Endocannabinoids Suppress Excitatory Synaptic Transmission to Dorsal Raphe Serotonin Neurons through the Activation of Presynaptic CB\textsubscript{1} Receptors

Samir Haj-Dahmane and Roh-Yu Shen

Research Institute on Addictions, University at Buffalo, State University of New York, Buffalo, New York

Received March 25, 2009; accepted July 9, 2009

ABSTRACT

Endocannabinoid signaling in the dorsal raphe (DR) has recently been implicated in the regulation of anxiety and depression. However, the cellular mechanisms by which endocannabinoids (eCBs) regulate the excitability of DR 5-hydroxytryptamine (serotonin; 5-HT) neurons remain poorly understood. In the present study, using whole-cell recording from DR 5-HT neurons, we examined the effects of eCBs on glutamatergic synapses in the DR. We found that the eCB anandamide decreased the amplitude of evoked excitatory postsynaptic currents (eEPSCs). This effect was blocked by CB\textsubscript{1} receptor antagonist N-(piperidin-1-yl)-5-(4-iodophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM 251) and mimicked by (R)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethylamine mesylate (WIN 55,212-2), a CB\textsubscript{1} receptor agonist. The inhibition of eEPSC amplitude was associated with an increase in the paired-pulse ratio and coefficient of variance. Activation of CB\textsubscript{1} receptors also reduced the frequency, but not the amplitude, of miniature excitatory postsynaptic currents, indicating that eCBs inhibit glutamate release in the DR. In addition, we found that depolarization of DR 5-HT neurons induced a transient inhibition of the amplitude of eEPSCs, termed depolarization-induced suppression of excitation (DSE). The induction of DSE required an increase in postsynaptic intracellular calcium and was due to a decrease in glutamate release. Furthermore, pharmacological studies showed that blockade of CB\textsubscript{1} receptors with AM 251 abolished the DSE. In contrast, activation of CB\textsubscript{1} receptors with WIN 55,212-2 mimicked and occluded the DSE, indicating that depolarization of DR 5-HT neurons triggers eCB release, which in turn mediates the DSE. Together, these results indicate that eCBs play a role in modulating glutamatergic synaptic transmission to DR 5-HT neurons.

Endocannabinoids (eCBs) are lipid messengers that produce their physiological effects via the activation of specific cannabinoid receptors (Devane et al., 1992; Mechoulam et al., 1995; Sugiura et al., 1995). Two cannabinoid receptors have been cloned, CB\textsubscript{1} (Matsuda et al., 1990) and CB\textsubscript{2} receptors (Munro et al., 1993). Both receptors are coupled to the per-
deletion or pharmacological blockade of CB1 receptors in anxiogenic effects (Are ´valo et al., 2001; Genn et al., 2004), (Berrendero and Maldonado, 2002; Marco et al., 2004) and increased anxiety related behavioral responses (Are ´valo et al., 2001; Haller et al., 2004). Furthermore, pharmacological enhancement of eCB signaling through the blockade of eCB hydrolysis and/or uptake reduces anxiety-related behaviors (Kathuria et al., 2003).

5-Hydroxytryptamine (serotonin; 5-HT) neurons of the dorsal raphe (DR) are critically involved in the regulation of mood, including anxiety (for review, see Gordon and Hen, 2004). Results from anatomical studies have shown that the DR expresses CB1 receptors (Herkenham et al., 1991; Matsuda et al., 1993; Tsou et al., 1998; Häring et al., 2007) and the enzymes responsible for the synthesis and metabolism of eCBs (Egertová et al., 2003), suggesting that eCBs may play an important role in the regulation of the 5-HT system. Consistent with this prediction, activation of CB1 receptors has been shown to inhibit 5-HT release (Nakazi et al., 2000; Tzavara et al., 2003) and to modulate the firing activity of DR 5-HT neurons in vivo (Bambico et al., 2007). Furthermore, behavioral studies in animal models have suggested that the anxiolytic-like effects of eCBs are mediated, at least in part, through modulation of the 5-HT system (Griebel et al., 2005, Marco et al., 2004). Despite the critical role of eCBs and 5-HT systems in regulating emotional states, little is known about the cellular mechanisms by which eCBs modulate the excitability of DR 5-HT neurons. In this study, we show that although eCBs and CB1 receptor agonists have no effect on the intrinsic excitability of DR 5-HT neurons, they strongly inhibit glutamatergic synaptic transmission to DR 5-HT neurons. Most importantly, we find that DR 5-HT neurons can release eCBs in an activity-dependent manner, which in turn mediate retrograde inhibition of glutamate release in the DR.

Materials and Methods

Slice Preparation. All experimental procedures were performed in accordance with the University at Buffalo Institutional Animal Care and Use Committee guidelines. Brainstem slices containing the DR nucleus were prepared from 3- to 4-week-old male Sprague-Dawley rats using a standard method (Haj-Dahmane, 2001). In brief, rats were anesthetized with halothane and killed by decapitation. The brain was quickly removed and placed in ice-cold choline chloride-based ACSF solution of the following composition: 110 mM choline-Cl, 2.5 mM KCl, 0.5 mM CaCl2, 7 mM MgSO4, 1.25 mM NaH2PO4, 26.2 mM NaHCO3, 11.6 mM sodiumL-ascorbate, 3.1 mM sodium pyruvate, and 25 mM glucose, and equilibrated with 95% O2, 5% CO2 for 30 to 45 min at 35°C. 5-HT neurons were prepared from 3- to 4-week-old male Sprague-Dawley rats in accordance with the University at Buffalo Institutional Animal Care and Use Committee guidelines. Stainless steel concentric bipolar stimulating electrode (inner pole diameter, 25 μm; FHC Inc., Bowdoinham, ME) was placed 50 to 100 μm dorsolateral to the recording site. Excitatory postsynaptic currents (EPSCs) were evoked with a single square-pulse (intensity, 5–20 V; duration, 100–200 μs) delivered at 0.1 Hz in neurons voltage-clamped at −70 mV. All recordings were performed in the presence of picrotoxin (100 μM) and strychnine (20 μM) to block GABAa and glycine receptors, respectively. For depolarization-induced suppression of excitation (DSE) experiments, evoked excitatory postsynaptic currents (eEPSCs) were evoked at 0.33 Hz before (8 to 10 eEPSCs) and after membrane depolarization to 0 mV (3–5 s duration) from a holding potential of −70 mV (30–40 eEPSCs). Membrane currents were amplified with an Axoclamp 2B amplifier (Molecular Devices, Sunnyvale, CA), filtered at 3 kHz, digitized at 20 kHz with Digidata 1200 (Molecular Devices), and acquired using the pClamp 9 software (Molecular Devices). Access resistance (10–20 MΩ) was monitored online using 5-mV hyperpolarizing voltage steps (200-ms duration). Recordings were discarded when the access resistance changed by >10%.

Data Analysis. The amplitude of eEPSCs was determined by measuring the average current during a 2-ms period at the peak of each eEPSC and subtracted from the average baseline current determined during a 5-ms window taken before the stimulus artifact. All eEPSC amplitudes were normalized to the mean baseline amplitude recorded for at least 10 min before drug application. For paired pulse experiments, paired stimuli were given at 30-ms intervals. The paired pulse ratios (PPR = eEPSC2/eEPSC1) were averaged for at least 60 trials in the absence and presence of WIN 55,212-2. For the determination of the coefficient of variance (CV), the background noise variance obtained from interleaved traces without stimulus was subtracted from the eEPSC amplitude variance obtained from 60 consecutive trials in the absence and presence of WIN 55,212-2. The CV was then given by the following ratio: (Var of eEPSC/Var of noise)/ (eEPSC mean amplitude). The magnitude of DSE was calculated using the mean amplitude of four consecutive eEPSCs taken before depolarization (Amplitude baseline) and three eEPSCs collected immediately after membrane depolarization (Amplitude DSE): DSE (%) = 100 (1 – (Amplitude baseline/Amplitude DSE)).

Glutamate-mediated miniature EPSCs (mEPSCs) were recorded in the presence of tetrodotoxin (1 μM), picrotoxin (100 μM), and strychnine (20 μM) and analyzed with Mini Analysis Software (Synaptosoft, Decatur, GA). The synaptic events were selected using amplitude threshold (5 pA, rise time (1 ms), and area threshold (30 fC). All selected events were further visually inspected to prevent

endocannabinoids Regulation of 5-HT Neurons 187

Downloaded from jpet.aspetjournals.org at ASPET Journals on July 8, 2017
noise from compromising the analysis. Results in the text and figures are presented as mean ± S.E.M. Statistical analysis was conducted using the Student’s paired t test for within-group comparison and the unpaired t test for comparisons between groups. The nonparametric Kolmogorov-Smirnov (K-S) test was used for comparisons of mEPSC distribution. p < 0.05 was considered statistically significant.

Most chemicals and drugs were obtained from Sigma-Aldrich (St. Louis, MO); Picrotoxin, strychnine, GYKI 52466, anandamide, WIN 55,212-2, AM 251, O-1602, and d-AP5 were purchased from Torcis Cookson (Ellisville, MO); Tetrodotoxin and BAPTA tetracesium were purchased from Alomone Labs (Jerusalem, Israel) and Molecular Probes (Carlsbad, CA), respectively. All drugs were administered by bath application. To reach the steady state, anandamide and the CB1 receptor antagonist, slices were preincubated with AM 251 for at least 30 min before testing the effect of CB1 agonists on the excitability of DR 5-HT neurons. We found that bath application of anandamide (10–30 μM) did not significantly affect the resting membrane potential of DR 5-HT neurons (n = 10; Fig. 1A). The average resting membrane potentials measured before and during anandamide application were −60 ± 3.5 and −59 ± 2.9 mV, respectively (n = 10; p > 0.05; Fig. 1C). Similarly, administration of anandamide did not alter the evoked spike activity (n = 8; Fig. 1B) nor the membrane input resistant (control, 368 ± 11 MΩ; anandamide, 380 ± 14 MΩ; n = 8; p > 0.05; Fig. 1C). Similar results were also obtained using the synthetic CB1 receptor agonist WIN 55,212-2. Thus, administration of WIN 55,212-2 (10 μM) did not affect the resting membrane potential (control, −61.5 ± 2.5 mV; WIN 55,212-2, −62.9 ± 3.9 mV; n = 9; p > 0.05; Fig. 1C) or the membrane input resistance (control, 368 ± 9 MΩ; WIN 55,212-2, 375 ± 13 MΩ; n = 9; p > 0.05; Fig. 1C). These findings indicate that activation of CB1 receptors with either eCBs or synthetic agonists exert no direct effects on the intrinsic excitability of DR 5-HT neurons.

eCBs Inhibit Glutamatergic Transmission to DR 5-HT Neurons via CB1 Receptors Activation. Previous in vivo and in vitro electrophysiological studies have shown that the electrical activity of DR 5-HT neurons is under the control of glutamatergic inputs and that modulation of glutamate synapses in the DR plays an important role in the overall regulation of serotoninergic transmission (Pan and Williams, 1989; Levine and Jacobs, 1992). Therefore, we next investigated whether eCBs could modulate excitatory synaptic transmission to DR 5-HT neurons. As reported previously (Haj-Dahmane and Shen, 2005), in the presence of picrotoxin (100 μM) and strychnine (20 μM) to block GABAA and glycine receptors, respectively, local electrical stimulations of the DR evoked fast eEPSCs in DR 5-HT neurons voltage-clamped close to their resting membrane potential (−70 mV). These eEPSCs were blocked by the selective AMPA receptor antagonist GYKI 52466 (50 μM), indicating that they were mainly mediated by AMPA receptors (n = 7; Fig. 2A). Bath application of anandamide (30 μM) induced a strong reduction in the amplitude of eEPSCs. On average, the amplitude of eEPSC was reduced to 58 ± 8% of baseline (n = 8; p < 0.01; Fig. 2B). The anandamide-induced depression of eEPSC amplitude persisted even after extensive washout, which is probably due to the high lipophilicity of anandamide.

Because anandamide is also an agonist for vanilloid receptors (Zygmun et al., 1999; Smart et al., 2000), the inhibition of eEPSC amplitude could be attributed to the activation of vanilloid receptors, cannabinoid receptors, or both. To distinguish between these possibilities, we tested the effect of anandamide in the presence of AM 251, a selective CB1 receptor antagonist. We found that pretreatment of slices with AM 251 (3 μM) abolished the anandamide-induced depression of eEPSC amplitude (102 ± 6.5% of baseline; n = 8; p = 0.25; Fig. 2A), suggesting that it was mediated by activation of CB1 receptors.

Results

Effects of eCBs on the Intrinsic Excitability of DR 5-HT Neurons. To examine the impact of eCBs on the excitability of DR 5-HT neurons, we first tested the effect of anandamide on the resting membrane potential of DR 5-HT neurons. We found that bath application of anandamide (10–30 μM) did not significantly affect the resting membrane potential of DR 5-HT neurons (n = 10; Fig. 1A). The average resting membrane potentials measured before and during anandamide application were −60 ± 3.5 and −59 ± 2.9 mV, respectively (n = 10; p > 0.05; Fig. 1C). Similarly, administration of anandamide did not alter the evoked spike activity (n = 8; Fig. 1B) nor the membrane input resistant (control, 368 ± 11 MΩ; anandamide, 380 ± 14 MΩ; n = 8; p > 0.05; Fig. 1C). Similar results were also obtained using the synthetic CB1 receptor agonist WIN 55,212-2. Thus, administration of WIN 55,212-2 (10 μM) did not affect the resting membrane potential (control, −61.5 ± 2.5 mV; WIN 55,212-2, −62.9 ± 3.9 mV; n = 9; p > 0.05; Fig. 1C) or the membrane input resistance (control, 368 ± 9 MΩ; WIN 55,212-2, 375 ± 13 MΩ; n = 9; p > 0.05; Fig. 1C). These findings indicate that activation of CB1 receptors with either eCBs or synthetic agonists exert no direct effects on the intrinsic excitability of DR 5-HT neurons.

eCBs Inhibit Glutamatergic Transmission to DR 5-HT Neurons via CB1 Receptors Activation. Previous in vivo and in vitro electrophysiological studies have shown that the electrical activity of DR 5-HT neurons is under the control of glutamatergic inputs and that modulation of glutamate synapses in the DR plays an important role in the overall regulation of serotoninergic transmission (Pan and Williams, 1989; Levine and Jacobs, 1992). Therefore, we next investigated whether eCBs could modulate excitatory synaptic transmission to DR 5-HT neurons. As reported previously (Haj-Dahmane and Shen, 2005), in the presence of picrotoxin (100 μM) and strychnine (20 μM) to block GABAA and glycine receptors, respectively, local electrical stimulations of the DR evoked fast eEPSCs in DR 5-HT neurons voltage-clamped close to their resting membrane potential (−70 mV). These eEPSCs were blocked by the selective AMPA receptor antagonist GYKI 52466 (50 μM), indicating that they were mainly mediated by AMPA receptors (n = 7; Fig. 2A). Bath application of anandamide (30 μM) induced a strong reduction in the amplitude of eEPSCs. On average, the amplitude of eEPSC was reduced to 58 ± 8% of baseline (n = 8; p < 0.01; Fig. 2B). The anandamide-induced depression of eEPSC amplitude persisted even after extensive washout, which is probably due to the high lipophilicity of anandamide.

Because anandamide is also an agonist for vanilloid receptors (Zygmun et al., 1999; Smart et al., 2000), the inhibition of eEPSC amplitude could be attributed to the activation of vanilloid receptors, cannabinoid receptors, or both. To distinguish between these possibilities, we tested the effect of anandamide in the presence of AM 251, a selective CB1 receptor antagonist. We found that pretreatment of slices with AM 251 (3 μM) abolished the anandamide-induced depression of eEPSC amplitude (102 ± 6.5% of baseline; n = 8; p = 0.25; Fig. 2A), suggesting that it was mediated by activation of CB1 receptors.
Endocannabinoids Regulation of 5-HT Neurons 189

CB1 Receptors Inhibit Glutamate Release in the DR.
In other brain regions, activation of CB1 receptors has been shown to inhibit synaptic transmission by reducing neurotransmitters release (Shen et al., 1996; Auclair et al., 2000; Azad et al., 2003). To test whether a similar mechanism also mediates the depression of EPSCs observed in DR 5-HT neurons, we examined the impact of CB1 receptor activation on the PPR and CV, two parameters that measure changes in neurotransmitter release at central synapses (Perkel and Nicoll, 1993; Dobrunz and Stevens, 1997). As illustrated in Fig. 3B, two consecutive stimuli with a 30-ms interstimulus interval induced a pair of EPSCs, with the second EPSC amplitude larger than the first EPSC. This paired pulse facilitation was observed in most DR 5-HT neurons examined (n = 8; Fig. 3C). Administration of WIN 55,212-2 (10 μM) reduced the amplitude of both EPSCs. However, the inhibition of the first EPSC was more profound than the second EPSC, resulting in a significant increase in the PPR (PPR control, 1.25 ± 0.09; PPR WIN 55,212-2, 1.98 ± 0.125; n = 8; p < 0.05, paired t test; Fig. 3D). This effect was totally blocked by AM 251 (3 μM, Fig. 3C; in AM 251, PPR control = 1.32 ± 0.035, PPR WIN 55,212-2 = 1.12 ± 0.023). The WIN 55,212-2-induced inhibition of EPSCs was also associated with an increase in the CV (control, 0.189 ± 0.013; WIN 55,212-2, 0.369 ± 0.033; n = 8; p < 0.05, paired t test; Fig. 3E). The increase in PPR and CV suggests that the depression of EPSC induced by the activation of CB1 receptors is mainly mediated by a presynaptic mechanism.

To determine the locus of CB1 receptor-mediated suppression of glutamatergic synaptic transmission, we tested the effect of WIN 55,212-2 on the frequency and amplitude of mEPSCs, which reflect action potential-independent glutamate release. Bath administration of WIN 55,212-2 (10 μM) reduced the frequency of mEPSCs (n = 8; Fig. 4A) and resulted in a significant rightward shift of the cumulative distribution curve of mEPSC frequency (p < 0.01, K-S test; Fig. 4B). The average mEPSC frequency was reduced from 2.88 ± 0.65 Hz in the control condition to 1.32 ± 0.39 Hz in the presence of WIN 55,212-2 (p = 0.05; paired t test; Fig. 4C). In contrast, bath application of WIN 55,212-2 did not significantly affect the amplitude distribution of mEPSCs (n = 8; p > 0.05, K-S test; Fig. 4E). The average amplitudes of all detected events recorded in the absence and presence of WIN 55,212-2 were 19.89 ± 4.6 and 21.78 ± 4.3 pA, respectively (n = 8; p > 0.05, paired t test; Fig. 4F). Together, these results demonstrate that the activation of CB1 receptors inhibits glutamate release from glutamatergic terminals impinging on DR 5-HT neurons.

Glutamate Synapses of DR 5-HT Neurons Express DSE. During the course of this study, we found that a strong membrane depolarization of DR 5-HT neurons also induced an inhibition of eEPSC amplitude. As illustrated in Fig. 5A, in a DR 5-HT neuron voltage-clamped at −70 mV, a brief (5-s) depolarizing voltage step from −70 to 0 mV resulted in a reduction of the subsequent eEPSC amplitude. The eEPSCs from 11 of 15 neurons tested were depressed by more
than 50%, although in the remaining neurons (4 of 15 neurons) membrane depolarization to 0 mV resulted in a very modest reduction of the eEPSC amplitude (less than 5%). On average, the amplitude of eEPSCs measured immediately after membrane depolarization was reduced to 57.4 ± 5.6% of the baseline level (n = 15; p < 0.05; Fig. 5B). The suppression of eEPSC amplitude was short lasting, as the amplitude of eEPSC recovered to predepolarization level with a time constant of 30.5 ± 8.8 s (n = 15; Fig. 5B). This form of short-term synaptic plasticity is called DSE and has been reported previously at glutamate synapses in other brain areas such as the cerebellum (Kreitzer and Regehr, 2001), hippocampus (Ohno-Shosaku et al., 2002), and the ventral tegmental area (Melis et al., 2004).

Previous studies have shown that DSE observed in other brain areas requires an increase in postsynaptic intracellular calcium (Kreitzer and Regehr, 2001; Melis et al., 2004). To determine whether an increase in postsynaptic intracellular is required for the induction of DSE in DR 5-HT neurons, we examined the effect of buffering postsynaptic intracellular calcium with BAPTA (25 mM) on the magnitude of DSE. We found that dialysis of neurons for at least 10 min with BAPTA (25 mM) containing internal solution prevented the induction of DSE (control, 52 ± 1.5% of the baseline; BAPTA, 102 ± 3.5% of the baseline; n = 8; p < 0.05; Fig. 6A). These results indicate that the rise in postsynaptic intracellular calcium induced by membrane depolarization is required for the induction of DSE in DR 5-HT neurons.

In theory, an increase in postsynaptic intracellular calcium could inhibit the amplitude of eEPSCs by reducing the number, function, or both of postsynaptic AMPA receptors; inhibiting glutamate release; or a combination of these effects. To address this issue, we measured the PPR before and during DSE to assess changes in the probability of neurotransmitter release. As illustrated in Fig. 6B, in all neurons tested, DSE was associated with an increase in PPR. On average, the mean PPR increased from 1.14 ± 0.06 during DSE (n = 8; t-test; Fig. 6B). This finding indicates that DSE was mediated by a presynaptic mechanism involving a decrease in glutamate release.

The above-mentioned results suggest that although DSE is initiated by a rise in postsynaptic intracellular calcium, it is mediated by presynaptic decrease in glutamate release. One possible interpretation of these findings is that depolarization of DR 5-HT neurons and the subsequent increase in intracellular calcium induces the release of retrograde signals that inhibit glutamate release. Several retrograde signals have been shown to modulate synaptic transmission at central synapses, including eCBs (for review, see Alger, 2002), which have been shown to mediate DSE in other brain areas (Kreitzer and Regehr, 2001; Melis et al., 2004).
Therefore, we examined whether the induction of DSE in DR5-HT neurons is mediated by retrograde eCB messengers. To that end, we tested the effect of CB1 receptor antagonist AM 251 on the magnitude of DSE. As illustrated in Fig. 7A, in control condition a depolarizing voltage step of DR 5-HT neurons elicited a strong DSE (51.5 ± 1.5% of baseline; \( n = 9; p < 0.05 \) paired \( t \) test). Bath administration of AM 251 (3 \( \mu M \)) did not significantly affect the baseline amplitude of eEPSC but abolished the induction of DSE (98.8 ± 3.5% of baseline; \( n = 9; p < 0.05 \)). These results indicate that activation of CB1 receptors is necessary for the DSE induction and that eCBs are probably the retrograde messengers released from DR 5-HT neurons.

Unexpectedly, in addition to blocking DSE, we found that in the presence of AM 251, depolarization of DR 5-HT neurons elicited a transient potentiation of eEPSC amplitude. This depolarization-induced potentiation of excitation (DPE) developed within 15 s after membrane depolarization (156.7 ± 5.3% of baseline; \( n = 9; p < 0.05 \); Fig. 7A) and lasted for approximately 60 s. Similar forms of potentiation of synaptic transmission have been reported previously at GABA synapses in the cerebellum and have been attributed to the retrograde release of glutamate acting on presynaptic NMDA receptors (Duguid and Smart, 2004). Therefore, to test whether a similar mechanism also mediates DPE in the DR, we examined the effect of \( \alpha \)-AP5 (50 \( \mu M \)), an NMDA receptor antagonist, on the magnitude of DPE. We found that blockade of the NMDA receptors had no significant effects on the magnitude of DPE (control, 156.7 ± 5.3% of baseline; \( \alpha \)-AP5, 152.8 ± 3.8% of baseline; \( n = 5; p < 0.05 \); Fig. 7B). These results indicate that the induction of DPE in the dorsal raphe is unlikely to be mediated by retrograde release of glutamate acting on presynaptic NMDA receptors. Future studies are required to precisely determine the mechanism underlying the DPE.

To further test whether DSE is mediated by retrograde eCBs acting on CB1 receptors, we examined the ability of CB1 agonist to occlude DSE. As expected, bath application of the CB1 receptor agonist WIN 55,212-2 (10 \( \mu M \)) induced an inhibition of eEPSC amplitude (\( n = 6; \) Fig. 7C). It is most important that in the presence of WIN 55,212-2, depolarization of DR 5-HT neurons failed to induce an additional inhib-
finding that CB1 agonists mimic and occlude DSE along with mental protocol induced a stable DSE (because in the absence of WIN 55,212-2 the same experiment cannot be attributed to a rundown phenomenon, 0.05; Fig. 7D). The blockade of DSE during perfusion of WIN larization from 10 ms. Bottom, time course and magnitude of DSE induced by 5-s depolarization (DSE), and during recovery (recovery). Calibration: 100 pA, (four trials) recorded before (baseline), immediately after membrane depolarization from −70 to 0 mV in a representative DR 5-HT neuron. B, summary plot of the average magnitude and duration of the DSE from all DR 5-HT neurons tested (n = 15).

Fig. 5. Glutamate synapses of DR 5-HT neurons express DSE. A, depolarization of a DR 5-HT neuron from −70 to 0 mV (5-s duration) elicits a transient depression of eEPSC amplitude. Top, average eEPSC traces (four trials) recorded before (baseline), immediately after membrane depolarization (DSE), and during recovery (recovery). Calibration: 100 pA, 10 ms. Bottom, time course and magnitude of DSE induced by 5-s depolarization from −70 to 0 mV in a representative DR 5-HT neuron. B, summary plot of the average magnitude and duration of the DSE from all DR 5-HT neurons tested (n = 15).

bition of eEPSC amplitude (Fig. 7D). On average, the magnitude of DSE induced by membrane depolarization in the absence and presence of WIN 55,212-2 was 51.5 ± 2.3% of baseline and 8.9 ± 6.5% of baseline, respectively (n = 6; p < 0.05; Fig. 7D). The blockade of DSE during perfusion of WIN 55,212-2 cannot be attributed to a rundown phenomenon, because in the absence of WIN 55,212-12 the same experimental protocol induced a stable DSE (n = 4; Fig. 7E). The finding that CB1 agonists mimic and occlude DSE along with the observation that CB1 antagonist abolishes DSE induction clearly indicate that retrograde eCB messengers mediate DSE at glutamate synapses of DR 5-HT neurons.

Discussion

The functional interaction between the eCB and 5-HT systems plays a critical role in the regulation of stress-related behaviors and emotional states. Thus, understanding the detailed mechanisms by which eCBs modulate the excitability of DR 5-HT neurons has important functional implications. In this study, we show that the eCB anandamide inhibits glutamatergic synaptic transmission to DR 5-HT neurons. This inhibitory effect is mainly mediated by the activation of presynaptic CB1 receptors and involves an inhibition of glutamate release. We have also shown that depolarization of DR 5-HT neurons can elicit the synthesis and release of eCBs, which in turn act as retrograde messengers on presynaptic CB1 receptors and mediate DSE. As such, the results of this study unravel a previously unsuspected mechanism by which 5-HT neurons modulate glutamatergic synaptic transmission in the DR.

Activation of CB1 Receptors Inhibits Glutamate Release in the DR. Early in vivo electrophysiological studies examining the effects of eCBs on the excitability of DR 5-HT neurons have reported that low systemic doses of eCBs or CB1 receptor agonist increase the firing activity of DR 5-HT neurons, whereas high systemic doses of CB1 agonist reduce the activity of these neurons (Gobbi et al., 2005; Bambico et al., 2007). The authors of these studies show that although the inhibitory effect is not mediated by CB1 receptors, the increase in the firing activity seems to be induced by activating these receptors located in the prefrontal cortex, which provide excitatory inputs to DR 5-HT neurons (Bambico et al., 2007). However, it is unclear how the activation of CB1 receptors, which has been shown to inhibit the excitability of prefrontal cortex pyramidal neurons and reduce glutamatergic synaptic transmission (Auclair et al., 2000), can lead to an increase in the firing activity of DR 5-HT neurons. Contrary to these studies, using intracellular recording in brain slices preparation, we show that eCBs (e.g., anandamide) profoundly inhibit glutamatergic synaptic transmission to DR 5-HT neurons without changing their intrinsic membrane properties. This inhibitory effect is exclusively mediated by the activation of CB1 receptors. Blocking CB1 receptors with AM 251 abolishes the eCB-mediated depression of eEPSCs. In addition, activation of CB1 receptors with WIN 212,212-2 mimics the effect of eCBs in that it induces a profound inhibition of eEPSC amplitude, an effect blocked by AM 251. Based on these pharmacological results, we concluded that eCBs suppress glutamatergic synaptic transmission to DR 5-HT neurons via the activation of CB1 receptors located in the DR area. This conclusion is consistent with previous binding and immunohistochemical studies showing the expression of CB1 receptors in the DR (Herkenham et al., 1991; Matsuda et al., 1993; Tsou et al., 1998; Häring et al., 2007).

Although results from previous anatomical studies have consistently reported the expression of CB1 receptors in the DR (Herkenham et al., 1991; Matsuda et al., 1993; Tsou et al., 1998; Häring et al., 2007), their exact subcellular distribution remains unknown. The findings that eCBs and synthetic CB1 receptor agonists have no direct postsynaptic effect on the intrinsic membrane properties of DR 5-HT neurons, along with the observation that the activation of these receptors presynaptically inhibits glutamate release (see discussion below), indicate that these receptors are unlikely to be located on DR 5-HT neurons but rather on glutamatergic terminals impinging on DR 5-HT neurons. However, future anatomical studies are required to further confirm this notion.

Several findings from the present study indicate that CB1 receptor-induced inhibition of eEPSC amplitude is mediated.
by a decrease in glutamate release. First, the inhibition of eEPSC is consistently accompanied by an increase in both the PPR and CV, which generally indicates a reduction in the probability of neurotransmitter release. Second, activation of CB1 receptors inhibits the frequency but not the amplitude of mEPSCs, which further indicates a decrease in glutamate release. These results are consistent with previous studies showing that activation of CB1 receptors regulates neuronal excitability mainly by inhibiting neurotransmitters release, including glutamate in the cortex (Auclair et al., 2000), amygdala (Azad et al., 2003), hippocampus (Shen et al., 1996), and ventral tegmental area (Melis et al., 2004).

**DR 5-HT Neurons Can Synthesize and Release eCBs.** An interesting finding of the present study is that depolarization of DR 5-HT neurons induces a DSE, a short-term depression of eEPSC amplitude. Examination of the cellular mechanism underlying DSE induction reveals that it is mediated by calcium-dependent eCB release from 5-HT neurons, which in turn stimulates presynaptic CB1 receptors and inhibits glutamate release. This conclusion is supported by the observation that the depression of eEPSC amplitude during the DSE is consistently associated with an increase in PPR, indicating an inhibition of glutamate release. Furthermore, buffering postsynaptic intracellular calcium with the fast calcium chelator BAPTA blocks the induction of DSE. Finally, blockade of CB1 receptors with AM 251 abolishes the DSE. Conversely, activation of CB1 receptors with WIN 55,212-2 not only mimics but also occludes the induction of DSE, indicating that it is mediated by eCBs released from DR 5-HT neurons. A similar DSE mediated by retrograde eCB signaling has been reported previously at glutamate synapses in several others brain area (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2002; Melis et al., 2004), indicating that eCB-mediated retrograde modulation of synaptic transmission is a widespread mechanism that enables postsynaptic neurons to fine tune the strength of their synaptic inputs.

The DSE observed at glutamate synapses of DR 5-HT neurons decays with a time constant of approximately 30 s, which is similar to the decay of DSE observed at parallel fiber Purkinje neuron synapses of the cerebellum (Tanimura et al., 2009) and mossy fiber granules neuron synapse of the dentate gyrus (Chiu and Castillo, 2008). Such decay time constant is somewhat slower than decay of the eCB-mediated depolarization induced suppression of inhibition observed at GABA synapses (Wilson and Nicoll, 2001). This probably reflects a difference in eCBs clearing between glutamate and GABA synapses in the central nervous system.

The finding that depolarization of DR 5-HT neurons can trigger the synthesis and release of eCBs is in agreement with previous reports showing that the DR expresses the enzymes responsible for eCB metabolism (Egertová et al., 2003) and contains a high concentration of eCBs, including anandamide (Petrosino et al., 2007). It is interesting to note that recent in vivo biochemical studies have reported that the concentration of eCBs (i.e., anandamide) in the DR is correlated to the level of excitability of DR 5-HT neurons in that an increase in the firing activity of these neurons is usually associated with an increase in the concentration of anandamide (Palazzo et al., 2006; Petrosino et al., 2007). Similarly, we have previously shown that activation of orexin receptors, which induce a profound increase in the excitability of DR 5-HT neurons, triggers the release of eCBs (Haj-Dahmane and Shen, 2005). Taken together, these studies combined with the present findings strongly support the notion that DR 5-HT neurons can synthesize and release eCBs in an
activity-dependent manner and that the release of these messengers plays a more prominent role in the regulation of central 5-HT neurotransmission than previously anticipated.

Functional Significance. Glutamatergic inputs to the DR have been shown to play an important role in regulating the in vivo firing and pacemaker activity of 5-HT neurons. Blockade of AMPA or NMDA receptors has been shown to reduce the firing activity of DR 5-HT neurons recorded in vivo (Levine and Jacobs, 1992). Therefore, the CB₁ receptor-induced decrease in glutamatergic synaptic transmission to DR 5-HT neurons reported in the present study would probably lead to a decrease in the overall firing activity of DR 5-HT neurons, and presumably result in an inhibition of central 5-HT transmission. Consistent with this notion, recent in vivo neurochemical studies have reported that activation of CB₁ receptors reduces 5-HT release in the projection area of the DR 5-HT neurons (Nakazi et al., 2000; Tzavara et al., 2003). In contrast, blockade of CB₁ receptors has been shown to facilitate 5-HT release (Tzavara et al., 2003). The present findings showing that activation of CB₁ receptors inhibits excitatory synaptic transmission to DR 5-HT neurons provides a potential cellular mechanism by which cannabinoid receptors suppress central 5-HT transmission.

DR 5-HT neurons are an integral part of the neural circuits that control anxiety-related behaviors (for review, see Gordon and Hen, 2004). Thus, exposure to a variety of stressors has been shown to increase the activity of DR 5-HT neurons (Grahn et al., 1999; Maier and Watkins, 2005). In addition, in animal models of anxiety, drugs that reduce serotonergic

---

**Fig. 7.** DSE in the DR is mediated by retrograde eCB messengers. A, CB₁ receptor antagonist AM 251 abolishes the induction of DSE. Top, superimposed eEPSC traces taken before and immediately after membrane depolarization in the control condition and in the presence of AM 251 (3 μM). Calibration: 100 pA, 10 ms. Bottom, summary plots of the magnitude and time course of DSE obtained in the absence (●; n = 8) and the presence of AM 251 (○; n = 8). Note that blockade of CB₁ receptors abolishes DSE and unmaskss a transient potentiation of eEPSCs (DPE). B, blockade of NMDA receptors had no significant effect on the magnitude of DPE. Top, superimposed eEPSC traces taken at the time indicated by numbers in the lower plot. Bottom, summary plot of the magnitude of DPE in the absence (●; n = 8) and the presence of D-AP5 (50 μM, ○; n = 5). C, CB₁ receptor agonist WIN 55,212-2 mimics and occludes DSE. Top, superimposed eEPSC traces taken before (●) and immediately after membrane depolarization (○) in the control condition (left traces) and in the presence of WIN 55,212-2 (10 μM, right traces). Bottom, effect of WIN 55,212-2 on the amplitude of eEPSC recorded before (F) and after membrane depolarization (M). Note that in the presence of WIN 55,212-2, there is no additional depression of eEPSC amplitude after membrane depolarization. D, summary plot of the effect of WIN 55,212-2 on the magnitude of DSE. Note that bath application of WIN 55,212-2 almost abolished DSE induction (n = 7, p < 0.05). E, time course of DSE recorded in the absence of WIN 55,212-2. Note that the DSE exhibits no significant run down during the duration of the recording.
activity can have an anxiolytic effect (Graeff et al., 1997), whereas drugs that increase the activity of DR 5-HT neurons exhibit anxiogenic effect (Hammack et al., 2003). Accordingly, the eCB-induced decrease in glutamatergic transmission to DR 5-HT neurons and the subsequent inhibition of 5-HT transmission may contribute, at least in part, to the anxiolytic effects induced by eCBs. More importantly, because DR 5-HT neurons can release eCBs in an activity-dependent manner, it is tempting to speculate that exposure to a variety of stressors, which have been shown to activate DR 5-HT neurons (Maier and Watkins, 2005), could lead to an increase in eCB release in the DR. Such an increase in eCB signaling may play a role in the regulation of stress-related anxiety behaviors. However, future studies examining the impact of stressors on eCB release in the DR are required to explore this possibility.

References
Trzavar ET, Davis RD, Perry KW, Li X, Salhoff C, Bymaster FP, Witkin JM, and

Endocannabinoids Regulation of 5-HT Neurons 195

 Downloaded from jpet.aspetjournals.org at ASPET Journals on July 8, 2017


Address correspondence to: Dr. Samir Haj-Dahmane, Research Institute on Addictions, University at Buffalo, 1021 Main St., Buffalo, NY 14203. E-mail: dahmane@ria.buffalo.edu