Effects of Sustained Gamma-Hydroxybutyrate Treatments on Spontaneous and Evoked Firing Activity of Locus Coeruleus Norepinephrine Neurons

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Background: *Gamma-hydroxybutyrate is currently used to promote nighttime sleep in the treatment of narcolepsy; however, it is also a drug of abuse ("Liquid Ecstacy") associated with a withdrawal syndrome with anxiety features. Of interest, the activity of locus coeruleus neurons is a reflective index of these above mentioned behavioral states.*

Methods: *Using in vivo extracellular unitary recordings, sustained administration of gamma-hydroxybutyrate (40 mg/kg/day via minipump implanted subcutaneously) on the spontaneous and sensory-evoked burst firing of locus coeruleus norepinephrine neurons was assessed in rats.*

Results: *A 2-day and 10-day gamma-hydroxybutyrate administration decreased the spontaneous firing activity of locus coeruleus neurons by 52% and 54%, respectively, when compared with controls. A similar degree of attenuation on evoked burst firing of norepinephrine neurons also occurred in these rats (2-day gamma-hydroxybutyrate: 47% and 10-day gamma-hydroxybutyrate: 58%), when compared with controls. In contrast, rats treated with gamma-hydroxybutyrate for 10 days followed by removal of the minipump for 36 hours resulted in a 33% augmentation in spontaneous locus coeruleus activity as compared with controls. Furthermore, a robust 79% increase in burst firing in response to paw-pinch was exhibited in theses rats.*

Conclusions: *Chronic gamma-hydroxybutyrate treatment inhibits the spontaneous and sensory-evoked burst firing of locus coeruleus norepinephrine neurons, whereas these indices are enhanced during drug withdrawal. The alteration in norepinephrine activity during chronic gamma-hydroxybutyrate administration may contribute to the ability of this agent to induce sleep and regulate narcoleptic episodes. Enhanced norepinephrine activity during withdrawal may be related to symptoms of anxiety on rapid termination of this drug in abusers.*

Key Words: Anxiety disorders, withdrawal, addiction, sleep, drug abuse

Gamma-hydroxybutyrate (GHB) is a metabolite of γ-aminobutyric acid (GABA) and is produced naturally in the brain through the semialdehyde reduction pathway (for a review, see Maitre et al 2000; Szabo et al 2003). Succinic semialdehyde dehydrogenase (SSADH) deficiency is a rare autosomal recessively inherited defect that leads to the accumulation of GHB (Gibson et al 1998). The clinical presentation of this disease includes significant behavioral disturbances and psychosis (hallucinations, disabling anxiety, aggressive behavior, and sleep disorder), which is accompanied by white-matter hyperintensities of the globus pallidus, thalamus, and brainstem in these patients (Pearl et al 2003). Pharmacologically, GHB binds not only to specific GHB receptors (Benavides et al 1982; Hechler et al 1992; Snead and Liu 1984) but has been largely regarded to mediate its central nervous system (CNS) effects though GABA type B ($GABA_B$) receptors (Bernasconi et al 1999; Emri et al 1996; Erhardt et al 1998; Lingenhoehl et al 1999; Waldmeier 1991; Williams et al 1995), with recent findings indicating a GABA type A ($GABA_A$) receptor interaction as well (Cammalleri et al 2002; Follesa et al 2003; Lobina et al 1999; Schmidt-Mutter et al 1998). More specifically, GHB binds reversibly to GHB receptors, now reported to be G-protein coupled (Ratomponirina et al 1995) with evidence for both a high (k_i = 95 nmol/L) and low (k_i = 16 μmol/L) affinity site, suggestive of two receptor populations. While GABA fails to bind GHB receptors, GHB can bind $GABA_B$ receptors (k_i = 80–120 μmol/L) with a much weaker affinity.

Currently, GHB (sodium oxybate is the official generic name; Xyrem) is a Food and Drug Administration (FDA) approved drug prescribed as a treatment for narcolepsy that is believed to regulate sleep architecture (Lammers et al 1993; Scrima et al 1989). On the other hand, because of its anxiolytic, antidepressive, euphoriclike, and sedative effects (mostly from case reports and testimonials), GHB is ingested knowingly or is given unknowingly as a date-rape drug (Stillwell 2002). Due to its increasing abuse (commonly referred to on the streets as "Liquid Ecstacy"), GHB has recently been reclassified as a Schedule I abusive drug (Bernasconi et al 1999; Kam and Yoong 1998). When GHB is abused for a considerable period of time, a withdrawal syndrome often follows. It is associated with characteristic features of anxiety, being similar to presentation of benzodiazepine and opiate withdrawal (Dyer et al 2001; Miotto et al 2001).

The locus coeruleus (LC) is the major norepinephrine (NE) cell group in the mammalian brain and is responsible for 90% of the NE innervation of the forebrain and 70% of the total NE content in the brain. Activity of the LC is integral in the regulation of sleep-wake cycles, behavioral states linked to attention and vigilance, as well as psychiatric disorders and their treatment (Aston-Jones et al 2001; Jones 1998; Szabo and Blier 2001b). For instance, administration of the α_2-adrenoceptor antagonist yohimbine produces hyperactivity of LC NE neurons and precipitates anxiety in healthy volunteers, panic attacks in panic disorder patients, and withdrawal in opiate-dependent patients (Charney and Heninger 1986; Charney et al 1984; Stine et al 2002). In turn, agents which attenuate the activity of this nucleus

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correlate with reduced anxiety, opiate-withdrawal attenuation, and sedation leading to sleep or unconsciousness (Szabo and Blier 2001b; Aston-Jones et al 2001).

Given that LC activity is important in regulating many of the aforementioned behavioral states that GHB influences, it was deemed interesting to evaluate the impact of this drug on the firing activity of neurons within this nucleus. The spontaneous firing activity of LC NE neurons was assessed in rats receiving GHB in a sustained fashion for 2 days, 10 days, and 36 hours following washout of the drug in 10-day treated animals. The latter was an attempt to mimic the withdrawal syndrome reported in chronic GHB abusers. Furthermore, as the response to sensory inputs may be altered when administered GHB, it was important to assess the responsiveness of LC neurons to an external stimulus in the above mentioned GHB experiments.

Methods and Materials

Animals

The experiments were carried out in male Sprague Dawley rats (Charles River, Raleigh, North Carolina), weighing between 300 g and 325 g, which were kept under standard laboratory conditions (12:12 light-dark cycle with access to food and water ad libitum). Rats were anesthetized with chloral hydrate (400 mg/kg, intraperitoneal [IP]) and mounted in a stereotaxic apparatus (David Kopf Instruments Tujunga, California). Supplemental doses (100 mg/kg, IP) were given to prevent any nociceptive reaction to pinching of a hind paw or the tail.

Short-Term, Long-Term, and Withdrawal Treatments

Rats were anesthetized with halothane for subcutaneous implantation of osmotic minipumps (ALZA, Palo Alto, California). Two groups of rats were treated with GHB (Sigma Chemicals St. Louis, Missouri) (40 mg/kg/day) for 2 days or 10 days. Groups of rats were treated with the vehicle for GHB (.9% sodium chloride [NaCl]) in each treatment group to act as respective controls. The rats were tested with the minipumps in place or 36 hours following removal. The effects of other doses as well as different time points of GHB administration and removal on LC activity were not assessed.

Electrophysiological Experiments

A burr hole was drilled 1 mm posterior to lambda and 1 mm lateral to midline for LC neuron recordings. Extracellular unitary recordings of LC NE neurons were conducted with single-barreled glass micropipettes preloaded with fiberglass filaments (to facilitate filling) being pulled in a conventional manner, with the tips broken back to 1 μm to 3 μm and filled with a 2 mol/L NaCl solution. Their impedance range was between 2 MΩ and 4 MΩ, which provides stable recordings and a signal-to-noise ratio easily allowing action potential discrimination from the baseline. Locus coeruleus NE neurons were recorded with micropipettes lowered at −.7 mm interaural and 1.1 mm to 1.4 mm lateral. Spontaneously active NE neurons of the LC were identified using the following criteria: regular firing rate (1–5 Hz) and positive action potential of long duration (.8–1.2 milliseconds) exhibiting a characteristic burst discharge in response to nociceptive pinch of the contralateral hind paw. Norepinephrine neurons were recorded for at least 1 minute to establish basal firing rate. To determine possible changes in the spontaneous firing activity of NE neurons, four to five electrode descents were carried out through this nucleus in control and treated rats.

Sensory-Evoked Response to an External Stimulus

The last electrode descent through the LC in each rat was dedicated to assessing sensory-evoked response on NE neuron firing to an external stimulus. Upon a stable firing rate (usually obtained after 1 minute), the contralateral paw was compressed with a surgical forceps that had a rounded end to avoid tissue damage on compression. This compression lasted approximately 1 second with equal pressure being applied to the paw of rats; once the opposite sides of the forceps made contact with each other, the forceps were then released. This is similar to the paradigm detailed by Grant and Weiss (2001). Of interest, it has also been reported that the number of elicited bursts is largely independent of paw-compression intensity (Simson and Weiss 1987). For each neuron tested, the value of burst firing activity assigned to a NE neuron was composed of an average of three paw-pinch trials, each of which was separated by at least 15 seconds.

Statistical Comparison

All results were expressed as mean (± SEM) of single neuron values. Statistical comparisons of values obtained in treated and control rats were carried out using Kruskal-Wallis one-way analysis of variance followed by post hoc Dunn method, where multiple comparisons versus control group were performed.

Results

Effects of GHB on the Spontaneous Firing Activity of LC Neurons

Rats receiving the saline vehicle for 2 days and 10 days, to act as respective controls for the GHB treated animals, did not result in a difference in LC spontaneous firing activity when compared with each other. These data were therefore merged and correspond to a single control group. Sustained 2-day and 10-day GHB treatments attenuated the spontaneous firing activity of LC neurons, whereas rats undergoing a 36-hour washout resulted in augmented NE neuron firing frequency (Figure 1). The 2-day and 10-day GHB treatments produced a significant 52% (range of firing: .2–2.2 Hz) and 54% (range of firing: .3–2.0 Hz) decrease in mean spontaneous firing rate, respectively. Rats treated with GHB for 10 days followed by a 36-hour washout resulted in a significant 33% increase in the mean spontaneous firing activity of LC neurons (range of firing: .6–6.6 Hz) when compared with that of control rats (range of firing: .5–4.4 Hz) (Figure 2). Systematic electrode descents into the LC yielded a trend for a decrease and increase in the number of LC neurons discharging spontaneously in rats treated with GHB or following removal of the drug, respectively, but did not reach statistical significance (Table 1).

Effect of GHB on the Sensory-Evoked Burst Firing of NE Neurons

Sustained 2-day and 10-day GHB treatments attenuated the sensory-evoked burst firing of LC neurons, whereas rats undergoing a 36-hour washout resulted in an increase in evoked firing rate (Figures 3 and 4). The 2-day and 10-day GHB treatments produced a significant 47% (range of firing: 1–5 Hz) and 58% (range of firing: 1–5 Hz) decrease, respectively, in the number of evoked bursts. Rats treated with GHB for 10 days following a 36-hour washout resulted in a significant 79% increase in the number of elicited bursts of LC activity (range of firing: 5–15 Hz) when compared with that of control rats (range of firing: 3–10 Hz) (Figure 4).

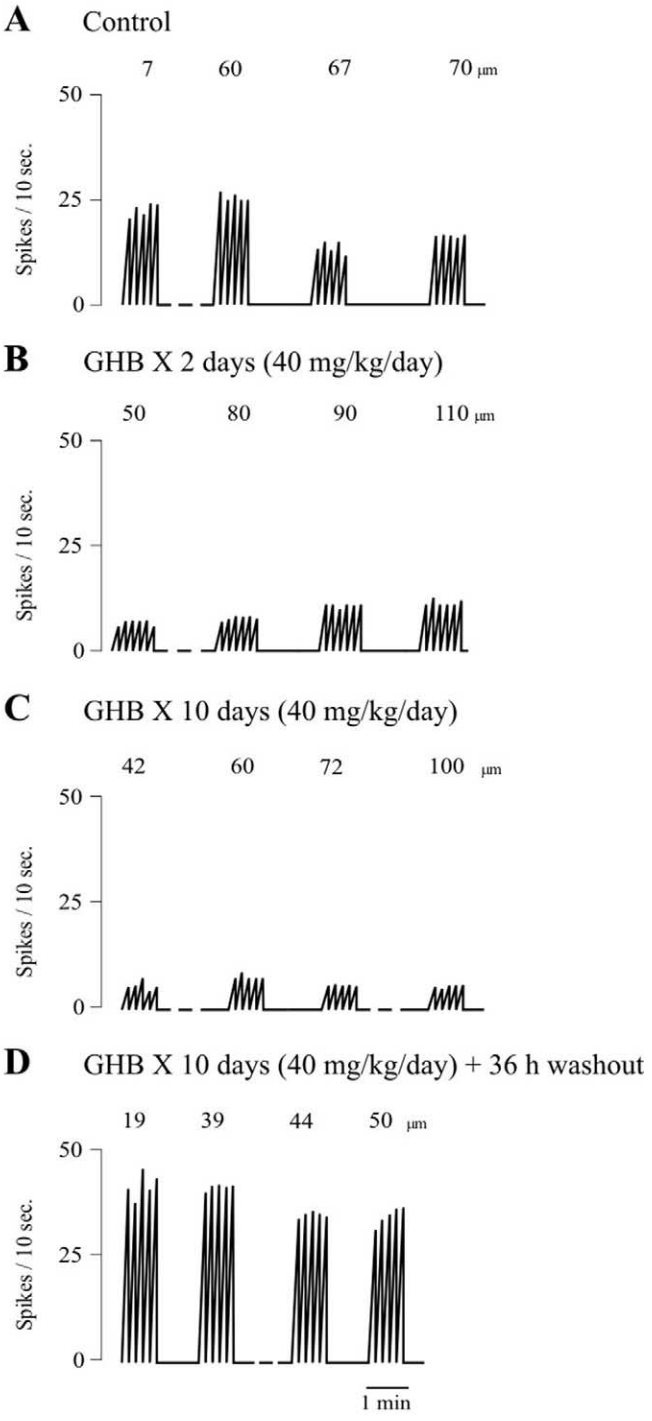

Figure 1. Integrated firing rate histograms of LC NE neurons, recorded in single electrode descent in the LC showing their spontaneous firing activity in control ($n = 4$) **(A)**, 2-day GHB treatment (40 mg/kg/day; $n = 4$) **(B)**, 10-day GHB treatment (40 mg/kg/day; $n = 4$) **(C)**, and 10-day GHB treated animals (40 mg/kg/day) where the minipump was removed for 36 hours ($n = 4$) **(D)**. Each spike corresponds to the number of discharges recorded per a 10-second time period. The dotted lines in between neurons indicate approximately 5-minute time laps. The number above each neuron indicates the depth from the floor of the fourth ventricle at which it was recorded. LC, locus coeruleus; NE, norepinephrine; GHB, gamma-hydroxybutyrate.

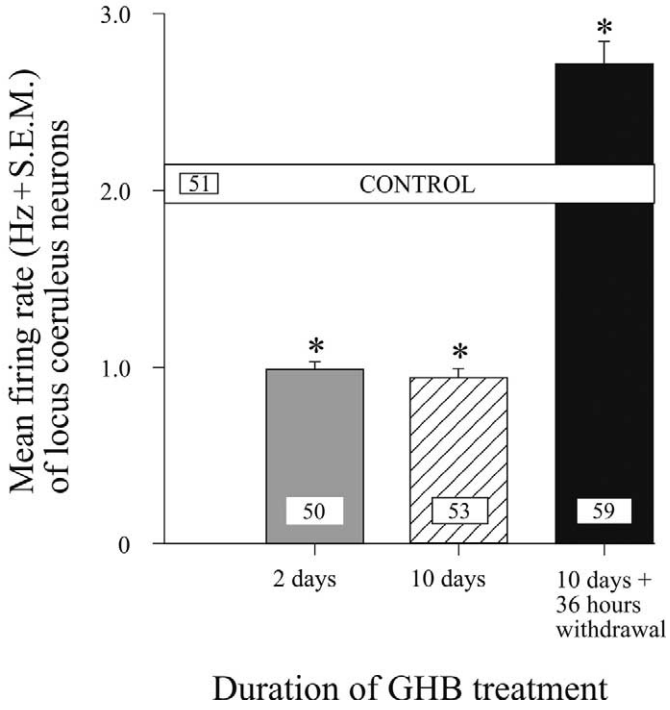

Figure 2. Effects of 2, 10, and 36 hours following removal of the minipump in 10-day GHB treatments (40 mg/kg/day) on the spontaneous firing activity of LC neurons. The control area represents the range (SEM × 2) of the mean firing activity of neurons recorded in control rats. *$p < .05$ (Dunn's method) when compared with the control value. The number of neurons recorded is displayed in each box. LC, locus coeruleus; GHB, gamma-hydroxybutyrate.

Discussion

A sustained 2-day and 10-day administration of GHB decreased the spontaneous firing activity of LC neurons, whereas GHB discontinuation assessed in the latter treatment group resulted in enhanced activity of this nucleus. The inhibitory action of GHB on LC neuron firing rate is consistent with reports of GHB being able to attenuate brain NE levels (Miguez et al 1988) and the ability of this agent to alter the metabolism of NE (Anden 1974; Gomes et al 1976). The exact mechanism by which GHB attenuates NE activity remains to be elucidated; however, the ability of GHB to activate $GABA_A$ and $GABA_B$ receptors may explain, at least in part, this effect because these receptors mediate an inhibitory influence on NE neuron firing (Shefner and Osmanovic 1991; Ennis and Aston-Jones 1989). Thus, GHB may attenuate the activity of this nucleus via direct activation of GABA

Table 1. Locus Coeruleus Norepinephrine Neurons in Controls and Treated Rats

	Average Number of Noradrenergic Neurons per Descent	Number of Descents	Number of Rats
Control	3.7 ± .4	14	4
GHB (40 mg/kg/day)			
2 days	3.1 ± .3	16	4
10 days	3.5 ± .5	15	4
10 days + 36 hours washout	4.5 ± .6	13	4

$p = .208$, among treatment groups using one-way analysis of variance. GHB, gamma-hydroxybutyrate.

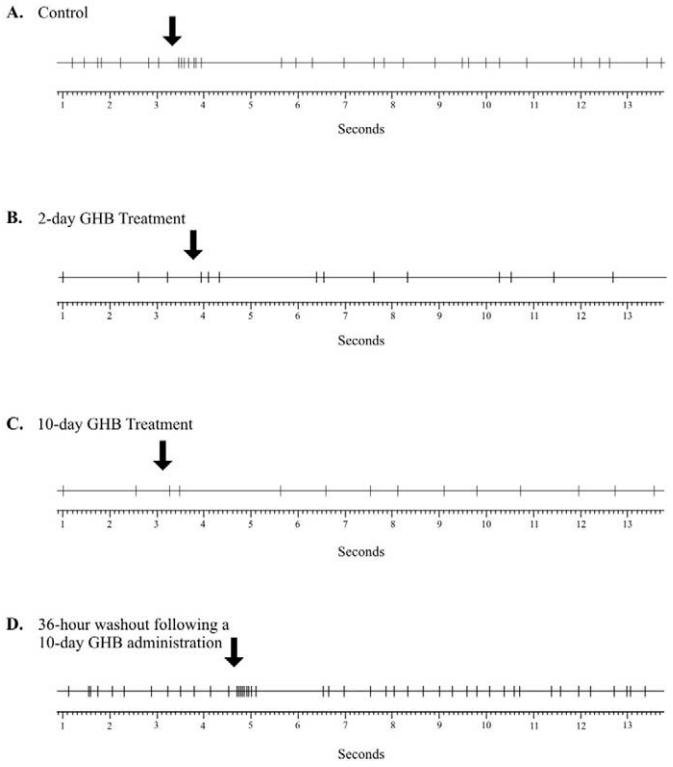

Figure 3. Recordings of LC NE neurons showing their spontaneous and evoked firing activities in control ($n = 4$) **(A)**, 2-day GHB treatment (40 mg/kg/day; $n = 4$) **(B)**, 10-day GHB treatment (40 mg/kg/day; $n = 4$) **(C)**, and 10-day GHB treated animals (40 mg/kg/day) where the minipump was removed for 36 hours ($n = 4$) **(D)**. Each vertical line represents an action potential from a single NE neuron that is discharging. The arrow signifies application of a brief paw-pinch. Note that paw-pinch produces an increase in NE neuron firing (sensory-evoked burst firing), followed by a period of poststimulus inhibition, and regain of the pacemakerlike firing activity characteristic of LC neurons. LC, locus coeruleus; NE, norepinephrine; GHB, gamma-hydroxybutyrate.

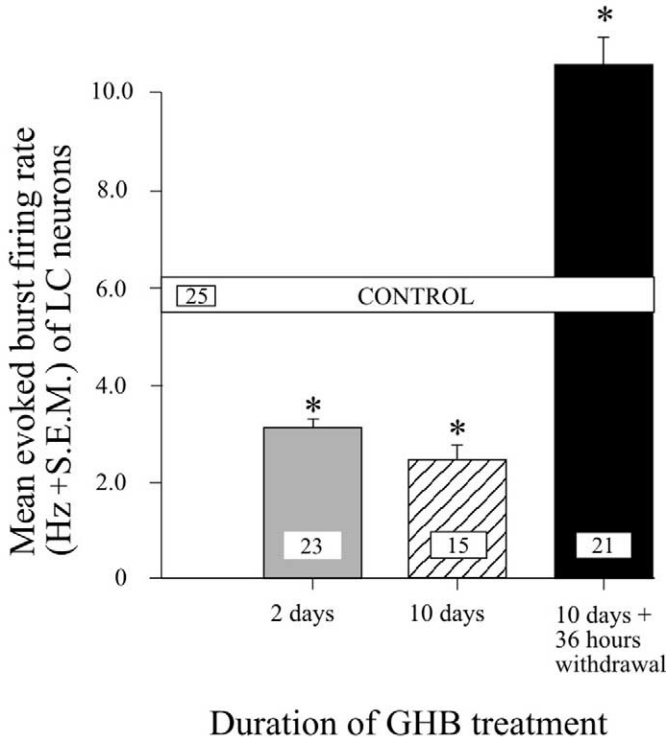

Figure 4. Effects of 2, 10, and 36 hours following removal of the minipump in 10-day GHB treatments (40 mg/kg/day) on the sensory-evoked firing activity of LC neurons. The control area represents the range (SEM × 2) of the mean activity of neurons recorded in control rats. *$p < .05$ (Dunn's method) when compared with the control value. The number of neurons recorded is displayed in each box. LC, locus coeruleus; GHB, gamma-hydroxybutyrate.

receptors on NE neurons or indirectly via alteration of neurons in complex circuitries through which LC activity is regulated (Jodo and Aston-Jones 1997; Szabo and Blier 2001a; Ennis and Aston-Jones 1988; Szabo and Blier 2001c). Studies using GABA receptor antagonists applied iontophoretically in the LC or injected systemically in GHB treated animals while recording the firing activity of NE neurons could help elucidate the mechanism by which GHB alters LC firing.

Endogenous GHB levels and binding sites have previously been reported to be highest in the pontomedullary region of the brain (Doherty et al 1978; Snead and Liu 1984), a region where the LC is located and where the predominant afferent regulation of this nucleus occurs (for a review, see Aston-Jones et al 1991). The distribution of GHB high-affinity binding sites in the rat brain does not match the distribution of $GABA_A$ or $GABA_B$ binding and appears to be specific for GHB (Maitre et al 2000). Recently, Gould et al (2003) published that newer ligands for the GHB receptor reveal highest binding in cortex and hippocampus and low levels in the LC, similar to that of previous reports utilizing radioactive GHB (Snead and Liu 1984; Hechler et al 1987, 1992); however, when the regional distribution of sites was tested by preparing synaptic membranes from various parts of the human and rodent brain, significant binding in the pons and hypothalamus was observed (Snead and Liu 1984). One possibility for this discrepancy could be that due to methodological differences, the pons and hypothalamus may have GHB receptors with lower affinity binding sites. Given that receptors specific for GHB are able to attenuate G-protein mediated cyclic adenosine monophosphate (cAMP) production (Snead 2000), if these receptors are present on LC neurons (or on neurons important to the afferent control of this nucleus), this may represent a mechanism whereby GHB is able to decrease NE neuron firing activity. In turn, augmented cAMP activity may also correspond to the signaling cascade for the GHB-induced augmentation on LC firing observed in rats chronically administered GHB undergoing a 36-hour washout period (Figures 1 and 2). Indeed, increased cAMP activity in the LC of rodents is observed in opiate withdrawal paradigms (Ivanov and Aston-Jones 2001; Kogan et al 1992; Rasmussen et al 1990), and reports of GHB being effective in treating opiate addicts in withdrawal may be germane to the ability of these agents to converge on similar intracellular signaling pathways (Gallimberti et al 1993; Nestler et al 1989a, 1989b). Experiments utilizing a biochemical assay for protein kinase A in the LC in animals undergoing GHB treatments and in withdrawal would shed light on this prospect.

A sustained administration of GHB decreased the number of burst firing of NE neurons to a contralateral paw-pinch (Figure 3). The inhibitory effect of GHB on evoked NE neuron burst firing in 2-day GHB treated animals was sustained during a 10-day GHB administration (Figure 4). The mechanism by which NE activity results in a burst-type firing pattern to paw-pinch can be influenced by many factors, including that of the activation of

a variety of receptors (Simson and Weiss 1987). More specifically, Ennis and Aston-Jones (1986) demonstrated that burst firing is due to glutamate, while α_2-adrenoceptors and/or calcium $(Ca)^{2+}$ channels are responsible for the inhibition of NE neurons following stimulus activation (Aghajanian et al 1977; Cedarbaum and Aghajanian 1976). Furthermore, α_2-adrenoceptors appear to be paramount to the production of burst firing of LC neurons to a paw-pinch independent of altered spontaneous firing rate (Simson and Weiss 1987). It is, however, not known whether an altered activation of α_2-adrenoceptors represents a mechanism in which GHB is able to produce its effects on paw-pinch induced LC burst firing or whether a constant increase of GABAergic tone in this nucleus or an action on glutamate, rather than an effect on adrenergic transmission, may underlie this phenomenon. Nonetheless, the ability of GHB to attenuate the perturbation of LC activity to an external stimulus may be important in the regulation of anxietylike symptoms that occur when chronic opiate or GHB abusers are in withdrawal (Gallimberti et al 1993; Goddard and Charney 1997). On the other hand, given that the α_2-adrenoceptor agonist clonidine is capable of curtailing these effects in the former (Gold 1993; Gold et al 1978), this agent may have some clinical utility in the management of GHB withdrawal.

In conclusion, the attenuation on NE neuron firing rate may be important to the anxiolytic and sedative effects associated with GHB (Miotto et al 2001), as LC activity is linked to these behavioral states (Jones 2003; Szabo and Blier 2001b). In turn, augmented LC activity may be likened to the anxiety symptoms chronic GHB abusers often exhibit when in withdrawal (Miotto et al 2001). This is consistent with agents or stimuli which increase LC activity being capable of triggering anxiety (Szabo and Blier 2001b), whereas anxiolytics which decrease LC activity, such as the benzodiazepines (Grant et al 1980) and barbiturates (Laurent et al 1983), are effective in reducing withdrawal symptoms associated with chronic GHB abuse (Dyer et al 2001; Miotto et al 2001). Furthermore, given that the clinical presentation of GHB withdrawal shares aspects similar with that of withdrawal from opiates, alcohol, and benzodiazepines, these drugs of abuse and potential treatments thereof may pharmacologically converge on common signaling mechanisms (Miotto et al 2001; Nestler et al 1989a, 1989b). For instance, GHB has been reported to substitute for the reinforcing effects of opiates in animal paradigms of drug addiction (Martellotta et al 1997), as well as being able to curtail the effects of opiate withdrawal in humans (Gallimberti et al 1993). Lastly, the ability of GHB to regulate the activity of NE neurons from internal (decrease in spontaneous firing activity) and external stimuli (paw-pinch evocation of burst firing) may also represent an important mechanism in the ability of this drug to restructure LC activity and regulate sleep-wake patterns in patients with narcolepsy. Further investigation of the mechanism(s) by which GHB alters LC activity and relevance into the production and treatment of the above mentioned disorders is warranted.

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