The spinal GABAergic system is a strong modulator of burst frequency in the lamprey locomotor network

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Abstract

The spinal network coordinating locomotion is comprised of a core of glutamate and glycine interneurons. This network is modulated by several transmitter systems including spinal GABA interneurons. The purpose of this study is to explore the contribution of GABAergic neurons to the regulation of locomotor burst frequency in the lamprey model. Using gabazine, a competitive GABA$_A$ antagonist more specific than bicuculline, the goal was to provide a detailed analysis of the influence of an endogenous activation of GABA$_A$ receptors on fictive locomotion, as well as their possible interaction with GABA$_B$ and involvement of GABA$_C$ receptors.

During NMDA-induced fictive locomotion (ventral root recordings in the isolated spinal cord), gabazine (0.1 to 100 µM) significantly increased the burst rate up to two-fold, without changes in regularity or “burst quality.” Gabazine had a proportionately greater effect at higher initial burst rates. Picrotoxin (1 to 7.5 µM), a less selective GABA$_A$ antagonist, also produced a pronounced increase in frequency, but at higher concentrations the rhythm deteriorated, likely due to the unspecific effects on glycine receptors. The selective GABA$_B$ antagonist, CGP55845 also increased the frequency, and this effect was markedly enhanced when combined with the GABA$_A$ antagonist gabazine. The GABA$_C$ antagonist TPMPA had no effect on locomotor bursting.

The spinal GABA system does, thus, play a prominent role in burst frequency regulation, in that it reduces the burst frequency by up to 50%, presumably due to presynaptic and somadendritic effects documented previously. It is not required for burst generation, but acts as a powerful modulator.

Keywords: GABA receptors, TPMPA, picrotoxin, gabazine, CGP 55845
Introduction

The control of movement depends crucially on central pattern generators (CPGs) and associated networks that coordinate many basic aspects of the motor repertoire (Grillner 1981, 2003; Kiehn and Butt 2003; Marder and Bucher 2001; Nusbaum and Beenhakker 2002). The lamprey brainstem–spinal cord provides a well-studied model system for vertebrate locomotion (Grillner 2003). In the lamprey locomotor CPG there are, in addition to the excitatory glutamatergic burst generating circuitry (Cangiano and Grillner 2003), inhibitory glycinergic mechanisms for production of right-left alternation (Buchanan and Grillner 1987; Cohen and Harris-Warrick 1984; Grillner and Wallén 1980). The core of the bilateral CPG is thus a glutamate–glycine network, but in addition there are several modulatory systems using GABA, dopamine, 5-HT and peptides for fine tuning of the locomotor network (see Grillner 2003).

This study is concerned with the contribution of the GABAergic system to the activity in the lamprey locomotor CPG. GABAergic neurons acting through GABA_B receptors are known to reduce the locomotor burst frequency, whereas the role of GABA_A receptors remains unclear (Tegnér et al. 1993, see below). Three types of GABAergic spinal neurons have been identified (Brodin et al. 1990): (1) multipolar cells in the lateral gray matter, (2) small bipolar cells located at the base of the dorsal horn and just under the dorsal columns, and (3) cells with ciliated processes extending into the central canal (Schotland et al. 1996) and with axons extending to the lateral edge of the spinal cord forming a plexus around the dendrites of stretch-sensitive edge cells (Grillner et al. 1984). GABA-immunoreactive fibers are generally ubiquitous within the gray and white matter (Christenson et al. 1991). A GABAergic locomotor-driven modulation of
presynaptic terminals of primary afferent fibers and interneuronal axons in the spinal network provides presynaptic inhibition (Alford et al. 1991; Alford and Grillner 1991; El Manira et al. 1996), phase-locked to the locomotor pattern. It is mediated through both GABA_A and GABA_B receptors. GABA_B receptors, additionally, act at the soma-dendritic level of interneurons and motoneurons and modify the intrinsic membrane properties (El Manira and Bussieres 1997; Matsushima et al. 1993; Wikstrom and El Manira 1998).

In a previous study, Tegnér et al. (1993) reported that agonists of GABA_A (muscimol and diazepam) and GABA_B (baclofen) receptors reduced the frequency of bursting, whereas, conversely, antagonists of GABA_A (bicuculline methiodide) and GABA_B receptors (phaclofen and saclofen) increased the frequency of bursting. GABA uptake blockers (nipecotic acid) and a benzodiazepine receptor agonist induced a pronounced slowing of the locomotor burst rate. These results, taken together, strongly indicate an endogenous role for the GABAergic synaptic transmission during locomotion.

Tegnér et al. (1993) showed that bicuculline methiodide caused an irregular motor pattern in addition to its effects on locomotor frequency. Subsequently, it was demonstrated that bicuculline methiodide, in addition to its effects on GABA_A receptors, also reduces the slow afterhyperpolarisation (Debarbieux et al. 1998; Johnson and Seutin 1997; Pfieger et al. 2002) due to a direct effect on calcium-dependent K^+ channels (K_{Ca}). Since a blockade of K_{Ca} channels profoundly affects the burst generation itself (El Manira et al. 1994; Hill et al. 1992), the conclusions reached, when using bicuculline methiodide as a GABA_A receptor antagonist, were thus invalidated. The main purpose of this study is therefore to elucidate the contribution of GABA_A receptors during fictive locomotion, using selective GABA_A antagonists (gabazine or picrotoxin). Concentrations of
picrotoxin or gabazine up to 100 µM do not affect the afterhyperpolarization (Seutin et al. 1997). Gabazine is a competitive (Chambon et al. 1985) and selective antagonist of the GABA_A receptor and has no known affects upon the glycine channel (Heaulme et al. 1986). A low affinity site exists to which gabazine binds noncompetitively (Heaulme et al. 1986). Picrotoxin is a non-competitive antagonist of GABA_A receptors interacting with the chloride channel of the GABA_A receptor. On reticulospinal neurons in lamprey, picrotoxin selectively blocked synaptic responses at concentrations below 20 µM (Matthews and Wickelgren 1979) and it had little effect on glycine responses in giant interneurons and Müller cells (Homma 1983; Homma and Rovainen 1978; Martin 1978). Above 20 µM it lowered glycine responses and ipsps in Müller cells. Picrotoxin has been reported to act non-selectively at higher doses in many systems, and affects glycine receptors (Davidoff and Aprison 1969; Yoon et al. 1998).

We also explored the possible role of GABA_C receptors, which have not previously been studied in the context of locomotion. GABA_C receptors have a widespread distribution including spinal cord, retina and the optic tectum (see Johnston et al. 2003). Like glycine receptors and other members of the ligand-gated chloride channel superfamily, some GABA_C receptors exhibit picrotoxin sensitivity (Dibas et al. 2002; Goutman and Calvo 2004). The highly specific antagonist of GABA_C receptors, TPMPA (Chebib and Johnston 1999), however, had no effect on the burst frequency.

We show here that the spinal GABAergic system is active during fictive locomotion and provides a marked depression of the output frequency of the locomotor CPG. This effect is mediated partially via GABA_A receptors, since the burst frequency
increased significantly up to nearly two-fold when selective GABA_A antagonists were administered, and partly via GABA_B receptors that further enhance these effects.

**Materials and Methods**

*Animals and preparation*

Adult lampreys (*Lampetra fluviatilis*) were handled according to Karolinska Institutet’s guidelines, and experiments were performed with permission from “Stockholms Norra försöksdjursetiska nämnd” (local ethical committee). The lampreys were held in aquaria at 5°C. Isolated notochord-spinal cord preparations were dissected from lampreys under anesthesia produced by 100-150 mg/l MS-222 (tricaine methane sulphonate). These preparations included pieces of spinal cord (10 to 25 spinal segments in length, up to 6 cm) taken from the region caudal to the gills and rostral to the dorsal fin. Pieces of spinal cord were mounted in Sylgard-lined chambers. The bath temperature was maintained between 1.3 and 7.7°C. For any given experiment, variations in temperature were controlled to within 2°C. Preparations were superfused with chilled, oxygenated HEPES-buffered saline consisting of (in mM): NaCl 138, KCl 2.1, CaCl_2 1.8, MgCl_2 1.2, glucose 4, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) 2 and L-glutamine 0.5 and pH adjusted to 7.4 with 1M NaOH to which N-methyl-D-aspartate (NMDA; 75, 100 or 150 µM) was added to induce locomotion. Superfusion rates used were 2.0 ml per minute for picrotoxin and gabazine, 1.0-1.2 ml/min for TPMPA, and 2.5 ml/min for the experiments in which two concentrations of NMDA were used and the effect of gabazine tested. In some experiments (two-level NMDA-excitation experiments), recordings were made from two ten-segment pieces
simultaneously. GABA<sub>A</sub> receptor antagonists were added to the superperfusate separately in the different experiments. Final solutions were adjusted to a pH of 7.4.

**Drugs**

The antagonists include picrotoxin, 0.5-100 µM (Sigma Aldrich, St. Louis); gabazine/SR95531 (6-Imino-3-(4-methoxyphenyl)-1(6H)-pyridazinebutanoic acid hydrobromide), 10<sup>-3</sup> to 10<sup>3</sup> µM (Tocris, Bristol, UK); and TPMPA, 1,2,5,6-Tetrahydropyridin-4-yl)methylphosphinic acid, 0.6-320 µM, (Tocris); the GABA<sub>B</sub> antagonist CGP55845, 20 µM, (Tocris); NMDA, N-methyl-D-aspartate (50 – 150 µM) (Tocris).

**Electrophysiology**

Recordings were made from ventral roots at least 4 segments from the cut edge of the spinal cord. Bursts of action potentials recorded from the ventral roots (fictive locomotion) were recorded using glass extracellular suction electrodes. The amplified signals (Differential AC Amplifier, model 1700, A-M Systems, Inc., Carlsborg, WA, USA) were band-pass filtered from 300 Hz to 500 Hz, or 100 Hz to 1kHz, depending on the experiment, and digitized at 2 to 5 kHz (Digidata 1200A and Axoscope 1.0 software, Axon Instruments, Union City, CA, USA).

**Experimental Protocols and Sampling**

In all experiments, three-minute records (five minutes in some early experiments) of burst frequencies and temperature of the bath were recorded every five minutes. Burst frequencies were measured by hand from 30-second samples of the above records, as a
rule the very first 30 seconds of a five minute sampling period. The reliability of this burst frequency measurement was double checked by Fast Fourier Transform analysis and from autocorrelograms in gabazine and picrotoxin concentration-response experiments involving burst quality, alternation quality and phase lag analyses. Burst frequencies were stable after two to five hours of exposure to 100 µM NMDA. The five 5-minute periods (viz., 25 minutes) immediately before the first drug administration were selected for the assessment, as described above, of stability of the pre-treatment, control burst frequency. Of twenty-two experiments, the standard deviations, SD, of the stabilized burst frequencies before the first treatment were all below 0.05 Hz (n = 22) except for one gabazine experiment where the SD was 0.076 Hz. For the concentration-response experiments with picrotoxin the 25-minute treatment periods were interposed with 25-minute washes with 100 µM NMDA in HEPES (control) superfusate. In the concentration-response experiments with gabazine, 25-minute treatment intervals were interposed with 50-minute washes as above. In the case of the latter two experiments, the highest burst frequency from a 30-second record of a 5-minute sample period at, or near, the change from drug to washout was generally chosen as representative. In approximately half the experiments the highest burst frequency occurred during the last part of the drug application, whereas in the remainder it had a lag into the wash period. In 80 different cases of presentations of different drugs at different concentrations, the rhythm broke down six times (3 picrotoxin experiments and 3 experiments involving CGP44845). In these cases, the highest burst frequency during the treatment period was used even if it was not within the last five minutes. Since the TPMPA burst frequencies showed little variation the average frequencies for the entire application periods were
used. In the experiments involving antagonism of GABA\textsubscript{B} receptors with CGP55845, the treatment periods with either 20 µM CGP55845 or 20 µM CGP55845 plus 10 µM gabazine were 120 minutes, 45 minutes, 90 minutes and 60 minutes long for experiments 1 through 4, respectively, (see Fig. 8) and mean burst frequencies were calculated from the 30-second records from five 5-minute sample periods at the end of the treatment period.

**Analysis**

Analysis of fictive locomotion employed Datapac (Run Technologies, Mission Viejo, CA, USA), for burst proportions, cycle durations, coefficient of variation (CV) of cycle duration, and Fast Fourier Transform analysis of burst frequency. Origin software was used (Northhampton, MA, USA) for time series analysis (cross- and autocorrelative), determinations of burst and alternation quality, as well as rostro-caudal phase lags, phase of alternation and burst frequency. Statistical analyses were performed with GraphPad Prism (San Diego, CA).

Burst quality was assessed both by CV of cycle duration and by autocorrelative processing of three to five minute long ventral root recordings. When correlated against itself, a single ventral root recording yields a maximum value of the autocorrelative correlation coefficient (arbitrary units) when initially there is no lag between the record and its identical copy (see inset, Fig. 4A). Sequential calculation of the correlation coefficient after repeated lag-shifts of the record with its copy yields a function of the value of the correlation coefficient versus the lag, or the autocorrelogram. The height of the first peak, $\alpha$, of the correlogram reflects the temporal periodicity, or rhythmicity, of
the bursts as well as the combined power of the signals (compound action potentials) making up the burst. Spikes occurring between bursts degrade the distinctness of the burst pattern and result in a diminished peak-to-trough difference \((\alpha - \beta)\) in the correlogram (see Fig. 4A inset). Dividing by \((\alpha + \beta)\) to adjust for relative differences in signal power, we employ a measure of burst quality (Cangiano and Grillner 2003) defined as

\[
BQ = \frac{\alpha - \beta}{\alpha + \beta}.
\]

Individual measurements of burst quality were normalized to control values for summarization (Fig. 4A). The cycle duration and, thus, the frequency of bursting can be determined from the time lag to the first peak of the autocorrelogram.

Cross-correlative analyses were made of contralateral ventral root recordings from the same spinal level (± 1 segment) producing cross-correlograms of the form depicted in the inset of Fig. 5A. In these cases, the correlation function tends to approach a local minimum at zero lag when there is the least overlap between bursts. Ideal alternation would be represented as maxima of the cross-correlogram symmetrically distributed on either side of the lag axis at -0.5 and +0.5. Similar to burst quality, an alternation quality, \(AQ\), can be operationally defined and quantified using the mean, \(\bar{\alpha}\), of the two peak values, \(\alpha_1\) and \(\alpha_2\), of the correlogram to either side of the \(x = 0\) axis. Thus,

\[
AQ = \frac{(\bar{\alpha} - \beta)}{(\bar{\alpha} + \beta)}.
\]
Intersegmental, or rostrocaudal, phase lag is the per-segment delay in the time of
bursting from a rostral segment to an ipsilaterally more caudal segment. Intersegmental
phase lag (IPL) is determined by cross-correlating the three minute recordings from
rostral and caudal ventral root recordings (see Fig. 5 B₁ inset). Generally, intersegmental
lag presents itself as a correlogram peak slightly offset from the lag = 0 axis by an
amount \( f \) representing the summed delay of bursts in successive segments. Dividing \( f \) by
the sum of both the average cycle period determined from the correlogram, \( \tau \), and the
number of segments between the recording electrodes, \( s \), yields the intersegmental phase
lag (expressed as a percentage). The convention of assigning positive or negative values
to phase lag were made consistent with those used previously whereby bursts occurring
later in more caudal segments, corresponding to forward swimming, are described with
positive phase lags (Wallén and Williams, 1984). Typical delays (converted from
correlative lag values) are in the order of 0.5-1% of the cycle period per segment. Our
preparations consisted of about 16 segments between ventral root electrodes. The phase
lag is then expressed as the per-segment delay (ms) divided by the cycle period (ms)
times 100%.

Statistics

Standard error of the mean (SEM) is reported as a ± value after the mean value.
Repeated measures ANOVAs were performed for analyses of burst characteristics for all
three drugs along with Dunnett’s \textit{a posteriori} test for multiple comparisons with the
control value. Tukey’s and Newman-Keuls \textit{a posteriori} tests were employed in
comparisons involving GABA\textsubscript{B} blockade with CGP55845. One- and two-tailed, paired t-
tests were employed as indicated in the text in comparisons involving treatment combinations with gabazine and/or CGP 55845. Two-tailed, paired t-tests were used for the comparison of all mean values of burst frequency in experiments involving two levels of NMDA with and without gabazine treatment. One- and two-tailed paired t-tests were employed as indicated in the text in comparisons involving treatment conditions with gabazine and/or CGP 55845. Statistical tests were performed on cycle duration (inverse) transformations and then reported as frequency values. SEM is reported as ± values after percentages. All bars represent SEM in relevant graphs.

Results

\textit{GABA} A antagonists and the frequency of fictive locomotion

To test the potential effect of GABA A antagonists on the pattern of fictive locomotion, we utilized the isolated spinal cord-notochord preparation superfused with NMDA (see Materials and Methods) and recorded changes in the ventral root burst activity (Fig. 1A-C). We subsequently applied GABA A antagonists at different concentrations in the NMDA solution. Both gabazine, a competitive antagonist and picrotoxin, a noncompetitive, chloride channel blocker, were administered.

\textit{a. Gabazine increases burst frequency}

Fig. 1A shows the alternating left-right locomotor pattern recorded from bilateral ventral roots at the same segmental level under control conditions (100 µM NMDA alone) and, in Fig. 1B, the increase in frequency occurring with the addition of 1 µM gabazine. The results from one complete experiment are shown in Fig. 1D. A progressive
Fig. 1. Effects of gabazine on burst frequency. A-C: Extracellular ventral root recordings from the right and left sides at the same or adjacent segmental level in control (A), in the presence of gabazine (B) and during washout (C). VR-R and VR-L = right and left side ventral root recordings, respectively. An increase in frequency from 1.88 Hz to 2.17 Hz can be observed with 1 µM gabazine followed by a return to near-control frequency during washout. D: The concentration-response record of a single experiment with increasing concentrations from control, 1.89 ± 0.003 Hz (n = 19), to a peak frequency value of 2.90 Hz with 100 µM gabazine. Recovery began 50 minutes after washout and the burst frequency returned to a stable, near-control burst frequency of 1.93 ± 0.002 Hz (n = 11) after 85 minutes post-washout. E: Summary of four experiments showing, for concentrations from 0.01 to 100 µM, significant changes in normalized burst frequency compared to control values (repeated measures ANOVA, performed on inverse transformation to cycle durations; six groups, F = 42.41, R² = 0.9339, P < 0.001). Dunnett's a posteriori test for multiple comparisons revealed a significant increase already at 0.01 µM gabazine (P < 0.05) and for all tested concentrations shown up to 100 µM (P < 0.001) where the burst frequency had increased, on average, to 90 ± 19% over the control values. Error bars = SEM.
rise in frequency from 1.9 to 2.9 Hz occurred when the gabazine concentration was increased from 0.01 to 100 µM. After a long period of washout (approximately 2 hours), the burst frequency recovered to the control level. From each experiment (as detailed in Materials and Methods), the burst frequency from near the end of each 25-minute treatment period was used to produce the summary histogram in Fig. 1E. This figure summarizes four experiments and shows that a significant increase (Dunnett’s a posteriori test, \( P < 0.05 \)) occurred already with a low level of gabazine (0.01 µM). At concentrations from 0.1 to 100 µM, the increase was highly significant (Dunnett’s, \( P < 0.001 \)) and, at the highest concentration, the mean increase in burst frequency reached 90 ± 19% (\( n = 4 \)). The prominent effects observed with gabazine suggest an important \( \text{GABA}_A \) inhibitory influence in the locomotor network.

\textbf{b. Picrotoxin increases burst frequency}

Also picrotoxin, a noncompetitive blocker of the chloride channel associated with the \( \text{GABA}_A \) receptor, elicited a marked concentration-dependent increase in burst frequency similar to that of gabazine (Fig. 2A-D). As for the gabazine experiments described above, the burst frequencies from a five minute period near the drug change to washout was used to produce the summary histogram in Fig. 2E. If at the highest concentrations the rhythm deteriorated, the last, and thus in all cases the highest, frequency before deterioration was measured. At 1 µM the increase in frequency was highly significant (Dunnett’s, \( P < 0.001, n = 4 \); Fig. 2E). The average increase in burst frequency with 7.5 µM was 100 ± 9% (\( n = 4 \)). Higher concentrations (7.5 to 10 µM) resulted in break down of the burst pattern in 3 of 4 preparations, with long periods of synchronous activity in the ventral
Fig. 2. Effects of picrotoxin on burst frequency. A-C: Recordings showing an increase in frequency from 1.87 Hz (A) to 2.02 Hz (B) with application of 1 µM picrotoxin followed by a washout to the control frequency of 1.85 Hz (C). D: Concentration-response record from a single experiment showing an increase in frequency from the control value, 1.86 ± 0.005 Hz (n = 6) to a maximum of 2.80 Hz at 7.5 µM picrotoxin just before the pattern deteriorated. Initial recovery began at 60 minutes after washout, regaining a measurable rhythm of 1.60 Hz, and stabilizing at a pre-treatment rhythm of 1.84 ± 0.004 Hz (n = 15) by 3 hours after washout. E: The mean normalized burst frequencies are shown for four experiments (ANOVA, six groups, F = 57.44, R² = 0.9504, P < 0.0001). Concentrations of 1.0 µM and above produced mean burst frequencies significantly different from the control value (***, P < 0.001, Dunnett’s multiple comparison test). At 7.5 µM, the mean, normalized frequency was 100 ± 9% higher than control. Error bars = SEM.
roots, similar to that observed with glycine receptor antagonists (Grillner and Wallén 1980; McPherson et al. 1994), and Fig. 6B below). These effects of picrotoxin are presumably nonspecific and can most likely be explained by an action on glycinergic chloride channels (see Introduction).

c. Gabazine has a greater effect on burst frequency at higher NMDA levels/burst frequencies

Spinal cord preparations were recorded as described in Materials and Methods. The protocol was as follows: 75 µM NMDA alone, 75 µM NMDA plus 10 µM gabazine, long wash in 75 µM NMDA (between two and three hours) during which frequencies in all cases returned to within 8% of the control, 150 µM NMDA alone, 150 µM NMDA plus 10 µM gabazine, and finally, a long washout in 75 µM NMDA alone, or 150 µM NMDA followed by 75 µM NMDA. Treatment periods were at least 30 minutes long and burst frequencies calculated, as previously described, from the last period or the highest frequency achieved before drug change. To investigate if the action of the GABA<sub>₆</sub> receptor is dependent on the control burst frequency, locomotor activity was induced by 75 µM and 150 µM NMDA. The control burst frequencies were, on average, 1.3 ± 0.09 Hz and 1.9 ± 0.12 Hz, respectively (n = 6) (Fig. 3). When adding 10 µM gabazine, the average burst frequency increased significantly (26%; P < 0.01) from 1.3 Hz to 1.7 ± 0.07 Hz (n = 6) with the lower concentration of NMDA, and at the higher level a greater increase from 1.9 Hz to 2.9 ± 0.17 Hz (50%, P < 0.005, n = 6) occurred. The GABA<sub>₆</sub> receptor antagonist thus enhances burst frequency at both levels of locomotor activity, but the effect is greater at higher burst frequencies (see Discussion).
Fig. 3. Effects of gabazine (GBZN) (10 µM) at two different concentrations of NMDA. Narrow hatched bars represent the burst frequencies from individual preparations exposed only to NMDA at either 75 µM, or 150 µM, n = 6. Heights of empty bars represent the burst frequencies of the same preparations exposed to their respective NMDA concentrations plus 10 µM gabazine. Wide, solid black and empty bars represent means (± SEM) of individual preparations. Upon application of 10 µM gabazine, the mean burst frequency of preparations treated with 75 µM NMDA increased significantly (paired t-test, two-tailed, six pairs, t = 4.920, df = 5, \( P = 0.044 \), *) by 26%, from 1.33 ± 0.09 Hz to 1.68 ± 0.07 Hz. Application of the same concentration of gabazine to preparations treated with 150 µM NMDA significantly increased (t = 5.835, df = 5, \( P = 0.0021 \), **) the burst frequency by 50%, from 1.93 ± 0.12 Hz to 2.90 ± 0.17 Hz. Mean burst frequencies for the 75 µM and 150 µM NMDA-only treatments differ significantly by 0.60 Hz, (paired t = 10.45, df = 5, \( P = 0.0001 \)). Likewise, the burst frequencies of gabazine treated preparations differed significantly by 1.22 Hz (t = 6.044, df = 5, \( P = 0.0018 \)).
Effects of GABA<sub>A</sub> antagonists on the burst pattern.

Not only is the frequency of ventral root bursting important to consider, but also the overall pattern of activity. This can be estimated by an index for burst quality (see Materials and Methods, Buchanan 1999a, b; Cangiano and Grillner 2003), the proportion of the locomotor cycle taken up by the burst and the regularity of bursting.

a. Effects on burst quality

The burst quality (Fig. 4A and Materials and Methods) was measured using an autocorrelation of three minutes of rectified and five-fold decimated ventral root burst activity and comparing the height of the first peak and first trough of the correlogram (see inset Fig. 4A). The sample periods chosen were identical to those used for the burst frequency analysis described above for gabazine and picrotoxin. Burst quality for all preparations was normalized to the control level (equal to a value of 1.0) and averaged to produce the mean values in Fig. 4. Neither gabazine nor picrotoxin (acting on chloride channels directly) produced a measurable effect on burst quality (ANOVA, \( P >> 0.05 \)). The strong suggestion of a deterioration of rhythm with picrotoxin treatment, however, prompted us to examine the issue further by analyzing the rhythm for alternation quality, as described below.

b. Burst proportion remained generally constant.

Burst proportion is the duration of the ventral root burst taken as a fraction of the cycle period. It was measured from the same sample records used to measure burst frequency above. Twenty to 48 cycles were used to determine a mean burst proportion. It generally
remains between 25 and 40% of the cycle duration during locomotion (Grillner 1974; Wallén and Williams 1984), regardless of the actual cycle duration. Fig. 4B shows variation of the normalized burst proportion at different levels of the two GABA_A antagonists used here (left-hand ordinate). No statistically detectable differences in absolute or normalized mean were found with gabazine or with picrotoxin (ANOVA, \( P > 0.05 \)).

Superimposed on the fast burst pattern, a slow modulation sometimes occurred (around 0.1 Hz or below, cf (Aoki et al. 2001) at higher concentrations of GABA_A antagonists. The slow modulation tended to show both patterns of right-left alternation and synchrony, as did the fast burst rate of normal fictive locomotion. In this superimposed slow rhythm the burst proportion could vary outside the 25 - 40% range.

c. Effects on the coefficient of variation of cycle duration

A second factor affecting the burst quality is the regularity of the burst pattern. The coefficient of variation of the cycle duration (CV; standard deviation/mean cycle duration) can be used as a measure of the regularity. Like burst proportion, CV of cycle duration was determined from the same five minute sample periods used to determine burst frequency in a treatment period. Twenty to 48 cycles were used to determine the mean value. In Fig. 4B, the CV of the cycle duration is normalized to the control value and averaged to produce mean CV of cycle durations. These values are plotted at different concentrations of gabazine and picrotoxin (right-hand ordinate). No changes in CV were observed (ANOVA, \( P > 0.05 \)).
Fig. 4. Effect of gabazine and picrotoxin (n = 4 for each) on burst quality, burst proportion and coefficient of variation. A: The unitless quantity of normalized burst quality is shown for gabazine (open circles) and picrotoxin (solid circles). The method of calculation is shown in the inset. BQ is calculated using the heights of the first peak a, and trough b, of an autocorrelogram (comparing a record of ventral root bursting with itself). The frequency of bursting is also evident from the position of the first peak of the correlogram along the x-axis of the inset. The burst quality showed no effect with increasing concentrations of either gabazine (ANOVA, seven groups, F = 0.8149, R² = 0.2136, P = 0.5726) or picrotoxin (ANOVA, six groups, F = 1.757, R² = 0.3694, P = 0.1824). B: The mean normalized burst proportion (top), and mean coefficient of variation (bottom), are shown for preparations treated with gabazine (open circles) and picrotoxin (solid circles). Burst proportion remained stable with gabazine treatment throughout the treatment regime (ANOVA, seven groups, F = 0.6399, R² = 0.1758, P = 0.6973) as well as for picrotoxin (ANOVA, six groups, F = 2.108, R² = 0.4127, P = 0.1209). Coefficient of variation of cycle duration (a measure of rhythmicity) displayed no effect of either gabazine (ANOVA, seven groups, F = 0.2122, R² = 0.0661, P = 0.9682) or picrotoxin (ANOVA, six groups, F = 0.3592, R² = 0.1069, P = 0.8684). Error bars = SEM.
**Effects on the quality of segmental coordination**

The pattern of right-left alternation in pairs of ventral roots at the same segmental level was analyzed, using cross-correlation of three minute records, as with burst quality, to arrive at a measure of the average quality of alternation (see Materials and Methods, and inset in Fig. 5A). Again, the sample periods were the same as used for determining burst frequency above.

The effects on the “quality of alternation” (AQ) for three gabazine and four picrotoxin experiments are shown in Fig. 5A along with the methods of analysis (Fig. 5A2). With gabazine, the quality of alternation (lower portion of Fig. 5A1) remains near the control level at all concentrations (ANOVA, \( P = 0.062 \)). The response profile for picrotoxin was different from that of gabazine: picrotoxin significantly depressed alternation quality (\( P = 0.0018 \)). The significant effects were at 5 and 7.5 \( \mu \)M picrotoxin (Dunnett’s, \( P < 0.001 \)), the latter showing a decline of 58% (Fig. 5A1).

For both gabazine and picrotoxin, the average phase value (Fig. 5A1, Phase) of alternation remained unaltered from its control value of 0.50 (± SEM for both drugs < 0.01, n = 4) across the various concentrations, ranging from approximately 0.4 to 0.6. Thus, neither gabazine nor picrotoxin had any effect upon phase of alternation (\( P >> 0.05 \)).

**Effects on intersegmental phase lag**

During locomotion there is an intersegmental rostro-caudal phase lag of around 1 % of the cycle duration per segment, regardless of the actual cycle duration (Cohen and Wallén 1980; Grillner 1974; Lansner et al. 1998; Zelenin et al. 2001). This phase lag remains in
the isolated spinal cord (Wallén and Williams 1984). As described in Materials and Methods, the cross-correlation of a rostral and an ipsilateral caudal ventral root bursting pattern yielded a correlogram function whose principal peak is shifted from the lag = 0 (ms) axis by the amount of delay between the bursts. When divided by the number of segments, this value yields a per-segment phase delay. The sample periods chosen were identical to those used for the burst and alternation qualities described above and the method of measurement is illustrated in Fig. 5B. In the present study, intersegmental phase lag remained essentially unresponsive to GABAergic antagonism (Fig. 5B1) for all picrotoxin concentrations (ANOVA, $P = 0.2222$) and concentrations of gabazine under 100 µM (Dunnett’s, $P > 0.05$). There was a moderate effects of doubling the average phase lag from 0.35% to 0.7% at the very highest concentration of gabazine (100 µM, Dunnett’s, $P < 0.001$, ANOVA, $P = 0.0090$). However, the general constancy of the phase lag remained for other concentrations of the drugs even though the frequency of bursting increased markedly with both gabazine and picrotoxin.

**Effects of very high concentrations of gabazine and picrotoxin on the locomotor activity**

With gabazine even at a very high concentration of 100 µM, the fast rhythmic alternating bursting was retained, as evident from Fig. 6A1-3 and the cross-correlogram in Fig. 6A4. In contrast, with a high concentration of picrotoxin the alternating motor pattern broke down, and changed to simultaneous bursts on the left and right ventral roots at a very slow rate (0.12 Hz, Fig. 6B2, B4). In three of four picrotoxin experiments, high concentrations at either 7.5 µM or 10 µM produced this pattern of bursting with a rhythm
Fig. 5. Effects on segmental bilateral and intersegmental coordination. A: Measurements of Alternation Quality, AQ, (A_1) determined using a cross-correlation analysis of long records, as well as an illustration (A_2) of the alternation phase delay between the left (L) and right (R) ventral roots. Phases of alternation (top graphs in A_1 and B_1) are shown for gabazine (open circles, n = 3, spurious changes in baseline precluded inclusion of a fourth replicate) and picrotoxin (solid circles, n = 4). The inset in A_1 shows the method for calculating AQ as described in Materials and Methods. Gabazine did not diminish the quality of alternation over the concentrations employed (ANOVA, seven groups, F = 1.399, R^2 = 0.4115, P = 0.2918). Picrotoxin, however, significantly reduced the alternation quality by 58 ± 0.09% (ANOVA, six groups, F = 6.694; r^2 = 0.6905; P = 0.0018). Dunnett’s a posteriori test revealed a significant effect of 5.0 and 7.5 µM picrotoxin on alternation quality (***, P < 0.001). Neither gabazine (ANOVA, seven groups, F = 0.1595, R^2 = 0.0505, P = 0.9844) nor picrotoxin (ANOVA, six groups, F = 0.415, R^2 = 0.1215, P = 0.8311) produced any change in the phase of alternation. B: The mean intersegmental phase lag per segment, IPL, between rostral and caudal segments, is shown individually for bath application of gabazine (open circles, n = 4) and picrotoxin (solid circles, n = 4) at different concentrations (B_1). IPL was measured by cross-correlating long rostral and caudal recordings ipsilaterally as explained in the text (see inset in B_1). The principle of intersegmental delay is illustrated in B_2 for ipsilateral rostral (R) and caudal (C) roots. The phase lag remained unchanged for all concentrations of gabazine except the highest (ANOVA, seven groups, F = 4.11, R^2 = 0.5781, P = 0.009, Dunnett’s a posteriori test at 100 µM gabazine, P < 0.001). No response to picrotoxin was observed (ANOVA, six groups, F = 1.592, R^2 = 0.3467, P = 0.2222). All error bars = SEM.
Fig. 6. Effects of high concentrations of gabazine and picrotoxin on burst frequency. Ventral root recordings (A1-3, gabazine and B1-3, picrotoxin) and their cross-correlograms during application (A4 and B4, respectively) are shown. At a concentration up to 100µM gabazine, ventral root bursting is maintained at a fast (3.4 Hz), alternating rhythm with peaks at (cycle) phase lag values near ± 0.5 of the cycle period (a total of 291 msec) as shown in the cross-correlogram (A4). In the case of picrotoxin at 10 µM, a slow ( = 0.1 Hz), synchronous rhythm is produced with the main peak at phase 0 (B4). In the case of an ideal, alternating rhythm, the lag (msec) between peaks of the correlogram reflects cycle period since the autocorrelative function must shift one-half cycle for the maximum overlap of the signals. In the case of the ideal, synchronous pattern, the cycle period is reflected by the lag between the main peak at the zero axis and the next peak or, more accurately, the average of the distances to the peaks in the positive and negative directions.
of about 0.1 Hz (Cangiano and Grillner 2003; Cohen and Harris-Warrick 1984). Such slow, synchronous rhythms are characteristic of a glycineric blockade, which presumably occurs with high doses of picrotoxin, as has been described previously for strychnine (Aoki et al. 2001; McPherson et al. 1994). In contrast, a very weak, slow modulation superimposed on the fast rhythm can sometimes be detected under control conditions (Brodin and Grillner 1985), and it could also be discerned after gabazine (Fig. 6A, middle traces).

**GABA<sub>C</sub> receptors are not involved in the regulation of burst frequency**

To explore whether GABA<sub>C</sub> receptors become activated during swimming, a selective GABA<sub>C</sub> antagonist, TPMPA, was administered in concentrations ranging from 0.6 to 320 µM, which encompasses the 10-50 µM used in rat brain slice and spinal cord (Kirischuk et al. 2003; Rozzo et al. 1999). The concentrations of TPMPA (Fig. 7) were presented in successive, 30-minute treatment periods without intervening washouts. Since the burst frequencies obtained upon exposure to TPMPA showed no clear change, the frequencies measured from each of the five periods of a treatment period were averaged to produce a single mean frequency. Summary of the burst frequencies averaged over the whole period are shown in (Fig. 7D). No effect on burst frequency was observed in four preparations even at the highest level of TPMPA ($p > 0.05$). This finding is important also in relation to the effects of picrotoxin, which has been reported to act nonspecifically also on GABA<sub>C</sub> receptors (Wang et al. 1995). The results thus suggest that GABA<sub>C</sub>
Fig. 7. Test of the influence of a GABA\textsubscript{C} antagonist on fictive locomotion. Examples of right (R) and left (L) ventral root bursts are shown for control (A) and a high concentration of TPMPA, 320 \textmu M (B) with no change in the frequency of bursting. C: The results of a typical experiment over time. No frequency change was observed over a range of TPMPA concentrations. D: Summary histogram of four preparations showing the mean burst frequency over all 8 treatment categories, which was 1.55 ± 0.010 Hz. There was no significant difference in burst frequency before and after TPMPA (ANOVA, eight groups, F = 0.5809, R\textsuperscript{2} = 0.1622, P = 0.7637). Error bars = ± SEM.
receptors do not contribute to the spinal pattern generation, and that the divergent effects of picrotoxin cannot be accounted for by an action on this receptor subtype.

_Endogenous GABA<sub>A</sub> and GABA<sub>B</sub> receptor activation is additive with regard to burst frequency regulation_

Tegnér et al. (1993) demonstrated that an administration of GABA<sub>B</sub> antagonists resulted in a marked increase of burst frequency. It was now important to establish whether the effects of GABA<sub>A</sub> antagonists documented here would add further to the GABA<sub>B</sub> induced effects. Using the protocol described in Materials and Methods, control (100 µM NMDA plus HEPES) was superfused before, between, and after the applications of 20 µM CGP55845, a GABA<sub>B</sub> antagonist, and/or 10 µM gabazine (Fig. 8B). Further, HEPES-only washes were superfused for one-half hour after either 20 µM CGP55845 alone or 20 µM CGP55845 plus 10 µM gabazine. These HEPES-only washes were followed by control NMDA/HEPES with burst frequencies very close to the control NMDA values (mean change compared to pre-treatment control = 1.8%, SD = 1.8%, n = 8). Fig. 8A<sub>1-4</sub> shows records of bursting during the treatment. Administration of 20 µM CGP55845 caused an acceleration of the locomotor burst pattern from 1.8 ± 0.2 Hz to 2.4 ± 0.3 Hz (n = 4, P < 0.01, Tukey’s and Newman-Keuls)(Fig. 8B) in confirmation of Tegnér et al. (1993). Adding 10 µM gabazine plus 20 µM CGP 55845 resulted in an additional increase to 2.8 ± 0.2 Hz (n = 4, P < 0.05, Newman-Keuls)(Fig. 8B). With regard to the locomotor system, the GABA<sub>A</sub> and GABA<sub>B</sub> receptor subtypes thus act in a synergistic fashion, which could not be assumed _a priori_, since the two receptor types
Fig. 8. Effects of simultaneous application of GABA\textsubscript{A} and GABA\textsubscript{B} antagonists on burst frequency. Records of ventral root bursts are shown during control, drug and washout conditions (A\textsubscript{1-4}). B: Normalized mean burst frequencies in four separate experiments, all in NMDA (100 µM): C = NMDA alone, CGP = the GABA\textsubscript{B} antagonist CGP55845 (20 µM), and CGP+GBZ = combined CGP55845 (20 µM) + Gabazine (10 µM). Mean frequency values were determined before normalization from the last five 5-min intervals of stable rhythm preceding the next drug change. Standard errors of the mean values of frequency ranged from 0.002 Hz to 0.030 Hz. For the four experiments taken together and calculated on mean values, treatment with CGP 55845 produced an expected increase in the mean value of burst frequency (2.42 ± 0.31 Hz) over the control NMDA-alone treatment (1.84 ± 0.18 Hz) (Repeated Measures ANOVA, one-tailed, \(P < 0.01\), Newman-Keuls, \(n = 4\)). Likewise, treatment with gabazine and CGP 55845 combined increased the mean frequency from 1.81 ± 0.17 Hz in NMDA alone to 2.80 ± 0.23 Hz (same tests, \(P < 0.001\)). Upon wash-out the burst frequency returned to 1.84 ± 0.19 Hz. The combination of CGP 55845 and gabazine produced a greater increase in mean burst frequency compared to that of CGP 55845 alone (repeated measures ANOVA, two-tailed, \(P < 0.05\), Newman-Keuls, \(n = 4\)).
could be expressed in different cellular compartments. In one of four experiments the locomotor rhythm became very irregular with the addition of 20 µM CGP55845 and in two out of four experiments the same effect occurred with the application of 10 µM gabazine plus 20 µM CGP55845. A regular burst activity was resumed after wash out.

**Discussion**

The main purpose of this study has been to explore whether an activation of GABA_A receptors takes place during fictive locomotion, and if so, which effects are exerted. The interpretation of the previous demonstration that biculline methiodide affected the frequency and regularity of locomotor activity (Aoki et al. 2001; Tegnér et al. 1993), had been compromised by the later finding that this GABA_A antagonist also affected K_Ca channels (Debarbieux et al. 1998; Johnson and Seutin 1997; Pflieger and Dubuc 2000), which importantly contribute to the locomotor pattern generation. The present study shows that gabazine, so far known to be a selective antagonist, causes a pronounced concentration-dependent increase of burst frequency up to a two-fold increase (Fig. 1E), without a concomitant change in burst quality, burst proportion or coefficient of variation (Fig. 4), and essentially no effect on intersegmental coordination (Fig. 5B). The gabazine blockade can thus, in contrast to bicuculline, selectively affect burst rate without changes in the regularity of locomotor activity.

Picrotoxin, the other GABA_A antagonist tested, is known to affect glycine activated Cl^- channels at higher doses. With picrotoxin we could confirm that the burst rate increased, but with higher doses the alternation quality decreased progressively, and at the highest dose the pattern converted to slow, synchronous, bilateral bursts (Fig. 6).
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6B₂B₄, which is similar to the effects of the glycine receptor antagonist strychnine (Aoki et al. 2001; McPherson et al. 1994). Our data thus suggest that with picrotoxin, a progressive concentration dependent blockade of glycine receptors occurs, as in other systems (Pflieger et al. 2002).

GABA_C receptors activate a related Cl⁻ channel with different and slow kinetics. This receptor subtype is expressed in a variety of tissues including the rodent retina, hippocampus and spinal cord (see Johnston et al. 2003). There is evidence that it contributes to the spinal network in neonatal rats (Rozzo et al. 1999). To investigate if GABA_C receptors are involved in the control of the locomotor network, we administered a selective antagonist, TPMPA. It did not, however, exert any effect, even at a very high dosage. Thus this receptor subtype does not appear to affect the locomotor network, provided of course that the antagonist is effective in the lamprey CNS. Further, with interference of picrotoxin on GABA_C receptors ruled out, our results support the evidence presented above that at high concentrations picrotoxin exerts effects on glycine receptors that contribute to the deterioration of rhythmic coordination. Having clarified this problem with picrotoxin, we base our conclusions preferentially on the results obtained with gabazine.

_Ctribution of the GABA_A component of the GABA system_

The endogenous GABA_A receptor activation thus contributes to a prominent slowing of the locomotor burst rate, both at low and high control burst rates (NMDA drive), but the effect is more prominent at higher burst rates (Fig. 3). Conversely, a potentiation of the endogenous GABA_A activation through activation of the benzodiazepine receptor with
diazepam, led to a further substantial slowing of the burst rate (20%) with maintained regular activity (Tegnér et al. 1993). The possible working range of the GABA_A component is thus substantial.

The endogenous GABA action via both GABA_A and GABA_B receptors

A potentiation of the endogenous action of the GABA system can be produced by administration of GABA uptake blockers (nipecotic acid), which results in a pronounced overall slowing of the burst rate by around 50% (Tegnér et al. 1993). This effect is partially reversed by GABA_B antagonists, with the remaining part being due to GABA_A receptors. In the present experiments we show that both GABA_B and GABA_A receptors contribute to the endogenous slowing of the locomotor burst activity (Fig. 8).

Mode of action of the GABAergic system

From previous studies (Alford et al. 1991), it is known that GABA interneurons are active in phase with the ipsilateral burst activity, as inferred from the phasic GABAergic presynaptic depolarization that occurs in the terminals of network interneurons. There are two types of GABAergic interneurons that are prime candidates for the effects observed here, the large multipolar subtype in the grey matter and the small dorsal bipolar subtype (Brodin et al. 1990). The former would appear the most likely, since it is large with numerous ramifications in the grey matter. The small bipolar subtype projects to the terminals of sensory afferents and co-expresses NPY (Bongianni et al. 1990; Parker et al. 1998). This subtype is most likely responsible for mediating presynaptic phasic inhibition
to sensory afferents (El Manira et al. 1997), but probably not to network interneurons, since they appear to have limited axonal ramifications.

The GABA system clearly exerts part of its burst rate effects through the presynaptic modulation via GABA$_A$ and GABA$_B$ receptors on network interneurons, which will decrease the excitatory drive. But most likely there are also direct effects on soma-dendritic level of network neurons. Administration of the GABA$_A$ agonist muscimol can stop network activity altogether, while the GABA$_B$ agonist baclofen slows the locomotor network activity profoundly (Grillner and Wallén 1980; Tegnér et al. 1993). Part of this latter action is through a depression of low and high voltage activated Ca$^{2+}$ channels and a concomitant decrease of the activation of K$_{Ca}$ channels, and thereby the afterhyperpolarization, and in part via reduction of Ca$^{2+}$-dependent postinhibitory rebound (Matsushima et al. 1993; Tegnér and Grillner 2000; Tegnér et al. 1998). These presumed postsynaptic effects would be expected to slow network activity by reducing the frequency adaptation, due to the afterhyperpolarization, thus prolonging burst periods, and also by reducing the postinhibitory rebound excitation, following each inhibitory phase leading to less activation of action potentials.

What is the role of GABAergic modulation of the locomotor system?

The locomotor system can, on one hand, generate locomotor activity when the GABA system is largely blocked, but the activity is then at a higher burst rate. The GABA system is thus not required for burst generation to occur, but it will extend the working range of the network in the lower end of the frequency span, without affecting the intersegmental coordination. The GABA action is larger at higher rates of locomotor
activity (Fig. 3). Whether the degree of GABAergic modulation is entirely intrinsic and driven by the spinal network or is subject to additional external modulation from the brainstem is so far unknown. The latter possibility could provide an additional way to modulate burst frequency without affecting intersegmental coordination. A similar GABA modulation of the locomotor network appears to occur in both mammals and amphibians, and may thus be a general vertebrate trait (Cazalets et al. 1998; Sillar et al. 2002).

It is interesting to compare with the spinal 5-HT system, another powerful intrinsic modulator, which is also activated from the locomotor network, and also contributes to a slowing of the locomotor burst rate due to an indirect action on $K_{Ca}$ channels and also via its effects on synaptic transmission (Harris-Warrick and Cohen 1985; Hill et al. 2003; Kozlov et al. 2001; Wallén et al. 1989; Zhang and Grillner 2000), (see Svensson et al. 2003). 5-HT, in lamprey and in other vertebrate locomotor networks, also promotes the occurrence of steady regular locomotor activity. In contrast to the GABA system, the 5-HT system generates an increased intersegmental phase lag with increasing levels of 5-HT (Matsushima and Grillner 1992), which would result not only in a lower locomotor frequency, but also in more than one undulatory locomotor wave along the body.

In conclusion, it is important to realize that these two modulator systems are both turned on when the locomotor network becomes active, both of which contribute to a slowing of the burst pattern and in addition modifies the network properties to generate a more stable locomotor rhythm.
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