Neuroscience 131 (2005) 745–758

DISTINCT ROLES OF GLYCINERGIC AND GABAERGIC INHIBITION IN COORDINATING LOCOMOTOR-LIKE RHYTHMS IN THE NEONATAL MOUSE SPINAL CORD

C. HINCKLEY,[a] B. SEEBACH[b] AND L. ZISKIND-CONHAIM[a]*

[a]*Department of Physiology and Center for Neuroscience, University of Wisconsin, Medical School, 1300 University Avenue, Madison, WI 53706, USA*

[b]*Department of Biology, University of Wisconsin, 1725 State Street, La Crosse, WI 54601, USA*

Abstract—The primary objective of our study was to examine the role of the inhibitory neurotransmitters glycine and GABA in modulating spontaneous activity and coordinating neurochemically induced locomotor-like rhythms in the mouse spinal cord. Motor outputs were recorded in lumbar ventral roots of 1-4-day old neonatal mice, and the function of glycinergic and GABAergic synapses in regulating spontaneous and induced activities was examined by suppressing synaptic inhibition using selective glycine or $GABA_A$ receptor antagonists. Strychnine (0.5 μM), a glycine receptor antagonist, did not change the pattern of spontaneous activity that consisted of random single spikes and discharges of variable durations and intervals. In contrast, blocking $GABA_A$ receptors with either picrotoxin (10 μM) or bicuculline (5 μM) triggered bilaterally synchronous, non-rhythmic discharges. These findings suggested that GABAergic synapses suppressed excitatory synapses, and their disinhibition synchronized spontaneous discharges between the two sides of the spinal cord. Locomotor-like rhythms alternating between the two sides of the spinal cord were triggered by the neurotransmitter agonists 5-HT, *N*-methyl-D,L-aspartic acid and dopamine. Blocking glycine receptors increased tonic discharges, and in most preparations it reduced the phase correlation between the alternating rhythms. Inhibiting $GABA_A$ receptor-mediated synapses synchronized the onset and prolonged the duration of rhythmic discharges. Intraburst alternating peaks were evident and those were suppressed by strychnine, suggesting that they were mediated via glycinergic synapses. Our findings indicated that GABAergic and glycinergic synapses played different roles in modulating neurochemically induced locomotion rhythms. GABAergic inhibition regulated the onset and duration of neurochemically induced locomotor-like rhythms, and glycinergic inhibition stabilized the pattern of the alternating rhythms. © 2005 Published by Elsevier Ltd on behalf of IBRO.

*Corresponding author. Tel: +1-608-263-3382; fax: +1-608-265-5512.
E-mail address: lconhaim@physiology.wisc.edu (L. Ziskind-Conhaim).
Abbreviations: CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CV, coefficient variance; D-APV, D-2-amino-5-phosphonovaleric acid; EGTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EPSCs, excitatory postsynaptic currents; $GABA_AR$, $GABA_A$ receptor; GlyR, glycine receptor; HEPES, *N*-(2-hydeoxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); IPSCs, inhibitory postsynaptic currents; mIPSC, miniature inhibitory postsynaptic current; NMA, *N*-methyl-D,L-aspartic acid; NMDA, *N*-methyl-D-aspartic acid; P, postnatal.

0306-4522/05$30.00+0.00 © 2005 Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2004.11.034

Key words: locomotor-like rhythm, bilateral rhythm coordination, disinhibition-induced discharges, GABA inhibition, glycine inhibition, mouse spinal cord.

Locomotion in vertebrates is generated by spinal networks that can function independently of central and peripheral synaptic inputs. In the isolated cord, hindlimb movements are represented by alternating left–right flexor- or left–right extensor-related motor rhythms. Fictive locomotion can be triggered by neurotransmitter agonists (Kudo and Yamada, 1987; Smith et al., 1988; Cazalets et al., 1992; Cowley and Schmidt, 1994; Kiehn and Kjaerulff, 1996; Whelan et al., 2000), stimulating peripheral afferent axons (Smith and Feldman, 1987; Lev-Tov et al., 2000; Marchetti et al., 2001; Bonnot et al., 2002b) or descending pathways (Atsuta et al., 1990; Magnuson and Trinder, 1997). The general consensus is that bilaterally alternating motor outputs are coordinated by reciprocal inhibitory interactions between rhythm-generating networks located on both sides of the spinal cord (Jankowska et al., 1967; Cowley and Schmidt, 1995; reviewed by Grillner and Wallen, 1985; Bonnot et al., 2002a; Butt et al., 2002b).

Previous studies have proposed that glycine (Kjaerulff and Kiehn, 1997; Kremer and Lev-Tov, 1997), GABA (Cazalets et al., 1994) or both inhibitory amino acids (Cowley and Schmidt, 1995; Beato and Nistri, 1999) are involved in the control of synaptic locomotor drive. The relative role of GABA and glycine in rhythm coordination shifts during embryonic development (Nakayama et al., 2002), paralleling the transition from GABAergic to glycinergic synapses on motoneurons (Gao et al., 2001). To determine the contribution glycine- and GABA-mediated synapses to rhythm coordinating networks, numerous studies have examined the effects of glycine and $GABA_A$ receptor ($GABA_AR$) antagonists on locomotor-like activity. Some of the ambiguity regarding their role in rhythm generation might have resulted from the non-specific actions of high concentrations of strychnine, glycine receptor (GlyR) antagonist, and picrotoxin, $GABA_AR$ antagonist, on $GABA_A$Rs and GlyRs, respectively (Yoon et al., 1998; Jonas et al., 1998; Tapia and Aguayo, 1998; Chattipakorn and McMahon, 2002). Therefore, it is conceivable that what was assumed to be selective inhibition of either glycinergic or GABAergic synapses actually resulted from suppression of at least a fraction of both receptor types.

Little is known about the effects of selective disinhibition on both spontaneous activity and neurochemically induced rhythms in the mouse spinal cord. Blocking inhibitory synapses by either bicuculline (20–30 μM) or strych-

nine (20 μM) elicited spontaneous, synchronous discharges of variable intervals (Bonnot and Morin, 1998). Strychnine (5 μM) also prolonged the interval between discharges induced by 5-HT (Branchereau et al., 2000). However, as stated above, data should be interpreted cautiously because of the potential non-selective actions of high concentrations of strychnine on GABA-mediated currents. It is intriguing that the effect of strychnine (0.3–10 μM) on spontaneous flexor–extensor activity pattern was dependent on the level of spontaneous activity: it synchronized flexor–extensor discharges in most spinal cord–hindlimb preparations that did not exhibit spontaneous EMG activity, but did not alter the activity pattern in spontaneously active preparations (Droge and Tao, 1993).

Our findings that selective suppression of glycinergic and GABAergic synapses has distinct effects on motor activity suggest that the inhibitory transmitters play different roles in regulating spontaneous activity and coordinating locomotor-like rhythms in the mouse spinal cord. A preliminary report of this study was published in an abstract form (Hinckley and Ziskind-Conhaim, 2002).

EXPERIMENTAL PROCEDURES

Spinal cord preparations

Experiments were performed using spinal cords of 1–4-day-old postnatal (P1–P4) Swiss Webster mice (Charles River Laboratories, Wilmington, MA, USA). The animal study protocol was approved by the Animal Use and Care Committee at University of Wisconsin–Madison and was in accordance with the NIH guidelines. Efforts were made to minimize the number of animals used and to reduce their suffering. The spinal cord was isolated using similar procedures to those used to isolate the rat spinal cord (Ziskind-Conhaim et al., 2003). Animals were anesthetized by hypothermia, decapitated and then eviscerated in ice-cold oxygenated extracellular solution. The cord with ventral and dorsal roots attached was isolated from thoracic segment T10 to sacral segment S2. The isolated spinal cord was equilibrated to room temperature for 30 min before it was placed in the recording chamber.

Transverse slices of the lumbar spinal cord were used for whole-cell patch clamp recordings (Gao and Ziskind-Conhaim, 1995). The isolated spinal cord was embedded in agar (2% in extracellular solution), and transverse slices, 350–400 μm thick were cut using a Leica VT1000S. Prior to whole-cell recordings, slices were incubated in extracellular solution at room temperature for 30–40 min. Slices were transferred into a recording chamber, which was mounted on the stage of an Olympus microscope (BX50WI), and were superfused with aerated extracellular solution at room temperature (20–22 °C).

Ventral root recording

Spontaneous motor output and induced locomotor-like rhythms were recorded in the intact en bloc spinal cord preparation. The isolated cord was pinned down to a Sylgard-coated recording chamber with the ventral roots up. The cord was continuously superfused with oxygenated extracellular solution at a rate of 2–5 ml/min at room temperature. Ventral root activity was recorded in contralateral L2 or L1 and ipsilateral L5 using tight fitting glass suction electrodes. Ventral root potentials were amplified with AC-coupled DAM-50 amplifiers (World Precision Instrument, Sarasota, FL, USA) and were recorded using Axotape software (Axon Instruments, Union City, CA, USA). The potentials were filtered at 1–3 kHz, digitized at 2–5 kHz and stored on a disk for off-line analysis.

Whole-cell patch clamp recordings

The actions of glycine and $GABA_A$ receptors antagonists on neuronal firing and spontaneous inhibitory postsynaptic currents (IPSCs) were examined in transverse slices. Action potentials were generated by intracellular current injection in unidentified ventral neurons in the area near the lateral motor column (Gao and Ziskind-Conhaim, 1998). The neurons were visualized using infrared–differential interference contrast optics. Whole-cell patch clamp recordings were carried out with electrodes pulled to tip resistances of 3–5 MΩ using a multi-stage puller (Sutter Instruments, Novato, CA, USA). Intracellular potentials were recorded with an Axoclamp 2B amplifier (Axon Instruments). IPSCs were recorded using a Multiclamp 700A amplifier (Axon Instruments Co). Potentials/currents were filtered at 3 KHz and digitized at 20 KHz.

Data analysis

Typically, in each experiment, random samples of 20 successive rhythmic bursts were analyzed off-line during a steady state phase when a consistent pattern was established. Rhythm properties were easier to analyze using rectified and smoothed traces of extracellular recordings. Smoothing was performed using adjacent averaging over 100–200 points (Origin 6, MicroCal, Northampton, MA, USA). Those traces were used to calculate the cycle period and burst interval, duration and amplitude. A burst was defined as the excitation above the baseline of smoothed potentials. Cycle period was calculated as the time between the onsets of two consecutive bursts. Burst duration was measured as the time between the onset of excitation and its return to baseline. Burst interval was estimated from the end of one burst to the onset of the next burst. Time to peak of spontaneous bursts was calculated as time from burst onset to its peak. Cycle regularity under various conditions was estimated by calculating the coefficient variance (CV; standard deviation divided by the mean) of the cycle period. Paired *t*-tests were used to determine the statistical significance ($P<0.05$).

Time series analysis was conducted to test the significance of the phase relations between two and three ventral roots. Analyses were carried out on random samples of 20 successive rhythmic bursts recorded during a steady state period (see above). Unless otherwise stated, cross-correlation analyses were performed using the rectified and smoothed data sampled at 10 Hz (lag=100 ms; Statistica 6; StatSoft, Tulsa, OK, USA). The correlation coefficient (r) was calculated at each kth lag (delay from time 0) to identify shifts at which the activity in the two data streams was in-phase (synchronous, values near 1.0), uncorrelated (values near 0; within the range of ±3-time the S.E., as plotted), or out-of-phase (alternating, values near −1.0). The kth lag is the interval from time 0, and is shown in the figures as delay in seconds. Analysis of spontaneous IPSC properties included peak amplitude, rise and decay times from 10 to 90% peak (Mini Analysis Program; Synaptosoft Inc., Decatur, GA, USA).

Solutions and drugs

Extracellular solution contained (in mM): NaCl 128, KCl 4, $CaCl_2$ 1.5, $MgSO_4$ 1, NaH_2PO_4 0.5, $NaHCO_3$ 21 and glucose 30. The solution was adjusted to pH 7.3 using NaOH, and the osmolarity was 315–325 mOsm. Whole-cell pipette solution contained (in mM): K gluconate 140, KCl 9, HEPES 10, EGTA 0.2, Mg-ATP 1, and GTP 0.1. The solution was adjusted to pH 7.2–7.3 using KOH, and the osmolarity was 290–305 mOsm. To increase IPSC frequency, some recordings were conducted in the presence of elevated extracellular KCl (10 mM), and NaCl concentration was reduced to 122 mM. The pipette solution for IPSC recordings contained (in mM): CsCl 149, HEPES 10, EGTA 0.2, Mg-ATP 1 and GTP 0.1. The solution was adjusted to pH 7.2–7.3 using