Endocannabinoids Suppress Excitatory Synaptic Transmission to Dorsal Raphe Serotonin Neurons through the Activation of Presynaptic CB₁ Receptors

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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 CB₁ receptor antagonist *N*-(piperidin-1-yl)-5-(4-iodophenyl)-4-methyl-1*H*-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-naphthalenylmethanone mesylate (WIN 55,212-2), a CB₁ receptor agonist. The inhibition of eEPSC amplitude was associated with an increase in the paired-pulse ratio and coefficient of variance. Activation of

CB₁ 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 CB₁ receptors with AM 251 abolished the DSE. In contrast, activation of CB₁ 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, CB₁ (Matsuda et al., 1990) and CB₂ receptors (Munro et al., 1993). Both receptors are coupled to the per-

tussis toxin-sensitive G protein G_{o/i} (Bayewitch et al., 1995; Felder et al., 1995). CB₁ receptors are predominantly expressed in the central nervous system (Herkenham et al., 1991; Matsuda et al., 1993; Egertová and Elphick, 2000). In contrast, CB₂ receptors were initially thought to be expressed mainly by immune and blood cells (Munro et al., 1993), but recent studies have reported the expression of these receptors in the central nervous system (Van Sickle et al., 2005; Ashton et al., 2006).

The cannabinoid system has been implicated in a myriad of physiological functions, including the regulation of emotional states. In humans, the recreational use of cannabis induces euphoria associated with a decrease in anxiety (Thomas,

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ABBREVIATIONS: eCB, endocannabinoid; 5-HT, 5-hydroxytryptamine (serotonin); DR, dorsal raphe; ACSF, artificial cerebrospinal fluid; QX314, 2-((2,6-dimethylphenyl)amino)-*N,N,N*-triethyl-2-oxoethanaminium; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; EPSC, excitatory postsynaptic current; DSE, depolarization-induced suppression of excitation; eEPSC, evoked excitatory postsynaptic current; WIN 55,212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate; CV, coefficient of variance; mEPSC, miniature excitatory postsynaptic current; K-S, Kolmogorov-Smirnov; GYKI 52466, 4-(8-methyl-9*H*-1,3-dioxolo[4,5-*h*][2,3]benzodiazepine-5-yl)-benzenamine hydrochloride; AM 251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; O-1602, 5-methyl-4-[(1*R*,6*R*)-3-methyl-6-(1-cyclohexen-1-yl)-1,3-benzenediol; D-AP5, D-(−)-2-amino-5-phosphonopentanoic acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; DPE, depolarization-induced potentiation of excitation; PPR, paired pulse ratio; NMDA, *N*-methyl-D-aspartate.

1996), whereas chronic cannabis abuse can lead to increased anxiety and panic attack (Hall and Solowij, 1998; Tournier et al., 2003). Similarly, behavioral studies in animals have shown that CB₁ receptor agonists can exert both anxiolytic (Berrendero and Maldonado, 2002; Marco et al., 2004) and anxiogenic effects (Arévalo et al., 2001; Genn et al., 2004), depending on the doses and behavioral models used. Genetic deletion or pharmacological blockade of CB₁ receptors increases anxiety related behavioral responses (Arévalo 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 sodium L-ascorbate, 3.1 mM sodium pyruvate, and 25 mM glucose, and equilibrated with 95% O₂, 5% CO₂. A block of brain tissue containing the area of the DR was dissected, and coronal slices (300–400 μ m) were cut using a Vibratome slicer (Lancer series 1000; Ted Pella, Reading, CA). Slices were transferred into a holding chamber containing regular ACSF: 119 mM NaCl, 2.5 mM CaCl₂, 1.3 mM MgSO₄, 1 mM NaH₂PO₄, 26.2 mM NaHCO₃, and 11 mM glucose continuously bubbled with a mixture of 95% O₂, 5% CO₂ and incubated for 30 to 45 min at 35°C. Slices were allowed to recover at room temperature for at least 1 h before recordings were conducted. After recovery, slices were transferred one at a time to a recording chamber mounted on a fixed

upright microscope and continuously perfused with standard ACSF saturated with 95% O₂, 5% CO₂ at 30 $\pm$ 1°C.

Whole-Cell Recordings. DR neurons were visualized using an upright microscope (BX 51 WI; Olympus, Tokyo, Japan) equipped with a differential interference contrast and infrared imaging system. Somatic recordings were obtained with patch electrodes (3–5 M Ω) filled with a solution containing 120 mM potassium gluconate, 10 mM KCl, 10 mM Na₂-phosphocreatine, 10 mM HEPES, 1 mM MgCl₂, 1 mM EGTA, 2 mM Na₂-ATP, and 0.25 mM Na-GTP, pH 7.3 (adjusted with KOH; osmolarity, 280–290 mOsmol/l). 5-HT neurons were identified using previously established criteria (Aghajanian and Vandermaelen, 1982; Haj-Dahmane, 2001). Synaptic currents were recorded with a cesium methanesulfonate-based solution of the following composition: 120 mM cesium methanesulfonic acid, 5 mM tetraethylammonium-Cl, 10 mM Na₂-phosphocreatine, 10 mM HEPES, 1 mM QX 314, 1 mM MgCl₂, 1 mM EGTA, 2 mM Na₂-ATP, and 0.25 mM Na-GTP, pH 7.3 (adjusted with CsOH; osmolarity, 280–290 mOsmol/l). In some experiments, to increase the calcium buffering capacity of the pipette solution, 25 mM cesium methanesulfonic acid was replaced with 25 mM BAPTA tetracesium salt. The estimated liquid junction potentials for the potassium gluconate- and cesium methanesulfonate-based pipette solutions were 5 and 15 mV, respectively. Membrane potentials were corrected for the liquid junction potentials.

Stimulation and Recordings. Stainless steel concentric bipolar stimulating electrode (inner pole diameter, 25 μ m; FHC Inc., Bowdoinham, ME) was placed 50 to 100 μ m dorsolateral to the recording sites. Excitatory postsynaptic currents (EPSCs) were evoked with a single square-pulse (intensity, 5–20 V; duration, 100–200 μ s) delivered at 0.1 Hz in neurons voltage-clamped at –70 mV. All recordings were performed in the presence of picrotoxin (100 μ M) and strychnine (20 μ M) to block GABA_A 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 M Ω) 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 EPSC 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 = EPSC₂/EPSC₁) 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_{eEPSC} – Var_{noise})/ (eEPSC mean amplitude). The magnitude of DSE was calculated using the mean amplitude of four consecutive eEPSCs taken before depolarization (Amp_{baseline}) and three eEPSCs collected immediately after membrane depolarization (Amp_{test}): DSE (%) = 100 (1 – (Amp_{test}/Amp_{baseline})).

Glutamate-mediated miniature EPSCs (mEPSCs) were recorded in the presence of tetrodotoxin (1 μ M), picrotoxin (100 μ M), and strychnine (20 μ M) 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

noise from compromising the analysis. Results in the text and figures are presented as mean $\pm$ 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 Tocris Cookson (Ellisville, MO). Tetrodotoxin and BAPTA tetraacetate 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 agonist were applied for at least 20 min. For the experiments using CB₁ receptor antagonist, slices were preincubated with AM 251 for at least 30 min before testing the effect of CB₁ agonists on the amplitude of eEPSCs.

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 μ M) 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 resistance (control, 368 ± 11 M Ω ; anandamide, 380 ± 14 M Ω ; $n = 8$; $p > 0.05$; 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 μ M) 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 M Ω ; WIN 55,212-2, 375 ± 13 M Ω ; $n = 9$; $p > 0.05$; 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 serotonergic 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 μ M) and strychnine (20 μ M) to block GABA_A 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 μ M), indicating that they were mainly mediated by AMPA receptors ($n = 7$; Fig. 2A). Bath application of anandamide (30 μ M) induced a strong reduction in the amplitude of eEPSCs. On average, the amplitude of eEPSC

Fig. 1. Anandamide has no significant effect on the intrinsic excitability of DR 5-HT neurons. **A**, current-clamp recording depicting the effect of anandamide on the resting membrane potential of DR 5-HT neuron. Calibration: 10 mV, 1 min. **B**, voltage response of DR 5-HT neuron to depolarizing (100-pA) and hyperpolarizing (-100 -pA) current pulses (600-ms duration) obtained in the absence (left traces) and presence of anandamide (30 μ M; right traces). Calibration: 40 mV, 200 ms. Note that bath application of anandamide (30 μ M) did not alter the resting membrane potential and the evoked activity of DR 5-HT neuron. **C**, summary histogram of the effect of anandamide and the synthetic CB₁ receptor agonist WIN 55,212-2 on the resting membrane potential (dashed bars) and membrane input resistance (black bars).

was reduced to $58 \pm 8\%$ 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 (Zygmunt 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 μ M) abolished the anandamide-induced depression of eEPSC amplitude ($102 \pm 6.5\%$ of baseline; $n = 8$; $p = 0.25$; Fig. 2A), suggesting that it was mediated by activation of CB₁ receptors.

Fig. 2. eCBs inhibit glutamate-mediated synaptic transmission to DR 5-HT neurons via CB₁ receptors. **A**, in the presence of GABA_A and glycine receptor antagonist, local stimulation of the DR elicits eEPSCs mediated mainly by AMPA receptors in DR 5-HT neuron voltage-clamped at -70 mV. Top, average eEPSC traces (60 trials) taken before and during bath application of the selective AMPA receptor antagonist GYKI 52466 (50 μ M). Calibration: 50 pA, 10 ms. Bottom, effect of GYKI 52466 on the amplitude of eEPSCs. **B**, anandamide inhibits eEPSC amplitude via the activation of CB₁ receptors. Top, superimposed eEPSC traces taken before and during bath application of anandamide (30 μ M) in control slices (left traces) and in slices treated with the CB₁ receptor antagonist AM 251 (3 μ M; right traces). Calibration: 50 pA, 10 ms. Bottom, summary graph of the effect of bath application of anandamide on the eEPSC amplitude obtained in the absence ($\bullet$; $n = 8$) and presence of AM 251 ($\circ$; $n = 8$). Note that the anandamide induced inhibition of eEPSC amplitude is blocked by AM 251.

To further examine the role of CB₁ receptor activation in the modulation of EPSCs, we tested the effect of WIN 55,212-2, a synthetic CB₁ receptor agonist, on the amplitude of eEPSCs recorded in control slices and slices treated with the CB₁ receptor antagonist AM 251 (3 μ M). As illustrated in Fig. 3A, bath application of WIN 55,212-2 (10 μ M) induced a profound inhibition of eEPSC amplitude recorded in control slices. On average, the amplitude of eEPSC was reduced to $53.5 \pm 3.8\%$ of the baseline level ($n = 8$; $p < 0.01$, paired t test; Fig. 3A). The depression of eEPSC amplitude induced by WIN 55,212-2 was totally blocked in slices pretreated with the CB₁ receptors antagonist AM 251 (3 μ M; $95.8 \pm 4.8\%$; $n = 8$; $p > 0.05$; Fig. 3A). In contrast to the effect of WIN 55,212-2, administration of O-1602 (10 μ M), a selective or-

phan G protein-coupled receptor 55 agonist, failed to alter the amplitude of eEPSCs ($93.5 \pm 5.5\%$ of baseline; $n = 5$; $p > 0.05$, paired t test; Fig. 3B). These results indicate that endogenous and exogenous cannabinoids inhibit glutamatergic synaptic transmission to DR 5-HT neurons through the activation of CB₁ receptors.

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 μ M) 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 μ M, 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 μ M) 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 ($n = 8$; $p < 0.05$, 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

Fig. 3. CB₁ receptor agonists inhibit glutamate-mediated eEPSCs by a presynaptic mechanism. A, summary plot of the time course and magnitude of the depression of eEPSCs induced by WIN 55,212-2 (10 μ M) obtained in control condition ($\circ$; $n = 8$) and in the presence of AM 251 (3 μ M; $\bullet$; $n = 8$). B, summary plot of the effect of the G protein-coupled receptor 55 agonist O-1602 (10 μ M) on the amplitude of eEPSCs. C, superimposed paired eEPSC traces recorded in the absence and presence of WIN 55,212-2 (10 μ M) at the time points indicated by number in A, in control slices and in slices treated with the CB₁ receptor antagonist AM 251 (3 μ M). Calibration: 50 pA, 30 ms. D, summary plots of the effect of WIN 55,212-2 (10 μ M) on the PPR obtained in control condition ($\circ$) and in the presence of AM 251 (3 μ M; $\bullet$). Note that WIN 55,212-2 induces a profound increase in PPR that is blocked by AM 251. E, summary graph of the effect of WIN 55,212-2 on the CV ($n = 8$; *, $p < 0.05$).

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 $57.4 \pm 5.6\%$ 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 prepolarization 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, $52 \pm 1.5\%$ of the baseline; BAPTA, $102 \pm 3.5\%$ 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 ± 0.03 in the control condition to 1.57 ± 0.06 during DSE ($n = 5$; $p < 0.05$, paired 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 regions (Kreitzer and Regehr, 2001; Melis et al., 2004).

Fig. 4. CB₁ receptor activation inhibits glutamate release in the DR. A, sample current traces showing mEPSCs recorded in control condition (left traces) and in the presence of WIN 55,212-2 (10 μ M; right traces). Calibration: 50 pA, 250 ms. B, plots of the cumulative distribution of the interevent interval for the neuron illustrated in A showing that bath application of WIN 55,212-2 significantly reduces the frequency of mEPSC ($p < 0.05$, K-S test). C, summary plots of the average mEPSC frequency recorded in control condition (□) and in the presence of WIN 55,212-2 (10 μ M, ■; $n = 8$; *, $p < 0.05$). D, plots of the cumulative distribution of mEPSC amplitude obtained in control condition (solid line) and in the presence of WIN 55,212-2 (dashed line). E, summary histogram of the average amplitude of mEPSCs recorded in control condition and in the presence of WIN 55,212-2. Note that WIN 55,212-2 has no significant effects on the amplitude of mEPSCs ($n = 8$; $p > 0.05$).

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 ($51.5 \pm 1.5\%$ of baseline; $n = 9$; $p < 0.05$ paired t test). Bath administration of AM 251 (3 μ M) did not significantly affect the baseline amplitude of eEPSC but abolished the induction of DSE ($98.8 \pm 3.5\%$ 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 ($156.7 \pm 5.3\%$ 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 D-AP5 (50 μ 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, $156.7 \pm 5.3\%$ of baseline; D-AP5, $152.8 \pm 3.8\%$ 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 μ 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 inhi-

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 $51.5 \pm 2.3\%$ of baseline and $8.9 \pm 6.5\%$ 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 CB_1 agonists mimic and occlude DSE along with the observation that CB_1 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 CB_1 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 CB_1 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 CB_1 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 CB_1 receptor agonist increase the firing activity of DR 5-HT neurons, whereas high systemic doses of CB_1 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 CB_1 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 CB_1 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 CB_1 receptors. Blocking CB_1 receptors with AM 251 abolishes the eCB-mediated depression of eEPSCs. In addition, activation of CB_1 receptors with WIN 25,212-2 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 CB_1 receptors located in the DR area. This conclusion is consistent with previous binding and immunohistochemical studies showing the expression of CB_1 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 CB_1 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 CB_1 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 CB_1 receptor-induced inhibition of eEPSC amplitude is mediated

Fig. 6. DSE in the DR requires an increase in postsynaptic intracellular calcium and is mediated by a decrease in glutamate release. **A**, buffering postsynaptic intracellular calcium abolishes the induction of DSE. Top, superimposed eEPSC traces recorded before and after membrane depolarization using a control intracellular solution (1 mM EGTA; left traces) and an intracellular solution containing 25 mM BAPTA (right traces). Calibration: 50 pA, 10 ms. Bottom, magnitude and time course of DSE obtained with an intracellular solution containing 1 mM EGTA (●; $n = 8$) and with an intracellular solution containing 25 mM BAPTA (○; $n = 8$). **B**, DSE is associated with an increase in PPR. Top, samples of pairs of eEPSCs taken before (baseline), during DSE (DSE), and after recovery (recovery). Calibration: 50 pA, 20 ms. Bottom, increase in PPR during DSE obtained in five DR 5-HT neurons ($n = 5$; *, $p < 0.05$).

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), indicat-

ing 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

Fig. 7. DSE in the DR is mediated by retrograde eCB messengers. **A**, CB_1 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 μ M). Calibration: 100 pA, 10 ms. Bottom, summary plots of the magnitude and time course of DSE obtained in the absence ($\bullet$; $n = 8$) and the presence of AM 251 ($\circ$; $n = 8$). Note that blockade of CB_1 receptors abolishes DSE and unmasks 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 ($\circ$; $n = 8$) and the presence of D-AP5 (50 μ M, $\bullet$; $n = 5$). **C**, CB_1 receptor agonist WIN 55,212-2 mimics and occludes DSE. Top, superimposed eEPSC traces taken before ($\circ$) and immediately after membrane depolarization ($\bullet$) in the control condition (left traces) and in the presence of WIN 55,212-2 (10 μ M; 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-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 CB_1 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 acti-

vation of CB_1 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 CB_1 receptors has been shown to facilitate 5-HT release (Tzavara et al., 2003). The present findings showing that activation of CB_1 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 (Grahm et al., 1999; Maier and Watkins, 2005). In addition, in animal models of anxiety, drugs that reduce serotonergic

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 eCBs release in the DR are required to explore this possibility.

References

- Aghajanian GK and Vandermaelen CP (1982) Intracellular recording from serotonergic dorsal raphe neurons: pacemaker potentials and the effect of LSD. *Brain Res* **238**:463–469.
- Alger BE (2002) Retrograde signaling in the regulation of synaptic transmission: focus on endocannabinoids. *Prog Neurobiol* **1** **68**:247–286.
- Arévalo C, de Miguel R, and Hernández-Tristán R (2001) Cannabinoid effects on anxiety-related behaviors and hypothalamic neurotransmitters. *Pharmacol Biochem Behav* **70**:123–131.
- Ashton JC, Friberg D, Darlington CL, and Smith PF (2006) Expression of the cannabinoid CB₂ receptor in the rat cerebellum: an immunohistochemical study. *Neurosci Lett* **396**:113–116.
- Auclair N, Otani S, Soubrie P, and Crepel F (2000) Cannabinoids modulate synaptic strength and plasticity at glutamatergic synapses of rat prefrontal cortex. *J Neurophysiol* **83**:3287–3293.
- Azad SC, Eder M, Marsicano G, Lutz B, Zieglgänsberger W, and Rammes G (2003) Activation of cannabinoid receptor type 1 decrease glutamatergic and GABAergic synaptic transmission in the lateral amygdala of the mouse. *Learn Mem* **10**:116–128.
- Bambico FR, Katz N, Debonnel G, and Gobbi G (2007) Cannabinoids elicit antidepressant-like behavior and activate serotonergic neurons through the medial prefrontal cortex. *J Neurosci* **27**:11700–11711.
- Bayewitch M, Avidor-Reiss T, Levy R, Barg J, Mechoulam R, and Vogel Z (1995) The peripheral cannabinoid receptor: adenylyl cyclase inhibition and G protein coupling. *FEBS Lett* **375**:143–147.
- Berrendero F and Maldonado R (2002) Involvement of the opioid system in the anxiolytic-like effects induced by delta (9)-tetrahydrocannabinol. *Psychopharmacology (Berl)* **163**:111–117.
- Chiu CQ and Castillo PE (2008) Input-specific plasticity at excitatory synapses mediated by endocannabinoids in the dentate gyrus. *Neuropharmacology* **54**:68–78.
- Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, and Mechoulam R (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* **258**:1946–1949.
- Dobrunz LE and Stevens CF (1997) Heterogeneity of release probability, facilitation and depletion at central synapses. *Neuron* **18**:995–1008.
- Duguid IC and Smart TG (2004) Retrograde activation of presynaptic NMDA receptors enhances GABA release at cerebellar interneuron-Purkinje cell synapses. *Nat Neurosci* **7**:525–533.
- Egertová M, Cravatt BF, and Elphick MR (2003) Comparative analysis of fatty acid amid hydrolase and CB1 cannabinoid receptor expression in the mouse brain: evidence of a widespread role of fatty acid amid hydrolase. *Neuroscience* **119**:481–496.
- Egertová M and Elphick MR (2000) Localization of cannabinoid receptors in the rat brain using antibodies to the intracellular C-terminal tail of CB₁. *J Comp Neurol* **422**:159–171.
- Felder CC, Joyce KE, Briley EM, Mansouri J, Mackie K, Blond O, Lai Y, Ma AL, and Mitchell RL (1995) Comparison of the pharmacology and signal transduction of the human cannabinoid CB₁ and CB₂ receptors. *Mol Pharmacol* **48**:443–450.
- Genn RF, Tucci S, Marco EM, Viveros MP, and File SE (2004) Unconditioned and conditioned anxiogenic effects of the cannabinoid receptor agonist CP 55,940 in the social interaction test. *Pharmacol Biochem Behav* **77**:567–573.
- Gobbi G, Bambico FR, Mangieri R, Bortolotto M, Campolongo P, Solinas M, Cassano T, Morgese MG, Debonnel G, Duranti A, et al. (2005) Antidepressant-like activity and modulation of brain monoaminergic transmission by blockade of anandamide hydrolysis. *Proc Natl Acad Sci U S A* **102**:18620–18625.
- Gordon JA and Hen R (2004) The serotonergic system and anxiety. *Neuromol Med* **5**:27–40.
- Graeff FG, Viana MB, and Mora PO (1997) Dual role of 5-HT in defense and anxiety. *Neurosci Biobehav Rev* **21**:791–799.
- Grahn RE, Will MJ, Hammack SE, Maswood S, McQueen MB, Watkins LR, and Maier SF (1999) Activation of serotonin-immunoreactive cells in the dorsal raphe nucleus in rats exposed to an uncontrollable stressor. *Brain Res* **826**:35–43.
- Griebel G, Stemmelin J, and Scatton B (2005) Effects of cannabinoid CB₁ receptor antagonist rimonabant in models of emotional reactivity I rodents. *Biol Psychiatry* **57**:261–267.
- Haj-Dahmane S (2001) D₂-like dopamine receptor activation excites rat dorsal raphe 5-HT neurons in vitro. *Eur J Neurosci* **14**:125–134.
- Haj-Dahmane S and Shen RY (2005) The wake-promoting peptide orexin-B inhibits glutamatergic transmission to dorsal raphe nucleus serotonin neurons through retrograde endocannabinoid signaling. *J Neurosci* **25**:896–905.
- Hall W and Solowij N (1998) Adverse effects of cannabis. *Lancet* **352**:1611–1616.
- Haller J, Varga B, Ledent C, and Freund TF (2004) CB₁ cannabinoid receptors mediate anxiolytic effects: convergent genetic and pharmacological evidence with CB₁-specific agents. *Behav Pharmacol* **15**:299–304.
- Hammack SE, Schmid MJ, LoPresti ML, Der-Avakian A, Pellymounter MA, Foster AC, Watkins LR, and Maier SF (2003) Corticotropin releasing hormone type 2 receptors in the dorsal raphe nucleus mediate the behavioral consequence of uncontrollable stress. *J Neurosci* **23**:1019–1025.
- Häring M, Marsicano G, Lutz B, and Monory K (2007) Identification of the cannabinoid receptor type 1 in serotonergic cells of raphe nuclei in mice. *Neuroscience* **146**:1212–1219.
- Herkenham M, Lynn AB, Johnson MR, Melvin LS, de Costa BR, and Rice KC (1991) Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. *J Neurosci* **11**:563–583.
- Kathuria S, Gaetani S, Fegley D, Valiño F, Duranti A, Tontini A, Mor M, Tarzia G, La Rana G, Calignano A, et al. (2003) Modulation of anxiety through blockade of anandamide hydrolysis. *Nat Med* **9**:76–81.
- Kreitzer AC and Regehr WG (2001) Retrograde inhibition of presynaptic calcium influx by endogenous cannabinoids at excitatory synapses onto the Purkinje cells. *Neuron* **29**:717–727.
- Levine ES and Jacobs BL (1992) Neurochemical afferents controlling the activity of serotonergic neurons in the dorsal raphe nucleus; microiontophoretic studies in the wake cat. *J Neurosci* **12**:4037–4044.
- Maier SF and Watkins LR (2005) Stressor controllability and learned helplessness: the role of the dorsal raphe nucleus, serotonin, and corticotrophin-releasing factor. *Neurosci Biobehav Rev* **29**:829–841.
- Marco EM, Pérez-Alvarez L, Borcel E, Rubio M, Guaza C, Ambrosio E, File SE, and Viveros MP (2004) Involvement of 5-HT_{1A} receptors in behavioral effects of the cannabinoid receptor agonist CP 55,940 in male rats. *Behav Pharmacol* **15**:21–27.
- Matsuda LA, Bonner TI, and Lolait SJ (1993) Localization of cannabinoid receptor mRNA in rat brain. *J Comp Neurol* **327**:535–550.
- Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, and Bonner TI (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* **346**:561–564.
- Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, Gopher A, Almog S, Martin BR, and Compton DR (1995) Identification of endogenous 2-monoglyceride, present in canine gut that binds to cannabinoid receptors. *Biochem Pharmacol* **50**:83–90.
- Melis M, Pistis M, Perra S, Muntoni AL, Pillolla G, and Gessa GL (2004) Endocannabinoids mediate presynaptic inhibition of glutamatergic transmission in rat ventral tegmental area dopamine neurons through activation of CB₁ receptors. *J Neurosci* **24**:53–62.
- Munro S, Thomas KL, and Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature* **365**:61–65.
- Nakazi M, Bauer U, Nickel T, Kathmann M, and Schlicker E (2000) Inhibition of serotonin release in the mouse brain via presynaptic CB receptors. *Naunyn-Schmiedeberg Arch Pharmacol* **361**:19–24.
- Ohno-Shosaku T, Tsubokawa H, Mizushima I, Yoneda N, Zimmer A, and Kano M (2002) Presynaptic cannabinoid sensitivity is a major determinant of depolarization-induced retrograde suppression at hippocampal synapses. *J Neurosci* **22**:3864–3872.
- Palazzo E, de Novellis V, Petrosino S, Marabese I, Vita D, Giordano C, Di Marzo V, Mangoni GS, Rossi F, and Maione S (2006) Neuropathic pain and the endocannabinoid system in the dorsal raphe: pharmacological treatment and interactions with the serotonergic system. *Eur J Neurosci* **24**:2011–2020.
- Pan ZZ and Williams JT (1989) GABA- and glutamate-mediated synaptic potentials in rat dorsal raphe neurons in vitro. *J Neurophysiol* **61**:719–726.
- Perkel DJ and Nicoll RA (1993) Evidence for all-or-none regulation of neurotransmitter release: implication for long term potentiation. *J Physiol (Lond)* **471**:484–500.
- Petrosino S, Palazzo E, de Novellis V, Bisogno T, Rossi F, Maione S, and Di Marzo V (2007) Changes in spinal and supraspinal endocannabinoid levels in neuropathic rats. *Neuropharmacology* **52**:415–422.
- Shen M, Piser TM, Seybold VS, and Thayer SA (1996) Cannabinoid receptor agonists inhibit glutamatergic synaptic transmission in rat hippocampal culture. *J Neurosci* **16**:4322–4334.
- Smart D, Gunthorpe MJ, Jerman JC, Nasir S, Gray J, Muir AI, Chambers JK, Randall AD, and Davis JB (2000) The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). *Br J Pharmacol* **129**:227–230.
- Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, Yamashita A, and Waku K (1995) 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem Biophys Res Commun* **215**:89–97.
- Tanimura A, Kawata S, Hashimoto K, and Kano M (2009) Not glutamate but endocannabinoids mediate retrograde suppression of cerebellar parallel fiber Purkinje cell synaptic transmission in young adult rodents. *Neuropharmacology* **57**:157–163.
- Thomas H (1996) A community survey of adverse effects of cannabis use. *Drug Alcohol Depend* **42**:201–207.
- Tournier M, Sorbara F, Gindre C, Swendsen JD, and Verdoux H (2003) Cannabis use and anxiety in daily life: a naturalistic investigation in a non-clinical population. *Psychiatry Res* **118**:1–8.
- Tsou K, Brown S, Sañudo-Peña MC, Mackie K, and Walker JM (1998) Immunohistochemical distribution of cannabinoid CB₁ receptors in the rat central nervous system. *Neuroscience* **83**:393–411.
- Tzavara ET, Davis RJ, Perry KW, Li X, Salhoff C, Bymaster FP, Witkin JM, and

- Nomikos GG (2003) The CB₁ receptor antagonist SR 141716A selectively increases monoaminergic neurotransmission in the medial prefrontal cortex: implications for therapeutic actions. *Br J Pharmacol* **138**:544–553.
- Van Sickle MD, Duncan M, Kingsley PJ, Mouihate A, Urbani P, Mackie K, Stella N, Makriyannis A, Piomelli D, Davison JS, et al. (2005) Identification and functional characterization of brainstem cannabinoid CB₂ receptors. *Science* **310**:329–332.
- Wilson RI and Nicoll RA (2001) Endogenous cannabinoids mediate retrograde signaling at hippocampal synapses. *Nature* **411**:588–592.

Zygmunt PM, Petersson J, Andersson DA, Chuang H, Sjørgård M, Di Marzo V, Julius D, and Högestätt ED (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* **400**:452–457.

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