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Neuromodulation via Conditional Release of Endocannabinoids in the Spinal Locomotor Network

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Summary

Endocannabinoids act as retrograde signals to modulate synaptic transmission. Little is known, however, about their significance in integrated network activity underlying motor behavior. We have examined the physiological effects of endocannabinoids in a neuronal network underlying locomotor behavior using the isolated lamprey spinal cord. Our results show that endocannabinoids are released during locomotor activity and participate in setting the baseline burst rate. They are released in response to mGluR1 activation and act as retrograde messengers. This conditional release of endocannabinoids can transform motoneurons and crossing interneurons into modulatory neurons by enabling them to regulate their inhibitory synaptic inputs and thus contribute to the modulation of the locomotor burst frequency. These results provide evidence that endocannabinoid retrograde signaling occurs within the locomotor network and contributes to motor pattern generation and regulation in the spinal cord.

Introduction

Endocannabinoids are lipophilic neuromodulators that can be released from postsynaptic neurons following depolarization or activation of certain G protein-coupled receptors such as metabotropic glutamate receptors (mGluRs) and muscarinic receptors (Brown et al., 2003; Chevaleyre and Castillo, 2003; Kim et al., 2002; Kreitzer and Regehr, 2001; Maejima et al., 2001; Ohno-Shosaku et al., 2002; Robbe et al., 2002; Varma et al., 2001; Wilson and Nicoll, 2001). When released from a postsynaptic site, they diffuse retrogradely to modulate synaptic transmission. Endocannabinoid-mediated retrograde signaling has been examined mainly with regard to synaptic transmission, where it can induce short- and long-term changes in synaptic strength (Carlson et al., 2002; Chevaleyre and Castillo, 2003; Freund et al., 2003; Gerdeman et al., 2002; Robbe et al., 2002). Activation of cannabinoid receptors has also been shown to interfere with experimental network oscillations in hippocampus (γ oscillations) (Freund et al., 2003; Hajos et al., 2000). The contribution of endocannabinoids to integrated network activity with relevant physiological function is, however, not yet well understood.

The spinal locomotor networks offer the possibility to determine the role of endocannabinoid retrograde signaling in the generation and regulation of integrated activity underlying locomotion. The basic components of these networks have been characterized in some detail in the lamprey and in the *Xenopus* tadpole (Buchanan, 2001; Grillner and Wallen, 2002; Roberts et al., 1998; Sillar et al., 1998). These networks consist of ipsilaterally projecting excitatory glutamatergic interneurons and contralaterally projecting inhibitory glycinergic interneurons. The glycinergic reciprocal inhibition allows alternating motor output that is characteristic for locomotion. A similar basic organization of the excitatory and inhibitory projections has been found in newborn rodents (Clarac et al., 2004; Kiehn and Butt, 2003; Nishimaru and Kudo, 2000). Intrinsic modulation operating within these networks plays a critical role in producing the final motor output (see Dale and Gilday, 1996; El Manira et al., 2002; Katz and Frost, 1996; Nusbaum et al., 2001).

In this study, we have used the lamprey spinal cord to examine the role of endocannabinoids in modulating the activity of the locomotor network. The intact lamprey spinal cord can be isolated in vitro, and the locomotor pattern, corresponding to swimming activity in the intact animal, can be elicited pharmacologically (Cohen and Wallen, 1980; Grillner et al., 1981). Receptors belonging to the three groups of mGluRs (I, II, and III) are present in the locomotor network and modulate the motor pattern (Alford et al., 2003; Cochilla and Alford, 1998; El Manira et al., 2002; Kettunen et al., 2002; Krieger et al., 1996). Glutamate released within the locomotor network activates the two subtypes of group I mGluRs (mGluR1 and mGluR5) and acts as an intrinsic modulator to regulate the burst frequency (Kettunen et al., 2002; Krieger et al., 1998, 2000).

Results of the present study show that endocannabinoids are released during locomotor activity and participate in setting the baseline burst rate. Their release can be triggered by activation of mGluR1, and they act to regulate inhibitory synaptic transmission and thereby the locomotor burst frequency. These results, previously reported in abstract form (P. Kettunen et al., 2002, Soc. Neurosci., abstract), provide insights into how separate modulatory systems interact to contribute to the patterning of activity in a motor network and show the likely functional relevance in the spinal cord.

Results

Activation of Cannabinoid Receptors Mimics the mGluR1-Induced Increase in the Locomotor Frequency

In the lamprey spinal cord, activation of mGluR1 increases the locomotor burst frequency through a potentiation of *N*-methyl-D-aspartate (NMDA) currents and a

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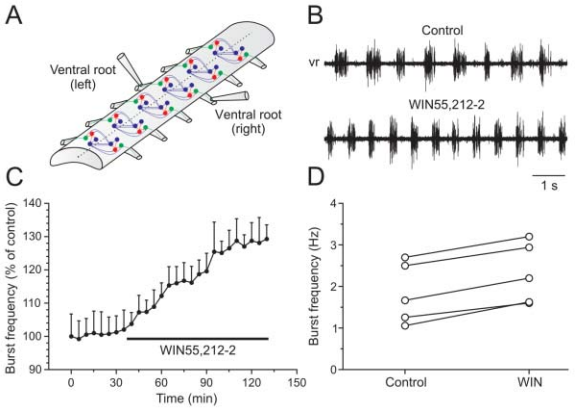

Figure 1. Activation of Cannabinoid Receptors Increases the Locomotor Burst Frequency

(A) The spinal cord contains the neuronal network underlying locomotion that consists of excitatory glutamatergic neurons (red) and inhibitory glycinergic neurons (blue). The axons of motoneurons (green) exit the spinal cord through ventral roots.

(B) Locomotor rhythm was induced by NMDA (control), and subsequent application of the cannabinoid receptor agonist WIN55,212-2 (5 μM) increased the burst frequency. Recording from a single ventral root is illustrated.

(C) Averaged data points from five experiments showing the change in burst frequency under control conditions and during application of WIN55,212-2.

(D) Graph summarizing the effect of WIN55,212-2 in five experiments. A significant ($p < 0.001$; $n = 5$) increase in the locomotor frequency was obtained in all the preparations tested.

depolarization of network neurons (Kettunen et al., 2003; Krieger et al., 1998, 2000). The increase in the burst frequency might also result from a release of endocannabinoids following activation of mGluR1. To test for this possibility, we first examined if the cannabinoid receptor agonist WIN55,212-2 (Pertwee and Ross, 2002) could mimic the mGluR1-induced increase in the locomotor burst rate. A notochord/spinal cord preparation was used, and fictive swimming pattern was induced by NMDA (50–100 μM). The changes in the locomotor rhythm were monitored by recording ventral root activity from both sides of the spinal cord (Figure 1A). Application of WIN55,212-2 (5 μM) during ongoing NMDA-induced fictive locomotion gradually increased the burst rate (Figures 1B and 1C). In total, WIN55,212-2 significantly ($p < 0.001$) increased the frequency of the locomotor rhythm from 1.8 ± 0.3 Hz to 2.3 ± 0.3 Hz ($n = 5$; Figure 1D). These results suggest that cannabinoid receptors exist in the spinal locomotor network and that their activation increases the frequency of the locomotor rhythm, thus mimicking the effect of mGluR1.

Endogenous Release of Cannabinoids within the Spinal Locomotor Network

If cannabinoid receptors are to play a physiological role in the spinal locomotor network, they should be activated by endogenously released cannabinoids during fictive locomotion. To test if endocannabinoids are released and to what extent they contribute to the baseline frequency of the locomotor activity, the cannabinoid receptor antagonists SR141716A and AM251 were administered (Pertwee and Ross, 2002). Fictive locomotion was first induced by NMDA (100 μM), and subsequent application of SR141716A (5 μM) decreased the frequency of the rhythm significantly from 2.8 ± 0.3 Hz to 2.0 ± 0.2 Hz after 2–3 hr ($n = 10$; $p < 0.001$; Figures 2A–2D). The decrease in frequency was accompanied by an increase in the burst duration from 125.7 ± 14.2 ms in control to 145.2 ± 13.9 ms in SR141716 (2–3 hr; $n = 10$; $p < 0.005$). Lipophilic antagonists like SR141716A display a slow speed of penetration into the notochord/spinal cord, and the resulting decrease in the locomotor burst frequency had a slow time dependence with no plateau reached after 2–4 hr of its application (Figures 2B and 2C). In experiments in which a shorter (30–45 min) application of SR141716A was used, the locomotor frequency recovered gradually during washout (data not shown). A similar effect on the locomotor frequency was obtained with the selective CB1 antagonist AM251, with a decrease in frequency from 1.9 ± 0.2 Hz to 1.3 ± 0.2 Hz after ~3–4 hr and to 0.7 ± 0.2 Hz after 7 hr application of the antagonist (1–5 μM; $n = 5$; $p < 0.001$). With prolonged application of AM251 (>6 hr), the cycle duration became less regular with missing ventral root bursts. These results indicate that endocannabinoids are released during ongoing fictive locomotion and contribute to setting the baseline burst frequency.

Cannabinoid Receptor Antagonists Block the Increase in the Locomotor Frequency Induced by WIN55,212-2

To ascertain that the agonist WIN55,212-2 and the antagonists SR141716A and AM251 act on the same cannabinoid receptor subtypes, we tested the effect of WIN55,212-2 on the locomotor burst frequency in the presence of these antagonists. SR141716A (5 μM) counteracted the increase in the locomotor burst frequency induced by WIN55,212-2 (5 μM; Figure 2E). In control preparations, WIN55,212-2 increased the frequency of the locomotor rhythm by 30.0% ± 6.5% ($p < 0.001$; $n = 5$; Figure 2F), while in preparations pretreated for 2–4 hr with SR141716A no increase in the locomotor frequency could be induced by WIN55,212-2 (Figures 2E and 2F). The slight decrease observed in Figure 2F (open bars) is presumably due to a blockade of the effect of the endogenously released endocannabinoids. Furthermore, application of WIN55,212-2 (5 μM), SR141716A (5 μM), or AM251 (5 μM), in the absence of NMDA, was unable to elicit any locomotor activity or induce any change in the membrane potential of motoneurons (MNs) (data not shown).

mGluR1 Modulates the Locomotor Pattern via Release of Endocannabinoids

The results above show that endocannabinoids are released within the spinal locomotor network and that a specific cannabinoid agonist mimics the increase in the frequency obtained with mGluR1 activation. We examined if mGluR1 actions could be mediated by release of endocannabinoids. Fictive locomotion was induced by NMDA, and the effect of the group I mGluR agonist (RS)-3,5-dihydroxyphenylglycine (DHPG) was tested in control and after blockade of cannabinoid receptors by SR141716A or AM251 (Figure 3). In control, application

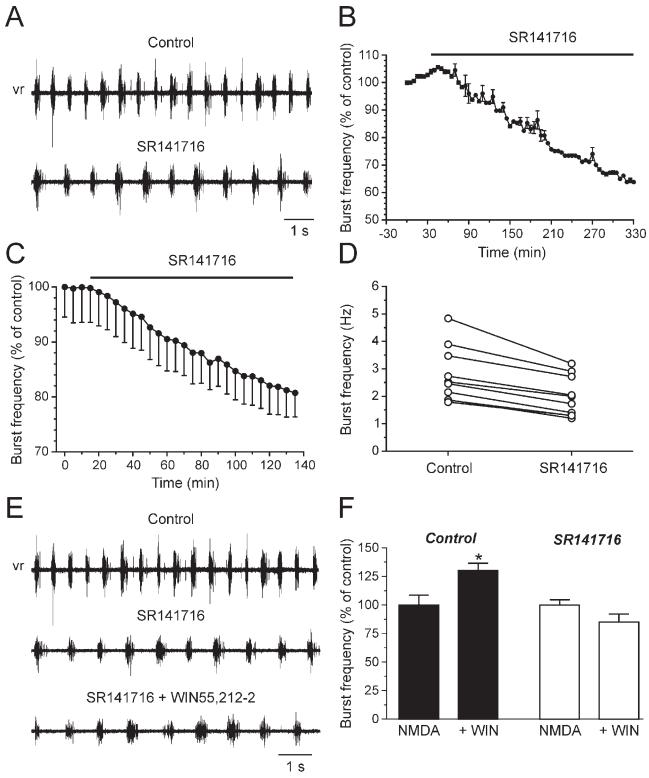

Figure 2. Release of Endocannabinoids during Fictive Locomotion

(A) Blockade of cannabinoid receptors by the antagonist SR141716 (5 μM) decreased the frequency of the locomotor rhythm. A single ventral root recording is illustrated.

(B) Plot of the burst frequency over time from the same preparation as that in (A) showing a gradual decrease during application of SR141716.

(C) Plot of averaged data from different preparations showing the time course of the decrease in the locomotor burst frequency during a 2 hr application of SR141716.

(D) A significant decrease ($p < 0.001$; $n = 10$) in the locomotor burst frequency was induced by SR141716 in all the preparations tested.

(E) During fictive locomotion induced by NMDA, application of the antagonist SR141716 (5 μM) decreased the frequency and blocked the effect of the agonist WIN55,212-2 (5 μM). This experiment shows that the agonist and antagonist used in this study act on the same receptor.

(F) The graph summarizes the effect of the cannabinoid receptor agonist WIN55,212-2 on the locomotor burst frequency in control preparations and in preparations preincubated with the antagonist SR141716. A significant increase ($p < 0.001$; $n = 5$) in the burst rate was obtained in control preparations (filled bars), whereas no increase ($p > 0.05$; $n = 5$) was induced in preparations preincubated with SR141716 (open bars). The slight decrease seen in the preparations treated with SR141716 is presumably due to a time-dependent effect of the antagonist.

of DHPG (100 μM) increased the locomotor burst frequency by 70.8% ± 18.0% ($n = 7$), which recovered after washout (Figures 3A, 3C, and 3D). Blockade of cannabinoid receptors by SR141716A (5 μM; 2–3 hr) significantly reduced the effect of DHPG on the frequency of locomotor rhythm, which increased only by 25.4% ± 5.9% ($p < 0.01$; $n = 7$; Figures 3B–3D). The decrease in the effect of DHPG was indeed due to the blockade of cannabinoid receptors and not to the repetitive application of this agonist. In a set of three experiments with two consecutive applications of DHPG in the absence of cannabinoid antagonists, similar increases in the locomotor frequency were obtained by the two applications. In these experiments, the first application of DHPG increased the burst frequency by 43.7% ± 12.4% ($n = 3$), and the second application increased the burst frequency by 42.7% ± 6.6% ($n = 3$; $p > 0.05$). These results suggest that the increase in the locomotor frequency obtained with mGluR1 activation involves release of endocannabinoids, which act as intrinsic modulators within the locomotor network.

Specificity of the Cannabinoid Agonist and Antagonists

Possible nonspecific effects of the cannabinoid agonist and antagonists were examined by testing their effect on the resting holding current, on mGluR1-induced inward current, and on NMDA current in dissociated spinal cord neurons. These currents showed some rundown but were not significantly altered during application of WIN55, 212-2, SR141716A, or AM251. The DHPG-induced current had an amplitude of 21.6 ± 1.8 pA in control and 20.8 ± 3.1 pA in WIN55,212-2 ($p > 0.05$; $n = 3$). In SR141716A, the amplitude of this current was 18.5 ± 4.0 pA compared to 21.6 ± 4.6 pA in control ($p > 0.05$; $n = 4$). There was no significant difference ($p > 0.05$) in the amplitude of DHPG-induced current in control (25.4 ± 7.0 pA; $n = 6$) and in AM251 (20.6 ± 6.7 pA; $n = 6$). No significant effects were seen on the NMDA-induced current by WIN55,212-2 (159.2 ± 31.1 pA [control] and 141.2 ± 23.0 pA [WIN]; $p > 0.05$; $n = 3$), by SR141716 (176.3 ± 15.4 pA [control]; 161.2 ± 15.2 pA [SR141716]; $p > 0.05$; $n = 4$), and by AM251 (191.8 ± 34.1 pA [control]; 180.2 ± 34.6 pA [AM251]; $p > 0.05$; $n = 3$). These results suggest that WIN55,212-2, SR141716A, and AM251 act specifically on cannabinoid receptors and that their effect on the locomotor frequency is not due to nonspecific actions on mGluR1 or NMDA receptors.

mGluR1 Activation Triggers Retrograde Signaling via Endocannabinoids in the Lamprey Spinal Cord

The mGluR1-induced increase in the locomotor frequency involving endocannabinoids could be mediated via retrograde modulation of synaptic transmission within

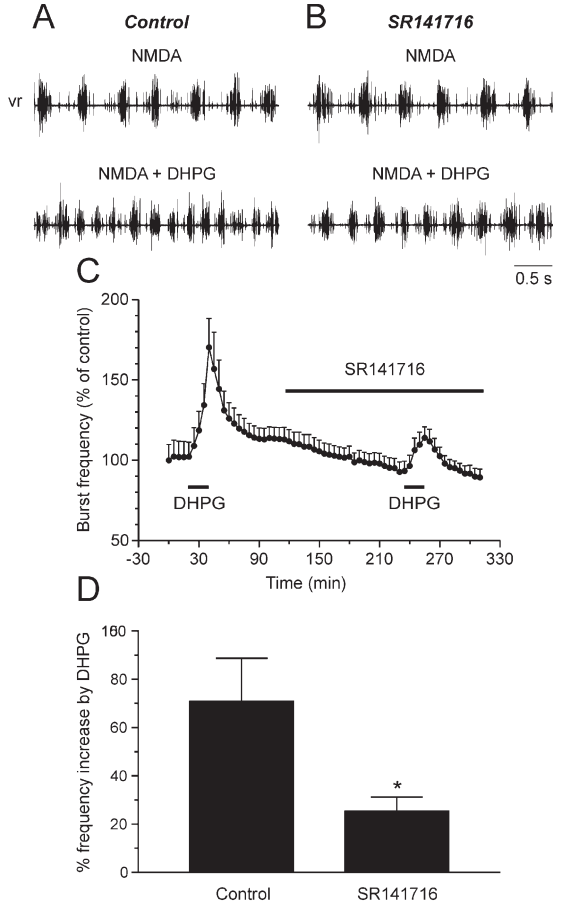

Figure 3. mGluR1 Increases the Locomotor Frequency via Release of Endocannabinoids

(A) The possible involvement of endocannabinoids in the mGluR1-induced increase of the locomotor frequency was examined. The group I agonist DHPG (100 μM) increased the locomotor frequency induced by NMDA ($p < 0.001$; $n = 7$).

(B) Blockade of cannabinoid receptors by SR141716 significantly reduced the increase in the frequency induced by DHPG ($p < 0.01$; $n = 7$).

(C) Plot of averaged data from seven preparations showing the effect of DHPG on the locomotor frequency in control and in the presence of SR141716.

(D) Summary of the data from all the experiments ($n = 7$) in which the effect of DHPG was tested before and after blockade of cannabinoid receptors. The increase in the locomotor frequency by DHPG was significantly reduced ($p < 0.01$; $n = 7$) after blockade of cannabinoid receptors by SR141716.

the locomotor network. One target for such a modulation is the glycinergic inhibitory synapse from contralaterally projecting interneurons underlying left-right alternation of locomotor activity. A reduction of glycinergic synaptic transmission with pharmacological (Cohen and Harris-Warrick, 1984; Grillner and Wallen, 1980) or surgical methods to separate left and right hemicords (Cangiano and Grillner, 2003) results in an increase in the locomotor frequency due to the decreased crossed inhibition. To examine if activation of the postsynaptic mGluR1 affected crossing-inhibitory synaptic transmission, intracellular recordings were made from identified MNs and crossing caudally projecting interneurons (CC-INs), while interneurons on the contralateral side were stimulated extracellularly. The spinal cord was completely isolated from the notochord with meninges removed, while the ipsilateral and medial parts of the spinal cord were lesioned to limit the spread of the stimulation (Figure 4A). In these experiments, NMDA, AMPA, and kainate receptors were blocked with AP5 (50 μM) and CNQX (30 μM), respectively. Activation of postsynaptic mGluR1 by DHPG decreased the amplitude of monosynaptic inhibitory postsynaptic potentials (IPSPs) elicited in identified MNs (Figures 4B1 and 4B2) and CC-INs (Figures 4C1 and 4C2) by extracellular stimulation of glycinergic interneurons. In MNs, the IPSPs had an amplitude of 5.9 ± 0.6 mV in control conditions, which was significantly depressed to 3.3 ± 0.5 mV in the presence of DHPG (100 μM; $n = 7$; $p < 0.001$; Figures 4B1 and 4B2). In CC-INs, the amplitude of IPSPs was 4.2 ± 0.4 mV in control and was decreased to 2.7 ± 0.2 mV in DHPG (100 μM; $p < 0.05$; $n = 3$; Figures 4C1 and 4C2). To determine if the depression of the glycinergic synaptic transmission mediated by mGluR1 required activation of cannabinoid receptors, the effect of DHPG was tested after the cannabinoid receptors were blocked by SR141716A. In preparations pretreated with the cannabinoid receptor antagonist SR141716A (5 μM), application of DHPG did not depress the amplitude of monosynaptic glycinergic IPSPs recorded in MNs (Figures 4D1 and 4D2) or CC-INs (Figures 4E1 and 4E2). In control conditions, the amplitude of the IPSPs was 5.8 ± 0.4 mV, which was not significantly different from that in DHPG, 5.7 ± 0.3 mV ($p > 0.05$; $n = 9$; six MNs and three CC-INs; Figures 4D and 4E). In these experiments, the membrane potential of the neurons examined was held at −58 mV and was not affected by the different agonists and antagonists.

The decrease in the IPSP amplitude induced by DHPG cannot be accounted for by a decrease in the input resistance of postsynaptic neurons, because this agonist is known to increase the input resistance (Kettunen et al., 2003). Furthermore, DHPG induced an increase in the paired-pulse ratio from 0.93 ± 0.30 to 1.14 ± 0.02 ($n = 4$; $p < 0.002$; Figures 5A and 5B). These data suggest that activation of group I mGluRs by DHPG induces release of endocannabinoids, which act via presynaptic mechanisms to depress inhibitory synaptic transmission within the locomotor network.

To further examine if DHPG depresses synaptic transmission by releasing endocannabinoids, experiments were performed to determine if the cannabinoid agonist WIN55,212-2 could occlude the effect of DHPG. Application of WIN55,212-2 (5 μM) alone decreased significantly the amplitude of the glycinergic IPSPs from 4.7 ± 0.6 mV in control to 3.1 ± 0.1 mV ($p < 0.02$; $n = 3$; Figure 5A). Application of DHPG in the presence of WIN55,212-2 (5 μM) did not induce any further decrease in the IPSP amplitude ($p > 0.05$; $n = 3$; Figures 5C and 5E), indicating that DHPG acts via release of endocannabinoids. In experiments in which DHPG was applied first, a decrease in the IPSP amplitude was induced from 4.8 ± 0.6 mV to 3.4 ± 0.4 mV ($n = 3$; $p < 0.05$), and the consecutive application of WIN55,212-2 further decreased the IPSP amplitude to 2.3 ± 0.6 mV ($n = 3$; $p < 0.05$; Figures 5D and 5E). These results suggest that the amount of endocannabinoids released by DHPG may not be high

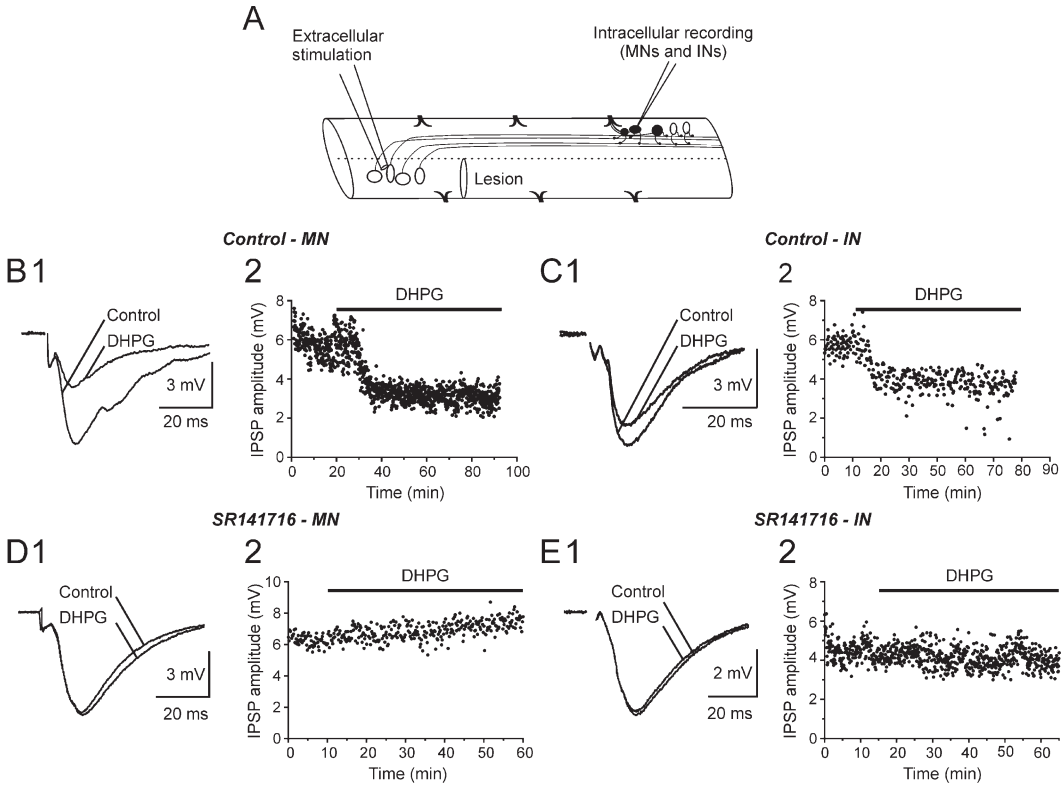

Figure 4. Endocannabinoids Are Released by Activation of Group I mGluRs and Depress Synaptic Transmission

(A) Monosynaptic IPSPs were elicited in postsynaptic motoneurons (MNs) and crossing interneurons (CC-INs) recorded intracellularly by stimulating axons of CC-INs with an extracellular suction electrode. Ionotropic glutamate receptors were blocked by AP5 (50 μM) and CNQX (30 μM).

(B1) The mGluR agonist DHPG (100 μM) significantly reduced the amplitude of the IPSPs elicited in a MN by stimulation of inhibitory CC-INs. The traces are averages of 20 sweeps.

(B2) Plot of the IPSP amplitude recorded in a MN before and after DHPG application.

(C1) The amplitude of the IPSPs elicited in a CC-IN was significantly reduced by the mGluR agonist DHPG (100 μM). The traces are averages of 20 sweeps.

(C2) Plot of the IPSP amplitude recorded in a CC-IN before and after DHPG application.

(D1) The cannabinoid antagonist SR141716 (5 μM) blocked completely the effect of DHPG on the amplitude of the IPSPs in a MN. No significant difference ($p > 0.05$) was seen on the amplitude of the IPSP between control and DHPG after treatment with SR141716. The traces are averages of 20 sweeps.

(D2) Plot showing that DHPG failed to reduce the IPSP amplitude in a MN when cannabinoid receptors were blocked with SR141716.

(E1) The cannabinoid antagonist SR141716 (5 μM) blocked the effect of DHPG on the amplitude of the IPSPs in a CC-IN. The traces are averages of 20 sweeps.

(E2) Plot showing that DHPG had no effect on the IPSP amplitude in a CC-IN when cannabinoid receptors were blocked with SR141716.

enough to activate all cannabinoid receptors and thus occlude completely the effect of WIN55,212-2 on inhibitory synaptic transmission.

Blockade of CB1 Receptor Increases Inhibitory Synaptic Transmission

To determine if endocannabinoids are released within the spinal cord in the absence of network activity, the effect of the CB1 antagonist AM251 was tested on the amplitude of IPSPs induced in MNs ($n = 4$; Figures 6A1 and 6A2) and CC-INs ($n = 4$; Figures 6B1 and 6B2) by stimulation of crossing inhibitory interneurons. Application of AM251 (5 μM) significantly increased the amplitude of the IPSPs from 5.1 ± 0.5 mV to 7.0 ± 0.9 mV in MNs ($n = 4$; $p < 0.03$; Figures 6C and 6D) and from 5.1 ± 0.4 mV to 6.8 ± 0.4 mV in CC-INs ($n = 4$; $p < 0.01$; Figures 6C and 6D). Thus, there appears to be a continuous modulation of inhibitory synaptic transmission within the spinal cord by endocannabinoids even in the absence of ongoing locomotor activity. The endocannabinoids might be released, at least partially, in response to activation of mGluR1 by endogenously released glutamate.

Retrograde Signaling of Endocannabinoids Is Mediated by mGluR1 Activation

To test if endocannabinoids are released from MNs and CC-INs following activation of postsynaptic mGluR1, we interfered with the signaling cascade of mGluRs in the postsynaptic neurons exclusively. To this end, we included the irreversible G protein inhibitor GDP-β-S (20 mM) in the recording sharp electrode. In neurons loaded with GDP-β-S, no significant decrease in the IPSP amplitude was induced by application of DHPG (100 μM;

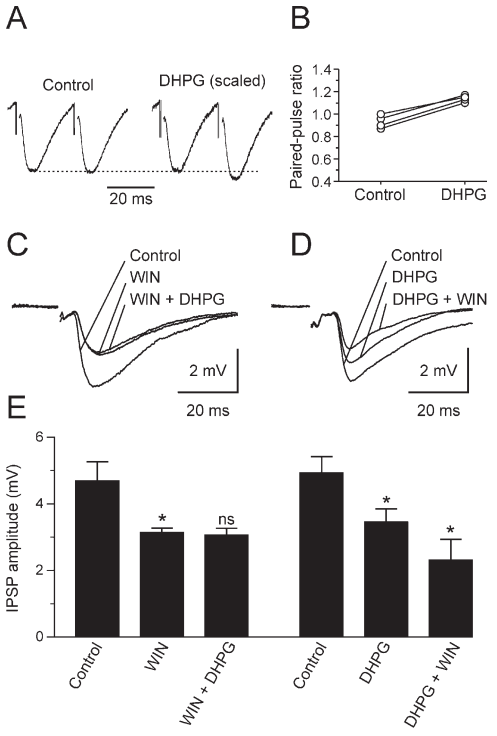

Figure 5. Activation of Cannabinoid Receptors Occludes the Depression of Synaptic Transmission by Group I mGluRs

(A) The decrease in the IPSP amplitude by DHPG was accompanied by an increase in the paired-pulse ratio. The trace in DHPG was normalized to the amplitude of the first IPSP in control.

(B) The paired-pulse ratio was significantly increased in DHPG compared to control ($n = 4$; $p < 0.002$).

(C) WIN55,212-2 (5 μM) reduced the amplitude of the IPSP induced by stimulation of CC-INs. Application of DHPG (100 μM) in the presence of WIN55,212-2 did not induce any further decrease in the IPSP amplitude. The traces are averages of 20 sweeps.

(D) DHPG alone reduced the amplitude of the IPSP, which was further decreased with the application of WIN55,212-2. The traces are averages of 20 sweeps.

(E) Summary graph of all experiments showing that WIN55,212-2 was able to occlude the effect of DHPG on the IPSP amplitude. On the other hand, DHPG reduced the effect of WIN55,212-2 on the IPSP amplitude but did not completely occlude it.

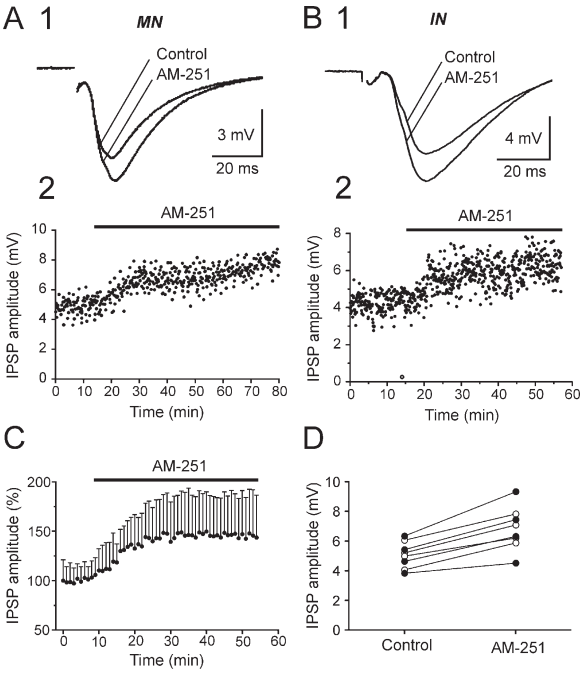

Figure 6. Endocannabinoids Modulate Inhibitory Synaptic Transmission

(A1) The CB1 antagonist AM251 (5 μM) increased the IPSP amplitude induced in a MN by stimulation of crossing inhibitory interneurons. The traces are averages of 20 sweeps.

(A2) Plot of the IPSP amplitude from one experiment showing the increase in the amplitude induced by AM251 in a MN.

(B1) The IPSP amplitude induced in a CC-IN by stimulation of crossing inhibitory interneurons was increased by AM251 (5 μM). The traces are averages of 20 sweeps.

(B2) Plot of the IPSP amplitude from one CC-IN showing the increase in the amplitude induced by AM251.

(C) Averaged data from all the experiments showing the time course of the increase in the IPSP amplitude by AM251.

(D) Application of AM251 significantly increased the IPSP amplitude in MNs (filled circles) and in CC-INs (open circles).

Figures 7A and 7B). In control conditions, DHPG decreased the amplitude of IPSPs in MNs by 44.5% ± 3.5% ($p < 0.02$; $n = 7$), whereas no significant decrease was seen in MNs loaded with GDP-β-S ($p > 0.05$; $n = 4$; Figure 5C). In CC-INs, DHPG decreased the IPSP amplitude by 34.0% ± 5.2% ($p < 0.05$; $n = 3$) in control but had no significant effect in CC-INs loaded with GDP-β-S ($p > 0.05$; $n = 3$; Figure 7C). These results thus suggest that activation of postsynaptic mGluR1 can induce release of endocannabinoids from MNs and CC-INs that act as retrograde messengers to depress inhibitory synaptic transmission within the locomotor network.

Discussion

Endocannabinoids within the Locomotor Network

In this study, we tested the role of cannabinoid receptors in an active neuronal network that expresses motor patterns that would normally result in locomotion in the intact animal (Wallen and Williams, 1984). Network neurons are thus activated during the appropriate phases of the locomotor cycle, and they receive rhythmic inhibitory and excitatory inputs from other network neurons. Their firing frequency is the result of the integrated network activity and is not imposed by artificial stimulus paradigms. This offered the possibility to test if endogenous cannabinoid retrograde signaling contributed to the generation and regulation of network activity that corresponds to locomotion. From our results, it can be inferred that endocannabinoids are released during fictive locomotion and act as endogenous neuromodulators that set the baseline frequency. Thus, blockade of cannabinoid receptors decreases the locomotor burst frequency, while conversely, their activation by a specific agonist increases that frequency. Moreover, the latter effect was abolished by specific cannabinoid receptor antagonists. Endocannabinoids are produced through hydrolysis of membrane lipid precursors in somata and dendrites (Freund et al., 2003; Piomelli, 2003; Sugiura et al., 2002). In the lamprey locomotor system, they appear to be released from MNs and CC-INs to act retro-

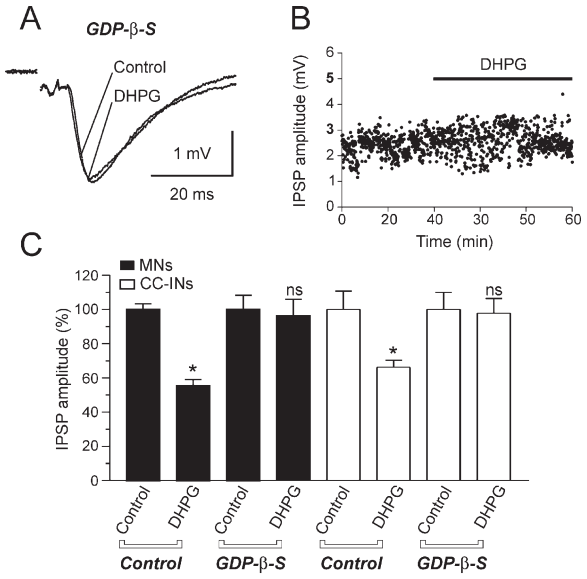

Figure 7. The mGluR Induced Release of Endocannabinoids Is Mediated by G Proteins in Both Motoneurons and Network Interneurons

(A) Loading postsynaptic neurons with the G protein inhibitor GDP-β-S (20 mM) blocked the reduction in the IPSP amplitude induced by DHPG (100 μM). The traces are averages of 20 sweeps.

(B) Plot showing the lack of an effect on the IPSP amplitude by DHPG in a neuron loaded with GDP-β-S.

(C) Graph showing reduction in the IPSP amplitude by DHPG in both motoneurons (filled bars) and crossing interneurons (CC-INs; open bars). This reduction was blocked in all neurons by injection of the G protein inhibitor GDP-β-S into the postsynaptic neurons.

gradely on inhibitory synaptic transmission from CC-INs that mediate left-right alternation. Endocannabinoids can thus be considered as intrinsic neuromodulators within the locomotor network, and their release is controlled by activation of mGluR1.

Mammalian Studies—Modulation of Synaptic Transmission

Studies in hippocampus and cerebellum slices have shown that endocannabinoids can be synthesized and released by depolarization of the membrane potential of neurons via a Ca^{2+}-dependent mechanism (Kreitzer and Regehr, 2001; Wilson and Nicoll, 2001). The released endocannabinoids act as retrograde messengers to depress synaptic transmission by inhibiting voltage-activated Ca^{2+} channels in presynaptic terminals (Kreitzer and Regehr, 2001; Wilson et al., 2001). In most studies, endocannabinoid signaling has been induced by fairly large and long-lasting depolarizations of postsynaptic neurons. Such depolarizations are unlikely to occur under physiological conditions. Release of endocannabinoids can also be mediated by activation of group I mGluRs (Brown et al., 2003; Chevaleyre and Castillo, 2003; Maejima et al., 2001; Ohno-Shosaku et al., 2002; Robbe et al., 2002; Varma et al., 2001). Thus far, the role of endocannabinoids has only been examined with regard to modulation of synaptic transmission, and their contribution to integrated network activity underlying motor behavior has not been determined.

Neuromodulation on Demand—mGluR1-Mediated Release of Endocannabinoids

mGluR1s are localized on postsynaptic soma and dendritic membrane in lamprey spinal cord neurons (Kettunen et al., 2003; Krieger et al., 1998, 2000; but see Cochilla and Alford, 1998). Activation of these receptors by endogenously released glutamate increases the frequency of the locomotor rhythm (Krieger et al., 1998, 2000). This increase in the frequency was initially thought to be mediated by a restricted postsynaptic action of mGluR1 via two mechanisms: first, through an interaction with NMDA receptors that increases their inward current and the associated Ca^{2+} influx (Krieger et al., 2000), and second, by blocking a leak conductance that results in the depolarization of the membrane potential of spinal cord neurons (Kettunen et al., 2003). The present study shows that mGluR1 in addition induces the release of endocannabinoids from neurons of the locomotor network that act as a retrograde messenger to depress inhibitory synaptic transmission onto MNs and CC-INs. The depression of inhibitory transmission controlling left-right alternation during locomotion is likely to mediate the increase in the locomotor frequency induced by mGluR1 and cannabinoid receptor activation (Figures 8A–8C). Previous studies, in which crossed glycinergic inhibition was reduced either pharmacologically or by lesions along the midline, also resulted in an increase in the frequency of the locomotor rhythm (Cangiano and Grillner, 2003; Cohen and Harris-Warrick, 1984; Grillner and Wallen, 1980). Endocannabinoids, in addition to depressing inhibitory synaptic transmission, might also affect the release of other modulators (e.g., dopamine and 5-HT) to change the frequency of the locomotor rhythm.

In the lamprey spinal cord, mGluR1 appears to exist on both excitatory and inhibitory neurons (Krieger et al., 2000). The released glutamate activates ionotropic receptors and underlies the cycle-by-cycle excitation. Activation of mGluR1 further boosts the depolarization of neurons by interacting with NMDA receptors and blocking leak channels. Our work suggests that, to ensure that the excitatory effect is not counteracted by a simultaneous increase of inhibitory inputs, mGluR1 induces the release of endocannabinoids, which causes a concomitant decrease of inhibitory inputs to network neurons via retrograde signaling, thereby ensuring that the excitatory effect of mGluR1 prevails at both the cellular and network levels (Figures 8B and 8C).

Ubiquity of Modulatory Potential within Neuronal Networks

Modulatory neurons have been classically defined as neurons releasing modulatory transmitters at their synaptic terminals that usually activate slow-acting G protein-coupled receptors. Our results suggest that the modulatory potential is not restricted to the subset of neurons that act in this way but in addition can include any neurons within a neuronal network that release substances such as endocannabinoids. Such a neuron can become a modulatory neuron by releasing endocannabinoids from its soma-dendritic membrane to affect its incoming synaptic inputs. Endocannabinoids can be synthesized "on demand" within the spinal locomotor

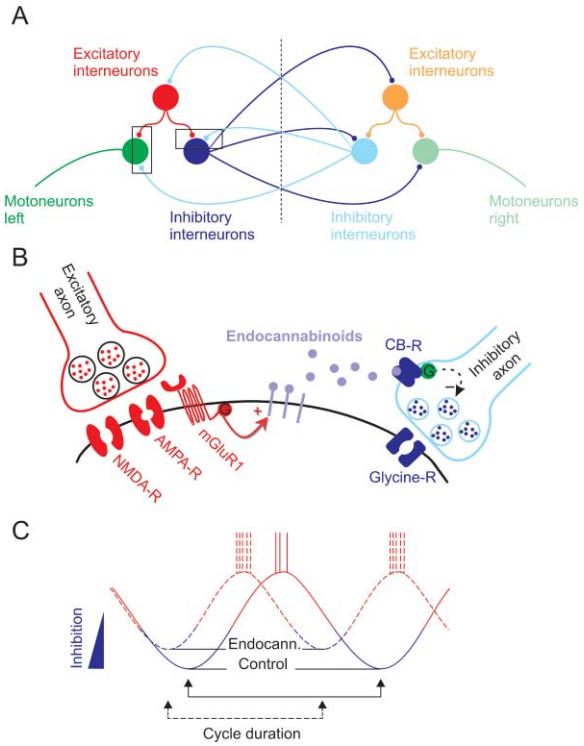

Figure 8. The Effect of Endocannabinoids on the Spinal Locomotor Network

(A) Diagram showing the known connectivity of excitatory glutamatergic neurons (red) and crossing-inhibitory interneurons (blue) within the lamprey spinal locomotor network. MNs (green) receive alternating excitatory and inhibitory inputs underlying the locomotor rhythm. The bright colors indicate the activated side, and dimmed colors represent the inhibited side during one locomotor cycle. The boxes drawn on the left MNs and CC-INs indicate the region enlarged in (B).

(B) Release of glutamate from excitatory interneurons act on NMDA and AMPA receptors to produce the cycle-by-cycle excitation, while the inhibition is mediated by glycine released from CC-INs. Glutamate, through activation of mGluR1, also acts as an endogenous modulator to increase the locomotor frequency. This is mediated partially through the release of endocannabinoids, which act as retrograde messengers to depress inhibitory synaptic transmission via presynaptic receptors (CB-R) and thereby reduce the glycinergic inhibition.

(C) During locomotion, spinal network neurons receive excitation (red) alternating with crossing inhibition (blue). The release of endocannabinoids (endocann., dashed trace) within the locomotor network can decrease the crossed inhibition as compared to control (solid trace). The reduction of inhibition induced by endocannabinoids combined with the increased excitation mediated by mGluR1 activation results in a decrease in cycle duration and thus an increased locomotor frequency. We propose that intrinsic modulation using on-demand release of endocannabinoids can be considered as an integral part of the pattern generation in the spinal cord.

network in response to activation of mGluR1 and thus transform MNs and network interneurons into modulatory neurons. The impact of endocannabinoid modulation relies on the distribution of cannabinoid receptors and their proximity to the release source. The distance over which endocannabinoids can diffuse have been estimated to be ~100 μm in the cerebellum (Kreitzer et al., 2002) and 20 μm in the hippocampus (Wilson and Nicoll, 2001). If a similar restricted diffusion occurred in the spinal cord, the effect of endocannabinoids could be limited mainly to the synaptic inputs impinging on a given neuron or part of a dendritic tree.

In vertebrates, in contrast to invertebrates, MNs have often been considered as mere output neurons (Mangan et al., 1994; Nusbaum and Beenhakker, 2002; see also Delgado-Lezama and Hounsgaard, 1999; Roberts and Perrins, 1995). The mechanism using on-demand release of endocannabinoids by activation of mGluR1 suggests that MNs also have a modulatory potential, allowing them to contribute to locomotor pattern generation. The proposed mechanism of activating a modulatory endocannabinoid capability through activation of specific receptors like mGluR1 may possibly be widespread in different vertebrate locomotor networks. It is still not known if the action of endocannabinoids is fast enough to act on synaptic transmission to MNs and CC-INs in a phasic manner during fictive locomotion or if it serves to mediate a tonic modulation of synaptic efficacy. Future experiments are required to determine the importance of endocannabinoid retrograde signaling in regulating synaptic transmission during the different phases of the locomotor cycle.

Experimental Procedures

Experiments were performed in vitro using the intact spinal cord from adult lampreys (*Lampetra fluviatilis*). All protocols were approved by the animal research ethical committee, Stockholm. Lampreys were anesthetized with MS 222 (100 mg/l; Sigma, St. Louis, MO) and eviscerated, and the lateral muscle walls were removed. The spinal cord and notochord were dissected and pinned in a cooled (8°C–12°C) Sylgard-lined experimental chamber continuously perfused with cooled physiological solution. The control solution was composed of 138 mM NaCl, 2.1 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 4 mM glucose, and 2 mM HEPES, bubbled with O_2 and pH adjusted to 7.4. To analyze the effects of different agonists and antagonists on the locomotor frequency, the spinal cord was left attached to the notochord, and the meninges were not removed. In this way, the ventral roots were left intact and were recorded from by gently placing en passant suction electrodes. This allowed long-lasting recordings (>24 hr) without damage to the ventral roots. Fictive swimming activity was induced in the notochord/spinal cord by adding 50–100 μM NMDA (Tocris, Bristol, UK) (Grillner et al., 1981) to the physiological solution. Alternating locomotor burst activity was recorded by glass suction electrodes from opposing ventral roots at their exits from the spinal cord. The burst frequency increased gradually with time, stabilized after 3–4 hr of perfusion with NMDA, and remained stable for several hours. Agonists and antagonists were added only after the burst frequency had been stable for at least 1 hr. Control preparations, in which no agonist or antagonist was applied, did not show any change in the locomotor burst frequency. One minute recordings of ventral root activity were sampled every 5 min throughout the experiment. The cycle duration, burst duration, and burst proportion (duty cycle) were measured and averaged from 50 to 100 consecutive cycles. The cycle duration was defined as the time interval between the onsets of two consecutive bursts, and the burst proportion was calculated as the ratio of the burst duration and the cycle duration. The burst frequency was calculated as the inverse of the averaged cycle duration.

Spinal neurons were dissociated from larval lampreys. MNs and interneurons were prelabeled for subsequent identification after dissociation by injecting fluorescein-coupled dextran amine (Molecular Probes, the Netherlands; Krieger et al., 2000). After 18 hr of incubation, the spinal cords were dissected from the notochord, cut in smaller pieces, and treated with collagenase (1 mg/ml; 30 min; Sigma) and then with protease (2 mg/ml; 45 min; Sigma), both dissolved in Leibovitz's L-15 culture medium (270 mOsm; Sigma) containing penicillin-streptomycin (2 μm/ml) (El Manira and Bussieres,

1997). The spinal cord was then triturated with a glass pipette in Leibovitz's solution. The solution with the fully dissociated tissue was distributed into 15 culture dishes (35 mm; Corning, VWR International, Stockholm) and incubated at 10°C for 2 days. Whole-cell patch-clamp recordings were made from isolated spinal cord neurons in culture using an Axopatch 200A (Axon Instruments). Dissociated cells were continuously perfused using a gravity-driven multibarreled perfusion system with the tip positioned close to the recorded neuron. The extracellular control solution contained 124 mM NaCl, 2 mM KCl, 1.2 mM $MgCl_2$, 5 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, with pH adjusted to 7.6. The electrode solution consisted of 113 mM KCH_3SO_3, 1.2 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, 3.9 mM adenosine 5′-triphosphate magnesium salt, 0.3 mM guanosine 5′-triphosphate, and 5 mM phosphocreatine, with pH adjusted to 7.6 with KOH. DHPG (100 μM; 10 s) and NMDA (200 μM; 5 s) were applied close to the recorded neurons in control and in the presence of the different cannabinoid agonists and antagonists. Recordings of the membrane currents were performed in gap-free mode. They were digitized using a Digidata 1320a interface, monitored, and stored with pClamp software.

The following drugs were added together with NMDA to the physiological solution: the group I mGluR agonist DHPG (100 μM; Tocris); the cannabinoid receptor antagonists SR141716A (1–5 μM) and AM251 (1–5 μM; Tocris); and the cannabinoid receptor agonist WIN55,212-2 mesylate (5 μM; Tocris). All the tested agonists and antagonists were dissolved as stock solutions in DMSO, except DHPG, which was dissolved in water. The same concentration of the solvent DMSO had no effect on the locomotor frequency and was always added to control solutions throughout the experiments.

Modulation of inhibitory transmission was also examined at the synaptic level. In these experiments, the spinal cord was completely isolated from the notochord, and the meninges were removed to allow for intracellular recordings. The isolated spinal cord was pinned with the ventral side up in a smaller recording chamber (one-third of the chamber used for the notochord/spinal cord preparation). Intracellular recordings were made from MNs with 3 M potassium acetate-filled thin-wall glass microelectrodes with a resistance of 15–30 MΩ. MNs were identified by recording their axonal action potentials in a one-to-one fashion from the corresponding ventral root using an extracellular suction electrode. CC-INs were identified by recording extracellularly their axonal action potentials two to three segments caudal and contralateral to the intracellular recording site. Then, a stimulating extracellular glass electrode was placed on the contralateral gray matter rostral to the recorded MN to activate contralaterally projecting descending inhibitory interneurons. A 0.5 ms stimulation pulse was given every 5 s. To ensure that only crossing descending interneurons were stimulated, the gray matter caudal to the stimulation site was cut, as well as the medial spinal cord, where the descending reticulospinal axons project. Ionotropic glutamate receptors were blocked by adding the NMDA receptor antagonist AP5 (50 μM; Tocris) and the AMPA receptor antagonist CNQX (30 μM; Tocris). Induced IPSPs recorded in the MNs were considered to be monosynaptic because they followed high-frequency stimulation (≥50 Hz) of the descending axons and also persisted in high-calcium/high-magnesium solution ($6\times Ca^{2+}$, $6\times Mg^{2+}$) (Krieger et al., 1996). Their glycinergic nature was also confirmed, as they were completely blocked by the antagonist strychnine (5 μM) (McPherson et al., 1994). The peak amplitude of the IPSPs was measured in control and in the presence of the group I mGluR agonist DHPG (100 μM). The contribution of activation of cannabinoid receptors in the modulation of glycinergic transmission by DHPG was tested in preparations in which cannabinoid receptors were blocked by preincubation with the antagonists SR141716A (1–5 μM) and AM251 (1–5 μM). Intracellular recordings were made in bridge mode or discontinuous current-clamp mode with an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA). Axon Instruments software (pClamp) was used for data acquisition and analysis on a PC computer equipped with an A/D interface (Digidata 1300). The values shown in the figures and given in the text correspond to mean ± SEM, and "n" represents the number of experiments. The significance was assessed with a paired Student's t test and defined as $p < 0.05$.

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