Abstract Effects of inhibitory neurotransmitters on the locomotor rhythm and pattern generation were investigated using an in vitro preparation isolated from the mudpuppy (Necturus maculatus). The preparation consisted of the first five segments of the spinal cord and the right forelimb attached by the brachial nerves. During N-methyl-D-aspartate (NMDA)-induced locomotion, the rhythmic motor output (EMG) was recorded unilaterally from elbow flexor and extensor muscles. While neither glycine nor \( \gamma \)-aminobutyric acid (GABA)-related substances induced locomotion in the absence of NMDA, they modulated NMDA-induced locomotion. Bath application of glycine and GABA suppressed the rhythmic motor pattern induced by NMDA. Addition of glycine receptor antagonist strychnine or GABA_A receptor antagonist bicuculline disrupted the phase relationship between antagonistic motor pools during ongoing locomotion, thereby changing the normal alternating pattern into synchronous EMG bursts. Both the GABA_A receptor agonist muscimol and GABA_B receptor agonist baclofen mimicked the effects of GABA as they either slowed down or stopped locomotion. Nipecotic acid, a GABA uptake blocker, had a similar effect. This suggested that an endogenous release of GABA modulated the locomotor rhythm. The endogenous release was antagonized by the GABA_A and GABA_B receptor antagonists bicuculline and CGP-35348, respectively. Immunocytochemistry revealed that glycine and GABA-positive neurons and fibers were present in mudpuppy spinal cord. Although the GABAergic neurons were more numerous than glycinergic neurons, both cell types contributed processes directed towards the white matter and occasionally towards the ependymal lining of the central canal. Our results suggest that inhibitory neurotransmitters exert powerful actions upon the neuronal network governing forelimb locomotion in the mudpuppy. The effects we observed may be mediated by a network of segmentally distributed glycinergic and GABAergic spinal neurons.

Key words GABA · Glycine · Central pattern generator · Spinal cord · Locomotion · In vitro

Introduction

Inhibition plays an important role in generating and/or coordinating rhythmic motor patterns throughout the animal kingdom. Reciprocal inhibition plays a crucial role in the control of the leech heart beat (Calabrese and Peterson 1983; Marder and Calabrese 1996), the generation of a rhythmic motor pattern in the lobster stomatogastric ganglion (Selverstone et al. 1983; Marder and Calabrese 1996), and the coordination of bilateral (Dale et al. 1990; Grillner and Matsushima 1991; Roberts et al. 1984) and tetrapod locomotion (Grillner 1981; McClellan 1996; Noga et al. 1993). Two principal neurotransmitters involved in mediating inhibitory processes are glycine and \( \gamma \)-aminobutyric acid (GABA). In vitro studies of the lamprey (Alford and Williams 1989; Cohen and Harris-Warrick 1984; Hagevik and McClellan 1994) and the Xenopus embryo (Roberts et al. 1984; Dale 1985) have shown that after blocking glycine receptors, rhythmic motor output can still occur in each half of their central nervous systems. Thus, glycinergic transmission is not necessary for generating the swimming rhythm. However, glycinergic crossed inhibition serves to couple the phase of independent rhythm generators in each half of the respective spinal cords, allowing the production of a coordinated alternating motor pattern typical of swimming. Similar findings have been reported in the cat (Noga et al. 1993; Pratt and Jordan 1987),

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neonatal mouse (Droge and Tao 1993), neonatal rat (Cowley and Schmidt 1995) and neonatal wallaby (Ho 1997). This suggests that glycine receptor activation is not required for motor rhythm generation but may mediate reciprocal antagonism underlying inter- and intralimb locomotor patterns.

Contrary to the role proposed for glycine, GABA appears to be more involved in providing for gain control and gating of the sensory input during ongoing locomotion. There is general consensus in both invertebrate (Clarac and Cattaert 1996; El Manira and Clarac 1991) and vertebrate (Dubuc et al. 1988; Stuart and Redman 1991) studies that a presynaptic GABAergic modulation of the sensory afferent transmission occurs during locomotion. In the lamprey, the efficiency of both the inhibitory and excitatory transmission is modulated phasically and can be gated at different levels by a combined action of GABA_A and GABA_B receptors. Acting in concert, pre- and postsynaptic GABA mechanisms have been shown to reduce the burst frequency and modify the intersegmental coordination (Tegnér et al. 1993). In neonatal rats, GABA affects both the duration and amplitude of the rhythmic motor output by activating GABA_A and GABA_B receptors (Cazalets et al. 1994). Furthermore, there is evidence that GABA affects network inter- and intralimb coordination (Cowley and Schmidt 1995; Kremer and Lev-Tov 1997). Together with glycine, GABA may also be involved in mediating recurrent inhibition (Schneider and Fyffe 1992).

In the present study we used an in vitro preparation of the mudpuppy to further clarify the role of inhibitory neurotransmitters in vertebrate locomotion. The mudpuppy, an aquatic amphibian, has a number of features that make it well suited for examining the neural mechanisms for generation and modulation of locomotor activity. This in vitro preparation stays alive for several days when superfused with cooled and oxygenated Ringer’s solution. Despite being an aquatic amphibian, the mudpuppy walks with an alternating quadrupedal gait characterized by robust and long-lasting EMG activity. Furthermore, it is the first adult in vitro preparation that allows simultaneous observation of the actual behavior (i.e., rhythmic movement of the forelimb) and recording of a motor output (EMG).

We demonstrate here that GABAergic and glycinergic systems are active during N-methyl-D-aspartate (NMDA)-induced locomotion in the mudpuppy. Their activity does not appear to be essential for the rhythm generation, but rather coordinates the alternation between flexors and extensors in a limb. Activation of glycine and GABA_A receptor seems to be more important for the rate control and the regularity of the EMG pattern. GABA_B receptor activity, on the other hand, alters the amplitude and frequency of EMG bursts, leaving the alternating pattern largely unaffected.

A preliminary report of this work has been published in abstract form (Jovanović et al. 1996b).

### Materials and methods

#### In vitro preparation

Experiments (n=52) were conducted on an in vitro spinal cord preparation isolated from adult mudpuppies as approved by the Animal Welfare Committee at the University of Alberta. Prior to dissection, the animals were anesthetized by immersion in a solution of 3-aminobenzoic acid ethyl ester (Sigma, 1 g/l). The skin and underlying muscles were removed and a dorsal laminectomy was performed from the first to the fifth vertebrae. The first five segments of the spinal cord (C1–C5) with the attached forelimb were then removed from the rest of the body and placed in a Sylgard-lined petri dish superfused with cooled (15°C) and oxygenated Ringer’s solution of the following composition (mM): 115 NaCl; 2 CaCl_2; 2 KCl; 1.8 MgCl_2; 5 hydroxyethylpiperazine ethanesulfonic acid (HEPES); pH 7.3; glucose, 1 g/l. While in the petri dish, the brachial plexus was exposed, the paraspinal muscles removed and bipolar Teflon-coated silver wires (75 µM) inserted into the elbow flexor (Brachialis) and extensor (Extensor ulnae) muscles for electromyographic (EMG) recording during locomotion. After a recovery period of approximately 1 h, the preparation was transferred to a Sylgard-lined recording chamber and placed dorsal side up. The spinal cord and the forelimb were then stabilized by pinning the vertebral column and the procoracoid cartilage to the base of the chamber. Throughout the course of the experiments the preparation was superfused with a cooled (15–18°C) and oxygenated Ringer’s solution at a flow rate of 5–10 ml/min.

Locomotion was induced chemically by adding 30–90 µM N-methyl-D-aspartic acid (NMDA) together with 10 µM t-serine to the superfusing solution. After a well-defined locomotor pattern was established, two types of experiment were performed.

#### Exogenous application of the drugs

To study the role of inhibitory neurotransmitters in the locomotor rhythm and pattern generation glycine, GABA and some of their respective agonists and antagonists were applied directly into the bath. In different experiments, the following drugs were used: GABA (0.75–900 µM), bicuculline methobromide (0.8–20 µM), muscimol (0.5–8 µM), baclofen (0.5–100 µM), CGP-35348 (5–100 µM), nipeptocic acid (0.05–4 mM), glycine (0.1–4 mM) and strychnine (1–100 µM). Each cycle of drug application was followed by a washout with Ringer’s solution. Following the washout, locomotion could be induced repeatedly by superfusing the preparation with NMDA and t-serine. The NMDA, t-serine, glycine and strychnine were obtained from Sigma Chemical (St. Louis, MO). The GABA uptake inhibitor nipeptocic acid, GABA_A antagonist bicuculline methobromide, GABA_A agonist muscimol and GABA_B agonist baclofen hydrochloride were obtained from Research Biochemical Inc. (Natick, MA). The GABA_B antagonist CGP-35348 was generously provided by Novartis Pharmaceuticals Canada Inc. All drugs were freshly prepared in distilled water and diluted to the appropriate concentration prior to use.

The effects elicited by applying different concentrations of drugs were observed as changes in the control cycle duration, normalized to 1, and in the flexor and extensor EMGs which were simultaneously recorded. EMG recordings were preamplified, rectified and filtered (10–300 Hz) and stored on a computer’s hard disk using commercially available data acquisition software (Axotape, Axon Instruments). All channels of data were digitally sampled at 50 Hz and later analyzed using a customized software package written by Drs. Ken Yoshida and Marc Bélanger. The EMG amplitude was determined as an average peak voltage of flexor and extensor bursts by using an analysis routine, custom written for Matlab v4.2 (The Math Works, Natick, MA). "n" refers to the number of experiments. The results regarding changes in the cycle duration were pooled and plotted in the figures as the means±SEM. These values were derived for each drug using a nonlinear least squares fit to a modified Michaels-Menten equation:

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T = \frac{1+CM/(C+C_{50})}{1+(C/1000)/(C+C_{50})} \tag{1}
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