Modulatory Effect of Substance P to the Brain Stem Locomotor Command in Lampreys

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INTRODUCTION

In all vertebrates, locomotion is generated by locomotor networks in the spinal cord that are controlled by supraspinal structures to adapt the locomotor movements to desired goals and the environment (Drew et al. 2004; Grillner et al. 1997, 1998; Jordan 1998; Mori et al. 2001). Neuromodulators, usually amines and peptides, have been shown to play an important role in these adaptations (Harris-Warrick and Marder 1997). The latter cells constitute the main descending pathway controlling locomotor activity (Brodin et al. 1985; Buchanan 2001; Deliagina et al. 2002). Whereas the DLR is under a GABAergic control from the ventral pallidum (Pombal et al. 1997), the forebrain inputs to the MLR remain unknown in lampreys. Similarly to higher vertebrates, there is a widespread distribution of tachykinin immunoreactive cell bodies and fibers in the lamprey CNS (Auclair et al. 2004; Nozaki and Gorbman 1985, 1986; Pombal et al. 1997) and the MLR (Brocard and Dubuc 2003; Le Ray et al. 2003; Sirotas et al. 2000). Electrophysiological evidence are showing that both the DLR and MLR of lampreys induce swimming through monosynaptic connections with reticulospinal (RS) cells (Brocard and Dubuc 2003; El Manira et al. 1997). The latter cells constitute the main descending pathway controlling locomotor activity (Brodin et al. 1988; Buchanan 2001; Deliagina et al. 2002). Whereas the DLR is under a GABAergic control from the ventral pallidum (Pombal et al. 1997), the forebrain inputs to the MLR remain unknown in lampreys. Similarly to higher vertebrates, there is a widespread distribution of tachykinin immunoreactive cell bodies and fibers in the lamprey CNS (Auclair et al. 2004; Nozaki and Gorbman 1985, 1986; Pombal et al. 1997; Van Dongen et al. 1986, 1990), the reticular formation (Garcia-Rill and Skinner 1987; Kinjo et al. 1990; Kungel et al. 1994; Sholomenko et al. 1991), ventral tegmental area (Kelley et al. 1979, 1985), and the substantia nigra (Kelley and Iversen 1978; Kelley et al. 1985) also induce or increase locomotor activity.

There are two supraspinal locomotor centers described in lampreys: the diencephalic locomotor region (DLR) (El Manira et al. 1997) and the MLR (Brocard and Dubuc 2003; Le Ray et al. 2003; Sirotas et al. 2000). Electrophysiological evidence are showing that both the DLR and MLR of lampreys induce swimming through monosynaptic connections with reticulospinal (RS) cells (Brocard and Dubuc 2003; El Manira et al. 1997). The latter cells constitute the main descending pathway controlling locomotor activity (Brodin et al. 1988; Buchanan 2001; Deliagina et al. 2002). Whereas the DLR is under a GABAergic control from the ventral pallidum (Pombal et al. 1997), the forebrain inputs to the MLR remain unknown in lampreys. Similarly to higher vertebrates, there is a widespread distribution of tachykinin immunoreactive cell bodies and fibers in the lamprey CNS (Auclair et al. 2004; Nozaki and Gorbman 1985, 1986; Pombal et al. 1997; Van Dongen et al. 1986, 1990; Weigle and Northcutt 1999; Weigle et al. 1996; Yanez et al. 1997) and the MLR (Brocard and Dubuc 2003; Le Ray et al. 2003; Sirotas et al. 2000). Electrophysiological evidence are showing that both the DLR and MLR of lampreys induce swimming through monosynaptic connections with reticulospinal (RS) cells (Brocard and Dubuc 2003; El Manira et al. 1997). The latter cells constitute the main descending pathway controlling locomotor activity (Brodin et al. 1988; Buchanan 2001; Deliagina et al. 2002). Whereas the DLR is under a GABAergic control from the ventral pallidum (Pombal et al. 1997), the forebrain inputs to the MLR remain unknown in lampreys. Similarly to higher vertebrates, there is a widespread distribution of tachykinin immunoreactive cell bodies and fibers in the lamprey CNS (Auclair et al. 2004; Nozaki and Gorbman 1985, 1986; Pombal et al. 1997; Van Dongen et al. 1986, 1990; Weigle and Northcutt 1999; Weigle et al. 1996; Yanez et al. 1997). This study was carried out to examine the possible role of substance P on the supraspinal locomotor command system in lampreys. The effects of the drug were examined on reticulospinal cells and on the occurrence of swimming in a semi-intact preparation. Bath applications of substance P induced sustained depolarizations occurring rhythmically in intracellularly recorded reticulospinal cells. Spiking activity was superimposed on the depolarizations and swimming was induced. The sustained depolarizations were abolished by tetrodotoxin, and substance P did not affect the membrane resistance of reticulospinal cells nor their firing properties, suggesting that it did not directly effect reticulospinal cells. To establish where the effects were exerted, successive lesions of the brain stem were made as well as local applications of the drug in the brain stem. Removing the mesencephalon abolished the sustained depolarizations, whereas large ejections of the drug in the mesencephalon excited reticulospinal cells and elicited bouts of swimming. More local injections into the mesencephalic locomotor region (MLR) also elicited swimming. After an injection of substance P, the current threshold needed to induce locomotion by MLR stimulation was decreased, and the size of the postsynaptic responses of reticulospinal cells to MLR stimulation was increased. Substance P also reduced the frequency of miniature spontaneous postsynaptic currents in reticulospinal cells. Taken together, these results suggest that substance P plays a neuromodulatory role on the brain stem locomotor networks of lampreys.

METHODS

Animals

Experiments were performed on larval (n = 41) and young adult (n = 3 newly transformed) sea lampreys, Petromyzon marinus (9–14 cm in length). The animals were collected from streams entering Lake Champlain and were kept in fresh water maintained at 8°C. Some of the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
the animals were purchased from ACME lamprey. All surgical and experimental procedures conformed to guidelines of the Canadian Institutes of Health Research and were approved by the University Animal Care and Use Committee. The animals were anesthetized with tricaine methanesulphonate (MS 222, 100 mg/l), incised along the ventral midline, and eviscerated. The dissection and experiments were performed in cold oxygenated Ringer (8–10°C) with the following composition (in mM): 130 NaCl, 2.1 KCl, 2.6 CaCl₂, 1.8 MgCl₂, 4 HEPES, 4 dextrose, and 1 NaHCO₃, at pH 7.4. A dorsal incision was made to expose the rostral spinal cord and the brain. All brain tissue rostral to the diencephalon was cut and removed. The spinal cord was sectioned between the fourth and fifth segments, and the isolated brain/spinal cord preparation was placed dorsal side up in a recording chamber continually perfused with Ringer at a rate of 4 ml/min. A semi-intact preparation was also used, in which the rostral end of the animal was dissected out as described in the preceding text and the tail was left intact to swim freely in the Ringer solution. More than 2 h was allowed between the end of the dissection and the beginning of recording sessions.

**Experimental procedures**

Intracellular recordings were made from RS neurons in the middle (MMRN, n = 63) and in the posterior (PRRN, n = 4) rhombencephalic reticular nucleus, using sharp glass microelectrodes filled with 4 M potassium acetate (80–130 MΩ). The signals were amplified by an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA; sampling rate: 2–10 kHz). Only RS neurons with a stable membrane potential (≥15 min after impalement) lower than −70 mV were included in the study. Current versus voltage (I–V) relationships were examined by injecting both hyper- and depolarizing current pulses in discontinuous current clamp mode (−4 to +2 nA, 500 ms, average of 3 trials). Repetitive firing properties were studied by increasing the injected depolarizing current (500-ms duration).

Using an axopatch 200B amplifier (Axon Instruments), whole cell patch-clamp recordings were also performed from RS cells. Patch electrodes (3–6 MΩ) were pulled from borosilicate glass capillaries (1.5 mm OD, 1.12 mm ID; World Precision Instruments, Sarasota, FL). The ionic composition of the internal solution was (in mM) 140 potassium gluconate, 5 NaCl, 2 MgCl₂, 10 HEPES, 0.5 EGTA, 3 ATP, and 6 GTP, at pH 7.4. Small RS cells, located in the caudal part of the MRRN, were recorded to obtain a better space clamp. The holding potential was set at −80 mV. After a seal formation (>1 GΩ), a brief suction allowed access to the intracellular space (Alford et al. 1995). Input resistance of the cell and access resistance were continuously monitored during the recording period. Cell recordings that showed >15% change in access resistance were discarded from further analysis. Spontaneous miniature postsynaptic currents (mPSCs) were recorded under tetrodotoxin (TTX). They were low-pass filtered at 2 kHz and digitized at 10 kHz.

The MLR was electrically stimulated with a glass-coated tungsten microelectrode (4–5 MΩ with a 10-μm tip exposure). Single stimuli of different intensities were delivered every 30 s to examine the synaptic inputs from the MLR to RS cells. Five traces (at each intensity) were computed and averaged to improve the signal-to-noise ratio. In semi-intact preparations, swimming was induced by repeated electrical stimulation of the MLR (1.5-ms square-wave pulses, 0.5–15 μA, at a frequency of 2 Hz) (see Brocard and Dubuc 2003; Sirotta et al. 2000). Each stimulation bout lasted 40 s, and the interval between successive trials was ≥3 min. The stimulus artifacts were clipped on the intracellular traces using a homemade software.

Chemical stimulation of the MLR was also performed by local pressure injections of substance P. In these experiments, the dorsal isthmus was cut to facilitate the placement of both the electrical and the chemical stimulation electrodes in the MLR on one side. The lesion did not induce differences in control of locomotion induced by the MLR. Once the MLR was localized by electrical stimulation, a glass micropipette containing substance P was first placed adjacent to the electrical stimulation one, by using a M-3333 micromanipulator (Narishige Group). Thereafter, its tip was lowered in the tissue at the same level as the electrical stimulation electrode. At the end of some experiments, the location of the injection was marked by injection of similar volume (0.5–10 nl) of Ringer solution containing horseradish peroxidase (HRP, 10%). The fixed brains (4% paraformaldehyde in 0.1 M phosphate buffer overnight) were cryoprotected with 30% sucrose, frozen, and cut at 25-μm thickness on a cryostat (American Optical, Buffalo, NY), and the sections were mounted on gelatinized slides and left to dry overnight. The sections were washed three times with PBS, and the presence of HRP was revealed by reacting the sections with 0.05% DAB and 0.01% H₂O₂ in PBS for 5 min. The sections were washed again three times with PBS and counterstained with cresyl violet. They were then dehydrated through graded concentrations of alcohol, cleared in xylene, and mounted with Entellan (VWR, Montréal, Québec, Canada). Photomicrographs were acquired with a Nikon Coolpix 995 digital camera custom-fitted on a Nikon Optiphot-2 microscope.

**Locomotion** was monitored using electromyographic (EMG) recordings. Pairs of Teflon coated stainless steel wires (diameter: 50 μm; California Fine Wire, Grover Beach, CA) were inserted into the myotomes between segments 20 and 25. The animals were relatively small, and thus it was often difficult to insert two pairs of wires into myotomes, one on each side. However, because we used semi-intact preparations, locomotor movements of the tail could be visualized to ascertain the quality of the swimming. The EMG signals were amplified (1,000 times), filtered (bandwidth: 30 Hz to 1 kHz) and acquired with a sampling rate of 5 kHz. They were then rectified and filtered (time constant: 10 ms) for analysis. The cycle period as well as the amplitude and the duration of the EMG bursts were measured using a homemade software. The period (swimming frequency) was measured as the interval between the onset of two consecutive bursts on the same side. For each testing session, the parameters were calculated by averaging ~10 consecutive cycles during a steady-state sequence of locomotion.

**Drugs**

All drugs were purchased from Sigma-Aldrich (Oakville, Ontario, Canada), kept as a concentrated stock solution, and dissolved to their final concentration in the Ringer solution. The following pharmacological agents were bath applied: substance P (1 μM), tetrodotoxin (TTX, 1 μM). Substance P was bath applied during 30 min only once in each preparation because it is known to produce long-lasting modulation of neuronal activity (Parker et al. 1998). Substance P (100 μM) was also pressure-ejected through a glass micropipette with a Picospritzer (General Valve, Fairfield, NJ). The inactive dye Fast Green was added to the drug solution to monitor the extent of the application. Control ejections of Ringer with Fast Green alone did not evoke postsynaptic potentials nor did it change the threshold of locomotion induced by MLR stimulation.

**Data acquisition and analysis**

Electrophysiological data were acquired through a Digidata 1322A interface with Clampfit 9.0 software (Axon Instruments). Intracellular signals were analyzed with Clampfit 9.0 software. Data are presented in the text and in the table as means ± SE. The input resistance was quantified by using the linear I–V relation. The rheobase was defined as the minimum current intensity needed to fire the cell. Several parameters related to the depolarizations induced by substance P were measured. The global activity of RS cells was quantified for fixed periods of time by measuring the area of the depolarizations defined as the surface under the intracellular trace. The area was measured from the resting membrane potential. The maximal amplitude of the sustained depolarizations was measured after filtering the spikes when
FIG. 1. Effects of a bath application of substance P (1 μM) on the activity of reticulospinal (RS) cells in the in vitro isolated brain/spinal cord preparation. A1: schematic representation of the experimental paradigm. A2: intracellular activity of a RS cell recorded continuously before and during bath application of substance P. A horizontal bar below the intracellular recording indicates the duration of the substance P application. Shaded areas in A2 are shown at a faster time scale in B, 1–3. Shaded area in B3 is shown at a faster time scale in C.
they occurred. The cycle period was defined as the time between the onsets of two consecutive depolarizations. The regularity of rhythmic activity was determined by computing the coefficient of variation of the period ([SD of period × 100]/mean period). The peak amplitude of the evoked postsynaptic potentials was defined as the largest voltage deflection from the resting membrane potential.

mPSCs were analyzed using MiniAnalysis software (Synaptosoft, Decatur, GA). The inter-mPSCs intervals and mPSCs amplitude were measured. The analysis of mPSCs was performed with a cumulative probability plot.

A Student’s paired $t$-test was employed for statistical analysis when two groups were compared and a one-way ANOVA followed by a Tukey test for multiple-group comparisons. The cumulative probability of the amplitude and inter-event interval of the mPSCs was compared using a Kolmogorov–Smirnov (K-S) nonparametric test, giving a measure of the relative dispersion between two distributions. Groups of data having a $P$ value <0.05 were considered significantly different.

**RESULTS**

**Effect of substance $P$ application on the brain**

Substance $P$ (1 $\mu$M) was bath applied and its effects were examined on five RS cells of the MRRN recorded intracellularly in five different in vitro isolated brain/spinal cord preparations (Fig. 1A). During the 10-min period preceding the application of substance $P$, spontaneous depolarizations occurred sporadically (Fig. 1, A2 and B1). The duration of spontaneous depolarizations was on average $11.4 \pm 2.2$ s, and their mean period was $79.4 \pm 7.5$ s. During the first minutes after the application of substance $P$, the depolarizations began to occur more frequently and a rhythm emerged (Fig. 1, A2 and B2), reaching a stable frequency during the following 5–10 min (Fig. 1, A2 and B3). At this time, rhythmic depolarizations occurred with a relatively constant period throughout the application of substance $P$ (Fig. 1B3). After those initial experiments, the effects of bath-applied substance $P$ were measured (for 10 min) only after the induced rhythm had stabilized. The depolarization lasted on average $39.1 \pm 5.4$ s and the mean period was $60.5 \pm 6.6$ s. Substance $P$ significantly decreased the coefficient of variation of the period ($P < 0.001$, paired $t$-test; Table 1). The depolarizations were characterized by two components: an early large, short-lasting depolarization (see asterisk in Fig. 1C) followed by a late sustained depolarization. In three of five preparations, membrane potential oscillations were superimposed on the sustained depolarizations (see arrowheads in Fig. 1C). The period of these oscillations was on average $2.4 \pm 0.14$ s, a value compatible with the range of periods seen during fictive locomotion (Brodin et al. 1985).

The occurrence of these fast oscillations was very rare under control condition. They occurred only in 1 of 37 spontaneous depolarizations.

The possibility that the fast oscillations superimposed to sustained depolarizations could underlie swimming behavior was examined in two semi-intact preparations. A Vaseline wall was built below the fourth spinal cord segment delimiting two distinct watertight perfusion compartments (Fig. 2A). Bath application of substance $P$ in the rostral compartment evoked rhythmic sustained depolarizations accompanied by swimming movements (Fig. 2B). After a 15- to 20-min application of substance $P$, the depolarizations induced in RS cells of the MRRN were similar to that observed in previous in vitro preparations ($P > 0.05$, Student’s $t$-test). The mean period of sustained depolarizations was $63.8 \pm 20.7$ s, and their duration tended to be shorter ($23.5 \pm 3.5$ s, $P = 0.15$, Student’s $t$-test). Swimming developed after spiking occurred in the recorded RS cell and lasted for as long as the cell remained depolarized (Fig. 2C). The swimming rhythm, synchronized with the membrane oscillations, had a mean cycle duration of $2.0 \pm 0.2$ s (see Fig. 2C).

**Effect of substance $P$ on RS cells**

Experiments were then performed to determine whether the excitation induced by substance $P$ resulted from either direct effects on the intrinsic properties of RS cells or from synaptic inputs to those cells. Substance $P$ was pressure ejected onto RS cells in the MRRN ($n = 5$) or the PRRN ($n = 4$). In none of the cases did it depolarize the recorded RS cells nor did the juxtions over the MRRN or PRRN elicit locomotor activity. Likewise, in the presence of TTX, pressure or bath applications of substance $P$ failed to evoke any responses of RS cells ($n = 9$ cells of the MRRN, Fig. 3A). For the 12 RS cells of the MRRN tested, there were no changes in the rheobase ($2.9 \pm 0.53$ vs. $2.9 \pm 0.42$ nA before and after substance $P$, respectively; $P > 0.05$, paired $t$-test), in the input resistance ($9 \pm 1.4$ vs. $9.4 \pm 1.4$ M$\Omega$ before and after substance $P$, Fig. 3B), or in the repetitive firing properties, even in neurons displaying fast repetitive discharges (Fig. 3C). The mean number of spikes induced by a 500-ms depolarizing pulse at three times the rheobase intensity was $11.4 \pm 1.2$ before, and $11.3 \pm 3.4$ after substance $P$ ($n = 10$ RS cells; $P > 0.05$, paired $t$-test, Fig.

**TABLE 1. Characteristics of depolarizations recorded in RS cells before and after bath application of substance $P$**

<table>
<thead>
<tr>
<th>Control</th>
<th>DI/BR/SP</th>
<th>DI/BR</th>
<th>Brain Stem</th>
<th>Rhombencephalon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration, s</td>
<td>11.4 ± 2.2</td>
<td>16.8 ± 7.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Area, mV·ms</td>
<td>0.93 ± 0.21</td>
<td>0.09 ± 0.18</td>
<td>0.11 ± 0.03</td>
<td>0.15 ± 0.003</td>
</tr>
<tr>
<td>CV**, %</td>
<td>73.2 ± 4.6</td>
<td>68 ± 15.3</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substance $P$</th>
<th>Duration, s</th>
<th>Area, mV·ms</th>
<th>CV**, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>39.1 ± 5.4</td>
<td>4.62 ± 0.54</td>
<td>15.5 ± 5.4</td>
</tr>
</tbody>
</table>

| Values are means ± SE, $n = 5, 5, 4, and 4$ for DI/BR/SP, DI, BR, brain stem and rhombencephalon, respectively. *DI/BR/SP: diencephalon/brain stem/spinal cord. ** CV: coefficient of variation. |
These results suggest that the sustained rhythmic depolarizations induced by bath applications of substance P do not result from direct effects on RS cells but more likely from increased synaptic inputs.

**Effect of substance P on different parts of the brain**

The effects of successive lesions on the ability of substance P to induce sustained depolarizations in RS cells were examined to localize the site of substance P effects. Because substance P was previously shown to have a powerful influence on lamprey spinal neurons (Parker and Grillner 1998; Parker et al. 1998), a complete transection was made at the caudal end of the rhombencephalon to remove the spinal cord in five preparations (Fig. 4A1). After the transection, the characteristics of the spontaneous activity were not affected (Table 1; $P > 0.05$, Student’s $t$-test). Without the spinal cord, substance P still induced sustained rhythmic depolarizations with a mean duration of $36.8 \pm 6.2$ s and mean period of $66.3 \pm 11.6$ s (Fig. 4A2, top; Table 1). The global activity, quantified by a cumulative measure of the area of the depolarizations, was also the same before and after removing the spinal cord (Table 1; $P > 0.05$, 1-way ANOVA). However, removing the spinal cord abolished the membrane potential oscillations superimposed on sustained depolarizations. These results strongly suggest that the oscillations result from efferent feedback from the central pattern generator and that the spinal cord does not contribute to the sustained depolarizations induced by substance P.
The diencephalon was then removed in addition to the spinal cord (Fig. 4B1). Under control conditions, the previously observed spontaneous activity was almost absent and was subthreshold in the four preparations tested (Fig. 4B2, top). After substance P, rhythmical sustained depolarizations were still induced in the RS cells (Fig. 4B2, bottom). The depolarizations lasted on average 48.5 ± 5.6 s, and the mean period was 76.6 ± 8.8 s. These values were not significantly different from those obtained when the spinal cord and/or the diencephalon were kept (Table 1; \( P > 0.05 \), 1-way ANOVA). Likewise, the mean area of the global activity induced by substance P did not change (Table 1; \( P > 0.05 \), 1-way ANOVA). However, removal of the diencephalon had a differential effect on the two components of the sustained depolarizations. Although the early large short-lasting component of the response was not affected, the maximum amplitude of the late sustained depolarizations was significantly depressed (63.4 ± 12.9 and 61.9 ± 12.5% of the amplitude observed in the in vitro preparations with and without spinal cord, respectively; \( P < 0.05 \), 1-way ANOVA). This reduction in amplitude was accompanied by an absence of spiking throughout the late sustained depolarization (Fig. 4B2, bottom).

Finally, the spinal cord and the mesencephalon were removed, isolating the rhombencephalon (Fig. 4C1). Under control condition, spontaneous activity was absent in the four preparations tested (Fig. 4C2, top). Bath application of substance P did not induce the sustained depolarizations but only rhythmical subthreshold short-lasting depolarizations (Fig. 4C2, bottom). The latter lasted on average 6.6 ± 1.3 s, and the mean period was 24.7 ± 2.3 s. Both the duration and the period of these depolarizations were significantly shorter than those observed in the in vitro preparations tested above (Table 1; \( P < 0.05 \), 1-way ANOVA).

**FIG. 3.** Effects of substance P on the input resistance and repetitive firing properties of RS cells. A: Intracellular recording from a RS cell in the presence of TTX (1 μM) and after pressure ejections of substance P (†) over the cell recorded. B1: Membrane voltage responses after injection of 500-ms-long current pulses of increasing amplitude (from –4 to 2 nA, with increments of 1 nA) under control condition and in the presence of substance P. Bottom: Injected currents. B2: The current versus voltage relationship for all recorded cells (n = 12). C1: Spiking of a cell at a current level of 3 times the rheobase before and after pressure application of substance P. C2: Plot of the number of spikes of 3 neurons as a function of the amplitude of the injected current. The latter is expressed as multiples of the rheobase.
0.01 for the duration, \( P < 0.05 \) for the period, 1-way ANOVA). In the same way, the mean area of the global activity was significantly less (Table 1; \( P < 0.001 \), 1-way ANOVA).

It should be noted that the occurrence of rhythmic depolarizations induced by substance P occurred throughout the drug application in any of the preparations used.

**Effect of substance P on the mesencephalon**

The previous results suggest that the occurrence of long-lasting depolarizations requires the mesencephalon. To further test this hypothesis, substance P (100 \( \mu \)M) was ejected into the mesencephalic ventricle in two in vitro preparations (Fig. 5A1). A large depolarization with membrane potential oscillations was elicited in the two RS cells recorded in the MRRN in control (top) and in the presence of the substance P (bottom).

![Diagram](image)

FIG. 4. Effects of a bath application of substance P (1 \( \mu \)M) on the activity of RS cells recorded from in vitro brain/spinal cord preparations reduced by different transection levels of the nervous system. A1–C1: schematic representation of the experimental paradigm. A1: the transection level was performed at the obex. B1: the transections were performed at the rostral end of the mesencephalon and at the caudal end of the rhombencephalon (obex). C1: the transections were performed at the rostral and caudal ends of the rhombencephalon (obex). A2–C2: intracellular recordings from a RS cell of the MRRN in control (top) and in the presence of the substance P (bottom).
of them, substance P ejection on the mesencephalon initiated swimming (Fig. 5B2). The latency from the moment of ejection to the depolarization of the RS cell was the same than that observed in the isolated brain/spinal cord preparations (22.2 ± 10.1 s; $P > 0.05$). Swimming occurred with a mean latency of 27.3 ± 9.5 s from the moment of ejection. A fast swimming
activity emerged with a cycle duration of 648 ± 90 ms and lasted 68.3 ± 14 s (Fig. 5B3). Our results strongly suggest that substance P activates either directly or indirectly some locomotor center in the brain stem, including the MLR.

Effect of substance P on the MLR

The MLR was first localized by electrical stimulation after which substance P (100 μM) was injected into it in seven semi-intact preparations (Fig. 6A). A representative site of injection is illustrated in Fig. 7. In five preparations, swimming activity was induced, whereas uncoordinated movements were observed in the other two. The latency from the moment of injection to the onset of depolarization in intracellularly recorded RS cells of the MRRN was 17.7 ± 1.7 s. Locomotion was initiated on average 24.2 ± 2 s after the injection. Compared with locomotion induced by substance P ejected over the mesencephalon, the duration of swimming tended to be shorter (37.4 ± 12.7 s, P = 0.15, Student’s t-test), and the swimming cycle duration tended to be longer (1.17 ± 0.25 s, P = 0.12, Student’s t-test, Fig. 6B and expanded records in Fig. 6C). Multiple applications of substance P, each separated by an interval of 5 or 10 min, lead to a rapid decrease in the ability of the drug to induce swimming. Only the first two or three applications were effective and several hours (3–24 h) were needed for the effect to recover. Local injections of the neuropeptide in other parts of the mesencephalon did not induce locomotion (n = 4 preparations).

**FIG. 6.** Effects of injecting substance P into the MLR on the initiation of locomotion in a semi-intact preparation. A: schematic representation of the experimental paradigm. B: intracellular recording of a RS cell (top) during swimming evoked by pressure injection of substance P (†) into the MLR. The EMG recordings (bottom) were performed at segmental level 20 ipsi- (iEMG) and contralateral (cEMG) to the intracellular recordings. † in B is shown at a faster time scale in C. Note that the 2nd burst of lesser amplitude (for each locomotor cycle) in the EMG recordings likely results from contralateral muscle contractions picked up by the EMG wires. D: histogram depicting the MLR stimulation threshold for swimming induction before and after injection of substance P into the MLR. The threshold values are expressed in percent of the control value.
Experiments \( (n = 7) \) were carried out to examine whether substance P modifies the threshold current \( (T) \) needed to initiate locomotion by electrical stimulation of the MLR. First, substance P was injected into the MLR to initiate a bout of locomotion while a tungsten microelectrode was already positioned in the MLR. In all cases, one RS cell of the MRRN was recorded intracellularly on the side of the MLR that was stimulated. At the end of the locomotion bout, when the membrane potential of the RS cell had returned to resting level, the MLR was stimulated to define the threshold current needed to induce locomotion. We observed that \( T \) decreased by a 22.5 \( \pm \) 21.1\% \( (n = 7 \) preparations, \( P = 0.053, \) paired \( t \)-test; Fig. 6D). This effect could last for \( >30 \) min after the injection of substance P into the MLR. At 1–1.5 T of control, the cycle duration of locomotion induced by MLR stimulation decreased significantly from 1.0 \( \pm \) 0.05 to 0.9 \( \pm \) 0.04 s \( (P < 0.01, \) paired \( t \)-test; Fig. 8 compare A, 1 with 2, and B, 1 with 2) after substance P. Furthermore, swimming activity persisted longer after turning off the MLR stimulation \( (30.3 \pm 7.8 \) vs. \( 14.4 \pm 5 \) s; \( P < 0.05, \) paired \( t \)-test; compare Fig. 8A, 1 with 2). In two of seven preparations, the faster swimming was accompanied with bursts of larger amplitude \( (\text{Fig. 8B}; 1 \) with 2). For stronger stimulation \( (\geq 2 \) T of control), however, the intensity of the swimming was similar before and after substance P.

Synaptic inputs from the MLR to RS cells were also examined by applying single stimulation shocks to the MLR \( (n = 7 \) experiments). Interestingly, at stimulation intensities \( (1–1.5 \) T of control) where substance P increased the swimming output, the peak amplitude of the evoked postsynaptic potentials increased by 33.9 \( \pm \) 13.3\% \( (P < 0.05, \) paired \( t \)-test; Fig. 8C, 1 and 2). However, at higher stimulation intensities \( (\geq 2 \) T), the amplitude of the synaptic responses did not significantly differ from controls \( (\text{Fig. 8C}; 1 \) and 3).

**Presynaptic effect of substance P on neurotransmitter release**

The following experimental protocol was carried out to determine whether an enhancement of presynaptic neurotransmitter release participates in the increase of RS inputs observed after bath application of substance P. Using whole cell voltage-clamp recordings, mPSCs were isolated by adding TTX to the perfusing Ringer \( (1 \) \( \mu \)M, 30 min). The monosynaptic response from the MLR was abolished by TTX indicating that the drug had acted. The mPSCs were recorded during 3 min before (control), during (starting at 30 min after onset of substance P superfusion) and after washout. Five neurons were examined. An example of continuous recordings of mPSCs before and after adding substance P to the bath is shown in Fig. 9A. Under control conditions, the mean number of mPSCs recorded was 2356 \( \pm \) 410 \( (\text{for a period of 3 min}) \). At a holding potential of \( \approx 80 \) mV, their mean interval was 89.9 \( \pm \) 15.3 ms and their mean amplitude was 16.4 \( \pm \) 1.1 pA. Substance P \( (1 \) \( \mu \)M) caused a decrease of 33.4\% in the number of mPSCs \( (1570 \pm 314, P < 0.01, \) paired \( t \)-test; compare Fig. 9A1 and A2). Therefore the mean interval between synaptic events increased to 141 \( \pm \) 28 ms \( (P < 0.05, \) Paired \( t \)-test; compare arrows in Fig. 9B1) without affecting the amplitude of mPSCs \( (15.8 \pm 1 \) pA, \( P > 0.05, \) Paired \( t \)-test; Fig. 9C1). As illustrated in Fig. 9C1, the decrease in the number of events was evenly distributed. Cumulative distributions of mPSCs amplitudes and inter-event intervals confirmed these observations \( (\text{Fig. 9B2 and C2}) \). The inter-event interval curve was shifted toward lower frequencies \( (\text{in all cases} \ P < 0.001, \) K-S test; Fig. 9B2) with no change in the cumulative amplitude curve for 4 out of 5 RS neurons \( (P > 0.05, \) K-S test; Fig. 9C2). The effect of substance P was long-lasting since recovery did not occur after a washout of more than 3 h. In 2 experiments in which TTX was perfused alone \( (\text{i.e., without substance P}) \), the mPSC inter-event intervals did not change with time \( (P > 0.05, \) K-S test).

**Discussion**

Results from this study show that bath-applied substance P induces sustained rhythmic depolarizations in RS cells. These depolarizations are associated with the occurrence of bouts of locomotor activity in a semi-intact preparation. The mesencephalon and, more specifically, the MLR appears to play a crucial role because local applications of substance P in the MLR produce a long-lasting depolarization in RS cells as well as swimming in a semi-intact preparation. Substance P also decreases the current threshold needed to induce locomotion by MLR stimulation and increases the size of the synaptic responses of RS cells to MLR stimulation. Collectively, these results suggest that substance P plays a neuromodulatory role on the locomotor networks in the brain stem of lampreys.

**Effect of substance P on the brain stem**

We show that bath applications of substance P induce sustained rhythmic depolarizations in RS cells as well as...
swimming bouts. In lamprey spinal motoneurons, substance P was previously shown to induce rhythmic depolarizations, which are dependent on a spinal network activity (Svensson et al. 2002). In our experiments, the precise location of the effects of substance P is not yet fully identified. The rhythmic sustained depolarizations induced by substance P persisted after removing the spinal cord and the diencephalon, but were abolished after removing the mesencephalon. This suggests that a large part of the neuronal circuitry involved is located in the mesencephalon. It appears that the MLR is a key component of the network because injections of the neuropeptide into this region elicited long-lasting depolarization in RS cells that were similar to those induced by a bath-application of substance P. Although the diencephalon was not essential for the sustained depolarizations, a contribution is likely. Indeed, spiking activity superimposed on the sustained depolarization was abolished after removal of the diencephalon, suggesting that a decrease in excitability occurred in the reduced preparation. It
is not clear why the sustained depolarizations occur recurrently with a rather constant period when substance P is bath applied. A rhythm was also observed in the isolated rhombencephalon but it was faster than that observed in brain stem preparations and the depolarizations were shorter in duration. Altogether, these observations suggest that brain stem networks influenced by substance P and impinging on RS cells are distributed. Inhibitory GABAergic or glycinergic inputs could be responsible for the pauses occurring between the sustained depolarizations. However, other mechanisms such as activity-dependent depression of the network (decrease of the synaptic transmission), previously described in relation to recurrent spontaneous activity in developing spinal networks, could also contribute (Tabak et al. 2001). Further experiments are needed to determine the mechanisms by which the sustained depolarizations occur recurrently.

**FIG. 9.** Effects of substance P on amplitude and frequency of mPSCs. In the presence of TTX (1 μM), whole cell recordings were performed on RS neurons, voltage clamped at ~80 mV. **A:** Representative continuous records showing mPSCs in control condition (**A1**) and in the presence of substance P (1 μM; **A2**). **B-C:** frequency histogram (**B1, C1**) and cumulative probability plots (**B2, C2**) of mPSCs of the same neuron showing the distribution of the inter-event interval (**B**) and the peak amplitude (**C**) in control and in the presence of substance P. Arrows in **B1** and **C1** indicate the mean values from the cell recorded before and after application of substance P. Note that the cumulative curve of the inter-event interval is shifted to the right whereas the cumulative amplitude curve for the same neuron is unchanged, indicating that substance P reduces the frequency of mPSCs without altering their amplitude. Histogram and cumulative probability plot analysis were constructed from 2784 and 2033 individual events in control and in the presence of substance P, respectively.
Effect of substance P on the MLR

In the present study, several lines of evidence suggest that substance P exerts an excitatory effect on MLR neurons. We now show that unilateral injections of the peptide into the MLR elicited locomotion as previously reported in cats and rats (Garcia-Rill et al. 1986, 1990). However, the swimming bouts tended to be shorter and displayed a lower frequency than those observed when substance P was injected in the mesencephalic ventricle. In the latter case, it is likely that the MLR from both sides was activated because the laterodorsal tegmental nucleus (one component of the lamprey MLR) is located close to the ventricular surface (Le Ray et al. 2003). The initiation of locomotion and the depolarization of RS cells were characterized by a delayed onset from the moment of injection. The delayed responses likely reflect the slow action mode of substance P through protein kinases as previously demonstrated in the modulation of the NMDA-evoked swimming or sensory inputs at the lamprey spinal cord level (Parker and Grillner 1999; Parker et al. 1997, 1998). Substance P also decreased the electrical threshold for the initiation of swimming by MLR stimulation. It is thus possible that substance P enhances the excitability of MLR cells by depolarizing them. Depolarizing effects of substance P have been reported in several classes of neurons of different animal species in the past (Le Gal La Salle and Ben-Ari 1977; Ptak et al. 2000; Snijdelaar et al. 2000; Svensson et al. 2002). Compatible with this view, was the increase in amplitude of the synaptic inputs from the MLR to RS cells. However, this occurred only for MLR stimulation at low intensity. By depolarizing MLR cells, substance P would cause a greater number of MLR cells to be recruited at low stimulation strengths. On the other hand, most MLR cells could already be recruited at higher stimulation intensities, and substance P would not cause additional recruitment of cells. We have previously shown that the amplitude of the responses evoked in RS cells by a single stimulation of the MLR increases gradually with the stimulation intensity to eventually reach a plateau (Brocard and Dubuc 2003). We have also reported that during repetitive stimulation of the MLR, the amplitude of the response is larger and results from a build-up of depolarization. Our data are consistent with the hypothesis that substance P increases the excitability of MLR cells. This would facilitate the build-up of depolarization in RS cells which in turn would decrease the MLR stimulation threshold to induce swimming. Swimming bouts would also be expected to be faster and longer. This is exactly what was observed with injections of substance P in the MLR (Fig. 8A). We also found that substance P was ineffective to induce swimming after several applications. The mechanisms by which this occurs is not known. However, a rapid internalization of substance P receptor previously described in the spinal cord could be involved (Honoré et al. 1999).

Altogether, our results suggest that MLR neurons in lampreys are sensitive to substance P. This is consistent with immunocytochemical data collected in rats, which show that substance P receptors are present in the two major components of the MLR, the nucleus cuneiformis and the pedunculopontine nucleus (Nakaya et al. 1994).

Electrophysiological and anatomical studies in mammals have shown that a major input to the MLR arises from the basal ganglia, and more particularly from the pallidum, the substan-

Effect of substance P on RS cells

Several observations in the present study suggest that substance P does not act directly on RS cells. (i) The depolarizations induced by substance P were abolished by TTX. (ii) The membrane properties of RS cells including resting potential, input resistance and repetitive firing capacity were not affected by the neuropeptide. (iii) The amplitude of mPSCs was not reduced by substance P, indicating the absence of postsynaptic changes in receptor sensitivity or ionic driving forces. We cannot exclude the possibility that substance P affects the distal dendrites of the large RS cells of the MRRN in a manner that was not detected by recording from the soma. However, 4 RS cells were recorded in the PRRN. These cells are smaller in size than those of the MRRN and were not depolarized by substance P. It appears that receptors activated by substance P were absent or very sparse on the cell body of RS cells.

On the other hand, substance P significantly decreased the frequency of the mPSCs in RS cells. The probability of neurotransmitter release from the presynaptic nerve terminals would thus be reduced by the neuropeptide. The cellular mechanisms by which this occurs are not yet understood. One possible mechanism may be similar to that described by Jafri and Weinreich (1996), where substance P hyperpolarizes vagal afferent somata by activating a calcium-dependent potassium current. In lampreys, it has also been found that four neuropeptides (peptide YY, cholecystokinin, calcitonin-gene-related peptide and neuropeptide Y) reduce the reticulospinal inputs onto spinal neurons at a presynaptic level (Parker 2000). Considering this and our present results, it would appear that presynaptic actions of neuropeptides would depress effects from the lamprey reticular formation by decreasing both the inputs and outputs of RS cells. Presynaptic modulation of RS cells could thus also participate in the control of locomotion. Under our intracellular recording conditions, Cl− currents were reversed and both excitatory and inhibitory mPSCs appeared as inward currents. Further experiments will be necessary to determine the nature of the neurotransmitter(s) presynaptically modulated.

Comparative aspects

A substance P-like neuropeptide was found in both Lampepetra fluviatilis and in Petromyzon marinus (Van Dongen et al. 1986; Waugh et al. 1994, 1995). In vertebrate phyla, the C-terminal part of substance P-related tachykinin is well conserved, probably allowing mammalian substance P to interact with tachykinin receptor(s) in the lamprey (Holmgren and
Jensen 2001). As discussed above, substance P has similar effects when injected into the MLR of rats or lampreys. However, some differences were found at the level of the reticular formation. According to their anatomical position, the MRRN and the PRRN, would be part of the pontine and bulbar reticular formation of higher vertebrates, respectively. In contrast with the present results, substance P injected into the medioventral medulla of the cat induces locomotion (Garcia-Rill and Skinner 1987). It also elicits long-lasting excitation of neurons recorded extracellularly in the pontine reticular formation of the rat (Kungel et al. 1994). A recent investigation using whole cell patch recordings demonstrates that substance P depolarizes these neurons at a postsynaptic level (Kohlmeier et al. 2002). These findings are supported by immunohistochemical data showing the presence of an antigen related to substance P receptor in a number of neurons in the pontine reticular formation (Nakaya et al. 1994). The difference between lamprey and higher vertebrates remains to be investigated. Previous studies have revealed that the distribution of the three tachykinin receptors is species-dependent (Boden and Woodruff 1994; Dietl and Palacios 1991; Mileusnic et al. 1999; Piggins et al. 2001; Saffroy et al. 1994). Thus we cannot exclude the existence of other tachykinin receptors on the surface of lamprey RS cells. Furthermore, the density and the distribution of tachykinin receptors could also change during the development from the larval to the adult period. Further investigations are needed to test these hypotheses.

In conclusion, our study indicates that substance P initiates locomotion in lampreys, presumably through the MLR. We propose that by increasing the excitability of MLR neurons, substance P potentiates the supraspinal locomotor command. Detailed electrophysiological and anatomical investigations are needed to identify the tachykininergic cells that project to the MLR and modulate the locomotor activity.

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