Modulation of calcium currents and membrane properties by substance P in the lamprey spinal cord

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Thörn Pérez C, Hill RH, Grillner S. Modulation of calcium currents and membrane properties by substance P in the lamprey spinal cord. J Neurophysiol 110: 286–296, 2013. First published April 24, 2013; doi:10.1152/jn.01006.2012.—Substance P is endogenously released within the locomotor network of the adult lamprey, accelerates the burst frequency of fictive locomotion, and reduces the reciprocal inhibition. Previous studies have shown that dopamine, serotonin, and GABA regulate calcium channels, which control neurotransmitter release, action potential duration, and slow afterhyperpolarization (sAHP). Here we examine the effect of substance P on calcium channels in motoneurons and commissural interneurons using whole cell patch clamp in the lamprey spinal cord. This study analyzed the effects of substance P on calcium currents activated in voltage clamp. We examined the calcium-dependent sAHP in current clamp, to determine the involvement of three calcium channel subtypes modulated by substance P. The effects of substance P on membrane potential and during N-methyl-d-aspartic acid (NMDA) oscillations were also analyzed. Depolarizing voltage steps induced inward calcium currents. Substance P reduced the currents carried by calcium by 61% in commissural interneurons and by 31% in motoneurons. Using specific calcium channel antagonists, we show that substance P reduces the sAHP primarily by inhibiting N-type (CaV2.2) channels. Substance P depolarized both motoneurons and commissural interneurons, and we present evidence that this occurs due to an increased input resistance. We also explored the effects of substance P on NMDA-induced oscillations in tetrodotoxin and found it caused a frequency increase. Thus the reduction of calcium entry by substance P and the accompanying decrease of the sAHP amplitude, combined with substance P potentiation of currents activated by NMDA, may both contribute to the increase in fictive locomotion frequency.

The modulatory effects of different monoamines, metabotropic receptors, and peptides on the locomotor network have also been studied extensively (Harris-Warrick and Cohen 1985; Kettunen et al. 2005; Krieger et al. 1998; Parker and Grillner 1999; Schotland et al. 1995; Wallén et al. 1989a; Zhang and Grillner 2000). Serotonin (5-HT), dopamine, and tachykinin immunoreactivity have been found along the lamprey spinal cord (Auclair et al. 2004; Brodin et al. 1988, 1990; Schotland et al. 1995; Van Dongen et al. 1985; Zhang and Grillner 2000; Zhang et al. 1996). When their receptors were pharmacologically blocked in the spinal cord in vitro preparation, the burst frequency and/or amplitude of the motor output was altered, suggesting that they are endogenously released (Harris-Warrick and Cohen 1985; Kemnitz 1997; Thörn Pérez et al. 2007; Wallén et al. 1989b). In many cases, this modulation has been shown to be mediated by direct or indirect effects on calcium channels (Hill et al. 2003; Matsushima et al. 1993; Wallén et al. 1989b; Wang et al. 2011). Calcium channels are particularly important in regulation of calcium-dependent potassium channels (KCa), which generate the slow afterhyperpolarization (sAHP) that has a key role in regulating spiking frequency, and therefore they have marked effects at the network level (El Manira and Bussieres 1997; Hill et al. 1992). In lamprey, the voltage-gated calcium channel subtypes N (CaV2.2) and P/Q (CaV2.1) are known to be involved in the regulation of KCa (Wikström and El Manira 1998) and in neurotransmitter release (Krieger et al. 1999), while L-type (CaV1.3) is important for the postinhibitory rebound depolarization (Matsushima et al. 1993; Wang et al. 2011). Inhibition of N-type (CaV2.2) and L-type (CaV1.1) channels can initiate a cascade of events that may induce changes in the firing frequency as well as on the network level (Buschges et al. 2000; Tegnér et al. 1997).

Substance P, the most studied peptide of the tachykinin family, binds to neurokinin 1 (NK1), a G protein-coupled receptor, and is capable of producing long-lasting and multiple effects. In mammals, substance P has been implicated in various responses such as respiration and pain (Ben-Mabrouk and Tryba 2010; Parenti et al. 2012). Several studies have revealed functional interactions (Santarelli et al. 2001) as well as colocalization (Nakamura et al. 2006) of substance P and 5-HT in the central nervous system. In rats, application of substance P during fictive walking is known to increase the locomotor frequency (Barthe and Clarac 1997).

Bath application of substance P in the lamprey brain stem potentiates the mesencephalic locomotor region and induces rhythmic sustained depolarizations in reticulospinal cells (Brocard et al. 2005). During fictive locomotion in the isolated spinal cord, application of substance P (1 μM, 20 min) increases the burst frequency (Parker et al. 1998), an effect that is dependent on the initial frequency (Thörn Pérez et al. 2007).

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The burst rate is enhanced by a reduction of the crossed inhibition, a substance P effect mediated by the endocannabinoid system (Thorén Pérez et al. 2009). The cellular mechanisms underlying the increase in burst frequency may also include a potentiation of the synaptic transmission via N-methyl-D-aspartic acid (NMDA) receptors and membrane depolarization, both shown to be mediated by substance P (Parker and Grillner 1998, 1999). Our goal in this study is to examine the effects of substance P on CaV channels, on the calcium channel subtypes involved in the activation of K_{Ca} during the sAHP, and on other membrane properties that may modulate the CPG in the lamprey spinal cord.

Our results show that substance P attenuates CaV currents and reduces the sAHP indirectly and primarily through an action on N-type (Ca_{V}2.2) calcium channels in motoneurons and commissural interneurons. We also provide results of relevance for the depolarization effect of substance P and include further evidence for a substance P-induced potentiation of the NMDA current, through an increase in the oscillation frequency of NMDA-induced tetrodotoxin (TTX) oscillations (Wallén and Grillner 1987), during substance P application. The possible involvement of these cellular mechanisms to the modulation of the locomotor network is discussed.

MATERIALS AND METHODS

Experiments were performed on the isolated spinal cords of young adult sea lampreys (Petromyzon marinus) in accordance with institutional guidelines of The Animal Research Ethical Committee, Stockholm, which approved all protocols (N114/09-N113/12).

Spinal cord preparation. Animals were anesthetized with tricaine methanesulfonate (MS 222, 100 mg/l; Sigma-Aldrich) decapitated, dissected, and kept at 4 – 8°C in a saline solution of the following composition (in mM): 137.9 NaCl, 2.1 KCl, 2.6 CaCl_{2}, 1.8 MgCl_{2}, 4 glucose, 5 HEPES. The pH was adjusted to 7.60 with 1 M NaOH, and the osmolarity was fixed to 270 mOsm with distilled water. The spinal cord and musculature of approximately 8 segments were pinned to a cooled sylgard-lined chamber that was inserted onto a cooled sylgard-lined chamber that was inserted onto a cooled sylgard-lined chamber that was inserted onto a cooled sylgard-lined chamber that was inserted onto a cooled sylgard-lined chamber that was inserted onto a cooled sylgard-lined chamber that was inserted onto a cooled sylgard-lined chamber that was inserted onto a cooled sylgard-lined chamber that was inserted onto a cooled sylgard-lined chamber that was inserted onto a cooled sylgard-lined chamber that was inserted onto a cooled sylgard-lined chamber that was inserted onto a cooled sylgard-lined chamber that was inserted onto a cooled sylgard-lined chamber that was inserted onto a cooled sylgard-lined chamber that was inserted onto a cooled sylgard-lined chamber that was inserted onto a cooled sylgard-lined chamber that was inserted onto a cooled sylgard-lined chamber that was inserted onto a cooled sylgard-lined chamber that was inserted onto a cooled sylgard-lined chamber that was inserted onto a cooled sylgard-lined chamber that was inserted onto a cooled sylgard-lined chamber that was inserted onto a cooled sylgard-lined chamber that was inserted onto a cooled sylgard-lined chamber that was inserted onto a cooled sylgard-lined chamber that was inserted onto a cooled sylgard-lined chamber. A lesion was made at the level of the gray matter of the contralateral side where the recordings were intended. Following this local incision, the rhombic dextran was permitted to retrogradely migrate caudally for at least 2 h. The protective meninx primitiva was removed, and the spinal cords were isolated and placed in a cooled microslicer with the ventral side up. A horizontal layer of about 40 μm above the gray matter was removed from the ventral surface to facilitate visibility and penetration of the patch electrode into the tissue (see Fig. 2A). The spinal cords were then pinned to a cooled sylgard-lined chamber that was inserted onto the stage of an upright microscope where motoneurons and commissural interneurons were identified by fluorescence, allowing also quantification of the cell body size using ImageJ software (National Institutes of Health) (see Fig. 2B). Solutions of pharmacological agents were bath-applied at a perfusion rate of 1 ml/min into a chamber volume of 1 ml. Data were acquired with Clampex software and analyzed using Clampfit (pCLAMP 9, Molecular Devices) and Spike2 4.16 software (Cambridge Electronic Design, Cambridge, UK). The oscillation frequency and rhythmic quality were analyzed by an autocorrelogram. The plateau duration was manually measured at the mid-amplitude value. The amplitude of the oscillations is related to the regularity, which is described with values that range between 0 and 1. Summary statistics and the values shown in the figures are reported as standard error of the mean (± SE), and “n” represents the number of experiments. The significance was determined using Student’s t-test with a 95% confidence interval.

Pharmacology. fictive swimming was induced by adding NMDA (Tocris, Bristol, UK) to the solution. A concentrated stock was kept frozen and was diluted to the final concentration during experiments (100 μM); Burst frequency increased gradually and stabilized after ≳3 h of NMDA perfusion. Substance P (Sigma-Aldrich) was added only after the burst frequency had become stable. Substance P was dissolved in water with 0.05 M acetic acid to prevent oxidation and 1% bovine serum albumin to increase the solution stability. Freeze 1 mM aliquots were stored at −20°C. They were dissolved in physiological solution to reach 1 μM concentration of substance P and applied for 20 min. Acetic acid alone at this final concentration had no effect on the locomotor frequency.

To visualize the calcium currents, sodium currents were blocked by extracellular application of TTX (1.5 μM, Sigma), α-CNToxon GVA (Tocris), a selective neurotoxin that blocks N-type (CaV2.2) calcium channels, was mixed with distilled water to a concentration of 0.1 mM and frozen in aliquots at −20°C. They were dissolved in physiological solution to reach 1 μM concentration when bath was applied. Cadmium chloride (500 μM, Sigma) was used to corroborate that the remaining currents were carried by calcium. Nimodipine (Tocris), an L-type (CaV1.1) calcium blocker, was dissolved in 70% alcohol and diluted with physiological solution to a final concentration of 10 μM during experiments. α-Agatoxin IV A (Sigma), a selective neurotoxin that blocks P/Q-type (CaV2.1) calcium channels, was mixed with distilled water to a concentration of 100 μM and frozen in aliquots at −20°C. They were dissolved in physiological solution to reach 400 nM concentration when bath was applied. Electrophysiology. For the extracellular experiments, glass suction electrodes were placed gently on contralateral ventral roots. Activity was recorded and amplified by a differential AC amplifier (model 1700, A-M Systems) and band-pass filtered between 100 and 500 Hz. The output from the amplifier was coupled to an A/D converter (Digidata 1320, Molecular Devices) at a sampling rate of 2.5 kHz. For the intracellular experiments, spinal neurons were recorded using patch electrodes pulled from borosilicate glass microcapillaries. Whole cell recordings were performed in current or voltage-clamp mode using a Multiclamp 700B amplifier (Molecular Devices). Bridge balance and pipette capacitance compensation were automatically adjusted. Patch electrodes had resistances of 5–10 MΩ and contained the following (in mM): 102.5 cesium methane sulfonate, 1 NaCl, 1 MgCl_{2}, 5 HEPES, 0.001 GTP, and 0.003 ATP. The pH was adjusted to 7.4 with CsOH, and the osmolarity to 250 mOsm with H_2O. Cells were voltage clamped at a holding potential of −60 mV, and currents were evoked by depolarizing steps applied at 10-s intervals. Linear leak and residual capacitive currents were subtracted except for analysis of input resistance. During current clamp, the intracellular solution for the sAHP experiments contained the following (in mM): 105 potassium-gluconate, 30 KCl, 10 sodium-phosphocreatine, 5 HEPES, 0.001 GTP, and 0.003 ATP. The pH was adjusted to 7.4 with KOH, and the osmolarity to 250 mOsm with H_2O. The membrane potential was held constant throughout the experiment by current injection. Action potentials were elicited by current pulses (0.5–2 ms), and the amplitude of the sAHP was monitored before and 10, 20, and 30 min after drug application. The amplitude of the AHP was measured at the peak level or, when this was not clear, at a constant time after the action potential (see dotted line in Fig. 4C) of 10 averaged traces in controls and after the full effect of the drugs had been reached. Traces with synaptic potentials during the sAHP were not included in the average. Since the effects of substance P and the calcium channel blockers were not reversible within reasonable times for whole cell recordings, only one neuron could be analyzed for each spinal cord preparation for all experiments with whole cell recording.
RESULTS

Modulation of the spinal network for locomotion by substance P. Fictive swimming is characterized by left-right alternation of ventral root bursts, and it can be evoked by NMDA perfusion of the spinal cord. The rhythmic bursting gradually develops into a stable burst frequency that is concentration dependent. In the adult species of *Lampetra fluviatilis* and *Ichthyomyzon uniscuspis*, activation of tachykinin receptors by exogenous application of substance P has been shown to enhance the burst rate and to modulate the motor activity (Parker et al. 1998; Thörn Pérez et al. 2007, 2009). As we studied the effects of substance P on calcium currents in young adult *Petromyzon marinus*, which offered excellent visibility necessary for whole cell patch recordings of labeled spinal neurons, we tested the effect of substance P during fictive locomotion also in this species. A 20-min application of substance P in the isolated spinal cord of *P. marinus* induced a slow modulation of the amplitude of the motor activity superimposed on a fast modulation. The burst frequency increased by an average of 15 ± 5% (*P* < 0.001, *n* = 4) and remained for more than 1 h (Fig. 1, A–C). Figure 1D shows the time course of the change in normalized frequency of four preparations. This result establishes a modulatory effect of substance P on the burst frequency in all three species. Based on this finding, we proceeded to study the modulatory effect of substance P on calcium currents in motoneurons and commissural interneurons in the spinal cord of *P. marinus*.

Morphology of motoneurons and commissural interneurons. Characterization of motoneurons and commissural interneurons provides a basis for dissecting the neuronal circuits that control the locomotion in the spinal cord. While observing the labeled cells, we found that these two types differ in soma size which might reflect their properties and roles. A comparison of the two-dimensional cell body area measured at the focal plane with the larger surface showed a significant difference (Fig. 2B) between the two types of neurons (see MATERIALS AND METHODS). The motoneurons were larger, and, in general, the commissural interneurons were found in a more lateral and dorsal position with respect to the motoneurons. A wide variation within each subgroup was observed. The somata of commissural interneurons varied between 50 and 600 μm² (average 338 ± 180 μm², *n* = 20), whereas those of motoneurons varied from 500 to 900 μm² (average 654 ± 110 μm², *n* = 19). The variation in each category may reflect that the measurement was performed in two dimensions, without a consideration of the three-dimensional perspective. Although the very smallest neurons are likely to be commissural interneurons and the very largest likely to be motoneurons, the overlap between the two groups is substantial.

![Figure 1](http://jn.physiology.org/) Substance P (SP) increases the locomotor burst frequency. A: representative example of stable ventral root bursting induced by 100 μM of *N*-methyl-D-aspartic acid (NMDA) in *Petromyzon marinus*. VR-L, ventral root left; VR-R, ventral root right. B: 20-min application of SP (1 μM) during fictive locomotion caused an acceleration of the frequency (*P* < 0.001). Fast and slow rhythms elicited by SP are shown. C: ventral root recording illustrating SP washout after 80 min. D: time course of the change in normalized frequency of four preparations with the application of SP. Error bars represent SE.
Lamprey commissural interneurons are essential for the left-right alternation of the motor activity during swimming (Buchanan 1982; Cangiano and Grillner 2003). They vary in their distribution along the spinal cord and transmitter phenotype. Immunohistochemistry studies have shown that 50% of these cells are glycinergic, while 29% were shown to be glutamatergic (Buchanan 1993; Mahmood et al. 2009; Mentel et al. 2008).

**Substance P causes a reduction in calcium currents in motoneurons and commissural interneurons.** To test whether the calcium entry was affected by substance P, neurons were voltage clamped at −60 mV, while sodium inward currents were blocked with TTX, and potassium outward currents were minimized by replacing K⁺ with Cs⁺ (see MATERIALS AND METHODS). Figure 3A shows an average of 10 sweeps as a control with inward currents induced by a depolarization step, thereafter followed by the same test after bath application of substance P. These currents were blocked by cadmium (Fig. 3B), indicating they were carried by Ca²⁺. In motoneurons, application of substance P (1 µM) clearly reduced the calcium conductance to 69 ± 6.7% of control (n = 7) (Fig. 3C). In commissural interneurons, a similar protocol also caused a reduction in calcium currents. Figure 3D shows a commissural interneuron where substance P reduced calcium conductance compared with control conditions. A tail following the depolarization pulse was observed in some motoneurons and commissural interneurons, indicating a delay in the closure of Ca²⁺ channels. Figure 3E shows single normalized traces, as well as the average of four cells’ reduction to 38 ± 9% of control caused by substance P application.

**Substance P reduces the sAHP in motoneurons through an action on N-type (CaV2.2) calcium channels.** Because substance P reduced calcium currents, it seemed likely that the sAHP would also be modulated since it is caused largely by intracellular Kᵥca of the SK-type (Cangiano et al. 2002; Hill et al. 1992; Wikström and El Manira 1998) and, to a lesser extent, by the opening of potassium channels activated by sodium (KᵥNa) (Wallén et al. 2007). Thus the sAHP is an indirect measure of calcium conductances that allows recording under current clamp conditions closer to the physiological state than with the channel blockers required during voltage clamp (Biro et al. 2006; Matsushima et al. 1993; McPherson and Kemnitz 1994; Parker and Grillner 1998; Wallén et al. 1989a; Wikström and El Manira 1998). We therefore chose this method to analyze the calcium channel subtypes modulated by substance P.

We first examined the effect of substance P on the sAHP in motoneurons. The membrane potential was held at approximately −50 mV (where the sAHP amplitude is usually higher than 1 mV) with current injection. A significant reduction in the peak amplitude of the sAHP was found after substance P application in all cells (Fig. 4A). Figure 4B shows the reduction as the percentage of control (66.3 ± 4%, n = 15). This result corroborates previous findings (Parker and Grillner 1998).

To test three different subtypes of high-voltage-activated calcium channels tentatively involved in the sAHP reduction caused by substance P, we tested specific calcium blockers: ω-conotoxin for N-type (CaV2.2), nimodipine for L-type (CaV1.1), and ω-agatoxin for P/Q-type (CaV2.1), alone and after application of substance P. The reduction caused by substance P alone is included in Fig. 4, D, F, and H, to compare with that in the presence of the channel blocker.

Bath application of the N-type calcium channel (CaV2.2) antagonist reduced the sAHP in motoneurons (Fig. 4, C and D) by 52.7 ± 6.3% (P < 0.005, n = 8). Adding substance P further reduced the sAHP by 21.1 ± 3.8% (P < 0.05, n = 8). The substance P effect is greater individually than when applied together with ω-conotoxin, suggesting that N-type calcium channels (CaV2.2) are partially responsible for the substance P effect. However, further reduction by substance P together with the blocker suggests that there is also a component modulated by substance P that is not mediated via N-type channels (CaV2.2).

We therefore tested the effects of the L-type calcium channel (CaV1.1) blocker nimodipine on the sAHP, which resulted in a significant reduction by 19.7 ± 6.1% (P < 0.05, n = 8, Fig. 4, E and F). When substance P was coapplied with the antagonist, a further reduction by 29.5 ± 7.4% (n = 8) was observed, which was not significantly different from the effects of substance P alone (Fig. 4F). Thus the changes in sAHP amplitude induced by nimodipine compared with control reveal a partial involvement of L-type calcium channels (CaV1.1) in the activation of Kᵥca which are not modulated by substance P. To test if P/Q-type calcium channels (CaV2.1) could account for the rest of the substance P decrease of the sAHP, substance P was also applied in the presence of ω-agatoxin, a P/Q-type calcium channel (CaV1.2) antagonist. The amplitude of the sAHP was unchanged during the application of the ω-agatoxin in three of four cells (Fig. 4G) (but see Wikström and El Manira 1998).
Substance P in the presence of the blocker reduced the sAHP in all four cells tested, but it was not significantly different with and without the blocker (Fig. 4H).

Taken together, by using calcium channel antagonists we show that the sAHP depends mainly on calcium influx through N-type (CaV2.2) channels and to a minor extent to L-type (CaV1) channels, and that substance P acts primarily on N-type (CaV2.2) but not L-type (CaV1) or P/Q-type (CaV2.1) channels. These results partially account for substance P modulation of the sAHP in motoneurons.

Substance P reduces the sAHP in commissural interneurons primarily through an action on N-type (CaV2.2) calcium channels. Within the commissural interneurons, we found a greater variety of the shape (duration and amplitude) of the AHP. Often, no distinct fast afterhyperpolarization could be separated from the sAHP type (cf., Fig. 5A) (Buchanan 1993). In cells exhibiting an sAHP, substance P application in 9 out of 10 cells showed a reduction (Fig. 5, A and B, 20 ± 7.8%, P < 0.05, n = 10), but more modest compared with motoneurons (Figs. 4B and 5B).

Which subtypes of calcium channels that may be involved in the sAHP reduction caused by substance P were also investigated. ω-Conotoxin alone reduced the sAHP by 48.9 ± 5.3% (Fig. 5C, P < 0.005, n = 8), indicating that N-type (CaV2.2) calcium channels are primarily responsible for the sAHP in commissural interneurons. The comparison between the effect of substance P in the absence and presence of ω-conotoxin showed a significant difference (5.2 ± 1.3%, P < 0.05, n = 8) (Fig. 5C), suggesting an involvement of this type of channel in the substance P effect. However, a remaining component is involved since a further reduction was observed by substance P. A significant but much smaller reduction in the AHP was seen with nimodipine (12 ± 5.1%, n = 52), indicating that L-type channels (CaV1) have a modest contribution to the KCa activation in commissural interneurons. On the other hand, substance P in the presence of the blocker reduced the sAHP, and there was no significant difference from the reduction caused by substance P alone. To investigate if P/Q-type (CaV2.1) CaV channels, ω-agatoxin was applied. No significant effect was seen on the sAHP amplitude with ω-agatoxin (Fig. 5D, n = 4), and the effect of substance P alone was similar to that in the presence of the calcium antagonist.

Substance P thus has a significant depressive effect on the sAHP (13%, P < 0.05), albeit much less than in motoneurons. As in motoneurons, the main contribution for KCa activation was through N-type calcium channels (CaV2.2).

Substance P depolarizes the membrane and increases the input resistance in motoneurons and commissural interneurons. Substance P application caused an increase in the input resistance in motoneurons (10 ± 5.2%, P < 0.05, n = 8) and commissural interneurons (8 ± 1.2%, P < 0.05, n = 4) (Fig. 6A). The input resistance of cells recorded was in the range of 100–500 MΩ. During voltage clamp, a negative holding current is required to maintain the holding voltage at −60 mV because the resting potential using Cs+ pipettes is more depolarized than those containing K+). Consistently, less current was needed for motoneurons and commissural interneurons to hold the voltage during substance P application (Fig. 6B), which is related to the increase in input resistance observed previously for both cell types. An increased input resistance is thus caused by substance P due to the decreased conductance of a resting outward current. The depolarization observed in

Fig. 3. Activation of tachykinin receptor reduces calcium currents in motoneurons and commissural interneurons. A: whole cell voltage-clamp records from a prelabeled motoneuron showing calcium currents elicited by a depolarizing pulse (lower trace) and with SP (upper trace). Vhold, holding potential. B: whole cell voltage-clamp records from a putative motoneuron with inward currents induced by a depolarization step. These currents were blocked by cadmium, indicating they were carried by Ca2+ channels, and that substance P acts primarily on N-type (CaV2.2) channels and to a minor extent to L-type (CaV1) channels.

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current clamp by application of substance P (4.2 ± 0.9 mV, 15 of 15 motoneurons, 7 of 10 of commissural interneurons, 3 ± 0.6 mV) is presumably related to this and to a decreased leak conductance responsible for maintaining the resting membrane potential.

**Substance P increases the frequency of NMDA-induced potential oscillations.** The increase in burst frequency by substance P during fictive locomotion has been suggested to be mediated partially by PKC-induced phosphorylation of NMDA receptors (Parker and Grillner 1999; Parker et al. 1998). We therefore tested the effect of substance P on the NMDA-induced membrane oscillation that occurs in the presence of TTX, as these oscillations have been suggested to be important in maintaining a steady rate of locomotor activity (El Manira et al. 1994; Wallén and Grillner 1987). It has been shown that Ca²⁺ entry is synchronized with subthreshold membrane potential oscillations during fictive locomotion (Bacskai et al. 1995; Viana di Prisco and Alford 2004; Wang et al. 2011) and can accumulate to activate KᵥCa channels that contribute to plateau termination (Wallén and Grillner 1987). Here we applied NMDA (100 μM) in the presence of TTX that elicited oscillations with trough potentials of −70 to −80 mV in motoneurons. Substance P application for 20 min resulted in an increase in the frequency of oscillations. The trough potentials were corrected to control values by current injection. Figure 7A shows an example of the NMDA-induced oscillations in TTX during control conditions and after substance P application. The oscillation frequency and rhythmic quality were analyzed by
autocorrelogram (see MATERIALS AND METHODS), as shown in Fig. 7B. The autocorrelogram displays oscillations of the period (T), established as 1/frequency. As can be seen in this graph, the frequency increased in the presence of substance P. The amplitude of the autocorrelogram oscillations is related to the regularity, and values closer to 1 indicate a more regular rhythm. The oscillation frequency increased in seven out of nine cells (Fig. 7C). The regularity improved in five out of nine cells, and the plateau duration increased in six out of nine cells. Taken together, these results show that substance P affects the NMDA-induced oscillations most likely through an effect on NMDA receptors (Parker and Grillner 1998) and may also be through low-voltage-activated calcium channels of the T-type (CaV3) or L-type (CaV1.3) (Lipscombe et al. 2004; Wang et al. 2013). At these potentials, high-voltage-activated calcium channels are mainly inactive. These potential oscillations occur during fictive locomotion (Sigvardt et al. 1985; Wallén and Grillner 1987), and a modulatory influence on these oscillations is relevant for the increase in the locomotor frequency caused by substance P.

DISCUSSION

Substance P modulates the sAHP via N-type calcium channels in motoneurons and commissural interneurons. Our voltage clamp experiments have shown that an application of substance P reduces the calcium currents in both motoneurons and commissural interneurons. These results highlight the possibility that the reduction of the sAHP caused by substance P is mediated by a reduction of calcium influx through CaV,

Fig. 5. NK1 receptor activation reduces the sAHP in commissural interneurons mainly through N-type calcium channels. A: whole cell current clamp records from prelabeled commissural interneurons showing the sAHP control (bottom trace) and a reduction of the sAHP after SP application (top trace). B: average data from 10 commissural interneurons showing a significant reduction of the sAHP after SP application.

Fig. 6. SP increases the input resistance of commissural interneurons and motoneurons. A: average and normalized data from 4 commissural interneurons and 7 motoneurons showing the input resistance before and after SP application. B: average and normalized data from 4 commissural interneurons and 7 motoneurons showing the current to hold the cell at −60 mV (in Cs+ pipettes) before and after SP application. **P < 0.01.
Another prominent effect of substance P is the potentiation of the current through NMDA receptor channels (Parker et al. 1998). NMDA receptors are postsynaptically located in motoneurons and interneurons. Inherent, TTX-resistant NMDA channels. Moreover, we confirm that the postspike afterhyperpolarization generated by KCa channels of the SK type (sensitive to apamin) is primarily due to entry of calcium via N-type calcium channels (CaV2.2) (Cangiano et al. 2002; Hill et al. 1992; Wikström and El Manira 1998), and that substance P acts to reduce the calcium entry via N-type channels (CaV2.2) and thereby regulates the amplitude of the sAHP (Fig. 8). N-type calcium channels (CaV2,2) may be localized closer to the KCa channels involved in the sAHP than the other subtypes of calcium channels. The lack of noticeable effect of substance P on the L-type (CaV1) or P/Q-type (CaV2.1) might be due to the fact that substance P does not modulate these channels or that they are not localized in the proximity of KCa to influence the sAHP. A small part (20%) of the sAHP is not due to apamin-sensitive KCa channels, but instead involves KNa (Cangiano et al. 2002; Wallén et al. 2007).

P/Q-type (CaV2.1) calcium channels have been reported to contribute to 20% of the sAHP, while N-type (CaV2.2) contributed 75%, but L-type (CaV1) did not appear involved (Wikström and El Manira 1998). In this study, we do not confirm a participation of P/Q channels (CaV2.1), but do instead report a small but significant reduction of the sAHP when blocking L-type channels (CaV1). The disparity in the results may be related to the fact that we used a different species and animals at a younger developmental stage (transformers), where the channel distribution may possibly differ (Baccaglini and Spitzer 1977; Norreel et al. 2003).

NK1 modulation on N-type calcium channels (CaV2.2) has been reported in rodents to act via two main pathways exerting different effects dependent on the activation site (Mitra-Ganguli et al. 2009). Both pathways involve arachidonic acid, a precursor of eicosanoids. The form of modulation depends on which CaV,β subunit is coexpressed with CaV2.2. When palmitoylated CaV,β2a is coexpressed, substance P enhances the N-current. In contrast, when CaV,β3 is coexpressed, substance P instead inhibits the N-current. The current findings thus could be explained by a mechanism involving CaV,β3.

Substance P depolarizes the cells and increases the frequency of NMDA-induced potential oscillations. We observed a depolarization upon substance P application in both motoneurons and commissural interneurons and also that substance P application results in an increased input resistance (Fig. 6A) at resting membrane potential in both cell types. The fact that we observed a depolarization with substance P, coupled with an increase in input resistance, means that the ion channels that are closing have a reversal potential negative to the resting potential, suggesting that this effect may be mediated by a decrease in potassium leak conductance, which is addressed in ongoing study in our laboratory. Two-pore domain potassium channels appear to be permeable to cesium (Lopes et al. 2000; Niemeyer et al. 2001). Previous studies on mammals have suggested that leak potassium channels are inhibited by substance P (Ptak et al. 2000; Talley et al. 2000).

Fig. 7. SP increases the frequency of NMDA-induced oscillations in tetrodotoxin (TTX). A: representative example of NMDA oscillations in a motoneuron induced by 100 μM of NMDA in 1.5 μM of TTX in control conditions and after 20-min application of SP (1 μM). B: the autocorrelation displays oscillations of the period (T) before and after SP application in one experiment (frequency = 1/T). C: oscillation frequencies normalized to control showing the effect of SP at 20-min application that caused an increase in the oscillation frequency in 7 of 9 motoneurons (P < 0.05, n = 9). *P < 0.05.

Fig. 8. Schematic illustration of the intracellular pathways proposed to modulate the sAHP following NK1 activation through SP. SP activates postsynaptic NK1 receptor, which in turn leads to a reduction of calcium currents via N-type (CaV2.2) calcium channels, and consequently a reduction on the activation of calcium-dependent potassium channels (KCa) is seen, resulting in a decrease of the action potential sAHP. Black arrows, new observations; gray arrows, established observations (Wikstrom and El Manira 1998). Thinness of the arrows represents the contribution to the KCa activation. NK1 receptor also potentiates the current through NMDA receptors (Parker et al. 1998) and may reduce the potassium conductance through leak channels (KNa leek; dotted arrow).
oscillatory activity has been demonstrated in both motoneurones and commissural interneurones (Wallén and Grillner 1987). These oscillations may play an important role during locomotor activity, particularly at low frequencies (Wallén and Grillner 1987), and are subject to modulation (Krieger et al. 2000). We show here that NK1 activation causes an increase in frequency of the NMDA-induced oscillations. The shape and frequency of the NMDA-induced TTX oscillation is voltage dependent and occurs approximately between −85 and −40 mV. In this interval, low-voltage-gated channels including L-type (CaV1.3) represent the main source of calcium entry (Wang et al. 2013) and contribute to the KCa activation together with NMDA channels (Wikström 1999). Thus it is likely that the potentiation of currents through NMDA receptor channels by substance P contributes to the frequency increase, together with other effects like the depolarization caused by substance P presumably via leak currents (Fig. 6).

Modulation of the spinal network for locomotion by substance P. Regulation of the locomotor burst frequency has been reported for several modulatory systems such as dopamine (McPherson and Kemnitz 1994; Schotland et al. 1995), 5-HT (Harris-Warrick and Cohen 1985; Wallén et al. 1989a), and GABAA (Alford and Grillner 1991; Matsushima et al. 1993; Schmitt et al. 2004). In each case, there is a reduction of the burst rate and prolongation of the bursts. A reduced sAHP amplitude can be one of several mechanisms accounting for this effect (Wallén et al. 1989a). This will result in a reduced spike frequency adaptation and reduced summation of sAHPs during the burst, which will result in longer bursts and lower burst rate (Wallén et al. 1989a). Substance P instead increases the burst rate, an effect mediated by a potentiation of NMDA currents (Parker et al. 1998), a reduction of crossed inhibition via endocannabinoids receptors (Thórn Pérez et al. 2009), membrane depolarization, and effects on inherent oscillating properties. The net effect of a decrease of the sAHP amplitude observed with substance P is thus not dominate in the overall effect of increased burst rate observed during fictive locomotion. In mammals, it has been reported that substance P is present in the brain stem (Mazzone et al. 1997) as well as the lumbar spinal cord. Substance P application during fictive walking in rat increases both the locomotor frequency and the duration of the bursts (Barthe and Clarac 1997). In the lamprey spinal cord, tachykinins, including substance P, are found in nerve fibers, cell bodies, and in a lateral plexus (Auclair et al. 2004; Fried et al. 1988; Van Dongen et al. 1986). In some cells, substance P is colocalized with 5-HT (Auclair et al. 2004; Van Dongen et al. 1986). Cells surrounding the lumen of the central canal and ventromedially have been shown to be immunopositive for substance P (Van Dongen et al. 1985). As dendrites of neurons in the spinal gray reach these locations or are traversed by fiber tracts emanating from them, substance P released there could modulate locomotion. Activation of the substance P system in the lamprey would result in more intensive swimming that could be useful under different conditions like, for example, migration for longer distances or swimming against water currents.

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No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: C.T.P. performed experiments; C.T.P. and R.H.H. analyzed data; C.T.P. and R.H.H. interpreted results of experiments; C.T.P. prepared figures; C.T.P. drafted manuscript; C.T.P., R.H.H., and S.G. edited and revised manuscript; R.H.H. and S.G. conception and design of research; R.H.H. and S.G. approved final version of manuscript.
TACHYKININS, Ca2+ CONDUCTANCE, LOCOMOTOR PATTERN GENERATION


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