Gamma-Hydroxybutyrate is a GABA$_B$ Receptor Agonist that Increases a Potassium Conductance in Rat Ventral Tegmental Dopamine Neurons$^1$

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ABSTRACT

$\gamma$-Hydroxybutyric acid (GHB) is an abused substance that occurs naturally in the basal ganglia. Electrophysiological recordings of membrane voltage and current were made to characterize the effects of GHB on dopamine neurons in the ventral tegmental area of the rat midbrain slice. Perfusion containing GHB caused a concentration-dependent membrane hyperpolarization ($E_{\text{rev}} = 0.88 \pm 0.21$ mV) and a reduction in input resistance ($E_{\text{rev}} = 0.74 \pm 0.21$ mV). The highest concentration of GHB studied (10 mM) hyperpolarized neurons by $20 \pm 3$ mV and reduced input resistance by $58\% \pm 9\%$. Changes in membrane potential and input resistance were blocked by the $\gamma$-aminobutyric acid antagonist CGP-35348 (300 $\mu$M), but neither bicuculline (30 $\mu$M) nor strychnine (10 $\mu$M) was an effective antagonist. Voltage-clamp recordings demonstrated that GHB (1 mM) evoked $80 \pm 6$ pA of outward current (at $-60$ mV) that reversed at $-110$ mV (in 2.5 mM K$^+$). Increasing concentrations of extracellular K$^+$ progressively shifted the reversal to more depolarized potentials. In tetrodotoxin (0.3 mM) and tetraethylammonium (10 mM), depolarizing voltage steps (to $-30$ mV) evoked calcium-dependent current spikes that were completely blocked by GHB (1 mM). These data suggest that GHB is an agonist at $\gamma$-aminobutyric acid receptors and would be expected to inhibit DA release by causing K$^+$-dependent membrane hyperpolarization.

GHB is present throughout the brain, the highest concentrations being found in the cerebral cortex, hippocampus, striatum (Snead, III, 1996; Vayer and Maitre, 1988) and ventral midbrain (Hechler et al., 1992). Although GHB is both a metabolite of GABA and a precursor for synthesis of this neurotransmitter (Vayer et al., 1985; Maitre, 1997; Hechler et al., 1997), GHB might also have a function, independent of GABA, as a neurotransmitter (Snead, III, 1977; Vayer et al., 1987; Cash, 1994). GHB is clinically useful as an anesthetic, but its sedative and mood-elevating properties have contributed to its use as a recreational drug (Galloway et al., 1997; Marwick, 1997). Despite its abuse potential, preliminary clinical data suggest that GHB may reduce craving for ethanol in alcoholics (Gallimberti et al., 1992) and that it may also reduce symptoms of opiate withdrawal (Gallimberti et al., 1994). A better understanding of its mechanism of action might help uncover new therapeutic uses and/or foster a greater appreciation of potential adverse effects.

Like many drugs of abuse (Di Chiara and Imperato, 1988), GHB significantly alters dopaminergic neurotransmission in the brain. Systemic injections of GHB to rats can significantly increase DA concentrations in striatum (Walters and Roth, 1972) and other brain regions (Da Prada and Keller, 1976; Nissbrandt and Engberg, 1996), but microdialysis studies generally show that systemically administered GHB reduces DA release (Feigenbaum and Howard, 1997; Nissbrandt et al., 1994). Whether GHB increases or decreases DA release depends partly on the dose and route of administration, the time at which levels are measured and the presence or absence of general anesthesia (Howard and Feigenbaum, 1996; Hechler et al., 1991). Spontaneous firing rates of DA neurons in vivo are generally reduced when GHB is administered systemically to rats, and perfusion of midbrain slices with GHB has been shown to hyperpolarize DA neurons recorded intracellularly (Harris et al., 1989). Although pharmacological data developed with the use of antagonists suggest that GHB inhibits DA neuronal activity by stimulating GABA$_B$ receptors (Engberg and Nissbrandt, 1993), an increase in amplitude of voltage-activated Ca$^{2+}$ spikes reported by one study (Harris et al., 1989) would be consistent with the hypothesis that GHB exerts an excitatory effect on DA neurons, which would facilitate DA release.

Although many studies suggest that GHB is an agonist at GABA$_B$ receptors (Da Prada and Keller, 1976; Engberg and

ABBREVIATIONS: ACSF, artificial cerebrospinal fluid; BMI, bicuculline methiodide; DA, dopamine; $E_{\text{rev}}$, reversal potential; GABA, $\gamma$-aminobutyric acid; GHB, $\gamma$-hydroxybutyric acid; SN, substantia nigra; TEA, tetraethylammonium; TTX, tetrodotoxin; VTA, ventral tegmental area.
Nissbrandt, 1993; Williams et al., 1995; Ito et al., 1995; Hosford et al., 1995; Xie and Smart, 1992), an autoradiographic binding study has shown that NSC-382, a GHB antagonist, does not effectively displace some GABA B receptor ligands in brain tissue (Snead, III, 1996). Moreover, this GHB antagonist reduces the ability of GHB to increase DA concentrations in striatum (Maitre et al., 1990). Therefore, the present series of experiments was designed to evaluate the pharmacological effects of GHB on membrane properties of DA neurons in the rat brain slice. DA neurons in the VTA were the focus of our investigation, because these neurons are known to mediate the rewarding effects of many drugs of abuse (Di Chiara and Imperato, 1988; Bozarth and Wise, 1981). Some of these results have been presented previously in abstract form (Madden and Johnson, 1996).

Materials and Methods

Tissue preparation. Male Sprague-Dawley rats (150–250 gm; Bantin and Kingman, WA) were housed according to National Institutes of Health guidelines. Briefly, each rat was anesthetized with halothane and killed by severing major thoracic vessels. The brain was quickly removed and submerged in aCSF that contained (in mM) NaCl (126), KCl (2.5), NaHPO4 (1.2), MgCl2 (1.2), CaCl2 (2.4), dextrose (10) and NaHCO3 (26), at pH 7.4. A 300-μm slice of the midbrain was cut horizontally on a vibratome, submerged in a tissue bath (0.5 ml) and bathed continually (2 ml/min) in aCSF equilibrated with 95% O2 and 5% CO2 at 36°C. The VTA was identified as the region lateral to the fasciculus retroflexus and medial to the medial terminal nucleus of the accessory optic tract.

Intracellular recording. Glass microelectrodes (O.D. 1 mm, I.D. 0.5 mm) were made on a P-87 Flaming-Brown (Sutter Instrument, Novato, CA) or a Narishige (Tokyo, Japan) micropipette puller. Microelectrodes were filled with KCl (2 M) and had resistances of 45 to 120 megohms. Measurements of membrane current and voltage were made with an Axoclamp-2B amplifier (Axon Instruments, Foster, CA) and recorded with Axotape (Axon Instruments) computer software, using an IBM-compatible personal computer. An active bridge circuit was used to ensure accurate measurement of membrane potential while current was passed through the micropipette; the bridge was frequently checked for proper balance by passing small (50-pA) current steps and monitoring the voltage output on an oscilloscope. Membrane input resistance was calculated by measuring the change in membrane potential evoked by small (20–100-pA) hyperpolarizing current pulses; membrane potentials were measured 30 to 50 ms after passing currents in order to avoid the influence of H-current.

Single-electrode voltage-clamp recordings were made at a gain of 0.5 to 2.5 nA/mV at a switching frequency of 2 to 4 kHz; head-stage voltage was monitored continuously on an oscilloscope. Relationships between membrane potential and current were studied in voltage clamp using pClamp software, a TL-1 analog/digital converter (Axon instruments) and an IBM-type computer. Current-voltage curves were obtained by measuring currents 30 to 50 ms after they were evoked by a voltage step. Chord conductance was measured by linear regression as the slope of the current-voltage plot in the linear range in response to small (20–100-pA) hyperpolarizing current pulses; membrane potentials were measured 30 to 50 ms after passing currents in order to avoid the influence of H-current.

Whole-cell recording. Patch electrodes were used to record currents under voltage clamp in the whole-cell configuration. Glass pipettes (O.D. 1.5 mm, thick wall) were pulled on a Flaming-Brown micropipette puller so that the initial resistance was 2 to 4 megohms and the outer tip diameter was about 1 μm. The internal (pipette) solution contained (in mM) K+ gluconate (125), NaCl (15), CaCl2 (1), MgCl2 (2), N-2-hydroxyethylpiperoxide-N′-2-ethanesulfonic acid (10), ethylene glycol-bis(β-amino-ethyl ether) N,N,N′,N′-tetraacetic acid (11), K3ATP (1.5) and Na3GTP (0.3). Osmolality was 290 mOsmol/l, and pH was 7.25. After forming a gigaseal and breaking into the cell, membrane currents were amplified by an Axopatch-1D amplifier (Axon Instruments). Series resistance (10–40 megohms) was compensated electronically 50% to 80%. Stated voltages are corrected for liquid junction potential (10 mV).

Drugs. All drugs were added to the perfusate. Drug solutions entered the recording chamber within 30 s of the turning of a stopcock, the delay being necessary for passage of the solution through a heat exchanger. Complete exchange of the bath solution occurred within 2 min. In constructing concentration-response curves, we allowed the effect of a given concentration of drug to wash out completely before the next concentration of drug was applied. A stock solution of DA HCl (Sigma, St. Louis, MO) was made daily and kept on ice to retard oxidation. BMI, strychnine, TTX and TEA were obtained from Sigma; CGP-35348 and GHB were purchased from Research Biochemicals Int. (Natick, MA); NCS-382 was from Torcis Cookson, Inc. (Ballwin, MO).

Statistics. Using the KaleidaGraph curve-fitting program on a Power Macintosh computer, we fitted concentration-response curves to the equation

\[ y = ax/(x + b) \]

where \( y \) is the magnitude of drug effect, \( a \) is the maximum drug effect, \( x \) is the concentration of drug and \( b \) is the EC50. An EC50 value was calculated for each neuron; data from all neurons were pooled in order to obtain an estimate of mean and S.E.M. Numerical data in the text and error bars in figures are expressed as means ± S.E.M.

Results

Identification of DA neurons. On the basis of visualization of landmarks with a dissection microscope, all impaled cells were located in the VTA (see “Materials and Methods”). A cell was categorized as a principal DA neuron in accordance with criteria described previously (Johnson and North, 1992). Briefly, dopamine cells fire spontaneously at 1 to 4 Hz, have relatively broad action potentials (1-ms duration at 50% maximum amplitude) and have time-dependent “sags” in membrane potential in response to hyperpolarizing current pulses (due to H-current). This type of neuron has been shown to contain tyrosine hydroxylase (Grace and Onn, 1988).

Effects on firing rate, membrane potential and input resistance. As shown in figure 1A, the spontaneous firing of action potentials was completely blocked by perfusion with 3 mM GHB (n = 5). GHB also hyperpolarized DA neurons in a concentration-dependent manner (fig. 1B). In order to prevent spontaneous action potentials, we applied a small amount of hyperpolarizing current (20–200 pA) to set the initial membrane potential to ~60 mV. The EC50 for membrane hyperpolarization was 0.88 ± 0.21 mM (n = 7), and the highest concentration of GHB perfused (10 mM) caused a hyperpolarization of 20 ± 3 mV (n = 4). GHB also reduced input resistance in a concentration-dependent fashion (fig. 2). The EC50 for reducing input resistance was 0.74 ± 0.21 mM (n = 7), and the highest concentration of GHB (10 mM) reduced resistance by 58% ± 9% (n = 4). Input resistance averaged 169 ± 7 megohms under control conditions (n = 10). Effects of GHB on firing rate, membrane potential and input resistance were completely gone 8 to 12 min after washout.

GHB activates GABAA receptor. As shown in figure 3, the GABAA receptor antagonist CGP-35348 (300 μM) completely prevented GHB (1 mM) from causing membrane hy-
perpolarization \((n = 4)\). In contrast, the GABA\(_A\) antagonist BMI \((30 \mu M; n = 5)\) and the glycine receptor antagonist strychnine \((0.1 \text{ or } 10 \mu M; n = 2; \text{ data not shown})\) had no effect on GHB \((1 \text{ mM})\)-induced hyperpolarization. These results suggest that GHB produces membrane hyperpolarization by activating GABA\(_B\) receptors.

**Single-electrode voltage-clamp experiments.** Under single-electrode voltage clamp, GHB \((1 \text{ mM})\) evoked \(80 \pm 6\) pA of net outward current at the holding potential of \(-60 \text{ mV} \) \((n = 3)\). As shown in figure 4A, this outward current was associated with an increase in chord conductance \((1.74 \pm 0.45 \text{ nS}, n = 3)\) as determined by measuring currents during delivery of a series of seven hyperpolarizing voltage steps. In an external \(K^+\) concentration of \(2.5 \text{ mM}\), currents evoked by GHB \((1 \text{ mM})\) reversed at \(-110 \pm 9 \text{ mV} \) \((n = 3)\); this \(E_{\text{rev}}\) equals that expected for \(K^+\) as calculated via the Nernst equation \((-105 \text{ mV})\). Figure 4B shows net currents produced by GHB recorded in different concentrations of extracellular \(K^+\); net currents were obtained by subtracting control currents from those recorded in GHB. Increasing concentrations of extracellular \(K^+\) \((2.5 \text{ to } 5, 7.5 \text{ and } 10 \text{ mM})\) progressively shifted the reversal potential to less hyperpolarized values \((n = 3)\), as expected for a current carried by \(K^+\) (fig. 4B).

**Whole-cell recordings.** Using patch pipettes to record currents under voltage clamp, we found that \(10 \text{ mM GHB evoked } 156 \pm 18 \text{ pA of outward current at } -60 \text{ mV} \) \((n = 3)\). As shown in figure 4A, this outward current was associated with an increase in chord conductance \((1.74 \pm 0.45 \text{ nS}, n = 3)\) as determined by measuring currents during
paired data from three neurons, we found that the increase in conductance produced by 10 mM GHB (3.39 ± 0.57 nS) was completely blocked by 300 μM CGP-35348 (n = 3). In contrast, the putative GHB antagonist NCS-382 (10 μM) did not significantly alter the increase in conductance produced by 10 mM GHB, judging by paired data from four neurons (P = .5). This concentration of NCS-382 was used because other investigators have shown that 10 μM NCS-382 selectively antagonizes the biological activity of GHB without significantly blocking GABA<sub>B</sub> receptors in brain slices (Maitre et al., 1990; Snead, III, 1996).

**Calcium currents.** During patch recordings in the whole-cell configuration, a 30-mV depolarizing voltage step (from −60 to −30 mV, 200 ms in duration) evoked 1 to 5 transient spikes of inward currents while being recorded in perfusate containing TTX (0.3 μM) and TEA (10 mM) (see fig. 5). GHB (10 mM) reduced the number of spikes from an average of 2.7 ± 0.5 spikes per depolarizing step to 0.5 ± 0.3 spikes (n = 7) (P < .01, t test). These spikes in current were presumed to be mediated by Ca<sup>2+</sup> because they were completely blocked by perfusate containing no added Ca<sup>2+</sup> (n = 2). As is also shown in figure 5, perfusate containing no added Ca<sup>2+</sup> also reduced the depolarization-activated outward current, which has been shown to be a Ca<sup>2+</sup>-activated K<sup>+</sup> current (Johnson and Seutin, 1997).

**Discussion**

These data indicate that GHB is a GABA<sub>B</sub> receptor agonist that causes membrane hyperpolarization by increasing K<sup>+</sup> conductance in DA neurons. Our results agree with those of others who made extracellular recordings of midbrain DA neurons in vivo (Engberg and Nissbrandt, 1993; Da Prada and Keller, 1976) and intracellular studies of hippocampal neurons in vitro (Xie and Smart, 1992). However, our results failed to support the hypothesis that some effects of GHB are mediated by non-GABA receptors that are sensitive NCS-382, a reputed GHB antagonist. We also found no evidence to support the study by Harris et al. (1989), who showed that GHB potentiated a voltage-activated Ca<sup>2+</sup> spike in DA neurons (Harris et al., 1989). In agreement with the work of others (Walters et al., 1973), our findings suggest that GHB will inhibit DA neuronal activity and thereby reduce DA release in tissues innervated by DA neurons.

Our finding that GHB reduces the ability of DA neurons to generate Ca<sup>2+</sup> spikes is consistent with its hyperpolarizing effect, because both processes would be predicted to reduce the release of DA from nerve terminals. It is possible that the inhibition of Ca<sup>2+</sup> spikes is mediated by a second-messenger system triggered by the binding of GHB to GABA<sub>B</sub> receptors. Because GABA<sub>B</sub> receptors are negatively coupled to adenyl cyclase, inhibition of protein kinase A activity with concomitant reductions in phosphorylation of intracellular proteins would be a reasonable hypothesis to explain reductions in Ca<sup>2+</sup> currents (Bowery, 1993). However, our finding could also be explained by the fact that the increase in K<sup>+</sup> conductance produced by GABA<sub>B</sub> receptor stimulation would tend to reduce our ability to clamp voltage in distal dendrites. Consequently, a reduction in Ca<sup>2+</sup> currents could be due to a reduced ability to move the voltage to a level that is sufficient to trigger the activation of these currents. At this time, it is not clear which of these explanations is correct.

One may wonder why such high concentrations of GHB (1–10 mM) were needed to alter neuronal activity, especially if GHB is an endogenous transmitter. Although GHB can be metabolized by enzymes in the brain to other compounds, including GABA (Vayer et al., 1985), the lack of effects mediated by GABA<sub>A</sub> receptors in our studies argues against a significant metabolic conversion of GHB to GABA in the brain slice. It is more likely that the active uptake of GHB by a transporter reduces its potency in the slice (Hechler et al., 1985; Benavides et al., 1982). Just as with GHB, mM concentrations of GABA are needed to affect membrane properties of DA neurons (Johnson et al., 1992), and this low potency can be attributed, in part, to active uptake by transporters (Shen et al., 1997). However, we concede that the need for mM concentrations of GHB to produce changes in membrane conductance is consistent with a pharmacological rather than a physiological action.

Our conclusion that GHB inhibits DA neuronal activity may be surprising, because it is generally acknowledged that the rewarding aspect of drugs of abuse is mediated by DA release (Di Chiara and Imperato, 1988). The GHB-induced hyperpolarization and inhibition of firing of DA neurons might be expected to conserve intracellular stores of DA. This expectation is consistent with the work of others, who showed that GHB reduces DA turnover (Walters and Roth, 1972) and increases DA concentrations in brain (Da Prada and Keller, 1976; Walters et al., 1973). Although we cannot rule out the possibility that the increased intracellular stores of DA are later released, it seems more likely that the reinforcing properties of GHB are mediated by a nondopaminergic mechanism. It is possible that the reinforcing property of GHB is similar to that of other sedative-hypnotic agents that do not necessarily cause DA release (Macdonald and Barker, 1979).

In conclusion, our results show that GHB hyperpolarizes DA neurons by a GABA<sub>B</sub> receptor-mediated increase in K<sup>+</sup> conductance. GHB also reduced depolarization-activated Ca<sup>2+</sup> spikes. Although none of these effects was prevented by NCS-382, a reputed GHB antagonist, our studies do not rule out the possibility that effects of GHB are mediated by non-GABA receptors elsewhere in the brain. Our findings suggest that GHB has an overall inhibitory effect on DA...
neuronal excitability that would be expected to reduce DA output from nerve terminals.

References


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