Endocannabinoids Mediate Tachykinin-Induced Effects in the Lamprey Locomotor Network

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Thörn Pérez C, Hill RH, El Manira A, Grillner S. Endocannabinoids mediate tachykinin-induced effects in the lamprey locomotor network. J Neurophysiol 102: 1358–1365, 2009. First published July 1, 2009; doi:10.1152/jn.00294.2009. The spinal network underlying locomotion in lamprey is composed of excitatory and inhibitory interneurons mediating fast ionotropic action. In addition, several modulator systems are activated as locomotion is initiated, including the tachykinin system and the metabotropic glutamate receptor 1 (mGluR1), the latter operating partially via the endocannabinoid system. The effects of mGluR1 agonists and tachykinins resemble each other. Like mGluR1 agonists, the tachykinin substance P accelerates the burst rate and reduces the crossed inhibition in an activity-dependent fashion. The present study therefore explores whether tachykinins also use the endocannabinoid system to modulate the locomotor frequency. By monitoring fictive locomotion, we were able to compare the facilitatory effects exerted by applying substance P (1 μM, 20 min), on the burst frequency before and during application of the endocannabinoid CB1 receptor antagonist AM251 (2.5 μM). By using two different lamprey species, we showed that the response to substance P on the burst frequency is significantly reduced during the application of AM251. To examine whether endocannabinoids are involved in the substance P–mediated modulation of reciprocal inhibition, the commissural axons were stimulated, while recording intracellularly from motoneurons. We compare the effect of substance P on the amplitude of the contralateral compound glycinergic inhibitory postsynaptic potential (IPSP) in control and in the presence of AM251. The blockade of CB1 receptors reduced the substance P–mediated decrease in the amplitude by 29%. The present findings suggest that the effects of substance P on the increase in the locomotor burst frequency and depression of IPSPs are mediated partially via release of endocannabinoids acting through CB1 receptors.

INTRODUCTION

The motor pattern underlying locomotion is coordinated by central pattern generator networks (CPGs) in all classes of vertebrates (see Grillner 2006). The locomotor CPGs are turned on from command regions in the brain stem via glutamatergic reticulospinal neurons (Alford and Dubuc 1993; Brodin et al. 1994; Dubuc et al. 2008; Ohta and Grillner 1989; Sirotà et al. 2000). The intrinsic function of the CPG networks has been elucidated in the lamprey and amphibian tadpoles (Buchanan and Grillner 1987; Cangiano and Grillner 2005; Cohen and Harris-Warrick 1984; Grillner et al. 1998; Roberts et al. 2008; Sillar and Roberts 1993). Reticulospinal neurons activate groups of CPG interneurons in the spinal cord, which generate the burst pattern, and the inhibitory glycinergic neurons ensure left–right alternation through reciprocal inhibition (Biro et al. 2008; Buchanan 1982; Cohen and Harris-Warrick 1984). The CPG network in lampreys consists of interneurons that act through ionotropic glutamate (N-methyl-d-aspartate [NMDA] and α-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid [AMPA]) and glycinergic receptors. In addition, numbers of metabotropic receptors contribute with a fine-tuning of the network properties by an action on neuronal and synaptic properties. They include different metabotropic glutamate receptors (mGluRs): the 5-hydroxytryptamine (5-HT) system, γ-aminobutyric acid type B (GABA_B) receptors, and tachykinins (Harris-Warrick and Cohen 1985; Kettunen et al. 2005; Krieger et al. 1994, 1998; Kyriakatos and El Manira 2007; Photowala et al. 2006; Thörn Pérez et al. 2007a; Zhang and Grillner 2000). mGluR1 receptors act partially via endocannabinoid signaling, through a depression of glycinergic synaptic transmission (Kettunen et al. 2005). The net effects of tachykinins and mGluR1 receptors on the network level resemble each other, in that substance P is also known to reduce the crossed inhibition in an activity-dependent fashion (Parker and Grillner 1999a). We therefore explored whether some of the effects of tachykinins in the lamprey CPG are mediated by endocannabinoids.

Tachykinins and endocannabinoids exert powerful control over the excitability in sensory and motor circuits in the spinal cord of the lamprey and other chordates (Farquhar-Smith et al. 2000; Hökfelt et al. 2001; Kettunen et al. 2005; Kyriakatos and El Manira 2007; Thörn Pérez et al. 2007a; Waugh et al. 1995). Tachykinin receptors—named NK1, NK2, and NK3—are G-protein coupled and have been characterized both pharmacologically and genetically. Substance P is the preferred ligand for the NK1 receptor, which is widely represented and distributed in the central and peripheral nervous system of chordates (Fried et al. 1988; Maggi and Schwartz 1997), including the lamprey (Van Dongen et al. 1985, 1986). Activation of NK1, as with the mGluR1 receptor, is known to produce diacylglycerol with the subsequent activation of protein kinase C (PKC) (Ferguson et al. 2008; Parker et al. 1998; Wajima et al. 2000). Eicosanoid ligands serve as endogenous agonists for CB1 and CB2, which are G-protein–coupled cannabinoid receptors. CB1 receptors are among the most abundant Gi/Go linked types in the brain. They have also been found in the spinal cord and are known to mediate retrograde signaling in the CNS (El Manira et al. 2008; Yoshida et al. 2002). CB2 receptors are primarily found on immune cells (Chevaleyre et al. 2006; Farquhar-Smith et al. 2000; Kano et al. 2009; Salio et al. 2002).

In the lamprey, a brief activation of tachykinin receptors by exogenous application of substance P to the spinal cord has both short- and long-term effects on the locomotor burst frequency (Parker et al. 1998; Svensson et al. 2001; Thörn...
Pérez et al. 2007a) and can modulate brain stem locomotor control as well (Brocard et al. 2005). Further, tachykinins are endogenously released during locomotor activity in that blockade of NK1 receptors reduces the burst frequency of fictive locomotion (Thörn Pérez et al. 2007a).

We show here that the effects of substance P on the network activity are appreciably reduced by a CB1 receptor antagonist. Furthermore, depression of the inhibitory postsynaptic potentials (IPSPs) in contralateral spinal neurons induced by substance P is markedly reduced by a CB1 antagonist. It is suggested that a component of the effects of substance P is mediated by activation of CB1 receptors. These results were previously reported in abstract form (Thörn Pérez et al. 2007b, 2008).

METHODS

Intact spinal cords from 65 adult lampreys of two species were used. *Lampetra fluviatilis* were collected in Ljusne, Sweden and *Ichthyomyzon uniculus* were obtained from Aquatic Supply (Marquette, IA). They were kept in separate aerated aquaria at a temperature of 5°C. All protocols were approved by the animal research ethical committee (Stockholm). Lampreys were anesthetized with tricaine methanesulfonate (MS 222, 100 mg/l; Sigma–Aldrich, Stockholm) and the viscera, musculature, and ventral half of the notochord were removed.

The spinal cord and notochord of around 10–12 segments from the region between the gills and the dorsal fin (Fig. 1A) were pinned to a Sylgard-lined chamber and continuously perfused with physiological solution at 8–10°C. The physiological solution for *L. fluviatilis* was composed of (in mM) 138 NaCl, 2.1 KCl, 1.8 CaCl2, 1.2 MgCl2, 4 glucose, and 2 HEPES, and it was bubbled for 20 min with O2 and pH adjusted to 7.4 with NaOH. The physiological solution for *I. uniculus* was composed of (in mM) 91 NaCl, 2.1 KCl, 2.6 CaCl2, 1.8 MgCl2, 4 glucose, and 23 NaHCO3, and it was bubbled with 5% CO2–95% O2 for 20 min, before the pH was adjusted to 7.65 with 1 M NaOH, and also during the experiment.

To analyze the effects of different agonists and antagonists on the locomotor frequency, the glycinergic and monosynaptic nature of the IPSP (in sensory afferents. The spinal cord was activated by electrical stimulation (100–300 μA), with a glass pipette electrode, by delivering a brief train of 10–11 pulses of 3 ms duration, 20 Hz every 10 s, to the ventral surface of the preparation near the midline. The intracellular electrode was placed contralaterally to this and four to five segments caudal to record responses, mainly from inhibitory crossed caudally (CC) projecting interneurons (Fig. 5A). Intracellular recordings were made with sharp electrodes pulled to a final resistance of about 50 MΩ when filled with 3 M potassium acetate and 0.1 M KCl. Intracellular signals were recorded using an Axoclamp 2A amplifier (Molecular Devices) in bridge mode, low-pass filtered at 1 kHz, and further amplified. The signals were digitized at a sampling rate of 6.7 kHz per channel by a Digidata 1320A (Molecular Devices) and stored on a PC using pClamp software (Molecular Devices).

Ionotropic glutamate receptors were blocked by adding the NMDA receptor antagonist d-2-amino-5-phosphonopentanoic acid (AP5, 50 μM; Tocris) and the AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 40 μM; Tocris). Crossing inhibitory premotor interneurons were monitored by recording their axonal action potentials extracellularly on the side contralateral and caudal to that of the stimulation. Motoneurons were identified by an extracellular spike in the ventral root recording (described earlier) in the same segment. The glycinergic and monosynaptic nature of the IPSP (in addition to the glutamatergic block) was further corroborated with the blockade of glycine transmission by the antagonist strychnine (5 μM; Sigma–Aldrich) and the constant delay between the stimulation artifact and the response. The peak amplitude of the IPSPs was measured and 100 sweeps were averaged in control and in the presence of the endocannabinoid antagonist AM251. The effects of the cannabinoid receptor agonist WIN55212-2 ([R(+)]-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo][1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-
methan-1-mesylate, 5 μM; Tocris Bioscience) were blocked by AM251, thus confirming the antagonist effect.

RESULTS

Blockade of CB1 cannabinoid receptors by AM251 attenuates the acute response of the locomotor network to substance P

Fictive swimming was induced in isolated spinal cord preparations by perfusion of NMDA (75–100 μM). After approximately 3 h, a stable pattern of activity was reached and the quality of the rhythmic activity, expressed by the coefficient of rhythmicity (Cr; see METHODS), indicated a high degree of regularity (Cr >0.6).

Activation of tachykinin receptors by exogenous application of substance P enhances the burst rate and modulates the motor activity in L. fluviatilis (Thorén Pérez et al. 2007a). These results were also obtained with a 20-min application of substance P (1 μM) in the isolated spinal cord of I. unicuspis. This response was used as a control to test for a possible role of endocannabinoid receptors on the effects exerted by substance P. As illustrated in Fig. 2, A and B, an application of substance P results in a significant (p < 0.001) increase in frequency of the locomotor rhythm from 2.5 ± 0.11 to 3.5 ± 0.12 Hz. A second application of substance P in the same spinal cord in the presence of the CB1 receptor antagonist AM251 did not result in an increased frequency (Fig. 2C, 2.1 ± 0.15 Hz). During the blockade with AM251, the average frequency as a rule decreases progressively, compared with the initial value (Kettunen et al. 2005). Figure 2D shows the time course of drug application in which, in addition to attenuation of the response to substance P, antagonizing CB1 also causes a steady decrease in frequency.

To test whether the decrease in the effect of substance P was indeed due to the blockade of cannabinoid receptors, and not to the previous application of substance P, two consecutive applications of substance P in the absence of cannabinoid antagonist were used as controls. In these experiments, the first application of substance P increased the burst frequency by an average of 33 ± 8.9% (p < 0.01, n = 10; Fig. 3A) for L. fluviatilis and 16 ± 4.3% (p < 0.01, n = 3, Fig. 3E) for I. unicuspis. The second application of substance P, after 3 h of washout, increased the burst frequency by an average of 20 ± 9.5% (p < 0.01, n = 10, Fig. 3A) in L. fluviatilis and 14 ± 8.6% (p < 0.01, n = 3, Fig. 3E) in I. unicuspis. Thus attenuation in a second application of substance P cannot account for the reduced effect in the CB1 antagonist.

Eight additional spinal cord preparations of L. fluviatilis and three of I. unicuspis were used to investigate the effect of AM251 on the second consecutive application of substance P. To compare the controls with those in AM251, averages of three to five recordings before and after application of substance P, alone and in AM251, were normalized to the steady-state control (Fig. 3, B and F). Due to the reduction in burst frequency by the blockade of CB1 receptors (Figs. 2D and 3, B and F), the decay was calculated using a linear regression and the values were extrapolated to subtract the projected effect of the antagonist. The values were then normalized and the percentage of change estimated. There was no significant difference between the samples during 30 min prior to the second application of substance P in the presence of AM251 (6.8 ± 7.0% in L. fluviatilis and 1.7 ± 1.2% in I. unicuspis). Figure 3, C and G shows the percentage of change in frequency after the second application of substance P in controls and in AM251 (p < 0.05, 14.9 ± 1.9 and 7.5 ± 2.1%, respectively, in L. fluviatilis; p < 0.001, 10 ± 1.5 and 1.75 ± 1.2%, respectively, in I. unicuspis) The increase in frequency was significantly lower in AM251 than that for the controls. Taken together, these observations suggest that the increase in the locomotor frequency obtained by substance P may include a modulation via CB1 receptors.

FIG. 1. Preparation, experimental configuration, and analysis protocols. A: extracellular suction electrodes were placed on the left and right ventral roots (VR-L, VR-R) of the isolated spinal cord at segments lying between the gills and the dorsal fin, as shown by the dashed lines on the whole animal illustration. B: a representative sample of the ventral root records taken during stable fictive locomotion induced by 75 μM N-methyl-D-aspartate (NMDA). Below each raw recording an event transformation is shown, which gives a quantitative illustration. C: the event autocorrelation displays oscillations of the period (T), established as 1/frequency. The amplitude of the oscillations is related to the regularity, which is described with values that range between 0 and 1, and was defined as the coefficient of rhythmicity [Cr = (A - B)/(A + B)].
Decrease in coefficient of rhythmicity induced by substance P is not affected by AM251

To investigate whether the blockade of endocannabinoid receptors had also affected the quality of the burst rhythm, we followed the change in the coefficient of rhythmicity (Cr, as defined in METHODS), induced by substance P alone and in the presence of AM251.

A 20-min application of substance P during stable swimming with regular rhythmicity (Cr > 0.6) resulted in a significant decrease in Cr by 24 ± 8.3% (p < 0.005, n = 10) for L. fluviatilis and 16 ± 3.1% (p < 0.05, n = 3) for I. unicuspis. This effect lasted for about 1 h and recovered to a similar control level after washout of substance P. A second application of substance P to the same spinal cords, 3 h after washout of the first application, induced a similar decrease in rhythmicity in both species (16 ± 6.5%, p < 0.05, n = 10, for L. fluviatilis; 20 ± 6.9%, p < 0.01, n = 3, for I. unicuspis). In the same way, we compared the Cr in the first and the second applications of substance P, with and without AM251. The Cr was not significantly different during the application of substance P with and without AM251 (Fig. 3, D and H), although the frequency was different (see Fig. 3, C and G). This might be explained by the different mechanisms underlying responses of the burst frequency and the coefficient of rhythmicity.

Blockade of CB1 cannabinoid receptors by AM251 obviates substance P-mediated depression of the IPSPs from commissural interneurons

The above-cited results show that the substance P-mediated increase in the locomotor frequency is significantly reduced by the CB1 receptor antagonist AM251. At the synaptic level, reduction of glycinergic inhibition with strychnine (Cohen and Harris-Warrick 1984; Grillner and Wallén 1980) or by surgical separation of the left and right halves of the spinal cord (Cangiano and Grillner 2003) results in an increase in the locomotor frequency. We therefore examined whether glycinergic inhibitory synapses from commissural interneurons underlying left–right alternation during locomotor activity could be modulated by substance P via CB1 receptors.

For this, intracellular recordings were made from motoneurons, whereas crossing axons and inhibitory interneurons on the contralateral side of the spinal cord were stimulated extracellularly with trains of 11 ± 1 pulses at 20 Hz (Alford and Grillner 1991; Parker and Grillner 1999a) (Fig. 4A). Because excitatory interneurons could also have been stimulated, the inhibitory component was isolated by blocking NMDA and AMPA receptors with AP5 (50 μM) and CNQX (40 μM), respectively.

The antagonistic action of AM251 was confirmed by the lack of effect of CB1 agonist WIN55212-2 (Fig. 4B), which reduces the IPSP amplitude in motoneurons (Kettenun et al. 2005). The glycinergic nature of the IPSP was corroborated by its blockade with the antagonist strychnine (Fig. 4D). In the presence of glutamate blockers, the amplitude of the IPSPs initially decreased during the first 5 min to subsequently remain stable (Fig. 4C). We therefore applied substance P only after ≥10 min of stable recording and took as control the data before the application.

In 9 of 12 cases, superfusion of substance P (1 μM) caused depolarization in motoneurons, which was compensated with current injection to maintain the cell at a stable membrane potential. In all cases, substance P caused a reduction in the amplitude of monosynaptic IPSPs elicited by the extracellular stimulation (Fig. 5A) and the first glycinergic IPSP was significantly reduced by 53 ± 8.7% (p < 0.05, n = 4).

`FIG. 2. The effect of substance P on ventral root burst frequency in the presence of an endocannabinoid antagonist. A: representative example of a stable ventral root bursting induced by 100 μM of NMDA in Lampetra fluviatilis. B: 20-min application of substance P (1 μM) during fictive locomotion caused an acceleration of the frequency (p < 0.01). C: 2nd 20-min application of substance P (1 μM) during fictive locomotion, following 2 h of blockade of endocannabinoid receptors by the antagonist AM251. Blockade of cannabinoid receptors by AM251 significantly reduced the increase in frequency induced by substance P (p < 0.001). D: time course of the change in frequency with the application of substance P alone or in AM251 (A, B, and C refer to the ventral root traces shown above).`
This depression was used as the control condition to be compared with the effect of substance P after the endocannabinoid receptors had been blocked by AM251 (2-5 μM) (Fig. 5B). In the preparations pretreated with AM251, the depression of the first IPSP caused by the application of substance P was significantly reduced by 24.3 ± 5.6% (*p < 0.05, n = 6, Fig. 5C and D).

These data suggest that the response to substance P is mediated partially by an endogenous release of endocannabinoids acting through presynaptic mechanisms to depress inhibitory synaptic transmission within the locomotor network. The fifth and the last IPSPs of the train were also analyzed and, in this case, no significant change was detected (Fig. 5D). Thus the effects of both substance P and its block by AM251 are primarily observed in the initial IPSP.

FIG. 3. The effect of endocannabinoids on the response to substance P. A and E: plots of averaged burst frequency over time from 2 different species showing similar responses to 2 consecutive 20-min applications of substance P (1 μM). B and F: averaged burst frequency showing a gradual decrease in the burst frequency in the presence of the cannabinoid antagonist, AM251 (2-5 μM); and during which the response of a 2nd application of substance P (20 min, 1 μM) was greatly attenuated. C and G: graphic illustration of the percentage of change in frequency with the 2nd application of substance P in control solution and in AM251 (*p < 0.05, **p = 0.001). D and H: graphic illustration of the percentage of decrease between Cr of the 2nd application of substance P in control solution and in AM251. No significant difference was detected.
DISCUSSION

The lamprey spinal cord allowed us to explore whether there is an interaction between tachykinins and endocannabinoids in an active network and at an inhibitory synapse. The results provide knowledge on possible inherent mechanisms playing a role in the coordination and modulation of rhythmic locomotor network activity. We show here that the effects of the tachykinin agonist substance P on the locomotor frequency and inhibitory synaptic transmission in the lamprey spinal cord are reduced if AM251 is applied, suggesting that the substance

P–induced effect may be elicited at least partially by a release of endocannabinoids.

Acute effect of substance P on the burst frequency is dependent on endogenous activation of CB1 receptors

Both substance P and endocannabinoid agonists increase the frequency of locomotor rhythm in the lamprey spinal cord and both are released endogenously during fictive locomotion (Kettunen et al. 2005; Kyriakatos and El Manira 2007; Thörn Pérez...
were essentially the same in both and the coefficient of rhythmicity.

is also suggested that by a different protein-synthesis–independent pathway, protein kinase C mediated spike modulation, it was suggested that protein kinase C receptors are involved in setting the level of network activity, whereas CB1 receptor activation may be necessary to maintain locomotion over extended periods.

Endocannabinoid-independent effects of substance P on locomotor network regularity

Application of substance P reproducibly and significantly reduced burst regularity (Cr) during fictive locomotion. It could be viewed as another aspect of the acute response to substance P. This effect, however, was not influenced by blocking cannabinoid receptors, whereas in the same experiments and during the same timeframe, the frequency was affected. The result is important in that it shows other components of the effect of substance P that do not involve cannabinoid receptors. Previous studies showed that NK1 receptors activate both adenylyl cyclase (AC) and phospholipase C (PLC) second-messenger systems (Maggi and Schwartz 1997). In an overview of the role of protein kinases in the tachykinin-mediated spike modulation, it was suggested that protein kinase C is responsible for the potentiation of NMDA receptor activity seen with exogenous application of substance P (Parker et al. 1998). It is also suggested that by a different protein-synthesis–independent pathway, protein kinase A modulates the burst regularity (Parker and Grillner 1999b) (Fig. 6). These two mechanisms might underlie the different responses of the burst frequency and the coefficient of rhythmicity.

The responses to substance P and the attenuation by AM251 were essentially the same in both _L. fluviatilis_ and _I. unicuspis_. Likewise, the substance P–induced irregularity in locomotor activity and the lack of effect by AM251 were similar in both species.

Interaction between substance P and CB1 receptors at inhibitory synapses

Paired recordings from CC interneurons to motoneurons had shown that substance P significantly depressed the monosynaptic IPSPs when activated at 20 Hz (Parker and Grillner 1999a). This response was used as a control in the present study to test for a possible role of endocannabinoids, although the stimulation was done extracellularly. In all cases, the first IPSP was significantly reduced following application of substance P. However, since there is synaptic depression in the control, the effect of substance P on the later pulses in the train would be less obvious. The difference between the first IPSP amplitude in control condition and with substance P (Fig. 5A) was significantly (p < 0.05) larger than the difference between the control condition and substance P in the presence of AM251 (Fig. 5B). From this we can infer that it is very likely that NK1 receptors use a mechanism similar to that of mGlur1, in which a reduced crossed inhibition is caused by endocannabinoid release.

Taken together, these observations suggest a possible scheme, shown in Fig. 6, for substance P activation of several pathways that could explain its effects on burst regularity and frequency, the latter involving endocannabinoid release.

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