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Feedback regulation of locomotion by motoneurons in the vertebrate spinal cord

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Motoneurons are known to be an essential component of central pattern generators in invertebrates, but it is only recently that they have been shown to play a similar role in vertebrate locomotor circuits. Here, we review early experiments implicating motoneurons in the genesis of spontaneous motor activity in development and more recent experiments identifying motoneurons as important regulators of locomotor activity in the adult zebrafish and in the neonatal mouse spinal cord. We discuss the mechanisms responsible for these actions, the experimental challenges in studying the role of motoneurons in the mammalian spinal cord and the functional significance of the excitatory influence of motoneuron activity on locomotor behavior.

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Introduction

The vertebrate spinal cord contains the essential circuitry for generating locomotion. The core component of this circuitry—the central pattern generator (CPG)—can produce the essential features of locomotor behavior in the absence of descending or afferent inputs [1]. It has long been assumed that the CPG for locomotion is comprised exclusively of spinal interneurons and that motoneurons function primarily as the output of the spinal cord [2]. This view was supported by anatomical and physiological evidence in the lamprey and zebrafish, showing that axial motoneurons possess few or no axon collaterals [3,4]. Consistent with this anatomical evidence, experiments in the isolated lamprey spinal cord showed that antidromic stimulation of the ventral roots had no effect on fictive swimming induced by NMDA [5]. By contrast, in limbed vertebrates, motoneurons have recurrent collaterals that synapse onto other motoneurons [6] and onto inhibitory interneurons (called Renshaw cells in mammals, R-interneurons in the chick embryo) within the spinal cord [7–9]. Because the target interneurons are inhibitory, they were not thought to have a significant role in locomotor rhythmogenesis.

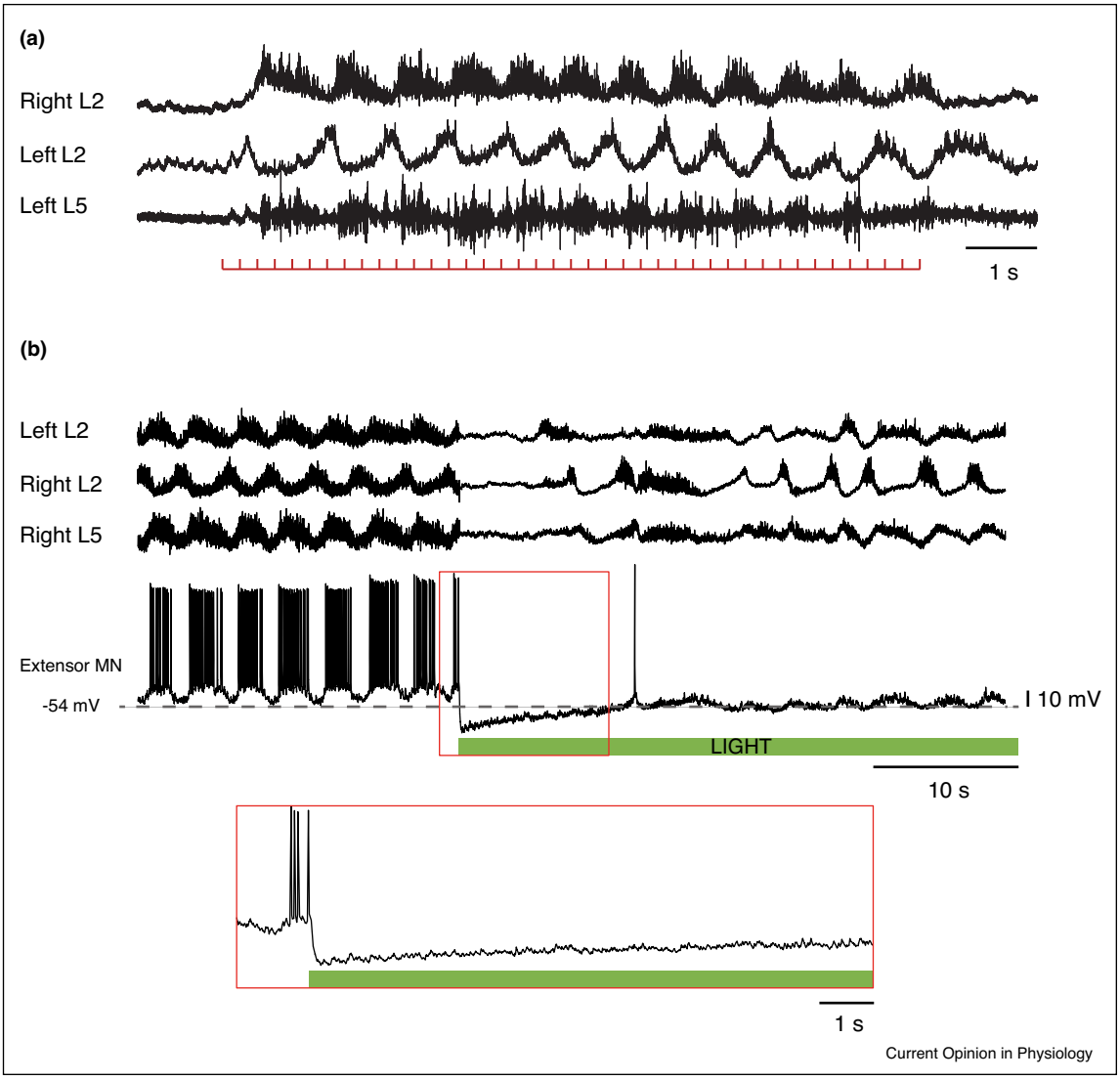
Motoneurons regulate rhythmic motor activity in the developing spinal cord

In the developing spinal cord, motoneurons have been shown to play an active role in regulating motor activity. In the isolated spinal cords of the chick [10] and mouse [11] embryos, stimulation of a ventral root can trigger an episode of rhythmic bursting. In the chick embryo, ventral root stimulation activated the spinal network through the synaptic connections of motoneurons with the avian homologue of the mammalian Renshaw cell [10]. In addition, at the onset of spontaneous episodes of rhythmic activity, calcium, and voltage sensitive dye imaging revealed that the earliest optical signals were detected in the ventrolateral part of the cord and spread throughout the cord from there [10,12,13]. The first indication that motoneurons might have access to the vertebrate locomotor CPG came from experiments on the tadpole showing that rhythmically active spinal interneurons, presumed to be part of the swimming CPG, received cholinergic synapses that were proposed at the time to originate from motoneurons [14]. However, the later demonstration that some spinal, glutamatergic interneurons also release acetylcholine rendered this assumption uncertain [15].

Motoneuron activity regulates locomotion and locomotor-like activity

The demonstration that motoneurons influence the mammalian locomotor CPG came from experiments in the neonatal mouse spinal cord, showing that stimulation of a ventral root or the sciatic nerve (sensory dorsal roots cut) could trigger an episode of locomotor-like activity [16] (Figure 1a). Surprisingly, the phenomenon was not blocked by cholinergic antagonists which could be partly explained by the finding that motoneurons release a second excitatory transmitter—that binds to glutamatergic receptors—in addition to acetylcholine at their central synapses with Renshaw cells [16,17]. Whether the transmitter is glutamate is not completely settled, because the expression of the known vesicular glutamate transporters at motoneuron terminals remains controversial [16–19] and aspartate is present at a higher level than glutamate [20]. Motoneurons also have excitatory connections with

Figure 1



(a) Motoneurons can trigger locomotor-like activity. Fictive locomotion evoked by ventral root stimulation (100 μA, 250 μs, 4 Hz) recorded from the right L2 and left L2 and L5 ventral roots in a P3 Wild type spinal cord. The signals were filtered to remove the stimuli artifacts and were high pass filtered at 0.1 Hz. The red trace below the recordings shows the train of stimuli. **(b)** Hyperpolarization of cholinergic neurons in a ChAT-Archaerhodopsin spinal cord transiently abolished the rhythmic synaptic drive to motoneurons and decreases the frequency of the rhythm. Locomotor-like activity evoked by 5 μM NMDA and 10 μM 5-HT recorded from the left L2 and right L2 and L5 ventral roots together with an extensor motoneuron in the right L5 segment. The green bar below the intracellular recording indicates the duration of the light. The part of the intracellular record delineated by the red rectangle has been expanded in the panel below to show the absence of rhythmic drive for the first 10–15 s after the light turns on. The data in (b) are adapted from Ref. [27••].

other motoneurons (at least in neonatal and juvenile mice) that have been reported to be mediated by both cholinergic and glutamatergic receptors [17] or exclusively by glutamate receptors [21•]. The difference in the findings may be due to the age of the animals which were older in the study finding exclusively glutamate-like transmission [21•]. Further evidence for an excitatory effect of motoneurons on locomotor networks came from studies in the neonatal rat [22] and mouse [23] showing that stimulation of a ventral root could accelerate locomotor-like activity induced by drugs, although in the rat this occurred rarely and only in the presence of noradrenaline. Machacek and Hochman [22] proposed that noradrenaline unmasked a connection between motoneurons and an unknown class of excitatory interneuron. They also showed that ventral root stimulation could sometimes entrain disinhibited bursting, another manifestation of the excitatory effects of ventral root stimulation on spinal networks. The ability of ventral root stimulation to entrain disinhibited bursting was also

demonstrated in the neonatal mouse spinal cord by Bonnot *et al.* [24] who used calcium imaging to show that, following a ventral root stimulus, optical activity often began in the vicinity of the motor nuclei and propagated dorso-medially from there to encompass the whole cord.

An important advance in understanding the role of motoneurons in modulating locomotor function came from experiments in the zebrafish showing that optogenetic hyperpolarization of motoneurons reduced the frequency and duration of swimming bouts in 3–4 week old zebrafish [25••]. In this animal, V2a interneurons project monosynaptically to motoneurons and are an important source of their locomotor synaptic drive [26]. A majority of the V2 neurons connect to motoneurons via bidirectional gap junctions at mixed-chemical electrical synapses. Through these gap junctions, motoneuron membrane potential can modulate transmitter release and the firing of the V2a interneurons. Accordingly, retrograde hyperpolarization of the V2a population by optogenetically hyperpolarizing motoneurons depresses V2a spiking activity and transmitter release thereby slowing and curtailing the swimming episode [25••]. For technical reasons, the demonstration that blockade of gap junctions eliminated the motoneuronal modulation of the swim bouts was not performed.

Using a similar optogenetic approach, it was shown that lumbar motoneurons can also regulate drug-induced locomotor-like activity in the neonatal mouse spinal cord. Falgairolle *et al.* [27••] expressed the inhibitory opsin archaerhodopsin into cholinergic neurons and in separate experiments into *islet*-1 expressing neurons. In both preparations, illumination of the lumbar spinal cord with green/yellow light slowed the locomotor rhythm (Figure 1b). Mixed chemical/electrical synapses are reported to be common in the adult mammalian spinal cord [28], so the question arises as to whether the regulation of locomotor-like activity in the neonatal mouse spinal cord also employs this mechanism of retrograde control. However, in contrast to the findings in the zebrafish, this effect did not appear to be mediated by gap junctions because it persisted in the presence of the gap junction blocker carbenoxolone, consistent with the earlier demonstration that carbenoxolone did not abrogate ventral root-evoked locomotor-like activity [16]. In addition, it has been shown that V2a neurons in the neonatal mouse spinal cord are not electrically coupled to motoneurons [21•] and that the modulatory effects of motoneuron activity on the locomotor-like rhythm appear to be mediated, in part, through a glutamatergic mechanism [27••].

Possible mechanisms for ventral root-evoked locomotor-like activity

The recent discovery that neonatal motoneurons have excitatory projections to spinal neurons other than Renshaw cells provides a potential mechanism for the excitatory effects of motoneurons on locomotor activity (Figure 2). Chopek *et al.* have shown that motoneurons have reciprocal, monosynaptic excitatory connections with V3 glutamatergic interneurons [29••]. The existence of these connections may explain the earlier observations of Ichinose and Miyata [30] and Schneider and Fyffe [31] who recorded ventral root-evoked EPSPs in motoneurons that had latencies similar to that of the recurrent IPSP, consistent with a disynaptic connection.

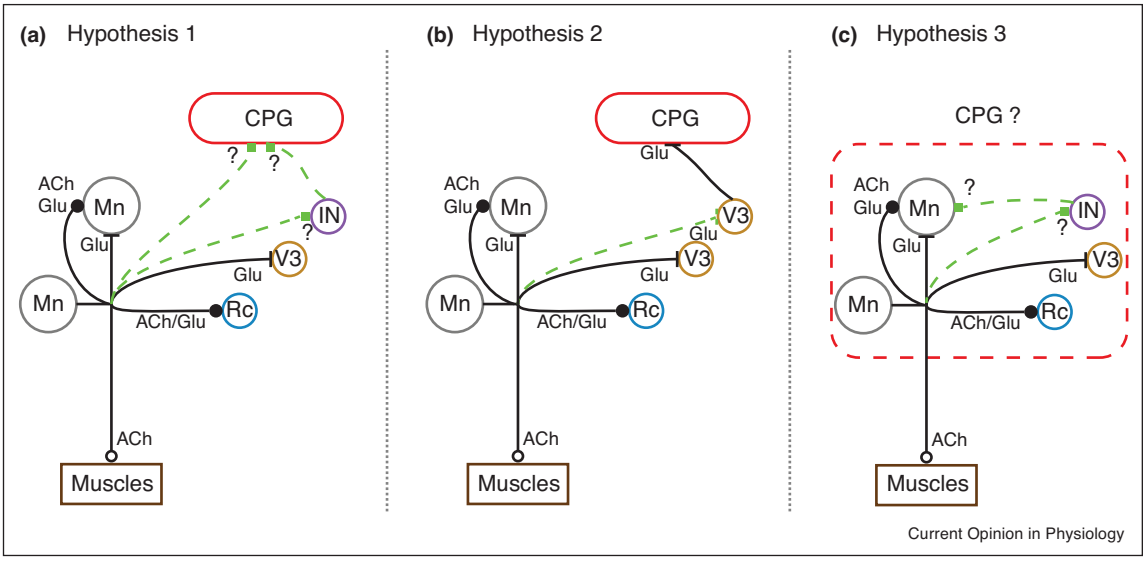
Whether or not these novel excitatory connections are responsible for the excitatory effects of motoneuron activity on the CPG is unclear. When V3 neurons are acutely silenced using the allatostatin receptor system, the locomotor-like rhythm is degraded so that the duration of the left and right flexor bursts becomes more variable as does the cycle period [32]. Furthermore, it is not known if activation of this population can initiate locomotor-like activity or the extent to which they regulate the frequency of locomotor-like activity. It is important to emphasize even if a particular cell class targeted by motoneurons does not underlie the excitatory effects of motoneurons on locomotion, the novel connection is likely to be functionally significant.

The excitatory effects of motoneuron activity on the locomotor CPG represent a form of positive feedback because motoneuronal activity increases the frequency of locomotor-like activity [22,23,27••]. Such a system is potentially unstable and may be balanced by an inhibitory pathway. The well-known connection of motoneurons to inhibitory Renshaw cells may serve this function although there is only indirect evidence to support such a role. This comes from experiments in mice in which a vesicular transporter for glutamate (VGluT2) was knocked out [23]. This transporter underlies ~90% of excitatory glutamatergic transmission in the spinal cord, and despite its absence, the isolated cord can still generate locomotor-like activity in response to drugs [23]. In this preparation, stimulation of a ventral root can block drug-induced locomotor-like activity indicating the potential presence of a negative feedback loop that may counteract the excitatory effects of motoneuron activity on the CPG.

Challenges in studying the feedback effects of motoneuron activity on spinal networks

One of the difficulties in studying the effects of motoneuronal effects on locomotor networks is that a unique molecular marker for motoneurons does not exist, which precludes the selective expression of the opsins into motoneurons. Song *et al.* [25••] used a Gal4s1020t zebrafish line in which the Gal-4 driver of halorhodopsin was expressed in motoneurons and also in GABAergic Kolmer–Agduhr cells. Optogenetic activation of the Kolmer–Agduhr cells can trigger swimming bouts in the zebrafish embryo [33]. Their influence in the experiments of Song *et al.* was minimized by the presence of the $GABA_A$ antagonist gabazine in the bathing medium. In

Figure 2



Hypothesized connections of motoneurons to the CPG in neonatal mice. Circles represent neurons: motoneurons (Mn), Renshaw Cell (Rc), V3 interneurons (V3) and an unknown interneuronal population (IN). Each axon has been labeled with their known transmitter(s): acetylcholine (ACh) and glutamate (Glu). Motoneuronal to motoneuronal connections show 2 axon collaterals to encompass different results in the literature [17,21•]. **(a) Hypothesis 1.** Motoneurons connect to the CPG either directly or through a projection to an unidentified interneuron (IN). **(b) Hypothesis 2.** V3 interneurons have been shown to receive monosynaptic inputs from motoneurons [29••] and to modulate the CPG [32]. Here we hypothesized that the ability of motoneurons to trigger locomotor-like activity and target the CPG is mediated through this pathway. **(c) Hypothesis 3.** Motoneurons and the CPG are traditionally seen as separate modules. In this schematic, we hypothesize that motoneurons are part of the CPG and that they might be playing a crucial role in activating and generating locomotion.

the ChAT-Archaerhodopsin mouse spinal cords used by Falgairolle *et al.* [27••], archaerhodopsin was expressed in autonomic preganglionic neurons and cholinergic interneurons in addition to motoneurons. To minimize the contribution of these non-motoneuronal cells to the light-induced effects on the locomotor rhythm, the experiments were repeated in the presence of nicotinic and muscarinic cholinergic antagonists. In these blockers, the inhibitory effects of light on the frequency of the locomotor-like rhythm were still present. Although this procedure would have also blocked cholinergic motoneuronal synapses, motoneurons also release an excitatory amino acid so that they could still exert an action on spinal networks. Of course, a similar argument applies to cholinergic interneurons that co-release acetylcholine and glutamate [15,19].

A second challenge in studying the excitatory actions of motoneurons on spinal and locomotor networks in the neonatal mouse cord is that the phenomenon is variable during the neonatal period and is not detectable in every experiment [22,24,34]. One possibility is that the effects may depend on the neuromodulatory state of the isolated cord. For example, Machacek and Hochman [22] showed that the excitatory effects of motoneuron stimulation were enhanced by noradrenaline and inhibited by serotonin. Humphreys and Whelan [34] showed that dopamine could block ventral root activation of the CPG and ventral root entrainment in the disinhibited cord. Why the neuromodulatory state of the cord should vary from one experiment to another is not clear, but it could depend on how the animals are raised and handled. Another contributing factor may be that the motoneuronal connections with the CPG are immature in the neonatal period. Unfortunately, it has been difficult to determine if the excitatory effects of motoneurons on locomotor activity are present in older cords because they lose viability when maintained *in vitro*. Nevertheless, it should be possible to establish if motoneuron projections to V3 interneurons are present in slice preparations of the adult cord.

Outstanding questions

There are many outstanding questions that remain to be addressed before we can achieve a complete understanding of how motoneurons interact with the mammalian locomotor CPG. For example, we do not know whether all classes of motoneuron are involved. Previous work reported that high stimulus intensities applied to the ventral roots were necessary to evoke locomotor-like activity, raising the possibility that the motoneurons responsible were of the slow type or were even gamma motoneurons [35•].

A second issue concerns how motoneuron stimulation triggers locomotor-like activity. V3 interneurons that are targeted by motoneurons have not been directly implicated in rhythmogenesis. Zhang *et al.* [32] reported that rhythmic motor activity persists when the population is chronically or acutely silenced. Moreover, intracellular recordings from V3 neurons do not reveal the presence of electrical properties consistent with cellular rhythmogenesis [32]. Of course, the V3 population is likely to be heterogeneous and perhaps the subpopulation targeted by motoneurons does exhibit these properties and is responsible for the ability of motoneurons to evoke fictive locomotion when stimulated (Figure 2b). Alternatively, another, as yet unknown, population of interneurons that is also targeted by motoneurons may be responsible (Figure 2a). Furthermore, unless this connection is not always present, it is difficult to account for the variability in evoking motor activity in either the normal or the disinhibited cord [22,24].

As discussed above, it is not known whether the excitatory effects of motoneuron activity on the CPG persist into adulthood. Early in the development of the tadpole and the zebrafish, motoneurons release glutamate at the neuromuscular junction [36,37], and this phenomenon is abolished or reduced later in development.

However, at motoneuron-Renshaw synapses in the adult rodent cord, glutamate or a glutamate-like neurotransmitter is co-released with acetylcholine, indicating that activation of glutamate receptors by motoneuron activity is conserved through development [38]. Determining the extent to which motoneurons regulate locomotion in adult animals, particularly *in vivo*, will be experimentally challenging. In the moving animal, any manipulations of motoneuronal activity will be complicated by the altered afferent feedback that accompanies changes in muscle activity.

An important and unresolved question is the extent to which motoneurons contribute to locomotor rhythmogenesis. Motoneurons are known to exhibit TTX-resistant, NMDA-induced oscillations of membrane potential [39], plateau potentials [40,41] and persistent inward and outward currents that can induce bursting [42]. Furthermore, none of the genetic disruptions and deletions of the canonical interneuronal precursors abolishes the locomotor rhythm. In some experiments, in which neonatal motoneurons were optogenetically hyperpolarized, rhythmic activity recorded from the ventral roots and intracellularly from individual motoneurons was abolished (Figure 1b), consistent with the idea that motoneurons might contribute to rhythmogenesis (Figure 2c). Resolution of this issue is a complicated, because it is difficult to distinguish between motoneuronal input as a requirement for CPG function compared to motoneurons as the origin of the rhythm. This difficultly applies not just to motoneurons but also to all classes of interneurons.

Like animals, human locomotion is thought to be generated by a spinal CPG, and it is likely the spinal circuitry is organized in a similar way [43]. If motoneurons do regulate motor function in humans, this will be of major significance for neurodegenerative motoneuron disorders and the recovery of locomotor function after spinal injury.

Conflict of interest statement

Nothing declared.

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