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Supraspinal control of locomotion: The mesencephalic locomotor region

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Abstract

Locomotion is a basic motor function generated and controlled by genetically defined neuronal networks. The pattern of muscle synergies is generated in the spinal cord, whereas neural centers located above the spinal cord in the brainstem and the forebrain are essential for initiating and controlling locomotor movements. One such locomotor control center in the brainstem is the Mesencephalic Locomotor Region (MLR), first discovered in cats and later found in all vertebrate species tested to date. Over the last years, we have investigated the cellular mechanisms by which this locomotor region operates in lampreys. The lamprey MLR is a well circumscribed region located at the junction between the midbrain and hindbrain. Stimulation of the MLR induces locomotion with an intensity that increases with the stimulation strength. Glutamatergic and cholinergic monosynaptic inputs from the MLR are responsible for excitation of reticulospinal (RS) cells that in turn activate the spinal locomotor networks. The inputs are larger in the rostral than in the caudal hindbrain RS cells. MLR stimulation on one side elicits symmetrical excitatory inputs in RS cells on both sides and this is linked to bilateral projections of the MLR to RS cells. In addition to its inputs to RS cells, the MLR activates a well-defined group of muscarinoceptive cells in the brainstem that feedback strong excitation to RS cells in order to amplify the locomotor output. Finally, the MLR gates sensory inputs to the brainstem through a muscarinic mechanism. It appears therefore that the MLR not only controls locomotor activity, but it also filters sensory influx during locomotion.

Introduction

The neural organization underlying locomotion – one of the most basic motor acts – is remarkably similar in different species of vertebrates. The muscle synergies responsible for propulsion are generated by neural networks in the spinal cord interacting with sensory signals (Grillner, 1981; Grillner, 1985; Rossignol 1996; Rossignol *et al.*, 2006). These spinal networks, known as Central Pattern Generators (**CPG**) for locomotion, are activated and controlled by specific supraspinal structures, which also receive sensory inputs (Armstrong 1986; Orlovsky *et al.*, 1999; Rossignol 1996; Rossignol *et al.*, 2006; Shik and Orlovsky 1976). The supraspinal control of locomotion includes forebrain structures, specific locomotor centers in the forebrain and brainstem, and command cells in the lower brainstem that activate the spinal CPGs (Fig. 1). The detailed contribution of forebrain structures to locomotion has not been resolved yet. However, the role of motor cortex is better known; it contributes to precision walking requiring an exact foot placement, such as on an uneven terrain (Beloozerova *et al.*, 1993a; Beloozerova *et al.*, 1993b; Bretzner *et al.*, 2005; reviewed in Drew *et al.*, 2008). The basal ganglia are believed to play a role in the selection of locomotor behaviors (Grillner *et al.*, 1997, 2008). A striking feature relative to the supraspinal control of locomotion is the presence of forebrain and brainstem *locomotor centers* specifically dedicated to initiating and controlling locomotion (for reviews see: Armstrong, 1986; Dubuc, 2009; Dubuc *et al.*, 2008; Grillner, 1988; Grillner, 1997; Jordan, 1998; Jordan, 1986; Orlovsky, 1999; Whelan, 1996). One such region is located in the diencephalon and another in the

mesencephalon. These two locomotor control centers are respectively referred to as the *Diencephalic Locomotor Region (DLR)*; El Manira *et al*, 1997; Grillner, 2008) and the *Mesencephalic Locomotor Region (MLR)*; Shik *et al*, 1966). This review article will focus primarily on the MLR and on recent findings obtained from one species of vertebrates, the lamprey.

The mesencephalic locomotor region (MLR) in vertebrates

The MLR has been identified in the 1960s by a research group in Moscow (Shik *et al*, 1966). This region receives inputs from the basal ganglia, the lateral hypothalamus and the medial hypothalamus through the periaqueductal gray matter (Jordan, 1998). The mammalian MLR consists of two nuclei, the nucleus cuneiformis (CN) and the pedunculo pontine nucleus (PPN). Electrical or chemical stimulation of the MLR induces bouts of locomotion (Shik, *et al*, 1966; Garcia-Rill *et al.*, 1985) via the activation of the reticulospinal (RS) pathways (Garcia-Rill and Skinner 1987; Orlovsky 1970; Steeves and Jordan 1984). The mechanisms that underlie the activation of the MLR when the basal ganglia are activated are not fully understood. It appears that the selection of a relevant motor program by the basal ganglia would rely on disinhibition (Hikosaka, 1991). It was proposed that the ventral pallidum and the SNr could act similarly and inhibit the MLR (Grillner *et al.*, 1998; Takakusaki, 2008). As such, locomotion would result from a disinhibition of the MLR by the ventral pallidum or the SNr. Experimental findings support this hypothesis. For instance, electrical stimulation of the SNr prevents MLR-induced locomotion (Takakusaki, 2003), suggesting that the SNr inhibits locomotor

activity. In addition, injections of the GABA_A antagonist bicuculline in the MLR induce bouts of locomotion (Garcia-Rill *et al.*, 1990).

Classically, electrical or chemical stimulation of the MLR in decerebrate cats elicits motor output that has been subdivided in two phases. First, there is an increase in muscle tone allowing the animal to fully support its weight; this is followed by the locomotor phase (Shik, *et al.*, 1966). In the first initial experiments describing the MLR of cats, it was found that the frequency of locomotion was graded in relation to the stimulation intensity. At low MLR stimulation intensities, the animals walked; as the intensity increased, they trotted and then galloped. This initial stunning observation provided the basis for qualifying this particular brainstem region as “dedicated to control a locomotor output”. The exact anatomical substrate of the MLR has been on the other hand subjected to debate. The most effective site to induce locomotion was a region comprising the CN and possibly a part of the PPN (Mori, 1989). Activation of the CN in a walking cat increased the speed of locomotion (Sterman and Fairshild, 1966). Similarly in rats, chemical activation of the PPN elicited only brief episodes of locomotion in comparison to those elicited by activation of the CN (Garcia-Rill, 1985; Garcia-Rill, 1990). The CN was thus proposed as the most effective site eliciting locomotion (for review see Grillner 1997). Whether a part or the entire PPN participated in the initiation of locomotion was not as clear; because of the proximity of these two structures, experimental results have been difficult to interpret. Several studies have tried to dissect the specific role of the CN and the PPN in the control of locomotion. Because locomotion is also strongly modulated by its behavioural context, it was

suggested that the MLR could be subdivided in different modules that would be activated in a context dependent manner (Sinnamon, 1993). For instance, it was proposed the MLR elicited locomotion in three different contexts and could therefore be subdivided into three main functional areas: “an exploratory system”, “an appetitive system” and “a defensive system” (Sinnamon, 1993). This concept of organization was supported by experimental findings. For example, injection of glutamate in the CN induced a sequence of freezing, darting and fast running (Mitchell, 1988a, 1988b). An “escape” locomotor behavior was observed both in cats and rats, when the CN was stimulated (Depoortere, 1990a, 1990b; Mori *et al.*, 1989; Sirota and Shik, 1973). On the other hand, injections of GABA antagonist in the PPN induced locomotion that was apparently more related to startle (Ebert and Ostwald, 1991; Garcia-Rill *et al.*, 1990). It was proposed that the PPN would itself be divided into two specific regions, a ventral and a dorsal component (Milner and Mogenson, 1988). The dorsal part of the PPN would be part of the MLR as a locomotor controlling region, whereas the ventral part would consist of a muscle tone inhibitory system (Takakusaki, 2003). Chemical activation of the dorsal part of the PPN was shown to increase locomotion in intact rats; an opposite effect was observed when ventral part of the PPN was activated (Milner and Mogenson, 1988). Functional MRI has recently revealed an increased BOLD signal in the MLR during mental imagery of walking and running in healthy volunteers (Jahn *et al.*, 2008). In addition, recent clinical trials have shown improvement in posture and gait after stimulating the pedunclopontine nucleus (a part of the MLR (Jordan, 1998) in Parkinson’s patients (Lozano, 2008; Mazzone, 2005; Stefani, 2007). There is

considerable new evidence that cholinergic mesencephalic neurons would be involved in gait and postural disorders in Parkinson's disease (Karachi *et al.*, 2010).

The MLR does not project directly to the spinal cord, but it activates hindbrain RS cells that in turn activate spinal cord locomotor networks (Garcia-Rill and Skinner, 1987a, 1987b Garcia-Rill, 1991; Grillner, 1981; for review see Rossignol, 1996). After its first discovery in cats (Shik *et al.*, 1966), the MLR was also identified in many other species of vertebrates including rats (Skinner and Garcia Rill, 1984), stingrays (Bernaudo, 1991), guinea pigs (Marlinsky, 1991), lampreys (Sirota *et al.*, 2000), salamanders (Cabelguen *et al.*, 2003) and rabbits (Musienko, 2008). The most remarkable feature of the MLR was the locomotor activity that increased in speed as the stimulation of the MLR was increased. This was seen in all the animal species investigated. Moreover, it was shown in salamanders that MLR stimulation elicited the two modes of locomotor activity displayed by these animals, walking and swimming. At low MLR stimulation, the motor output elicited was characterized by limb movements associated with stepping. As the MLR stimulation was increased, the stepping movements increased in frequency. With further increases in stimulation strength, the limbs moved under the animal's belly and swimming movements were elicited. Altogether, these observations indicate that the MLR is responsible for the initiation of swimming, walking, trotting or galloping in different species of vertebrates. Moreover, observations made in salamanders indicate that this brainstem region can control two different modes of locomotion in the same animal.

The supraspinal control of locomotion in lampreys

The lamprey model has provided first-hand information on the cellular mechanisms of vertebrate locomotion. The general organization of the lamprey nervous system is strikingly similar to that of mammals, but the presence of considerably fewer neurons results in a reduced complexity that has been very useful to examine the cellular mechanisms underlying motor behavior. The lamprey model has paved the way for several important discoveries. One of these is the detailed characterization of a vertebrate CPG for locomotion (Buchanan and Grillner 1987). The brainstem mechanisms responsible for initiating and controlling locomotion have also been successfully uncovered in lampreys with an array of *in vitro* techniques, with the added benefit of including all relevant brain structures needed for locomotor control, and the ability to monitor the active locomotor behavior.

One common feature of all vertebrate species is the crucial role played by RS cells in relaying MLR inputs to the spinal CPGs for locomotion. The RS cells receive peripheral and central inputs, integrate these signals, and generate a coherent descending motor command. In lampreys, RS cells have been described anatomically and physiologically. They constitute about 90% of the neurons projecting to the spinal cord (Bussi eres, 1994; Davis and McClellan, 1994a, 1994b; Stefani *et al.*, 2007). The lamprey RS cells are located in one mesencephalic reticular nucleus (MRN) and in three rhombencephalic reticular nuclei, the anterior (ARRN), the middle (MRRN) and the posterior (PRRN; Brodin *et al.*, 1988; Davis and McClellan, 1994a, 1994b; Nieuwenhuys, 1972, 1977; Swain *et al.*, 1993). There are around 2500 RS cells and $\approx 85\%$ of them reside in the

PRRN and MRRN (Bussi eres, 1994). There are clear homologies with reticular nuclei in other vertebrate species, including mammals (Cruce and Newman, 1984). The ARRN and MRRN are located in the “lamprey pons” and contain large RS cells (M uller cells; Rovainen, 1967) that send their axons medially in the spinal cord. These two nuclei are similar to the superior and middle reticular nuclei in fish, which are respectively homologous to nuclei pontis oralis and caudalis of mammals (Cruce and Newman, 1984). The PRRN, located in the “lamprey medulla oblongata”, contains RS cells that send axons laterally, similarly to the nucleus gigantocellularis in mammals (also discussed in Brocard and Dubuc, 2003). The axons of large RS neurons make synaptic contacts with several classes of spinal neurons and some of these are part of the locomotor CPG (Buchanan, 1982; Ohta *et al.*, 1989; Rovainen, 1974). The prevalent neurotransmitter is glutamate, although 5-HT and neuropeptides are also present in some RS cells (reviewed in Brodin *et al.*, 1988).

As in other species, the MLR was first characterized functionally in the lamprey by its ability to initiate and control locomotion when electrically stimulated (Sirota *et al.*, 2000). The neuroanatomical substrate of the MLR has remained more elusive, but recent studies have provided new insights relative to this. Stimulation experiments demonstrated that the brainstem area responsible for the control of locomotion (i.e., the MLR) is located at the mesopontine border, close to the wall of the mesencephalic ventricle (Brocard and Dubuc, 2003; Brocard *et al.*, 2004, 2010; Le Ray *et al.*, 2003; Sirota *et al.*, 2000; Smetana *et al.*, 2010). The most efficient area for eliciting locomotion is a region containing a group of cholinergic cells close to a large RS cell, I1. This area

corresponds to the caudal pole of the laterodorsal tegmental (LTD) nucleus. Increasing the MLR stimulation intensity elicited faster and faster swimming movements of greater amplitude (Sirota et al., 2000; Fig. 2).

The forebrain projections to the lamprey MLR have not been as extensively studied as in mammals. There are GABAergic inputs from the caudal portion of the medial pallium (Ménard *et al.*, 2007), a region that could correspond to the amygdala of mammals. As shown in mammals, the lamprey MLR is under a tonic inhibition. Locomotion induced by glutamate ejection in the MLR is suppressed by co-ejection of GABA receptor agonist. Moreover, the GABA receptor antagonist, gabazine elicits bouts of swimming when injected in the MLR of a semi-intact lamprey (Ménard *et al.*, 2007). The forebrain connections to the MLR as well as their neurochemical identity are still not fully identified.

Downstream effects of the MLR

Inputs to the MLR in lampreys are just starting to be explored, but the output projections from the MLR have been defined more extensively. The MLR projections are relayed within the hindbrain reticular formation. Several lines of evidence indicate that the MLR projections to RS neurons are monosynaptic. First, electrical stimulation of the MLR evokes short-latency EPSPs that are maintained during repetitive MLR stimulation at high frequency. Second, the synaptic responses in RS cells, in a Ringer's solution enriched in divalent cations, still followed a 25-Hz MLR stimulation (Brocard and Dubuc, 2003; Le Ray *et al.*, 2003).

Glutamatergic and Cholinergic MLR outputs

The MLR of mammals contains populations of cholinergic neurons (see Jordan, 1998). It is also the case in lampreys where cholinergic neurons have been found within the isthmic region (Pombal *et al.*, 2001). The possibility that the cholinergic neurons would be located within the MLR *per se* was addressed directly using immunohistochemical ChAT staining at the site of electrical stimulation that was found to initiate and control locomotion in lampreys (Le Ray *et al.*, 2003). Two distinct groups of ChAT-immunoreactive neurons were observed in an area that included the isthmus and the caudal mesencephalon. One group consisted of densely clustered cells located medially close to the ventricular border. Another group of cholinergic cells was more loosely distributed further rostrally and laterally within the tegmentum. Comparison with other species (cats: Mitani *et al.*, 1988; rats: Jones, 1990; and amphibians: Marin *et al.*, 1997) suggested that the first group would correspond to the laterodorsal tegmental nucleus (LDT), whereas the second one to the PPN. In mammals (Garcia-Rill and Skinner, 1987a; Lai *et al.*, 1999; Mesulam *et al.*, 1983; Skinner *et al.*, 1990), cholinergic neurons in these nuclei project to the reticular formation. This suggests a role for ACh in the MLR control of locomotion. However, numerous neurons projecting to RS cells are located in regions of the lamprey MLR that do not contain ChAT-positive neurons (Brocard *et al.*, 2010). This implies that other neurotransmitter systems may also be involved (see below). Electrophysiological experiments are also in accord with a role of cholinergic transmission in mediating MLR effects onto RS cells. In mammals and birds, local

injections of cholinergic agonists in the reticular formation elicit locomotion (Garcia-Rill & Skinner, 1987a; Sholomenko *et al.*, 1991). In these animal species however, a direct link between a cholinergic command originating from the MLR and locomotion was not established. The role of cholinergic inputs was examined in semi-intact preparations of lampreys. The MLR was stimulated at 5 Hz, and the evoked EPSPs in RS cells summed up until the threshold for spiking was reached and swimming activity was elicited (see also Sirota *et al.*, 2000). In the presence of the nicotinic antagonist D-tubocurarine (30-50 μM), applied selectively to the brainstem and not to the spinal cord using a partitioned recording chamber, the membrane potential of RS neurons remained mostly below spiking threshold and swimming was prevented, even under MLR stimulation intensity that would normally induced swimming. In fact, swimming could only be induced by significantly increasing the MLR stimulation intensity. In addition, the application of the selective cholinesterase inhibitor physostigmine (100 μM) to the "brainstem" chamber partition largely enhanced the MLR-evoked compound EPSPs and facilitated the RS cell depolarization and the occurrence of swimming (Le Ray *et al.*, 2003). The amplitude and slope of the MLR-evoked monosynaptic EPSP were largely, but not completely blocked by the nicotinic antagonists D-tubocurarine (30-100 μM) or α -bungarotoxin (0.1 μM). Adding a mixture of NMDA and non-NMDA glutamate receptor antagonists (200 μM AP5 and 25 μM CNQX, respectively) further reduced the EPSPs. Taken together, these results suggested that MLR inputs to RS cells use cholinergic and glutamatergic transmission. Additional support for this was the observation that a direct application of nicotinic receptor agonists in either the MRRN or the PRRN evoked swimming in a semi-intact

preparation. Comparable results were obtained in an *in vitro* isolated brainstem-spinal cord preparation in which fictive locomotion was induced by nicotinic receptor agonists. In addition, when applied on preparation already displaying a slow fictive locomotor activity under NMDA perfusion, cholinergic agonists speeded up the locomotor rhythm early after their application. This effect did not occur when the fictive locomotor rhythm had already stabilized to its faster rhythm under NMDA perfusion or after a previous ejection of the cholinergic agonist had already accelerated the fictive locomotor rhythm.

TTX-resistant depolarizing responses were generated in intracellularly recorded RS neurons by local application of nicotinic agonists onto the recorded cell. When repeated before the RS neuron membrane potential returned to resting value, the nicotinic responses showed summation properties allowing the neuron to reach spiking threshold and to generate a sustained firing of action potentials. Such temporal summation of responses may be an important for the slow buildup of RS cell depolarization and the delayed swimming onset that occur at low intensities of MLR stimulation. Indeed, in lampreys (Sirota *et al*, 2000) as in mammals (Garcia-Rill & Skinner, 1987b; Iwakiri *et al.*, 1995) several seconds of repeated MLR stimulation are required to induce locomotion. Furthermore, the onset delay of swimming decreases as the intensity or frequency of stimulation increases, probably due to the enhancement of RS depolarization by the nicotinic response summation. According to this, the buildup of the response to a 5 Hz stimulation of the MLR was dramatically reduced in the presence of a nicotinic antagonist and largely increased in the presence of physostigmine, which resulted in the

blockade or the facilitation of the initiation of swimming activity, respectively (Le Ray *et al*, 2003).

Differential MLR inputs to RS cells in the MRRN and PRRN

Although it is well established that the MLR elicits locomotor behavior by activating RS cells in different species of vertebrates, the detailed connectivity between the MLR and RS cells has remained unresolved. Such connectivity was examined in lampreys by comparing the recruitment of large MRRN and PRRN RS cells using paired intracellular recordings and increasing stimulation strength of the MLR (Brocard and Dubuc, 2003); such an approach was not used in any other vertebrate species previously. MRRN cells displayed spiking activity at low MLR stimulation strength, whereas PRRN cells begin to discharge at higher intensities, when MRRN cells have already reached their maximal spiking frequency. The respective contribution of the MRRN and PRRN in locomotor control was also investigated by selectively injecting a mixture of ionotropic glutamate receptor antagonists in each of the two reticular nuclei (Brocard and Dubuc, 2003). Injections over the entire MRRN prevented locomotion, even during MLR stimulation at high intensities. Injections over the PRRN only decreased locomotion intensity. According to these observations, RS cells receive differential inputs from the MLR, such that RS cells in the rostral hindbrain discharge more importantly at low swimming intensities and RS cells located more caudally begin spiking at higher swimming speeds.

Bilateral MLR inputs to RS cells

Another interesting aspect of the MLR is that it will elicit bilaterally symmetrical locomotion even when it is stimulated only on one side (Shik *et al.*, 1966; Shik and Orlovsky, 1976; Sirota *et al.*, 2000). This has been a feature observed in the different animal species investigated to date. A unilateral electrical (E) or chemical (C) activation of the MLR produces symmetrical locomotion in the guinea pig (C: Marlinsky and Voitenko, 1991), lamprey (E and C: Sirota *et al.*, 2000), rabbit (E: Musienko *et al.*, 2008), rat (E: Skinner and Garcia-Rill, 1984), salamander (E: Cabelguen *et al.*, 2003), and stingray (E: Bernau *et al.*, 1991; for review see Orlovsky *et al.*, 1999). One question relates to how a unilateral stimulation of the MLR is converted into a bilateral symmetrical locomotor bout? At which level (or levels) does the symmetry appear?

We recently addressed these questions in a study combining anatomical, electrophysiological, Ca²⁺ imaging, and kinematic analysis in lampreys. We found that MLR inputs to RS cells are at least partly responsible for the transformation of a unilateral MLR stimulation into bilaterally symmetrical locomotor output (Brocard *et al.*, 2010). Direct evidence for the symmetry of MLR inputs to RS cells was provided by simultaneously recording the intracellular responses of bilateral pairs of identifiable homologous RS cells from the MRRN and the PRRN to stimulation of the MLR on one side (Brocard *et al.*, 2010). The synaptic responses on both sides were very similar in shape, amplitude, and threshold intensity. Increasing the intensity of MLR stimulation produced a strikingly similar increase in the magnitude of the responses on both sides (Fig. 3). Because the technique of intracellular recordings limits our conclusions to a

small number of large-size paired RS cells, Ca^{2+} imaging experiments were performed on brainstem-isolated preparations. In accord to what been found in the large cells, a bilaterally symmetrical activation of smaller-sized RS cells of the MRRN and PRRN was seen when unilaterally stimulating the MLR.

Monosynaptic inputs from the MLR to RS neurons were known to be present in lampreys (Brocard and Dubuc, 2003). In Brocard *et al.* (2010), it was shown that MLR projects monosynaptically to RS cells not only on the ipsilateral, but also on the contralateral side. In a high-divalent cation solution, the synaptic responses of simultaneously-recorded homologous RS cells persisted and exhibited a constant latency during high-frequency stimulation. Moreover, during gradual replacement of normal Ringer's solution with a Ca^{2+} -free solution, the magnitude of responses showed a gradual reduction with a similar time course in the homologous RS cells. These results provided strong evidence for monosynaptic inputs from the MLR to RS cells on both sides.

Simultaneous recordings of homologous RS cells of the MRRN on both sides also revealed a symmetrical output in frequency when bouts of symmetrical swimming (Fig. 4) are generated by unilateral stimulation of the MLR in a semi-intact preparation (Brocard *et al.*, 2010). When increasing the MLR stimulation, the increase in discharge frequency was identical for the left and right RS cells, and the swimming frequency proportionally increased (Brocard *et al.*, 2010).

Anatomical experiments confirmed that, in the MLR on one side, neurons projecting to the left MRRN were intermingled with neurons projecting to the right

MRRN: a unilateral injection of a retrograde tracer into the MRRN revealed labeled cells bilaterally in the MLR (Brocard *et al.*, 2010; see also Sirota *et al.*, 2000). Bilateral injections of two different tracers in the MRRN revealed that the same MLR cells very rarely projected to both MRRN at the same time (Brocard *et al.*, 2010). The anatomical projections were bilaterally asymmetrical: retrograde markers injected in the MRRN on one side always revealed fewer labeled cells in the contralateral MLR, indicating that the descending projections from the MLR to RS neurons were slightly biased ipsilaterally (Brocard *et al.*, 2010). Because the MLR inputs to RS cells are perfectly symmetrical as well as the swimming behavior elicited upon a unilateral stimulation of the MLR, the anatomical asymmetry must then be physiologically compensated (Brocard *et al.*, 2010).

Bilateral descending projections from the MLR to RS neurons have also been described anatomically in the cat (Garcia-Rill *et al.*, 1983; Steeves and Jordan, 1984) and rat (Garcia-Rill *et al.*, 1986). Electrophysiological experiments in cats revealed bilateral inputs (Garcia-Rill and Skinner, 1987; Orlovsky, 1970). The bilateral anatomical projections seen in cats were on the other hand asymmetrical (Steeves and Jordan, 1984). Injections of [³H]proline and [³H]leucine into the MLR revealed descending neurons from the MLR that were located mainly on the ipsilateral side. In line with this anatomical observation, Garcia-Rill and Skinner (1987b) provided electrophysiological evidence in the cat that the MLR projected mainly to ipsilateral RS cells, that in turn projected to the ipsilateral spinal cord. Noga and colleagues showed that the pattern of post synaptic responses measured intracellularly in alpha-motoneurons (innervating flexor, extensor, or bi-functional muscles) in the L6-L7 spinal cord segments was similar

whether the ipsilateral or the contralateral MLR was stimulated. The segmental latency of the first locomotor EPSP detected in motoneuronal response was also similar on both sides (Noga *et al.*, 2003). They proposed that the slight asymmetry of the descending signal generated by unilateral MLR stimulation could be compensated by descending RS neurons projecting contralaterally directly or indirectly through spinal commissural neurons, to end up with a symmetrical motor output (Noga *et al.*, 2003). As indicated above, we found in lampreys an anatomical bilateral asymmetry in the projections between the MLR-RS cell projections. On the other hand, our physiological experiments revealed a striking symmetry in the MLR-RS inputs, suggesting that the anatomical asymmetry was fully compensated physiologically. Whether this is the case in mammals remains to be established. The physiological connections between the MLR in RS cells on both sides have not been examined directly. In an attempt to do so, Orlovsky used intracellular recordings of RS cells and stimulated the MLR on each side with two different stimulating electrodes (Orlovsky, 1970). Small differences in the positioning of the stimulating electrodes and/or differences in the impedance of the two stimulating electrodes could account for differences in the size of synaptic responses of RS cells on both sides.

It appears therefore that unilateral activation of the MLR produces a bilateral symmetrical locomotor output. The bilateral projections from the MLR to RS cells are likely to play a crucial role in the symmetrical locomotor activity. Further experiments are needed to establish the contribution of bilateral connections in the spinal cord to this symmetry as well as the possible contribution of sensory inputs. Moreover, it is not

known whether crossing connections innervating the RS cells themselves in the hindbrain could also strengthen the bilateral symmetry observed during locomotion induced by a unilateral MLR stimulation. These issues should be examined further.

A locomotor boosting mechanism within the brainstem

The MLR has traditionally been described as a neural control area in the brainstem, which activates specific populations of reticulospinal neurons to initiate and control locomotion. As such, the MLR has been considered as part of a serial control system for locomotion (Forebrain structures → MLR → RS cells → spinal CPGs)

Recently, we described in lampreys a parallel projection from the MLR to a group of hindbrain neurons that, in turn, provide additional excitation to reticulospinal cells to amplify the locomotor output. These interesting findings were made as we were examining the role of muscarinic receptor activation of RS cells (Smetana *et al.*, 2007). We found that lamprey RS cells were activated by bath application of a cholinergic muscarinic agonist. Muscarine elicited sustained membrane depolarizations (~ 5 s duration) in RS cells that recurred at periodicity of ~ 60s. Interestingly, the depolarizations occurred simultaneously in pairs of homologous RS cells recorded intracellularly and calcium imaging showed that entire populations of RS cells were activated synchronously. The sustained depolarizations were also associated with ventral discharges, suggesting they could somehow participate in locomotion.

The effects of muscarine disappeared when TTX was added to the perfusion Ringer's suggesting that muscarine was not acting directly onto RS cells, but most likely

through neurons that were presynaptic to the RS cells. Indeed, it was found that the depolarizations resulted from the activation a group of hindbrain muscarinoceptive interneurons that projected to RS neurons. Lesion studies as well as local muscarine injections revealed that the population of muscarinoceptive cells was located between the caudal border of the MRRN and rostral border of the PRRN. Bilateral injections of atropine in this region prevented the depolarization of RS neurons when muscarine was bath applied.

There were cells in this region showing immunoreactivity for muscarinic receptors. In addition, calcium imaging experiments revealed that cells in this region displayed sustained rises in intracellular calcium to bath application of muscarine. These calcium responses persisted in the presence of TTX (Smetana *et al.*, 2010). It was also shown that the muscarinoceptive cells provided strong bilateral glutamatergic inputs to the RS cells. Paired recordings of RS neurons and muscarinoceptive interneurons were carried out; depolarization of the muscarinoceptive cell induced short latency EPSCs in the RS cell, suggesting that the connections were monosynaptic (Smetana *et al.*, 2010). The physiological significance of these connections was then determined. First, it was found that these muscarinoceptive cells received inputs from the MLR that were blocked by the muscarinic receptor antagonist, atropine. The direct connectivity between the MLR and the muscarinoceptive neurons was also confirmed anatomically. Cells were labeled and the MLR and both sides after injecting biocytin into the muscarinoceptive cell region. These results confirmed that the MLR not only projected to RS neurons MLR, but it also projected to a group of muscarinoceptive cells that in

turn sent by natural excitation to RS neurons. This parallel pathway amplifies significantly the synaptic input received by the RS cells during the activation of the MLR. The specific role was confirmed using a semi-intact swimming preparation. Bilateral injections of atropine in the muscarinoceptive cell area considerably depressed the depolarization responses of the RS cells to MLR stimulation and modified the locomotor output (Fig. 5). The linear relation between swimming frequency and MLR stimulation intensity was dramatically depressed by the bilateral injection of atropine in the muscarinoceptive neurons area (Smetana *et al.*, 2010).

There is a strikingly linear relationship between the MLR stimulation intensity and locomotion frequency in many animal species. We have now found in lampreys that when the MLR is intensively activated, a population of muscarinoceptive cells located in the hindbrain is recruited to literally “boost” the locomotor output. Whether this mechanism is present in other vertebrate species remains to be determined. In rat, the ejection of cholinergic agonists (carbachol, methacholine and arecoline) in the medioventral medulla induces locomotion that is prevented by atropine, thus suggesting a role for muscarinic receptors (Kinjo *et al.*, 1990). The neural substrate is however still unknown in mammals.

Gating of sensory inputs on RS neurons by the MLR

During locomotion, sensory inputs shape the activity of the central generating and controlling neural networks according to external and internal constraints. In turn, the central networks gate sensory influx (Graham Brown, 1911; Grillner, 1973; Grillner and

Rossignol, 1978; Rossignol and Gauthier, 1980). Gating has been described extensively in the mammalian spinal cord (Hultborn, 2001; Krawitz *et al.*, 2001; Rossignol *et al.*, 1981), but the underlying cellular mechanisms have not been identified yet. In lampreys, we found that muscarinic receptor activation depressed sensory inputs to RS cells (Le Ray *et al.*, 2004). The efficacy of the sensory-motor connection between trigeminal afferents and RS neurons was then tested in the context of a MLR-induced locomotor behavior (Le Ray *et al.*, 2010). For this purpose, intracellular recordings were made from RS cells in isolated brainstem preparations while the trigeminal sensory root on one side was electrically stimulated at a low frequency (0.1 Hz). The trigeminal EPSP was monitored before and after a 3 Hz train of stimuli was applied to the MLR for 15 s. Consecutively to MLR tonic activation, a powerful depression (>60%) of the trigeminal EPSP was observed in RS cells (Fig. 6). On average, both the peak and amplitude of the synaptic responses were significantly decreased and showed a progressive recovery about 30 min *in vitro* (Le Ray *et al.*, 2010).

It was found that the depression of the trigeminal-evoked EPSPs also depended on the level of MLR activation (Le Ray *et al.*, 2010). Different frequencies of stimulation were randomly applied to the MLR in order to reproduce different levels of locomotor activity in the isolated brainstem. We found that the level of depression was linearly correlated to the frequency of the MLR stimulation. The higher was the MLR stimulation frequency, the stronger was trigeminal EPSP depression until a maximal depression was obtained at 7-8 Hz. Increasing the stimulation frequency further did not produce more

depression. Interestingly, the sensory depression was maximal for MLR stimulation frequencies that were sub-maximal for swimming speeds (see Sirota *et al*, 2000).

The time course of the depressive effects produced by the activation of the MLR suggested the involvement of metabotropic mechanisms, and because the MLR contains cholinergic neurons, the implication of muscarinic receptors was tested. Activating the MLR in the presence of the muscarinic receptor antagonists, atropine, MLR stimulation produced a far less depression of trigeminal EPSPs in RS cells (~15% EPSP reduction). There was still a residual depression suggesting that other neurotransmitter systems can be involved. Altogether, these results indicate that the MLR-induced depression of the RS neuron response to trigeminal nerve stimulation is largely mediated by muscarinic acetylcholine receptors. This is supported by the immunohistochemical demonstration of the presence of muscarinic receptors on RS cells (Le Ray *et al*, 2010).

Immunohistochemical labeling was also seen on cells located in the trigeminal descending tract (Northcutt, 1979), which was found to contain the second-order trigeminal sensory neurons that relay directly trigeminal inputs to RS neurons (Viana Di Prisco *et al*, 2005).

Local pressure application of ACh or its muscarinic agonist pilocarpine onto the recorded RS neuron or in the trigeminal relay area reproduced the depressive effects of the MLR activation onto the trigeminal EPSP, without affecting the membrane potential of the recorded RS cell (Le Ray *et al*, 2004). Conversely, EPSP enhancement was observed when atropine (or scopolamine, another muscarinic antagonist) was substituted for the muscarinic agonists in the ejection pipette or when it was bath-

applied. This suggests the existence of a tonic inhibitory control exerted via muscarinic receptors onto the trigemino-reticular connection. In addition, pre-incubation with atropine totally prevented the depressive effects of the muscarinic agonists. Several lines of evidence also indicate that the muscarinic modulation is predominantly exerted on the NMDA receptor-mediated component of the glutamatergic EPSP elicited by trigeminal stimulation, without affecting the glycinergic one (see Viana Di Prisco *et al.*, 1995): the depolarizing responses to direct application of NMDA onto the recorded RS cells were enhanced by atropine, whereas the responses to AMPA application were not. Moreover, blocking NMDA receptors with AP5 abolished the effects of muscarinic agonists and antagonists on the trigeminal-evoked EPSP; muscarinic drug applications usually had little effect on the early part of the synaptic responses. In contrast to most of the cases reported in the literature (Jiang and Dun 1986; Scanziani *et al.* 1995; Bellingham and Berger 1996; Smolders *et al.* 1997), the muscarinic modulation of the RS responses to glutamate relies mainly on postsynaptic mechanisms in lampreys (i.e., at the level of the RS neuron itself). Whether this is also the case within the trigeminal relay area where muscarinic modulation also clearly occurs will need to be examined.

As reported above, stronger trigeminal stimulation induces sustained depolarizations in RS neurons, which trigger swimming activity in the spinal cord (Viana Di Prisco *et al.*, 1997, 2000). There is evidence that a tonic muscarinic inhibition of this sustained activity occurs (Le Ray *et al.*, 2004): the duration of the depolarizing plateaus displayed a 5-folds increase when atropine was perfused on the brainstem; the threshold for inducing a sustained depolarization was reduced by half; the firing rate

during maximal responses was increased more than twice. Because the RS neuron input resistance, resting potential, and after-hyperpolarization were not affected by atropine perfusion, the enhancement of the sustained depolarizations likely resulted from a specific action on the synaptic response to trigeminal excitatory inputs. Interestingly, when depolarizations were evoked by local ejection of NMDA onto the recorded RS neuron, atropine unmasked membrane potential oscillations that occurred on top of the depolarizing plateau, and spiking occurred on top of each oscillation during the whole duration of the NMDA-evoked oscillatory behavior (Le Ray *et al*, 2004). The persistence of these oscillations under TTX suggested an intrinsic nature for the NMDA-induced activity. In the lamprey RS neurons, sustained depolarizations require the activation of NMDA receptor (Viana Di Prisco *et al*, 2000) by trigeminal inputs, and a 30-Hz electrical stimulation of a trigeminal nerve could also trigger membrane potential oscillations in RS cells in the presence of atropine (Le Ray *et al*, 2004). Experiments performed on the isolated hindbrain demonstrated that RS oscillations could also occur in the absence of atropine and were blocked by the local ejection of a muscarinic agonist onto the recorded RS neuron (Le Ray *et al*, 2004). Because the spinal cord was removed, the NMDA-induced oscillations could not result from the ascending spinal inputs (Dubuc and Grillner 1989; Vinay and Grillner 1993; Vinay *et al*. 1998a, b).

Conclusions

The supraspinal control locomotion relies in large part on neural regions within the brainstem and forebrain specifically dedicated to locomotion. The MLR is one such

region at the mesopontine border identified in all vertebrate species examined to date. It is believed to channel brainstem and forebrain inputs to then activate populations of RS cells in order to initiate and control locomotion. Examining the detailed downstream effects of the MLR in lampreys has yielded new information relative to the role of this region (Fig. 7). Inputs from the MLR to RS cells are strikingly symmetrical on both sides and this will play a significant role in generating symmetrical locomotion. The MLR does not only project to RS cells, but it also sends powerful inputs to a population of muscarinoceptive cells that provide additional excitation of RS cells, considerably amplifying locomotor output. In addition to controlling locomotor output, the MLR has now been shown to gate sensory inputs in the brainstem. The detailed mechanisms by which this sensory modulation operates have not been fully identified, but muscarinic receptors are involved. Future research will be needed to elucidate the detailed mechanisms involved as well as the functional importance of such sensory modulation.

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Figure Legends

Figure 1: The supraspinal control of locomotion. The general organization of the supraspinal control of locomotion has been described in mammals and some of the relevant structures and their connections are schematically illustrated on a sagittal view of the forebrain and brainstem. DLR: Diencephalic Locomotor Region; MLR: Mesencephalic Locomotor Region; RF: Reticular Formation.

Figure 2: The velocity of swimming movements is correlated to the intensity of MLR stimulation. EMG recordings of rostral, middle and caudal body segments during MLR-induced swimming at different intensities of stimulation (A panel) with sketches of the body swimming movements (B panel). The MLR was electrically stimulated with trains of stimuli of 1ms duration (10Hz pulses) at 1.5 μ A (A1, B1) 2 μ A, (A2, B2); 2.5 μ A (A3, B3). (Adapted from Sirota *et al.*, 2000).

Figure 3: Post synaptic potentials recorded in homologous RS cells on both sides in response to unilateral stimulation of the MLR. (A) Data from ipsilateral (ipsi) and contralateral (contra) reticulospinal (RS) cells from the middle rhombencephalic reticular nucleus (MRRN). (B) Data from ipsi and contra RS cells from the posterior rhombencephalic reticular nucleus (PRRN). (A1, A2, B1, B2) Bilateral graded responses recorded simultaneously from homologous RS cells to increasing intensity of stimulation of the MLR on one side. All traces are averages of three sweeps. **(A3, B3)** Relationship

between the intensity of MLR stimulation and the area of post synaptic potentials elicited in ipsilateral (black squares) and contralateral (blue circles) RS cells. Data from RS cells in the MRRN and in the PRRN are from different preparations. (Adapted from Brocard *et al.* (2010)).

Figure 4: Homologous reticulospinal (RS) cells display a symmetrical output in frequency when swimming is generated by a unilateral stimulation of the MLR. (A) Simultaneous paired intracellular recordings from homologous ipsilateral (ipsi) and contralateral (contra) RS cells of the middle rhombencephalic reticular nucleus (MRRN) during unilateral MLR electrical stimulation at 4.5 μ A in a semi-intact preparation of larval lamprey. The duration of the electrical stimulation applied to the MLR is indicated by the stimulation bar (Stim MLR) below the recordings. Note the enlargement illustrating the antiphasic relationship between ipsilateral and contralateral RS neurons. (B) Kinematic analysis (15 frames/s) of one representative swimming cycle elicited by unilateral MLR stimulation (4.5 μ A). Tracking positions of markers equidistantly distributed along the body of the animal using software analysis revealed that left and right maximal bending angles of the body are not statistically different, indicating the bilateral symmetry of swimming movements in response to unilateral stimulation of the MLR. (C) Discharge frequencies of homologous ipsilateral (black squares) and contralateral (gray dots) RS neurons in the same animal. Each dot illustrates the mean \pm SEM discharge frequency for a 20 s bout of MLR stimulation. Each intensity was tested three times. RS discharge frequencies are expressed in percentage of the maximal RS discharge frequency. (D)

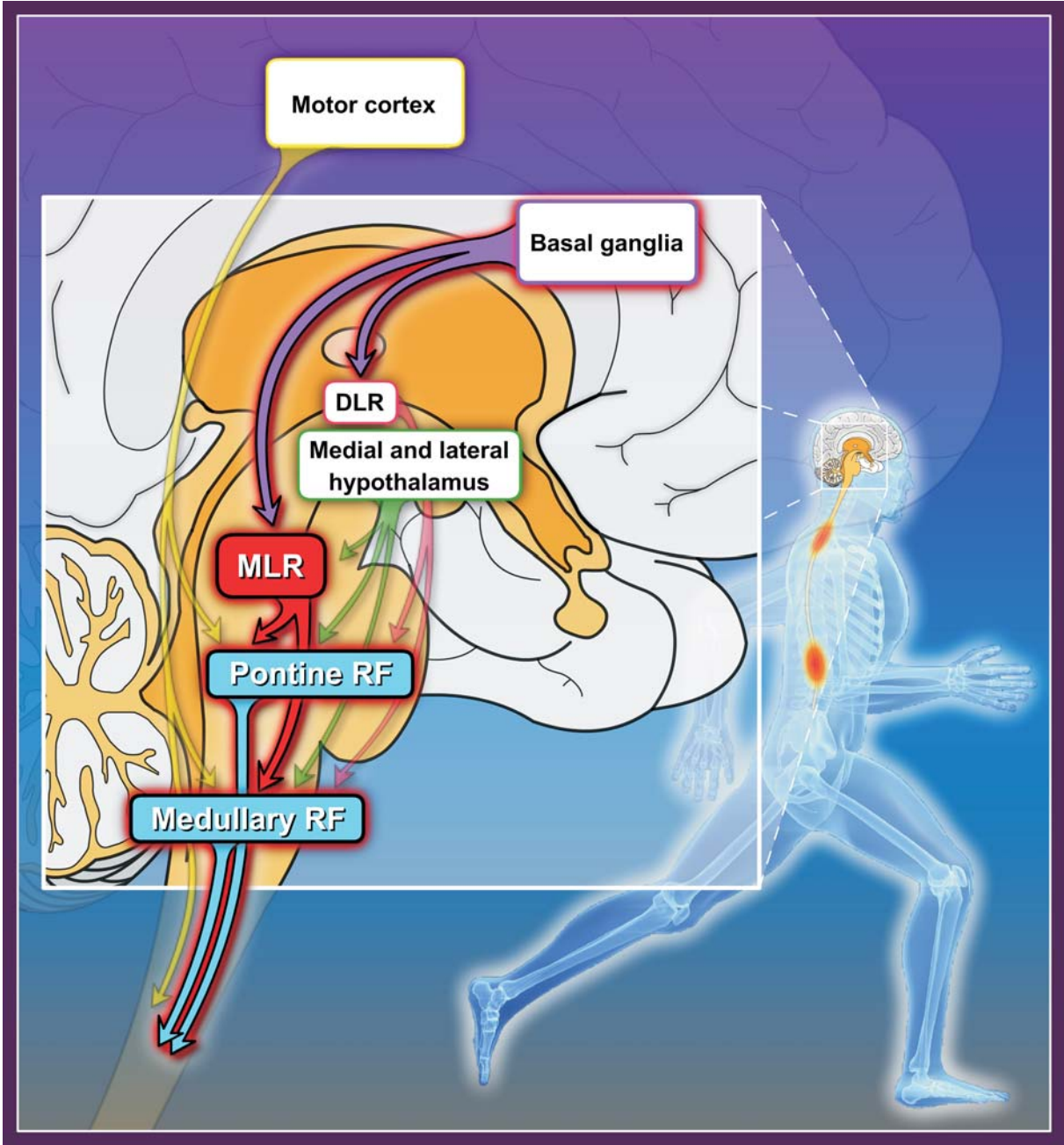
Relationships between the intensity of unilateral stimulation of the MLR and the discharge frequencies of ipsilateral (black squares) and contralateral (blue dots) RS neurons in five animals. Both ipsilateral and contralateral data followed a highly similar cubic polynomial function (black and blue solid lines, respectively). The dotted lines illustrate the prediction intervals for each fit at 95%. Data in A, B, C are from the same animal. (Adapted from Brocard *et al.*, 2010).

Figure 5: Inactivation of muscarinoceptive neurons slows down swimming movement velocity. (A1) Schematic representation of the experimental set-up. Swimming is induced in a semi-intact preparation by the stimulation of the MLR. The activity of RS neurons is recorded intracellularly and EMG is used to assess the swimming activity. After ejection of atropine over the muscarinoceptive neurons, the swimming frequency and the oscillation of RS neuron membrane potentials are slowed down in comparison to control (compare A2 left and right). (A3) Graphic representation of the swimming frequency as a function of MLR stimulation intensity. The ejection of atropine over the muscarinoceptive neurons prevent the production of fast swimming even when the MLR is stimulated at high intensity. (Adapted from Smetana *et al.*, 2010).

Figure 6: Synaptic transmission of trigeminal sensory nerve inputs to RS neurons is depressed by MLR stimulation. (A) Schematic representation of the experimental design. Stimulation (St.) electrodes are positioned within the MLR and on the trigeminal nerve, and the activity of a RS neuron is recorded using an intracellular electrode. (B, top) The

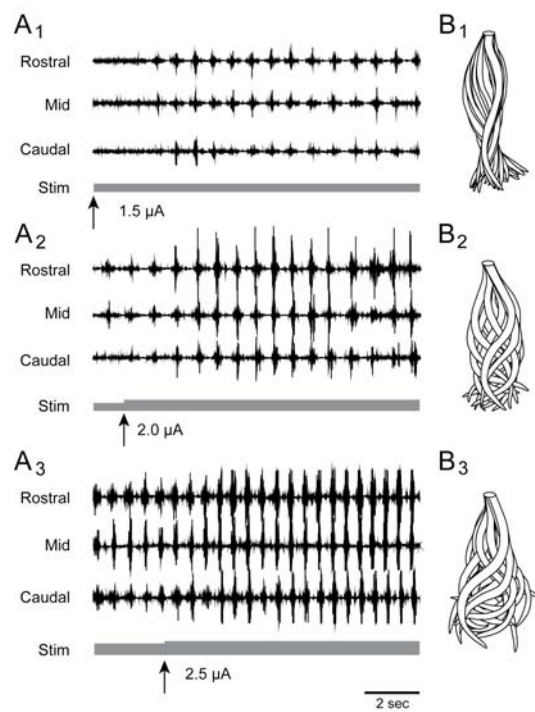
area of EPSP recorded from RS neurons in response to stimulation of the trigeminal nerve is depressed for several minutes by the stimulation of the MLR (arrow). To compare, see the difference between superimposed EPSP in control (1) and post-MLR-stimulation (2) conditions. (B, bottom) Perfusion of atropine (10 μ M) prevented the EPSP depression. (Adapted from Le Ray *et al.*, 2010).

Figure 7: Schematic representation of the proposed brainstem locomotor control circuitry, based on the results reviewed in this paper. A first series of experiments established the contribution of bilateral glutamatergic and cholinergic inputs from the MLR to RS neurons. Further experiments brought to light a group of muscarinoceptive cells located at the pontomedullary border that receives direct input from the MLR and increases RS cell activity and locomotor output. Finally, the MLR modulates sensory transmission in the brainstem by likely acting at the level of sensory relay cells in the lateral part of the brainstem.



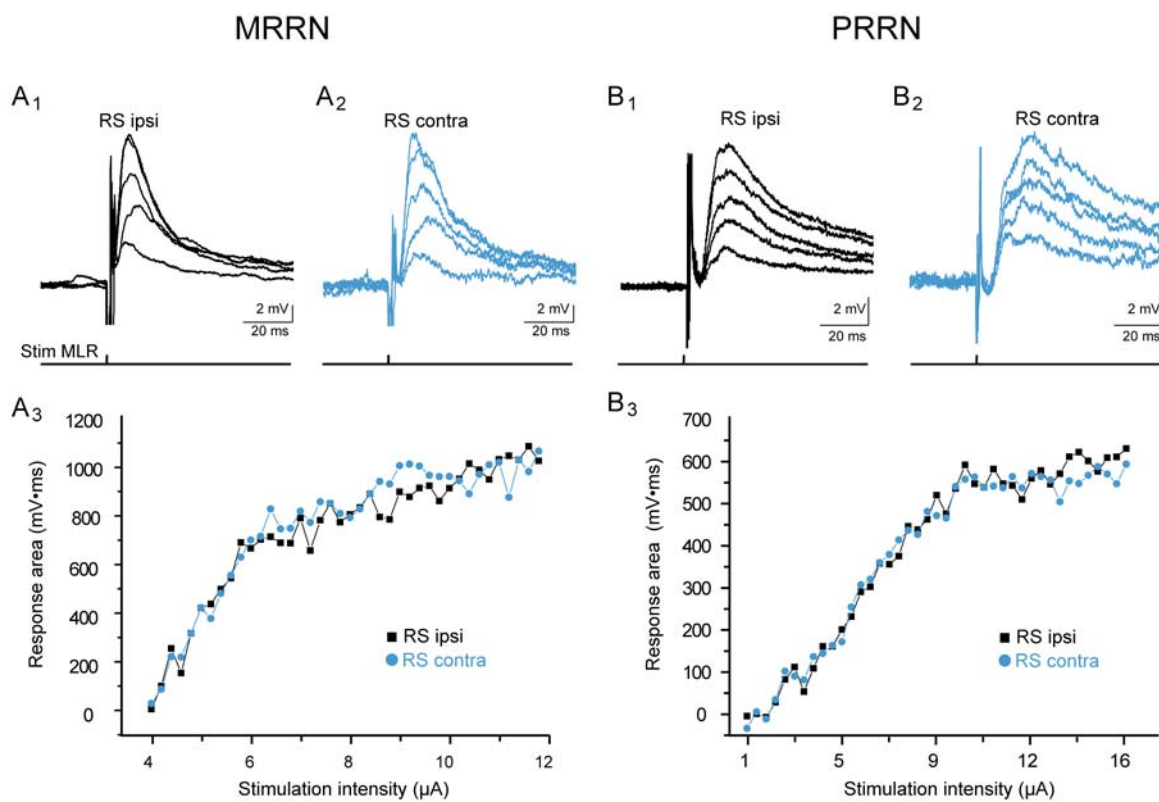
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Figure 1



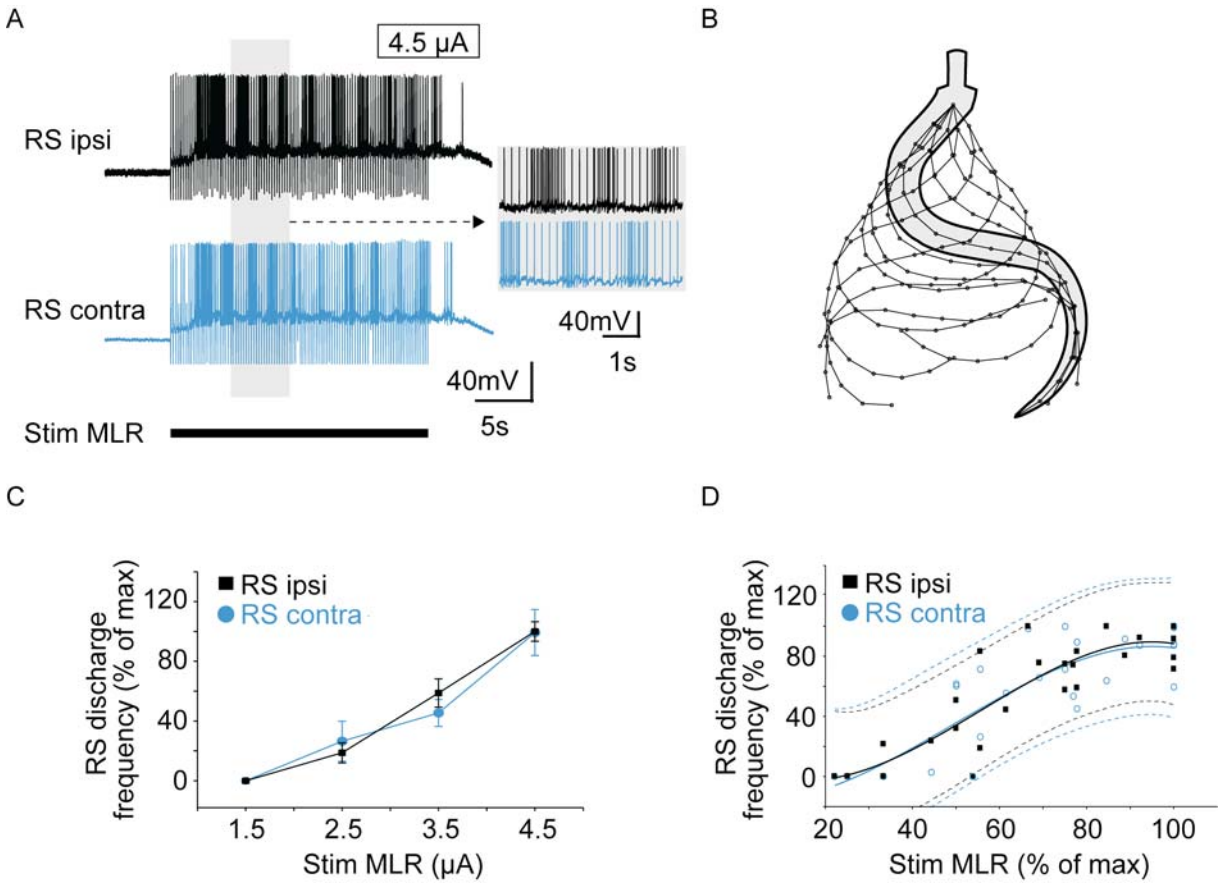
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Figure 2



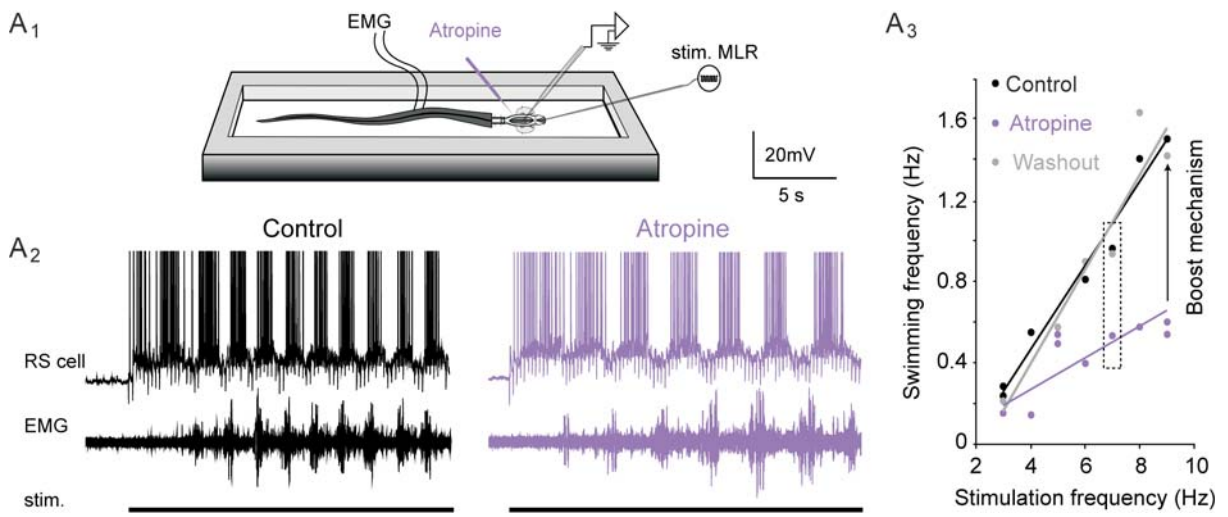
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Figure 3



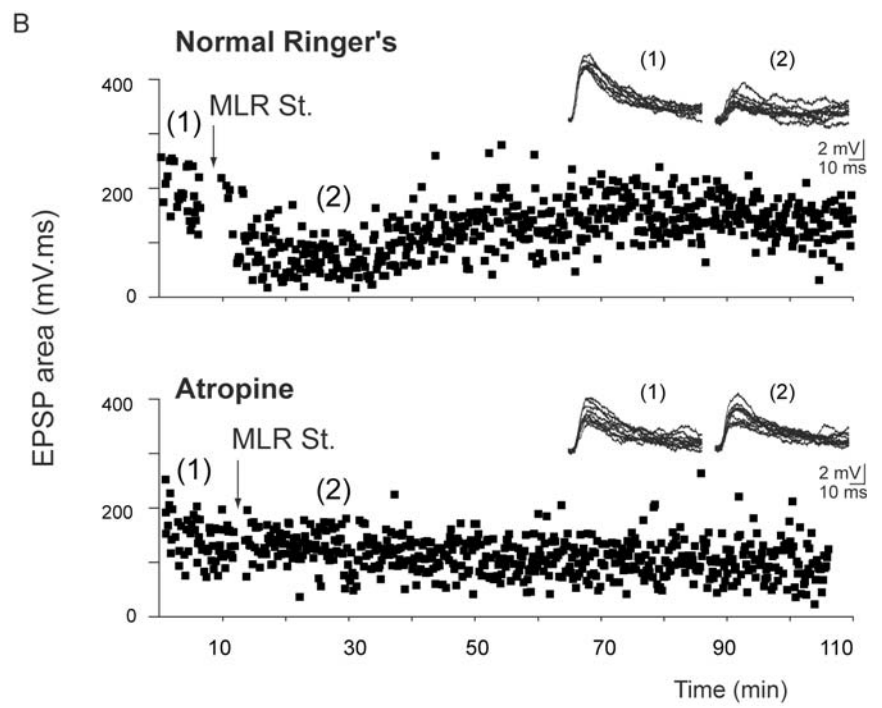
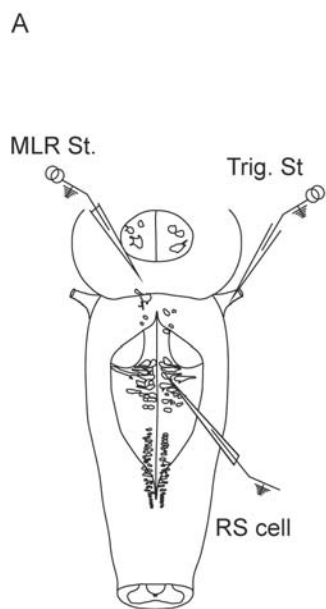
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Figure 4



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Figure 5



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Figure 6

