Development/Plasticity/Repair

Tonic and Transient Endocannabinoid Regulation of AMPAergic Miniature Postsynaptic Currents and Homeostatic Plasticity in Embryonic Motor Networks

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Endocannabinoid signaling has been shown to mediate synaptic plasticity by retrogradely inhibiting presynaptic transmitter release in several systems. We found that endocannabinoids act tonically to regulate AMPA miniature postsynaptic current (mPSC) frequency in embryonic motor circuits of the chick spinal cord. Further, strong postsynaptic depolarizations also induced a short-lived endocannabinoid-mediated suppression of mEPSC frequency. Unlike many previous studies, endocannabinoid signaling was not found to influence evoked transmitter release. The results suggest a special role for spontaneous glutamatergic mPSCs and their control by endocannabinoids in the developing spinal cord. We determined that blocking endocannabinoid signaling, which increases spontaneous glutamatergic release, increased spontaneous network activity *in vitro* and *in vivo*. Previous work in spinal motoneurons had shown that reducing spontaneous network activity (SNA) chronically *in vivo* led to homeostatic increases in AMPA and GABA mPSC amplitude (homeostatic synaptic plasticity). Blocking endocannabinoid signaling *in vivo*, and thus increasing SNA, triggered compensatory decreases of both AMPA and GABA mPSC amplitudes. These findings, combined with previous results, are consistent with the idea that this form of homeostatic synaptic plasticity is a bidirectional process in the living embryo. Together, our results suggest a role for tonic signaling of endocannabinoids as a potential mechanism to regulate the level of SNA, which is known to be critical for synaptic maturation in the embryonic spinal cord.

Introduction

Embryonic movements are produced by spontaneous bursts of spiking activity arising episodically in the developing spinal network. This spontaneous network activity (SNA) results from the highly excitable nature of the synaptic connections in the spinal circuitry, as both GABAergic and AMPAergic currents are excitatory at this stage. SNA is expressed in most, if not all, developing networks, suggesting a fundamental importance for the maturation of these neuronal circuits (Blankenship and Feller, 2010; O'Donovan et al., 1998). In the embryonic spinal cord SNA has been shown to be important for the proper development of the limbs, and motoneuron axon pathfinding (Gonzalez-Islas and Wenner, 2010; O'Donovan et al., 1998). In addition, we have recently shown that SNA is important in the regulation of synaptic strength through a form of homeostatic synaptic plasticity (Gonzalez-Islas and Wenner, 2006). When SNA was blocked *in vivo* in the chick from embryonic day 8 to 10 (E8–E10), the amplitude of AMPA and GABA miniature postsynaptic currents (mPSCs) was altered in a manner that should compensate for

activity blockade. On the other hand it is unknown whether homeostatic plasticity is triggered by chronic increases in the levels of SNA in the embryonic cord.

Additionally, a faster form of SNA-dependent synaptic plasticity also occurs in developing spinal motoneurons. Immediately following an episode of SNA, evoked and spontaneous glutamatergic synaptic responses are depressed, and then recover in the interepisode interval (IEI; Fedirchuk et al., 1999; Tabak et al., 2001; C. Gonzalez-Islas and P. Wenner, unpublished results). This postepisode depression and recovery appear to be at least partially mediated by changes in presynaptic release. The mechanism responsible for the SNA-dependent modulation of AMPAergic synaptic strength is not clear. We considered the possibility that endocannabinoid signaling, implicated in many different forms of synaptic plasticity, was involved. It is widely known that after strong depolarizations and metabotropic signaling, as would occur during episodes of SNA, neurons can synthesize and release endocannabinoids; the released endocannabinoids retrogradely activate a presynaptic G-protein-coupled cannabinoid receptor (CB1), which in turn inhibits neurotransmitter release (Alger, 2009; Heifets and Castillo, 2009; El Manira and Kyriakatos, 2010; Ohno-Shosaku et al., 2012). Further, previous work suggested that hippocampal SNA leads to endocannabinoid liberation, thus inhibiting presynaptic vesicle release (Bernard et al., 2005). We therefore hypothesized that episodes of SNA could trigger the release of endocannabinoids and reduce presynaptic glutamatergic release following the episode.

We show that endocannabinoid signaling did not appear to mediate the postepisode modulation of mEPSC frequency or evoked (action potential-dependent) glutamatergic responses.

Table 1. Effect of cannabinoid modulators on motoneuron resting membrane properties and on the mPSC parameters

Parameter	Control (n = 7)	AM 251 (n = 7)	Control (n = 15)	WIN 55,212-2 (n = 17)	Control (n = 9)	ACEA (n = 6)	Control (n = 5)	AM 404 (n = 5)	Control (n = 8)	LY 2183240 (n = 13)
Membrane potential (mV)	−63 ± 9.2	−66 ± 8.7	−65 ± 5.4	−71 ± 4.7	−71 ± 5.3	−65 ± 4.2	−59 ± 6.4	−62 ± 7.1	−61 ± 3.5	−60 ± 4.2
Membrane resistance (MΩ)	519 ± 76	487 ± 53	582 ± 56	573 ± 28	576 ± 89	521 ± 49	448 ± 62	472 ± 85	519 ± 38	530 ± 27
AMPA mPSC mean amplitude (pA)	8.4 ± 0.2	8.5 ± 0.5	15.2 ± 1.5	15.6 ± 1.4	16.8 ± 0.4	11.0 ± 0.3	8.8 ± 0.5	9.1 ± 1.5	12.8 ± 0.6	12.9 ± 0.7
AMPA mPSC frequency (Hz)[‡]	0.24 ± 0.03	**1.03 ± 0.03**	0.26 ± 0.08	**0.10 ± 0.02**	0.88 ± 0.24	**0.4 ± 0.08**	0.4 ± 0.04	**0.11 ± 0.02**	0.45 ± 0.14	**0.15 ± 0.05**
	(n = 7)	(n = 7)[‡]	(n = 8)	(n = 8)[‡]	(n = 4)	(n = 4)[‡]	(n = 5)	(n = 5)[‡]	(n = 5)	(n = 5)[‡]
AMPA mPSC decay time (ms)	4.8 ± 0.2	5.2 ± 0.3	4.9 ± 0.7	5.1 ± 0.6	6.7 ± 0.3	7.1 ± 0.4	7.4 ± 1.0	**9.3 ± 1.1**	5.6 ± 0.3	6.6 ± 0.5
GABA mPSC mean amplitude (pA)	13.3 ± 0.4	12.0 ± 0.6	23.7 ± 2.1	25.3 ± 2	24.4 ± 1.1	**14.5 ± 0.5**	17.8 ± 1.8	**11.5 ± 1.4**	n.m.	n.m.
GABA mPSC frequency (Hz)	0.57 ± 0.04	0.61 ± 0.04	0.28 ± 0.1	0.31 ± 0.08	0.57 ± 0.06	0.54 ± 0.07	0.52 ± 0.07	0.35 ± 0.11	n.m.	n.m.
GABA mPSC decay time (ms)	26.3 ± 0.8	27.4 ± 1.1	32 ± 4	33.6 ± 2.7	32.8 ± 0.1	**35.4 ± 0.1**	30.8 ± 1.3	**34.7 ± 1.1**	n.m.	n.m.

Bold numbers indicate significant differences ($p < 0.05$), n.m. indicates not measured. [‡]Denotes that the value was obtained from the same cells before and during drug addition (number of cells shown in parentheses). Averages are expressed as mean ± SEM Student's t test ($\alpha = 0.05$) was used to perform the statistical analyses of membrane potential, resistance, mPSC amplitude, frequency, and decay time.

Despite this, endocannabinoid signaling did mediate a transient depolarization-induced suppression of mEPSCs and had a profound effect on a tonic regulation of mEPSCs, demonstrating an importance of mEPSC regulation. Additionally, altering endocannabinoid signaling also influenced the frequency of SNA *in vitro* and *in vivo*, likely because of the changes in glutamatergic spontaneous release. By blocking endocannabinoid receptors *in vivo*, we also were able to demonstrate a form of compensatory synaptic plasticity following increases in SNA. The results suggest an important role for endocannabinoids in the maturation of motor networks.

Materials and Methods

Dissection. Experiments were performed on isolated spinal cords of stage 36 (Hamburger and Hamilton, 1951) White Leghorn chicken embryos of either sex. Eggs were incubated for 10 d (E10) (GQF Manufacturing) at 38°C. The lumbosacral spinal cord region, with attached spinal nerves, was dissected under cooled (15°C) oxygenated Tyrode's solution containing the following (in mm): 139 NaCl, 12 D-glucose, 17 NaHCO$_3$, 3 KCl, 1 MgCl$_2$, and 3 CaCl$_2$. Tyrode's solution was constantly bubbled with a mixture of 95% O$_2$-5% CO$_2$ to maintain a pH of ~7.3. After dissection, the cord was allowed to recover for at least 6 h in Tyrode's at 18°C. The cord was then transferred to a recording chamber and continuously perfused with oxygenated Tyrode's solution that was slowly heated to recording temperature (28°C). Stable spontaneous network activity was established before starting the experiments (constant burst frequency, every 10–12 min).

Immunoblot. Lumbosacral spinal cords were homogenized in radio-immunoprecipitation assay buffer supplemented with protease and phosphatase inhibitors (Sigma-Aldrich). Samples were then centrifuged at 16,000 g for 5 min to remove cell debris. Protein concentration was quantitated using BCA reagent (Pierce). Samples were separated on 4–15% SDS-PAGE, and blotted to a nitrocellulose membrane. The primary antibody against CB1 receptor was from Alomone Labs (rabbit anti-CB1R ACR-001). The secondary antibody was a horseradish peroxidase-goat anti-rabbit IgG from Sigma-Aldrich. The blot was visualized by ECL chemiluminescence (GE Healthcare). Blots were done in duplicate, and each sample represents a lysate of 2 different cords.

In ovo *drug application.* At E8, a window in the shell of the egg was opened to allow monitoring chick embryo movements and drug application, and 5 μM AM 251 (based on a 50 ml egg volume; Tocris Bioscience) in dimethyl-sulfoxide (DMSO) was applied onto the chorioallantoic membrane of the chick embryo. Modification of the spontaneous activity was monitored by assessing the duration of embryonic movements in a 5 min window at different times during treatment. Only eggs with a cardiac rate >100 beats/min at E10 were used (untreated E10 embryos: 156 ± 3, $n = 10$; treated E10 embryos: 162 ± 5, $n = 10$).

Recording of mPSCs. Whole-cell patch-clamp recordings were made from motoneurons localized between lumbosacral segments 1 and 3 (LS1–LS3) to acquire mPSC. Tetrodotoxin was not used to isolate mPSCs during the IEI, as the vast majority of these events were action potential

independent (Chub and O'Donovan, 2001; Gonzalez-Islas and Wenner, 2006). Motoneurons were antidromically identified by stimulating muscle nerves via tight-fitting glass suction electrodes. Patch-clamp tight seals (>2 GΩ) were obtained using electrodes pulled from thin-walled glass (World Precision Instruments) in two stages, using a P-87 Flaming/Brown micropipette puller (Sutter Instruments) to obtain resistances between 5 and 10 MΩ. Series resistance during recording varied from 15 to 20 MΩ among different motoneurons and was not compensated. Recordings were terminated whenever significant increases in series resistance (>20%) occurred. Standard extracellular recording solution for mPSCs contained the following (in mm): 139 NaCl, 5 KCl, 17 NaHCO$_3$, 3 CaCl$_2$, 1 MgCl$_2$, and 12 D-glucose, and pH was adjusted to 7.3 with NaOH. The extracellular solution was constantly bubbled with a mixture of 95% O$_2$-5% CO$_2$ to maintain a pH of ~7.3. The intracellular patch solution for mPSC recordings contained the following (in mm): 10 NaCl, 100 K-gluconate, 36 KCl, 10 HEPES, 1.1 EGTA, 1 MgCl$_2$, 0.1 CaCl$_2$, 1 Na$_2$ATP, and 0.1 MgGTP; pH was adjusted to 7.3 with KOH. The junction potential was determined for each experiment (approximately −13 mV) and corrected off-line. Motoneuron GABA and AMPA mPSCs were separated based on their kinetics, as shown previously for this preparation (Gonzalez-Islas and Wenner, 2006); thus no antagonists were added to the bath to isolate mPSCs (unless specified in the results). Glycine receptor antagonists were not used since glycinergic mPSCs were not observed in chick embryo motoneurons at the stages studied (Gonzalez-Islas and Wenner, 2006). No NMDA-sensitive currents could be detected when motoneurons were held at −70 mV and with 1 mm MgCl$_2$ in the bath (Gonzalez-Islas and Wenner, 2006). mPSCs were acquired on an EPC 8 patch-clamp amplifier (Heka Elektronics), digitized on-line using PClamp 10 (Molecular Devices), and analyzed off-line with MiniAnalysis (Synaptosoft).

Recording of ventral root potentials. Tight-fitting glass suction electrodes were used to record and stimulate the ventral roots, dorsal roots, and ventrolateral funiculus (VLF) as described previously (O'Donovan, 1989; O'Donovan and Landmesser, 1987; Xu et al., 2005). These ventral root recordings represent the electrotonically degraded motoneuron population potentials as shown originally in the 1940s (Eccles, 1946; Brooks et al., 1948) and later using glass suction electrodes as sucrose gap recordings (Roberts and Wallis, 1978; Lüscher et al., 1979; Brink et al., 1981), and more recently in the *in vitro* preparation (O'Donovan, 1987; Wenner and O'Donovan, 1999; Butt and Kiehn, 2003). The signals were amplified (1000×) and filtered (DC to 0.3–5 kHz) by an AC/DC differential amplifier (A-M Systems), and digitally recorded using Axograph acquisition software (Molecular Devices). Further analyses of the data were performed off-line. The ventral root response amplitude was measured following stimulation of the dorsal root or VLF.

Statistics. Data from averages are expressed as mean ± SEM. Student's t test ($\alpha = 0.05$) was used to perform the statistical analyses of mPSC amplitudes, frequencies, and decay times. Best-fit analyses were performed for cumulative distributions using a Kolmogorov–Smirnov test ($\alpha = 0.05$). Statistical analysis of mEPSCs (see Figs. 3D, 7), evoked responses (see Fig. 6), IEI (see Fig. 8), and same cell comparisons of Table 1 were performed using a paired t test.
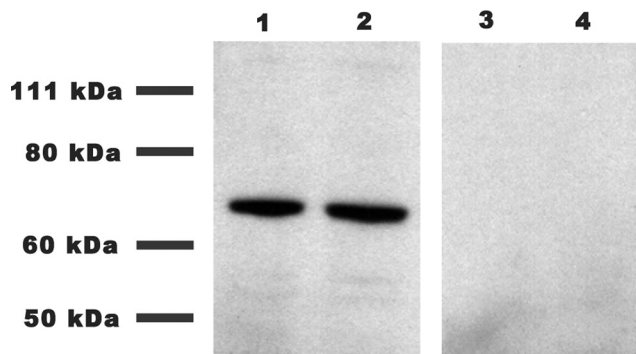
Figure 1. CB1 receptors are present in the developing chick spinal cord. Western blots showing the presence of CB1 in E10 chick embryo spinal cords. Lanes 1 and 2, Total spinal cord lysates incubated with CB1 receptor antibody. Lanes 3 and 4, Total spinal cord lysates incubated first with epitope peptide and then CB1 receptor antibody to show specificity of the antibody.
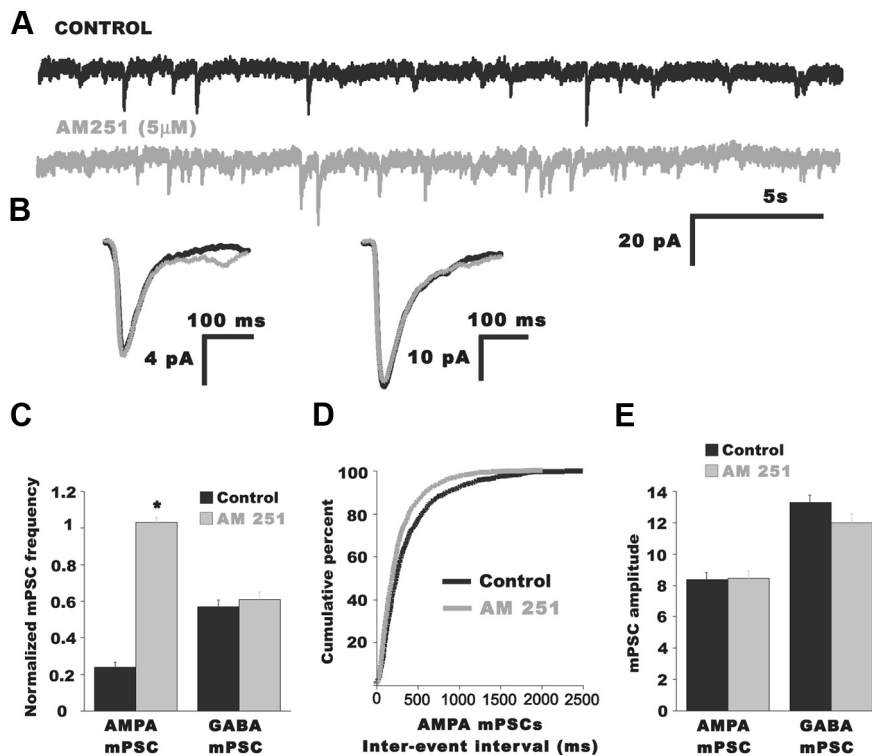

Figure 2. Blocking CB1 receptors increased AMPA mPSC frequency. **A**, Representative traces of mPSCs from an E10 motoneuron before (black trace) and after bath application of the CB1 inverse agonist AM 251 (5 μM; gray trace). **B**, Superimposed average traces of AMPA mPSC (left) and GABA mPSC (right) before (black trace) and after bath addition of AM 251 (gray trace). No change in mPSC kinetics was found in the presence of AM 251. **C**, Summary of the effect of AM 251 on mPSC frequencies. Only AMPA mPSC frequency increased significantly after AM 251 addition. *$p < 0.01$. **D**, Cumulative distribution plot showing the reduction of the IEI after AM 251 addition. **E**, Bar graph showing AM 251 had no effect on mPSC amplitudes.

Drugs. AM 251 (*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichloro phenyl)-4-methyl-1*H*-pyrazole-3-carboxamide); AM 404 (*N*-(4-hydroxyphenyl)-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamide); WIN 55,212-2 mesylate ((*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo [1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate); ACEA (*N*-(2-chloroethyl)-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamide); and LY 2183240 (5-[(1,1′-biphenyl]-4-yl)methyl]-*N*,*N*-dimethyl-1*H*-tetrazole-1-carboxamide) were purchased from Tocris Bioscience. KCl was purchased from Fisher Scientific and potassium D-gluconate from Alfa Aesar. All other salts and compounds were purchased from Sigma-Aldrich.

Results

Antagonizing endocannabinoid receptors increased AMPAergic mPSC frequency

Our initial hypothesis was that the large depolarizations produced by episodes of SNA evoke the release of endocannabinoids and activate CB1 receptors, resulting in the weakening of AMPAergic synaptic strength. Thus, we first biochemically verified that CB1 receptors were present in the developing chick spinal cord. Specific CB1 receptor antibodies recognized a single band in Western blots from E10 chick embryo spinal cords (Fig. 1).

Next, we tested the possibility that the endocannabinoid signaling would influence AMPA mPSCs. We bath applied an inverse agonist of the CB1 receptor AM 251 (5 μM) while recording whole cell from antidromically identified motoneurons in the isolated E10 spinal cord preparation. Indeed, after adding AM 251 (>20 min), AMPA-mPSC frequency was robustly increased (Fig. 2; Table 1; $p = 0.005$) without changes in AMPA mPSC amplitude (Fig. 2B,E; Table 1; $p = 0.2$) or kinetics (Fig. 2B; Table 1; $p = 0.13$). Further, AM 251 had no effect on GABA mPSC frequency (Fig. 2C), amplitude (Fig. 2B,E; Table 1), or kinetics (Fig. 2B; Table 1). These results suggested that ongoing glutamatergic quantal release was tonically inhibited by CB1 activation.

Activation of endocannabinoid signaling decreased AMPAergic mPSC frequency

To determine whether exogenous CB1 activation could reduce the frequency of AMPA mPSCs, we bath applied the CB1 agonist WIN 55,212–2 mesylate (WIN), and recorded mPSC frequency. Surprisingly, AMPA mPSC frequency was no different in control cells and cells exposed to WIN for ≥60 min (1–10 μM, control -0.47 ± 0.06, $n = 7$; WIN -0.59 ± 0.10, $n = 7$). However, we found that AMPA mPSC frequency was significantly reduced in individual motoneurons after a 20 min bath application of WIN (Fig. 3D,E; Table 1; $p = 0.04$). Thus, WIN reduced mEPSC frequency within the same cell after 20–40 min, but not in separate populations of cells that were recorded before and ≥60 min after WIN application. These findings suggest that AMPA mPSC frequency is initially reduced in the presence of WIN, but that mEPSC frequency then recovers in a compensatory manner (see Discussion). WIN did not appear to influence AMPA mPSC amplitude or GABA mPSC amplitude or frequency (Fig. 3; Table 1).

To further confirm that cannabinoid agonists can reduce the frequency of AMPA mPSCs we used another CB1 receptor agonist, ACEA. Bath application of ACEA (5 μM) reduced AMPA mPSC frequency 20–40 min after bath application of the drug (Fig. 4A,E; Table 1; $p = 0.0001$). In this case, we did not observe the recovery of AMPA mPSC frequency (≥60 min in drug) found with WIN. ACEA had no effect on GABA mPSC frequency (Fig. 4E, left), but did
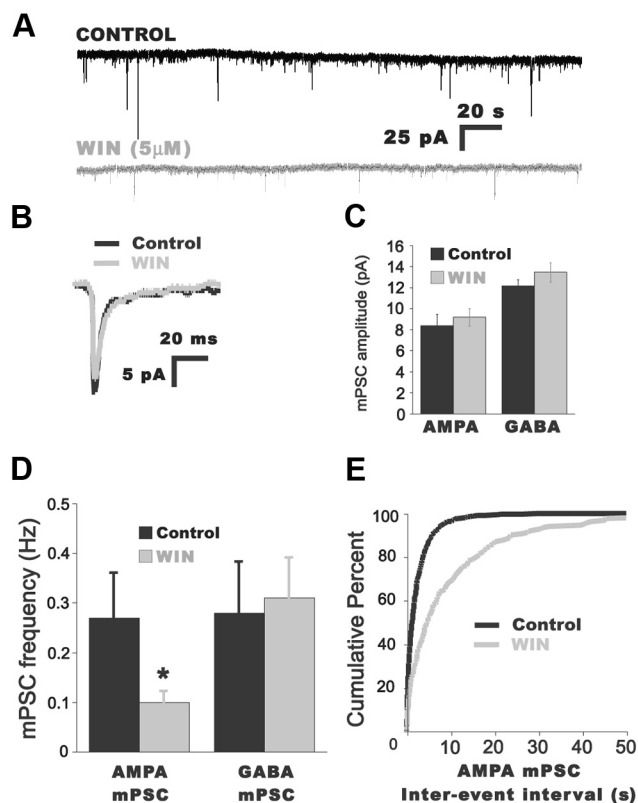
Figure 3. Increasing CB1 receptor activation reduces AMPA mPSC frequency. *A*, Representative traces of AMPA mPSCs from a motoneuron before (black trace) and 20 min after bath addition of the CB1 receptor agonist WIN (5 μM; gray trace). *B*, Superimposed traces of averaged AMPA mPSCs before (black trace) and after bath addition of WIN (gray trace). *C, D*, Summary of the effects of WIN (1–10 μM) on AMPA and GABA mPSC amplitude (*C*) and frequency (*D*). *$p < 0.05$. *E*, Cumulative distribution plot showing the increase in the IEI (reduction in frequency) after addition of 5 μM WIN for the entire distribution of AMPA mPSCs.

significantly reduce the amplitude of both AMPA and GABA mPSCs (Fig. 4C,E; $p = 0.01$ AMPA mPSC, $p = 0.01$ GABA mPSC). We hypothesized that the effect of ACEA on amplitude was not through the CB1 receptor, but rather through some nonspecific interaction. To test this we recorded mEPSCs in the presence of ACEA and AM 251 to block any CB1 receptor contribution. Figure 4 E (right) shows that normalized AMPAergic mPSC amplitude was reduced by ACEA to the same extent whether CB1 signaling was blocked or not, suggesting this effect was not mediated by the CB1 receptor. On the other hand, Figure 4 E (left) shows that the reduction in AMPAergic mPSC frequency produced by ACEA was blocked when CB1 signaling was blocked by addition of AM 251. This finding suggests that the effect of ACEA on mEPSC frequency was through the CB1 receptor.

The CB1 agonists demonstrated that CB1 activation could reduce AMPA mPSC frequency. Application of the inverse agonist demonstrated that inhibition of CB1 signaling could increase mEPSC frequency; this increase could occur through the blockade of endocannabinoid activation of the CB1 receptor or by blocking a constitutively active CB1 receptor in the absence of endocannabinoids. To determine whether endocannabinoids are released and activate the CB1 receptor in the embryonic spinal cord, we introduced drugs that are thought to elevate endogenously produced endocannabinoids. Therefore, we bath applied 20 μM AM 404 (Pertwee et al., 2010). After at least 20 min in AM 404, AMPA mPSC frequency was significantly reduced (Fig. 5C,D; Table 1; $p = 0.001$), while amplitude was unaffected (Fig. 5B,E; $p = 0.5$). AM 404 reduced GABA mPSC amplitude ($p =$
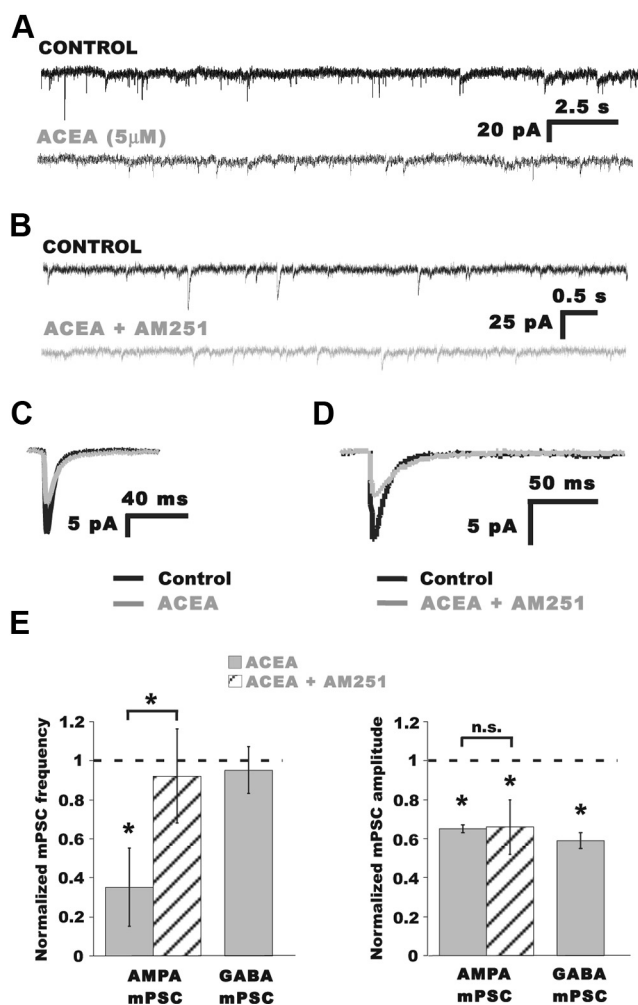

Figure 4. The CB1 agonist ACEA decreased AMPA mPSC frequency. *A*, Representative traces of mPSCs from a motoneuron before (black trace) and 20 min after bath application of the CB1 receptor agonist ACEA (5 μM; gray trace). *B*, Representative traces of mPSCs from a motoneuron in normal Tyrode's (black trace) and 20 min after simultaneous bath application of the CB1 receptor agonist ACEA and the CB1 antagonist AM 251 (5 and 5 μM, respectively; gray trace). *C*, Superimposed traces of averaged AMPA mPSCs before (black trace) and after bath addition of ACEA (gray trace). *D*, Superimposed traces of averaged AMPA mPSCs before (black trace) and after simultaneous bath addition of ACEA and AM 251 (gray trace). *E*, Summary of the effects of ACEA and ACEA + AM 251 on normalized AMPA mPSC frequency (left) and amplitude (right). The reduction of AMPA mPSC frequency following addition of ACEA (Table 1) was blocked when CB1 signaling was inhibited with AM 251 (1.10 ± 0.05 Hz control; 0.81 ± 0.22 Hz in ACEA + AM 251; $n = 5$). A slight reduction in mEPSC frequency was observed in AM 251 + ACEA compared with controls ($p = 0.03$, paired t test) as would be expected given the dramatic reduction in mEPSC amplitude, dropping some mEPSCs below our ability to detect them. Both AMPA and GABA mPSC amplitudes were significantly reduced after ACEA (Table 1). Unlike frequency, however, mEPSC amplitude was reduced by ACEA to the same extent whether CB1 signaling was blocked by AM 251 or not (12.1 ± 1.4 pA control; 8.0 ± 1.1 pA in ACEA + AM 251; $n = 5$). *$p \leq 0.05$.

0.03; Fig. 5 B,E) but did not appear to have a significant effect on GABA mPSC frequency (Fig. 5C).

Since it has been previously shown that AM 404 may also activate vanilloid receptors, we used another highly potent blocker of anandamide uptake that additionally inhibits the enzyme responsible for anandamide hydrolysis (fatty acid amide hydrolase), LY 2183240. We found that increasing the endocannabinoid concentration by bath applying 1 μM LY 2183240 significantly reduced AMPA mPSC frequency ($p = 0.03$), but had no effect on amplitude (Table 1). These experiments were per-
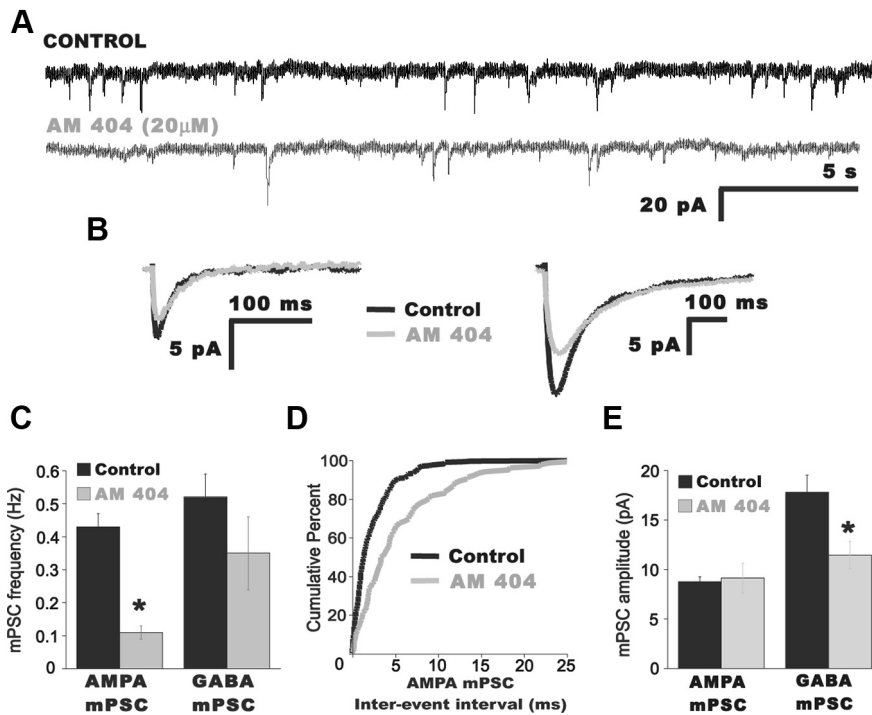
Figure 5. Increasing endocannabinoid signaling decreased AMPA mPSC frequency. **A**, Representative traces of mPSCs from a motoneuron before (black trace) and 20 min after bath addition of the endocannabinoid reuptake blocker AM 404, 20 μM (gray trace). **B**, Superimposed traces of averaged AMPA mPSC (left) and GABA mPSC (right) before (black trace) and after bath addition of AM 404 (gray trace). **C**, Summary of the effect of AM 404 on mPSC frequency. *$p = 0.001$. **D**, Cumulative distribution plot showing the increase in the AMPA mPSC IEI (reduction in frequency) after AM 404 addition. **E**, Summary of the effect of AM 404 on mPSC amplitude. No effect on AMPA mPSC amplitude was found in AM 404 but GABA mPSC amplitude was significantly reduced. *$p = 0.003$.

formed in the presence of gabazine (5 μM) to isolate AMPA mP-SCs, and the potential effects of LY 2183240 on GABA mPSCs were not evaluated.

Did endocannabinoids modulate glutamatergic transmission following SNA?

Following an episode of SNA, AMPA currents (evoked and mPSC frequency) were reduced, and then recovered in the IEI (Fedirchuk et al., 1999; Tabak et al. 2001; C. Gonzalez-Islas unpublished; Figs. 6A,B, 7A–C). Therefore, we reasoned that during episodes of SNA endocannabinoids could be liberated as a result of strong depolarizations and metabotropic signaling associated with SNA. During the IEI endocannabinoids would be progressively cleared and CB1-mediated suppression of glutamatergic release would be reduced. If probability of release from glutamatergic terminals was modified by endocannabinoids released during episodes, then we would expect that blocking CB1 receptors would significantly increase evoked glutamatergic currents and reduce episode-dependent modulation of these currents. Thus, we measured ventral root potentials while stimulating interneurons projecting through the VLF every 1 or 2 min. Ventral root potentials represent subthreshold electrotonically degraded population potentials from motoneurons (Fig. 6A; see Materials and Methods). The VLF contains predominately glutamatergic and GABAergic interneurons, many of which make direct synaptic connections onto motoneurons. We measured the strength of the glutamatergic connections by blocking GABAergic synaptic transmission (10–20 μM gabazine), while stimulating the VLF, and recording short latency potentials in motoneurons through ventral root recordings (Fig. 6A,B). The episode-dependent modulation of glutamatergic synaptic strength was clearly observed. When we

added AM 251 to block CB1 receptors there was no change in the average amplitude of the potential (97.7%; $p = 0.75$, $n = 5$; Fig. 6B,D), or its episode-dependent modulation (amplitude after episode normalized to amplitude before episode—46.5 ± 9.3% before AM 251, 43.3 ± 13.5% after AM 251; $p = 0.69$, paired t test). We also looked at a glutamatergic pathway that does not modulate in the IEI (Fedirchuk et al., 1999) by stimulating sensory neurons that travel through the dorsal roots, while recording the response in the homonymous ventral root (also in the presence of gabazine). Again, there was no change in the evoked response (Fig. 6C,D; 102.8%, $p = 0.56$). In addition, we saw no changes when we increased CB1 signaling with AM 404 while assessing the average amplitude of the VLF-evoked potential (Fig. 6B,D; 87.6%, $p = 0.16$), its episode-dependent modulation (amplitude after episode normalized to amplitude before episode; 28.0 ± 4.9% before AM 251, 25.7 ± 4.7% after AM 251; $p = 0.27$, paired t test; Fig. 6B), or the average dorsal root-evoked response (Fig. 6C,D; 129.3%, $p = 0.29$). Together, the results suggest that while endocannabinoid signaling strongly regulated AMPAergic mPSC frequency, it did not play a significant role in the regulation of evoked glutamatergic responses, either at baseline or through episode-dependent modulation.

Although we did not observe an endocannabinoid-dependent effect on the modulation of evoked glutamatergic responses, it was still possible that there could be an effect on the modulation of AMPAergic mPSCs. We tested this possibility by recording mEPSCs using whole-cell electrodes; we anticipated that endocannabinoids could mediate the suppression of mEPSCs even though we were holding the cells at −70 mV due to the fact that metabotropic signaling was still intact, and because voltage-clamp will not be perfect when dendrites are involved. To test this idea, we divided the IEI into six consecutive 2 min bins and measured AMPA mPSC frequency in each bin. In Figure 7B we plotted AMPA mPSC frequency obtained at each interval for cords before and after bath application of 5 μM AM 251 to block CB1 activation. We then assessed the rate of recovery of AMPA mPSC frequency through the slope of the frequency interval plot. We found that overall AMPA mPSC frequency was increased; however, there was still a very clear SNA-dependent modulation of AMPA mPSC frequency (Fig. 7B; control: $m = 0.03$ Hz/min, $r = 0.94$; following AM 251: $m = 0.08$ Hz/min, $r = 0.93$, $n = 6$). When we normalized mEPSC frequency values to the last interval before an episode it was again clear that the modulation was observed when CB1 signaling was blocked, although there may have been a slight, but not significant inhibition of the rate of recovery in the presence of AM 251 (Fig. 7C; slopes were not significantly different, $m = 0.09$ Hz/min, $r = 0.94$, in control and $m = 0.06$ Hz/min; $r = 0.93$ in AM 251). Further, if we compared the suppression of mEPSC frequency in the first 2 min period (normalized to last period before an episode) before and after AM 251, the values were still not significantly different (0.50 ± 0.09 vs 0.65 ± 0.21 Hz, $p = 0.52$). The results suggested that the
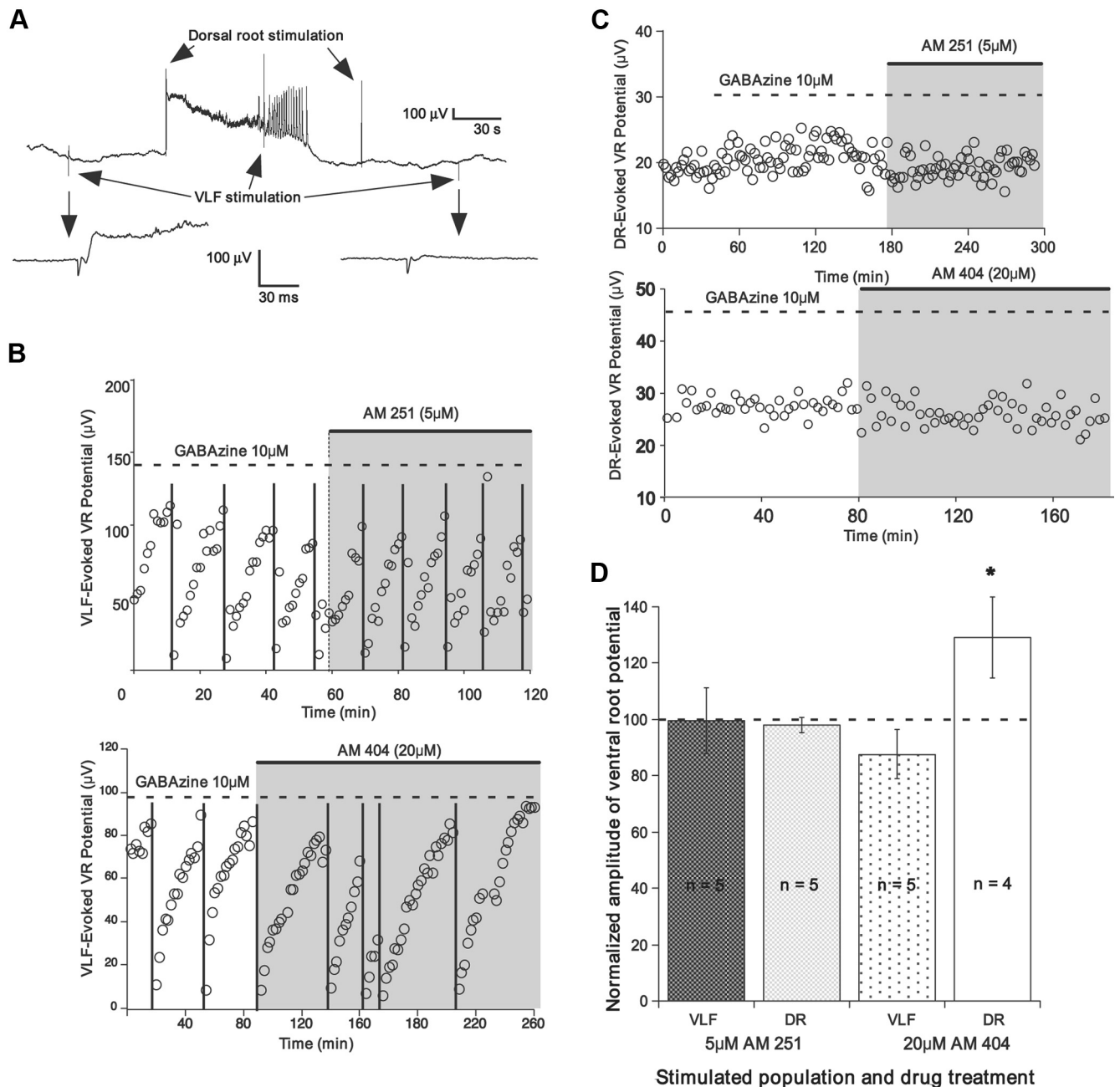
Figure 6. CB1 signaling does not influence evoked glutamatergic responses. *A*, Ventral root (VR) recording showing the alternation of dorsal root (DR) and VLF stimulation. Ventral root recordings expanded from VLF stimulations before and after an episode of SNA are shown below. *B*, Plot of the amplitudes of VLF-evoked glutamatergic responses in motoneurons, as measured from the ventral root. The responses modulate in an episode-dependent manner, dropping after an episode (vertical lines) and recovering in the IEI. Application of AM 251 or AM 404 had no effect on these responses. *C*, Dorsal root-evoked responses are not modulated by SNA, and are unaffected following bath application of AM 251 or AM 404. *D*, Summary of data showing the applied drugs had no effect on VLF- and dorsal root-evoked responses. Twenty dorsal root-evoked values were averaged per preparation in the presence of the drug (20 min after addition of drug) and normalized to last 20 values before drug. VLF-evoked values from two IEIs in the presence of the drug (20 min after addition of drug) were averaged and normalized to the average value of the responses in the last two IEIs before the drug.

activation of CB1 by an SNA-dependent release of endocannabinoids could not explain the observed depression and recovery of AMPA mPSC frequency during the IEI, although we cannot rule out a small contribution from CB1 signaling.

Next we considered the possibility that the depolarization associated with SNA could produce endocannabinoid signaling, but that the depression of spontaneous release might be too short lived to effect mEPSCs in the IEI. Large depolarizations have been shown to induce the production and release of endocannabinoids in other systems, which then act to reduce presynaptic glutamate release through CB1 receptors (depolarization-induced suppres-

sion of excitation; DSE). Therefore, we tested if strong depolarizations could suppress mEPSC frequency right after the depolarization (Fig. 7D). We found that following a depolarizing step to 0 mV (60 s, in TTX), mEPSC frequency in motoneurons was significantly reduced in the first, but not the second minute after the step (Fig. 7E; 64.6% first minute, $p = 0.037$; 96.7% second minutes, $p = 0.63$, $n = 6$). Consistent with DSE being mediated by endocannabinoid signaling, we found that no such depression of mEPSC frequency occurred in the presence of AM 251 (Fig. 7E; 104.0% first minutes, $p = 0.75$; 87.7% second minutes, $p = 0.30$, $n = 6$). The results suggested that there were two
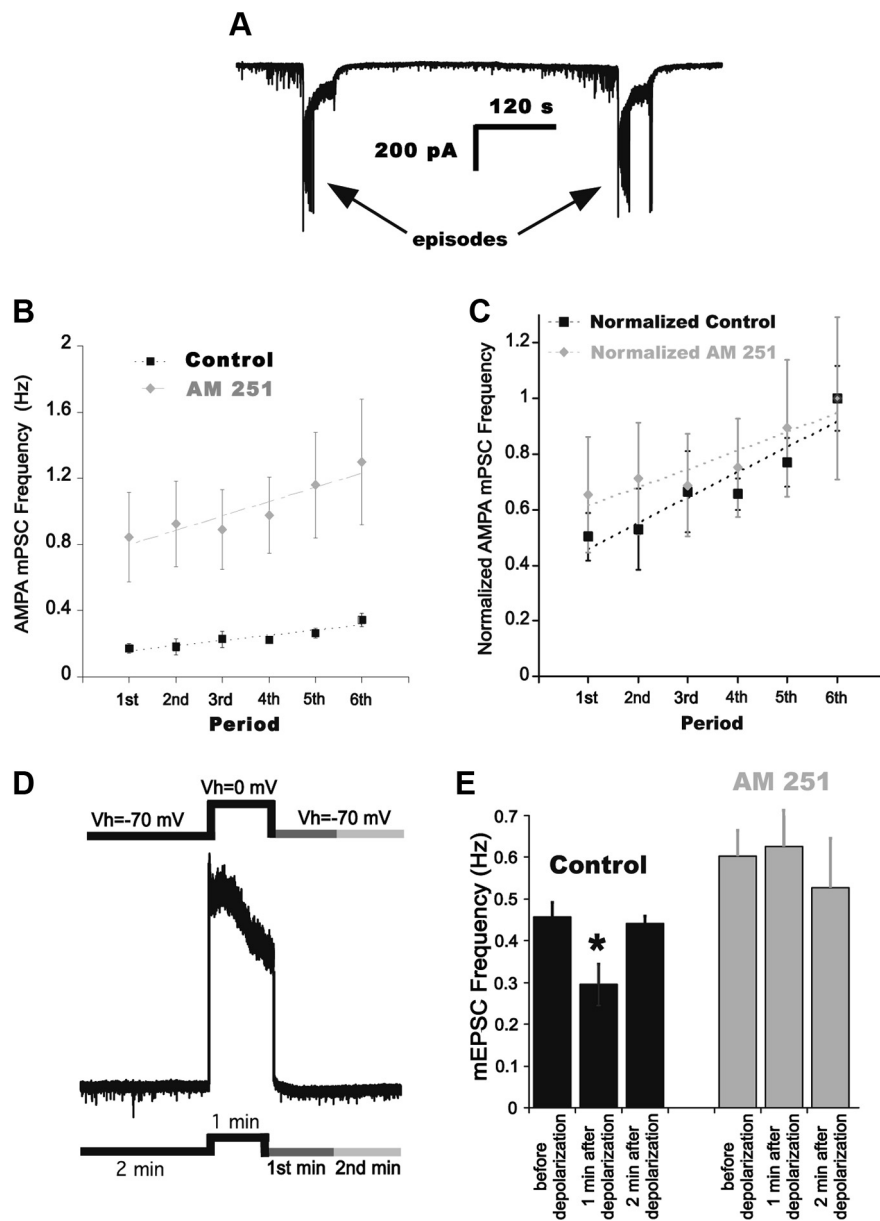
Figure 7. Endocannabinoid signaling and the modulation of AMPA mPSC frequency. *A*, Whole-cell recording of a motoneuron shows two consecutive episodes and the modulation of mPSCs in the IEI. *B*, Plot of average AMPA mPSC frequency for consecutive 2 min bins through the IEI. Plots represent values before (black squares) and after (gray diamonds) bath application of 5 μM AM 251 for six different cells. The slopes were obtained from fitting the averaged data to linear functions. *C*, Normalizing AMPA mPSC frequencies to the frequency in the last 2 min period of the IEI shows that the gradual increase in AMPA mPSC frequency is slightly reduced but is still present after the addition of AM 251. *D*, Schematic representation of the protocol followed for DSE. AMPA mPSC frequency was measured for 2 min holding the cell at −70 mV, then stepping to 0 mV for 1 min. AMPA mPSC frequency was then measured in the first and second minute after depolarization. *E*, Summary of the effect of 0 mV step on AMPA mPSC frequency in the first and second minute following depolarization. A significant reduction in AMPA mPSC frequency was observed in the first, but not second minute in control conditions (black bars). *$p <$ 0.04. Bath application of AM 251 (gray bars) prevented this form of DSE.

forms of endocannabinoid signaling. The first was likely associated with SNA, but only transiently expressed. The second form of endocannabinoid signaling appeared to be independent of SNA, acting to tonically suppress spontaneous, but not evoked, vesicle release.

It is possible that the terminals giving rise to mEPSCs and the stimulated terminals that underlie evoked release could be separate populations. However, we are stimulating many different classes of glutamatergic interneurons and glutamatergic sensory neurons and we would expect some overlap with the spontaneous population.

Rather, we considered the possibility that CB1 activation could influence spontaneous release independently of evoked release in the same terminal. CB1 activation typically suppresses evoked release by inhibiting voltage-gated calcium channels in the terminal. We tested the calcium dependence of VLF- and dorsal root-evoked responses in the ventral root by comparing ventral root responses in normal (3 mM) then low (1 mM) external calcium. As expected, evoked release was calcium-dependent; the VLF-evoked response was depressed in low calcium (17.4 ± 0.9, 3 mM/10.0 ± 0.5, 1 mM/15.8 ± 1.6 μV, back to 3 mM; $n = 7$, $p < 0.0001$). The dorsal root-evoked response was also depressed in low calcium (13.4 ± 0.7, 3 mM/8.2 ± 0.3, 1 mM/15.3 ± 1.6 μV, back to 3 mM; $n = 7$, $p = 0.002$). On the other hand, low calcium had no effect on mEPSC frequency (0.43 ± 0.3 Hz, 3 mM; $n = 8$/0.40 ± 0.4 Hz, 1 mM; $n = 6$, $p = 0.59$). Since spontaneous release is not altered by lowered calcium, endocannabinoid-mediated suppression of mEPSC frequency must occur through a calcium-independent pathway. These results suggested that evoked and spontaneous release could be regulated through distinct pathways.

Endocannabinoid signaling influenced the frequency of SNA in the isolated spinal cord
The finding that SNA-dependent modulation of AMPA mPSC frequency and evoked glutamatergic responses were unaffected by endocannabinoid signaling was surprising, but suggested endocannabinoids were important for the regulation of AMPAergic mPSCs, specifically. The function of endocannabinoid signaling in the embryonic spinal cord might then be to set the levels of miniature AMPAergic transmission tonically by reducing the probability of spontaneously released glutamatergic vesicles. We considered the possibility that the frequency of AMPAergic mPSCs might influence the frequency of SNA. We tested this idea by altering AMPAergic mPSC frequency by applying modulators of the CB1 receptor while monitoring the frequency of SNA in the isolated *in vitro* spinal preparation. Blocking CB1 receptors with AM 251 significantly increased SNA frequency (Fig. 8, reduced the IEI, 11.8 ± 1.2 min control, 9.4 ± 0.8 min AM 251, $n = 7$, $p = 0.004$). Moreover, increasing endocannabinoid tone with AM 404 produced an increase in the IEI (Fig. 8; 10.4 ± 0.9 min control, 13.7 ± 0.5 min AM 404, $n = 5$, $p = 0.002$).

***In ovo* blockade of CB1 receptors increased SNA and caused homeostatic increases in AMPA and GABA mPSC amplitude**
We tested whether these increases in SNA observed *in vitro* could be extended to the embryo *in vivo*. We injected AM 251 onto the
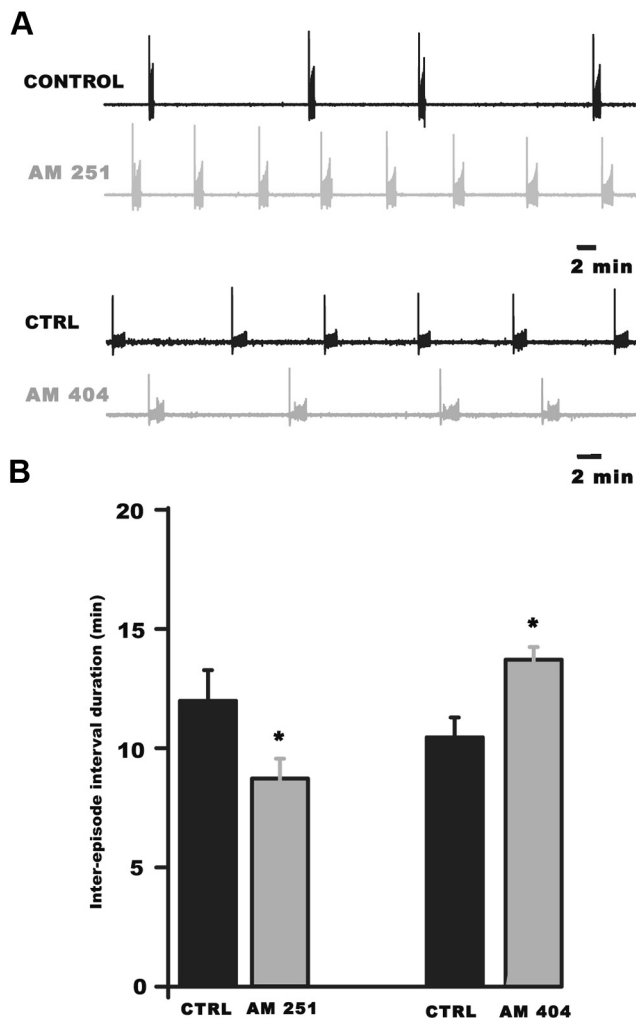
A

B

Figure 8. Endocannabinoid signaling influences embryonic spontaneous network activity in the chick spinal cord *in vitro*. ***A***, Example traces of ventral root recordings showing the expression of SNA before (control, black) and after the addition of endocannabinoid signaling modulators (gray). ***B***, Bar graph summarizing the effects of activation or inhibition of CB1 receptors on the duration of the IEI of SNA in isolated spinal preparations. Antagonizing CB1 receptors with AM 251 (5 μM) increased AMPA SNA frequency (decreased IEI) *in vitro*. Increasing endocannabinoid signaling with AM 404 significantly decreased SNA frequency (increased IEI) *in vitro*. *$p < 0.005$.

Figure 9. Chronic blockade of CB1 receptors increased embryonic limb movements *in ovo*. Plot of the average embryonic kicking time *in ovo* in 5 min observation periods during the 2 d treatment with vehicle (DMSO black) and 5 μM AM 251 dissolved in DMSO (gray). Limb movements started to increase in AM 251-treated embryos 12 h after injection and remained more active than controls during the rest of the 2 d long treatment.

chorioallantoic membrane at E8 and measured embryonic movements until E10 (Fig. 9). Like the results in the *in vitro* cord, embryonic movements were increased after AM 251 was added to the egg; however, this did not occur until 12 h after the *in ovo* injection. This delay is likely to occur due to the immature development of the glutamatergic system at E8 (Milner and Landmesser, 1999). We have previously shown that blocking SNA *in ovo* from E8–E10 through lidocaine infusion leads to a homeostatic increase in GABA and AMPA mPSC amplitude. The observation that embryonic movements, and therefore SNA, were increased with AM 251 provided us with the opportunity to test if this increase in SNA *in vivo* homeostatically reduced AMPAergic and GABAergic mPSC amplitudes. At E8, we again injected AM 251 (5 μM) into the egg to increase SNA *in ovo*. AM 251 was allowed to act for 2 d and at E10, spinal cords from AM 251-treated embryos were isolated and mPSCs amplitude in spinal motoneurons were measured. After chronic AM 251 treatment, AMPA-mPSC amplitude was significantly reduced
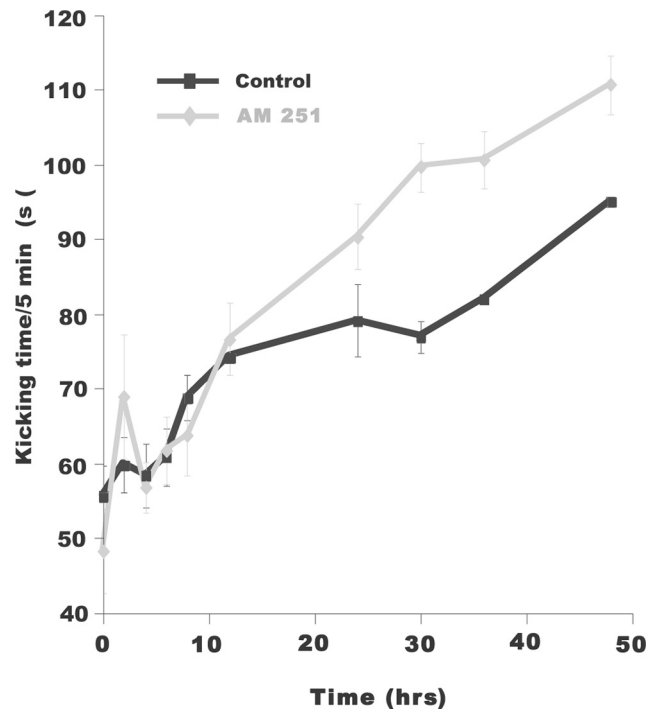
compared with untreated controls (Fig. 10; control, 13.8 ± 2.1 pA; AM 251, 11.9 ± 0.7 pA; $p = 0.04$, $n = 6$). GABA mPSC amplitude was similarly reduced after the levels of activity were increased with AM 251 (Fig. 10; control, 27.4 ± 2.8 pA; AM 251, 16.9 ± 1.2 pA; $p = 0.03$, $n = 6$). No significant effect was found in AMPA mPSC frequency after AM 251 treatment (Fig. 10; control, 0.17 ± 0.04 Hz; AM 251, 0.23 ± 0.05 Hz; $p = 0.3$, $n = 6$). In contrast, GABA mPSC frequency was significantly increased compared with controls (Fig. 10; control, 0.44 ± 0.08 Hz; AM 251, 0.79 ± 0.04 Hz; $n = 6$, $p = 0.02$). While both AMPA and GABA mPSC amplitude were reduced following increases in SNA *in ovo*, the amplitude distributions did not undergo synaptic scaling (see Fig. 10 legend; see Discussion).

Discussion

Embryonic cannabis exposure has been associated with hyperactivity and cognitive impairment in animal models and humans (Huizink and Mulder, 2006; Fried, 2002; Campolongo et al., 2009). This is consistent with the widespread nature of endocannabinoid signaling in several developing systems (Middleton and Protti, 2011; Penzo and Peña, 2009; Jiang et al., 2010; Bernard et al., 2005; Li et al., 2009). Here, we demonstrate that the endocannabinoid signaling system does not play a role in the modulation of glutamatergic synapses during the IEI. However, CB1 signaling does mediate a transient depolarization-induced inhibition of mEPSC frequency, along with a tonic suppression of glutamatergic spontaneous events. Further, we show that endocannabinoid signaling alters the frequency of SNA *in vitro* and *in vivo*. Blockade of CB1 *in vivo* chronically increases the frequency of SNA
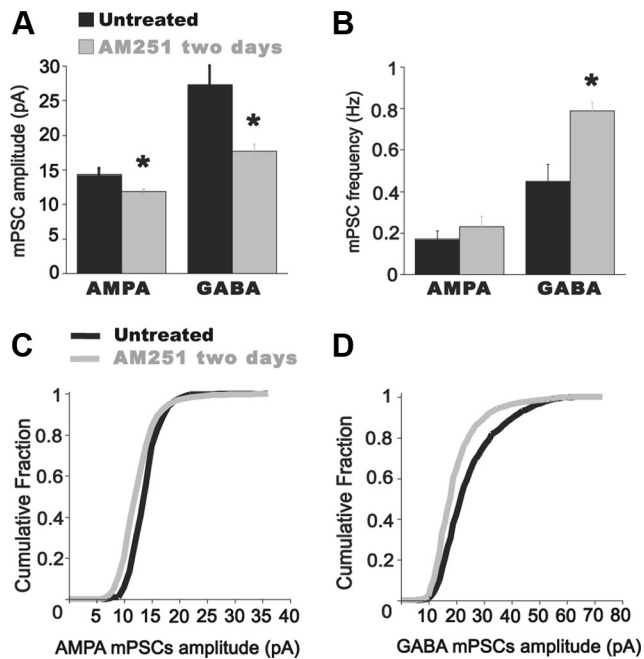
Figure 10. Chronic blockade of CB1 receptors induce compensatory reductions of AMPA and GABA mPSC amplitude in embryonic spinal motoneurons. *A*, Summary of the effect on mPSCs of 2 d blockade of CB1 receptors *in ovo* with AM 251. AMPA and GABA mPSC amplitude is decreased compared with controls. *p < 0.05. *B*, Bar graph showing that GABA mPSC frequency is significantly increased after chronic treatment with AM 251. *p = 0.02. AMPA mPSC frequency was unaffected by the treatment. *C*, *D*, Cumulative distribution of mPSC amplitudes for control (treated with DMSO for 2 d; black line) and for AM 251-treated embryos (gray line). AMPA and GABA mPSC amplitudes did not scale across their distributions following AM 251 treatment (Kolmogorov–Smirnov $Z = 4.44$; $p < 0.001$ for AMPA mPSC amplitude distribution; Kolmogorov–Smirnov $Z = 5.18$; $p < 0.001$ for GABA mPSC amplitude distribution).

leading to a compensatory reduction in excitatory GABA and AMPA mPSC amplitude.

Endocannabinoid signaling mediates a transient glutamatergic plasticity, but this does not contribute to the IEI plasticity

Following an episode of SNA, evoked glutamatergic currents and AMPA mPSC frequency were depressed, but then recovered in the following IEI (Figs. 6, 7) (Fedirchuk et al., 1999; Tabak et al., 2001). We hypothesized that the spiking activity and neurotransmission associated with SNA would enhance endocannabinoid signaling and reduce glutamatergic probability of release until endocannabinoid signaling was weakened in the IEI as endocannabinoids were cleared from synapses. This hypothesis was not supported by our findings, since the SNA-dependent modulation of glutamatergic currents was unaffected by blocking CB1 receptors. It is unlikely that other cannabinoid receptors mediate this form of glutamatergic plasticity, as increasing endocannabinoid levels with AM 404 similarly had no effect on glutamatergic modulation. We did find that strong depolarizations, as would occur during episodes of SNA, did lead to an endocannabinoid-mediated depression of mEPSC frequency, but this effect was only observed within 60 s of the depolarization. Therefore, endocannabinoids may not contribute to the glutamatergic modulation observed in the IEI due to their transient nature, possibly resulting from a rapid endocannabinoid clearance. Thus, other mechanisms must exist that mediate the SNA-dependent modulation of glutamatergic transmission, such as nitric oxide (Kyriakatos and El Manira, 2007).

Endocannabinoid signaling tonically inhibits AMPAergic mPSCs while having no observable effect on evoked glutamatergic currents

Our results suggest that endocannabinoid signaling tonically inhibits glutamatergic mPSCs. Blocking endocannabinoid signaling dramatically increased mEPSC frequency, while activating the CB1 receptor suppressed mEPSCs. These results favor the possibility that endocannabinoids are constitutively released at some base level in an SNA-independent manner, and thus tonically inhibit spontaneous glutamatergic vesicle release, thereby lowering AMPAergic tone. While several other studies have demonstrated a tonic endocannabinoid inhibition of presynaptic release of GABAergic transmission (Losonczy et al., 2004; Oliet et al., 2007; Zhu and Lovinger, 2010; Inada et al., 2010; Neu et al., 2007; Hentges et al., 2005; Farkas et al., 2010; Kim and Alger, 2010), this is the first demonstration of tonic inhibition of glutamatergic release (Alger, 2012).

In addition to an influence on AMPAergic mPSC frequency, different endocannabinoid signaling modulators also had effects on mPSC amplitude. For instance, AM 404 produced a significant reduction of GABA mPSC amplitude, and ACEA reduced AMPAergic and GABAergic mPSC amplitude. These effects appear to be due to the nonspecific effects of the drugs (Fig. 4*E*). AM 404 has been reported to activate the vanilloid receptor TRPV1 (Zygmunt et al., 2000) and ACEA can inhibit voltage-gated calcium channels (Pertwee et al., 2010). Further, the transient effect of WIN, compared with ACEA, may be due to the rapid internalization/desensitization of CB1 receptors, which has been reported following WIN application (Hsieh et al., 1999; Jin et al., 1999; Coutts et al., 2001; Kouznetsova et al., 2002). We cannot speak with certainty about the influence of nonspecific effects, as the results are mixed. However, we are confident in the interpretation that endocannabinoid signaling reduces the base level of AMPAergic mPSCs, because all of the results with different modulators were consistent in this regard.

While endocannabinoid signaling had a clear effect on glutamatergic spontaneous release, it had no detectable effect on evoked responses of two different glutamatergic inputs to motoneurons. When spontaneous and evoked currents are assessed in the same study, they are typically influenced by endocannabinoid signaling in similar ways (Wilson and Nicoll, 2002; Wamsteeker et al., 2010; Chevaleyre et al., 2007; Hentges et al., 2005). To our knowledge, this is the first direct demonstration that endocannabinoids inhibit presynaptic spontaneous release, while having no effect on evoked release. One recent study in the embryonic tectum demonstrated that mEPSC frequency, but not evoked currents, was suppressed in response to postsynaptic depolarization, but this was not mediated by endocannabinoid signaling (Penzo and Peña, 2011). Several studies suggest that spontaneous and evoked vesicle release can be regulated independently through separate pathways (Chung et al., 2010; Geppert et al., 1994; Groffen et al., 2010), although it is not clear whether this is accomplished through distinct pools for spontaneous and evoked release (Denker and Rizzoli, 2010). Our study suggests that endocannabinoid signaling regulates spontaneous release in a calcium-independent manner. It appears that spontaneous and evoked release have distinct regulatory pathways in the embryonic spinal cord, as evoked glutamate release is not influenced by endocannabinoid signaling but is calcium dependent.

The functional role of endocannabinoid signaling in the embryonic spinal cord

Tonic suppression of spontaneous glutamatergic release is quite different from the more established role of endocannabinoid signaling in presynaptic short and long-term plasticity (Alger, 2009; Heifets and Castillo, 2009; El Manira and Kyriakatos, 2010). Thus, endocannabinoid signaling could have a unique function in the developing spinal network. In the current study we have shown that endocannabinoid signaling regulates the frequency of spinal SNA *in vitro* and *in vivo*, likely by inhibiting spontaneous glutamatergic release. We have previously shown that episodes are triggered by motoneuron discharge, which recruits spinal interneurons (Wenner and O'Donovan, 2001). Motoneuron discharge typically arises from minis, so an increase in mEPSCs (AM 251) could reduce the IEI by initiating more spikes and triggering the episode earlier. In the developing hippocampus, endocannabinoid signaling reduced the frequency of giant depolarizing potentials (a hippocampal form of SNA) by CB1 inhibition of excitatory GABAergic transmission presynaptically (Bernard et al., 2005). In the motor circuitry of the adult lamprey spinal cord, endocannabinoid signaling increased the frequency of locomotor bouts of activity by presynaptically reducing inhibitory transmission onto motor and interneurons (Kettunen et al., 2005; Kyriakatos and El Manira, 2007; El Manira and Kyriakatos, 2010). It will be important to understand how spinal circuit CB1 signaling transitions from the embryo (suppresses SNA via tonic mEPSC inhibition) to adult (enhances rhythms through evoked IPSC inhibition). SNA in the embryonic cord has been shown to be important in the development of limbs, motoneuron axon pathfinding, and synaptic maturation (O'Donovan et al., 1998; Hanson and Landmesser, 2004; Gonzalez-Islas and Wenner, 2006; Blankenship and Feller, 2010). Therefore, these SNA-dependent developmental features could be regulated by endocannabinoid signaling via its effect on SNA frequency.

In addition to a role in setting SNA frequency, endocannabinoids may have functional significance by selectively controlling glutamatergic spontaneous release. Glutamatergic mPSCs appear to have important functional roles themselves (Sutton and Schuman, 2009). Spontaneous mPSCs can influence firing rates in electrically compact neurons (Carter and Regehr, 2002), and stabilize excitatory synaptic function (Sutton et al., 2006; Jakawich et al., 2010; Frank et al., 2006). Finally, mPSCs can regulate the activity of postsynaptic signaling pathways differently than that controlled by action potential-triggered neurotransmission (Sutton et al., 2007).

Increasing SNA through chronic blockade of endocannabinoid signaling reduces AMPA and GABA mPSC amplitude

Synaptic scaling is a form of homeostatic plasticity that increases or decreases the strength of all synapses onto a neuron by a scaling factor to stabilize firing rates (Turrigiano, 2012). As discussed above, reductions of SNA *in ovo* led to homeostatic increases in depolarizing GABAergic and AMPAergic mPSC amplitude in embryonic chick motoneurons (Gonzalez-Islas and Wenner, 2006). Studies in culture have suggested that this synaptic scaling process is bidirectional, responding to both decreases and increases in activity (Turrigiano et al., 1998). Because we were able to increase activity *in ovo* through application of a CB1 receptor antagonist, we were able to show that chronic increases in SNA *in vivo* reduce AMPAergic and GABAergic mPSC amplitude in a compensatory direction. This demonstrates a synaptic plasticity in the living embryo that appears to be homeostatic, although we do not actually demonstrate homeostasis in this study. Regardless, we show a compensatory plasticity

that is bidirectional [changes in synaptic strength to both increases, this study, and decreases (Gonzalez-Islas and Wenner, 2006) in network activity]. On the other hand, the mPSC amplitude distributions did not scale (mPSC amplitude distributions from control and endocannabinoid-treated motoneurons did not appear to be multiplicatively related). The lack of scaling in this process suggests that the compensatory synaptic responses to increases versus decreases in SNA likely involve distinct mechanisms. As discussed above, the finding that endocannabinoid signaling regulates SNA levels, and therefore synaptic maturation, highlights the potential impact of perturbations to endocannabinoid signaling during embryonic development.

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