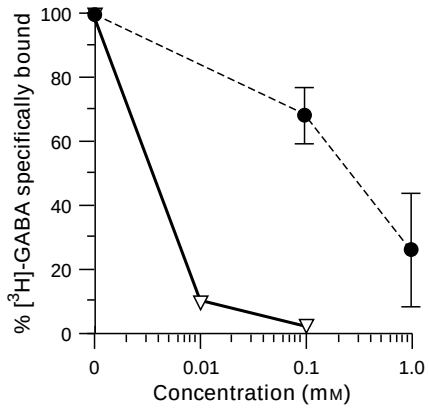


**Figure 4** Inhibition by (−)-HA-966 of the specific binding of [$^3$H]-GABA to $GABA_B$ receptors. (▽) Effect of GABA and (●) effect of (−)-HA-966 on [$^3$H]-GABA bound. Each point represents the mean of five separate determinations, each performed in triplicate; vertical lines show s.e.mean.

the drug persist in nigral brain slices, a preparation presumably devoid of extrinsic afferent input. Although similar in some respects to results obtained in the intact animal, differences between the pharmacological profile of (−)-HA-966 *in vitro* and *in vivo* offer insights into the possible mechanisms underlying the electrophysiological effects of the drug. For example, the transient increase in firing rate typically preceding onset of the inhibitory actions of (−)-HA-966 *in vivo* (Shepard & Lehmann, 1992) was never observed *in vitro*. Based on these findings, it seems reasonable to conclude that the excitatory effects of the drug are mediated through one or more afferent pathways which are lost during preparation of the tissue slices. On the other hand, the ability of (−)-HA-966 to inhibit neuronal firing rate *in vitro* would seem to imply that this component of the response is mediated locally – possibly through a direct interaction with nigral dopaminergic neurones.

In agreement with earlier *in vivo* studies (Shepard *et al*., 1995), high concentrations of (−)-HA-966 (0.5 – 1 mM) proved capable of completely suppressing neuronal activity in a percentage of cells. Although of similar apparent efficacy, qualitiative differences appear to exist in the potency of the drug between *in vivo* and *in vitro* preparations. Thus, while the average intravenous dose of (−)-HA-966 needed to inhibit dopamine cell activity by 50% in the chloral hydrate anaesthetized rat averaged 5.7 mg $kg^{-1}$ (Shepard *et al*., 1995), relatively high concentrations of the drug ($\approx$450 $\mu$M) were required to produce similar effects *in vitro*. Although comparison of $EC_{50}$ and $ED_{50}$ estimates cannot be made directly, it seems unlikely that a single intravenous dose of less than 50 $\mu$mol $kg^{-1}$ (15 $\mu$mol/300 g rat) could result in tissue concentrations approaching 0.5 mM. Further evidence of the attenuated potency of (−)-HA-966 *in vitro* was obtained from a direct comparison of its effects with those of the prototypical $GABA_B$ agonist, (±)-baclofen. Bath application of (±)-baclofen led to a concentration-dependent reduction in dopaminergic neuronal activity. Although qualitatively similar to the effects of (−)-HA-966, (±)-baclofen was over 800 fold more potent in inhibiting dopamine cell firing rate *in vitro* ($EC_{50}$ = 0.54 $\mu$M vs. 444 $\mu$M, respectively).

One explanation for the apparent discrepancy in the potency of (−)-HA-966 between *in vivo* and *in vitro* preparations concerns the potential influence of chloral hydrate on the responsiveness of nigral dopaminergic neurones to (−)-HA-966. In addition to potentiating the inhibitory effects of a variety of direct-acting dopamine agonists (Kelland *et al*., 1989; 1990), recent studies have indicated that chloral hydrate may increase the sensitivitiy of nigral dopaminergic neurones to the rate decreasing effects of $\gamma$-hydroxybutyrate *in vivo* (Diana *et al*., 1991). Preliminary data have also shown that the inhibitory effects of (−)-HA-966 are attenuated in ketamine-anaesthetized rats (Shepard *et al*., 1993). Consistent with these observations, coapplication of a low concentration of chloral hydrate (10 $\mu$M) was found to enhance significantly the inhibitory effects of (−)-HA-966 *in vitro*, although the magnitude of the inhibition produced by (−)-HA-966 in the presence of chloral hydrate was only slightly greater than that observed under control conditions. However, it should be noted that the extent of synergism between these drugs was probably affected by the low concentrations of anaesthetic and a retarded rate of formation of the active metabolites of chloral hydrate (Breimer, 1977).

Despite the low affinity of (−)-HA-966 for $GABA_B$ binding sites, the results of the present study indicate that the inhibitory effects of (−)-HA-966 *in vitro* may derive from a direct interaction with these receptors. Thus, (−)-HA-966 was found to displace the specific binding of [$^3$H]-GABA to $GABA_B$ receptors in a concentration-dependent manner. It is also conceivable that in addition to its weak agonist properties, (−)-HA-966 acts to release endogenous GABA onto $GABA_B$ receptors. The comparatively low potency of the drug in inhibiting dopamine cell firing (relative potency $\sim$0.001(±)-baclofen) was reflected by similar differences between the affinity of GABA and (−)-HA-966 for the $GABA_B$ binding site (relative potency $\sim$0.0005 GABA). Although of significantly lower potency than either (±)-baclofen or GABA, the inhibitory effects of (−)-HA-966 were completely reversed by the $GABA_B$ antagonists, CGP-35348 (100 $\mu$M) and 2-hydroxysaclofen (500 $\mu$M). These concentrations are equivalent to or in some cases well below those previously shown to antagonize fully $GABA_B$-mediated postsynaptic potentials or the inhibitory effects of local application of baclofen in brain slices (Olpe *et al*., 1990; Hausser & Yung, 1994; Benardo, 1995; Guyon & Leresche, 1995; Li & Guyenet, 1995; Morishita & Sastry, 1995). Notably, reversal of the inhibitory effects of (−)-HA-966 produced by CGP-35348 was frequently accompanied by an overshoot in activity resulting in firing rates that were often higher than control values. Modest excitatory effects were also observed in response to CGP-35348 applied in the absence of (−)-HA-966 suggesting that endogenous GABA levels in the brain slice are sufficiently high to maintain tonic activation of $GABA_B$ receptors. Nevertheless, the magnitude of the excitation produced by CGP-35348 (<10%), since firing rate is a linear function of excitability (Silva & Bunney, 1988; Grace, 1991; Yung *et al*., 1991), was not sufficient to account for the ability of the drug to reverse the inhibitory effects of (−)-HA-966 completely.

In summary, (−)-HA-966 was found to inhibit the activity of nigral dopaminergic neurones in a concentration-dependent fashion. Although the effects of the drug *in vitro* could be attributed to its ability to act as a weak $GABA_B$ agonist, this mechanism is unlikely to account for the potent inhibitory effects exhibited by low doses of (−)-HA-966 *in vivo*.

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