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# Cannabinoid receptor activation modifies NMDA receptor mediated release of intracellular calcium: Implications for endocannabinoid control of hippocampal neural plasticity

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ABSTRACT

Chronic activation or inhibition of cannabinoid receptors (CB1) leads to continuous suppression of neuronal plasticity in hippocampus and other brain regions, suggesting that endocannabinoids may have a functional role in synaptic processes that produce state-dependent transient modulation of hippocampal cell activity. In support of this, it has previously been shown *in vitro* that cannabinoid CB1 receptors modulate second messenger systems in hippocampal neurons that can regulate operation of intracellular processes including receptors which release calcium from intracellular stores. Here we demonstrate in hippocampal slices a similar endocannabinoid action on excitatory glutamatergic synapses via modulation of NMDA-receptor mediated intracellular calcium levels in confocal imaged neurons. Calcium entry through glutamatergic NMDA-mediated ion channels increases intracellular calcium concentrations by modifying release from ryanodine-sensitive channels in endoplasmic reticulum. The studies reported here show that NMDA-elicited increases in Calcium Green fluorescence are enhanced by CB1 receptor antagonists (i.e., Rimonabant), and inhibited by CB1 agonists (i.e., WIN 55,212-2). Suppression of endocannabinoid breakdown by either reuptake inhibition (AM404) or fatty-acid amide hydrolase inhibition (URB597) produced suppression of NMDA-elicited calcium increases comparable to WIN 55,212-2, while enhancement of calcium release provoked by endocannabinoid receptor antagonists (Rimonabant) was shown to depend on the blockade of CB1receptor mediated de-phosphorylation of Ryanodine receptors. Such CB1 receptor modulation of NMDA elicited increases in intracellular calcium may account for the respective disruption and enhancement by CB1 agents of trial-specific hippocampal neuron ensemble firing patterns during performance of a short-term memory task, reported previously from this laboratory.


## 1. Introduction

The possible cellular bases for the chronic effects of cannabinoid treatment in recent investigations are related to cannabinoid receptor modulation of synaptic activity in specific neuronal systems (Fowler et al., 2010; Glickfeld and Scanziani, 2006; Hashimotodani et al., 2008; Kim and Alger, 2010; Martin-Garcia et al., 2010). Prior studies have revealed, interactions between cannabinoid CB1 receptor activation and reductions in synaptic transmission in some systems (Chevaleyre and Castillo, 2003; Falenski et al., 2007; Fortin

*Abbreviations:* ACSF, artificial cerebrospinal fluid; AM281, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1H-pyrazole-3-carboxamide; AM404, *n*-(4-hydroxyphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide; AMPA, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; cAMP, 3′,5′-cyclic adenosine monophosphate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DMSO, dimethylsulfoxide; JZL184, 4-nitrophenyl-4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate; MK801, dizocilpine: (+)-5-methyl-10,11- dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione; NMDA, *n*-methyl-D-aspartic acid; PKA, cAMP-dependent protein kinase; Rmbt, rimonabant (original designation SR141716A): 5-(4-chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-*N*-(piperidin-1-yl)-1*H*-pyrazole-3-carboxamide; Rp-cAMPS, (Rp)-adenosine-3′,5′-cyclic-*S*-(4-bromo-2,3-dioxobutyl)monophosphorothioate; RyR, ryanodine-sensitive intracellular calcium channel; Sp-cAMPS, (Sp)-adenosine-3′,5′-cyclic-*S*-(4-bromo-2,3-dioxobutyl)monophosphorothioate; TTX, tetrodotoxin – octahydro-12-(hydroxymethyl)-2-imino-5,9:7,10a-dimethano-10aH-[1,3]dioxocino[6,5-d]pyrimidine-4,7,10,11,12-pentol; URB597, (3′-(aminocarbonyl)[1,1′-biphenyl]-3-yl)- cyclohexylcarbamate; URB602, [1,1′-biphenyl]-3-yl-carbamic acid, cyclohexyl ester; WIN, WIN 55,212-2: (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de)-1,4-benzoxazin-6-yl]-1-napthalenylmethanone.

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et al., 2004; Pertwee, 2005). A direct action of endocannabinoids on synaptic transmission provides possible explanations for noted incidences of behavioral change due to modulated release of neurotransmitters or modification of related cellular processes (Foldy et al., 2006; Kim and Alger, 2004; Losonczy et al., 2004; Wilson et al., 2001). Here we provide direct evidence for cannabinoid modulation of hippocampal glutamatergic activation, by showing that NMDA-receptor mediated release of intracellular calcium ($Ca^{++}$), imaged in hippocampal slices, is reduced via CB1 receptor activation by endocannabinoids. The following report shows that the ability of NMDA receptor gated $Ca^{++}$ ions potentiate calcium-sensitive endoplasmic reticulum Ryanodine (RYR) receptors to release intracellular calcium (Li et al., 2006). This ability is reduced by activation of CB1 receptors. The results support the theory that CB1 receptor activation attenuates synaptic processing in hippocampal neurons by decreasing the potential to modulate the release of intracellular calcium in circumstances where such flexibility is critical for adapting to requirements for successful memory encoding and retrieval (Deadwyler et al., 2007; Deadwyler and Hampson, 2008).

Recent studies have demonstrated the selective disruption of different types of memory following treatment with cannabinoid receptor agonists such as WIN 55,212 (Abush and Akirav, 2009; Kim and Alger, 2010; Manwell et al., 2009). Prior investigations from this laboratory have demonstrated the negative influence of endogenous cannabinoids on the modulation of hippocampal memory encoding in ensembles of neurons, by revealing improved performance in the presence of CB1 receptor antagonists which increased the range of ensemble encoding on a trial-by-trial basis (Deadwyler et al., 2007). The results presented here support the above observations by demonstrating that CB1 receptor activation and inhibition alter intracellular calcium release provoked by excitatory synapses in hippocampus.

## 2. Methods

### 2.1. Hippocampal Slices

Preparation of hippocampal slices was similar to that described in previous reports (Hampson et al., 2003; Zhuang et al., 2005a, 2005b). Slices were obtained from Sprague–Dawley rats aged 10–17 days, following NIH guidelines. The brain was rapidly removed to a chilled (4°) oxygenated (95% $O_2$, 5% $CO_2$) artificial cerebrospinal fluid (ACSF) solution containing (mM) NaCl (126), $NaHCO_3$ (20), KCl (5), $MgCl_2$ (2), $CaCl_2$ (2.5), glucose (10), HEPES (20). Transverse slices (250–300 μm) were cut with a vibratome (Leica VT1000S); the hippocampus was dissected free and suspended in oxygenated ACSF at 30° for 1 h.

Slices were transferred to a darkened Petri dish containing 13 μM Calcium Green acetoxymethyl ester-ACSF solution (50 μg Calcium Green 1 AM, Molecular Probes; reconstituted with 20% pluronic acid in dimethylsulfoxide (DMSO); Pluronic F-127, Molecular Probes) and incubated at room temperature with oxygenation for 45 min, rinsed in fresh ACSF and maintained in the dark at room temperature in oxygenated ACSF. Individual slices were then transferred to a temperature controlled recording chamber (Warner Instruments; Hamden, CT), held in place with a harp slice anchor, and perfused continuously with gravity fed oxygenated ACSF solution at a rate of 1.8 ml/min.

### 2.2. Calcium imaging

Imaging was performed on CA1 pyramidal cells with a Nikon E800 upright confocal microscope equipped with a water-immersion objective, a Hamamatsu-Orca-ER digital camera and a Perkin Elmer Ultraview spinning disc confocal system. The "Nipkow" spinning disk confocal technique provides rapid confocal imaging over a very large area, allowing subsecond imaging of a 1 mm square of tissue (Rutter et al., 2006). Calcium Green emission images (500–600 nm) were acquired using monochrome laser excitation at 488 nm with no emission wavelength filtering (Maravall et al., 2000). The images were stored at 0.3 s intervals, with a piezoelectric motor "stepping" the focal plane to acquire 40 vertical slices (2.5 μm depth) per field, thus producing a complete 3-D image every 12 s. Images were "flattened," combining multiple depth slices, to allow analysis of whole neural soma captured in the 3-D confocal image.

Intracellular calcium was assessed via change in cell soma fluorescence ($\Delta E$, emission image density) plotted as a function of baseline ($E_0$) Calcium Green fluorescence (Paredes et al., 2008). The change in fluorescence, $\Delta E/E_0$, correlates to percentage change in intracellular calcium concentration (Maravall et al., 2000; Paredes et al., 2008; Wilms and Eilers, 2007) using single line (488 nm) laser excitation without requiring ratiometric measurement (e.g., Fura-2 (Paredes et al., 2008)). All slices were recorded and initially tested under baseline ACSF treatment conditions during which no change in intracellular calcium occurred (Step A – ACSF perfusion for 6.0 min). They were then perfused with *n*-methyl-D-aspartic acid (NMDA) to elicit baseline intracellular calcium increases (Step B – ACSF perfusion for 3.0 min, 10 μM NMDA perfusion for 2 min, followed by ACSF for 5.0 min). To test the modulation of NMDA-elicited calcium, slices were pre-exposed to the experimental compound for 10.0 min via perfusion with media containing only the compound (Step C) then switched to media containing the compound + NMDA (concentration as in Step B) and exposed for 2.0 min, after which perfusion with media containing only the experimental compound was continued for an additional 5.0 min (Step D). Changes between the control ACSF solution and the experimental solutions were achieved by a solenoid valve switching system (Warner Instruments) to ensure no interruption would occur in the perfusion of the slice. Confocal calcium imaging was continuous during Steps A–D to show the time course and percentage of change in NMDA-induced fluorescence following pre-exposure or reversal of effects of other (experimental) compounds.

### 2.3. Data analysis

Changes in intracellular calcium-dependent Calcium Green fluorescent image density (Fig. 1A) were measured for Regions of Interest (ROIs) corresponding to CA1 cell soma (Fig. 1B). The fluorescence change ($\Delta E$) was divided by baseline fluorescence ($E_0$ – mean of first 120 s; Fig. 1C) to normalize across cells and correct for photobleaching. Mean (percentage) $\Delta E/E_0$ across cells is plotted over time to indicate timecourse; the mean of peak % $\Delta E/E_0$ was statistically compared across cells using ANOVA. To ensure adequate sampling, a minimum of 3 cells (soma, Fig. 2B) were measured per hippocampal slice, with 5–10 slices tested for each treatment. All slices were tested with a minimum of one control (NMDA only) and one test drug, while some slices were tested with a second drug to assess blockade or reversal of the initial drug. In the latter case, control experiments showed no more than 5% variance in mean $\Delta E/E_0$ with repeated NMDA exposure, and complete washout of drug effects after 10 min perfusion.

### 2.4. Drug preparation

The CB1 cannabinoid receptor antagonist rimonabant (SR141716A) was obtained from the National Institute on Drug Abuse (NIDA); all other drugs were obtained from commercial sources (Sigma–Aldrich and Tocris). Soluble drugs, NMDA, NBQX – 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione, CNQX – 6-cyano-7-nitroquinoxaline-2,3-dione, MK801 – (dizocilpine) (+)-5-methyl-10,11- dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate, ryanodine, Sp-cAMPS – (Sp)-adenosine-3′,5′-cyclic-*S*-(4-bromo-2,3-dioxobutyl)monophospho rothioate, Rp-cAMPS – (Rp)-adenosine-3′,5′-cyclic-*S*-(4-bromo-2,3-dioxobutyl) monophosphorothioate, PKA catalytic subunit, were prepared as dilutions in the ACSF bathing solution. Other drugs, WIN 55,212-2 – (R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de)-1,4-benzoxazin-6-yl]-1-napthalenyl methanone, Rimonabant – 5-(4-Chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-*N*-(piperidin-1-yl)-1*H*-pyrazole-3-carboxamide, AM281 – 1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1H-pyrazole-3-carboxamide, AM404 – *N*-(4-Hydroxyphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide, URB597 – (3′-(aminocarbonyl)[1,1′-biphenyl]-3-yl)- cyclohexylcarbamate, URB602 – [1,1′-biphenyl]-3-yl-carbamic acid, cyclohexyl ester, and JZL184 – 4-nitrophenyl-4-(dibenzo[d][1,3] dioxol-5- yl(hydroxy)methyl)piperidine-1-carboxylate were prepared as 10 mM stock in ethanol, then diluted in ACSF to final concentration and exposed to a constant stream of nitrogen to evaporate residual ethanol. All drugs were delivered by bath perfusion at 37 °C.

## 3. Results

### 3.1. Cannabinoid receptors modulate neuronal calcium release

Slices of hippocampal tissue were saturated with Calcium Green AM (Sigma), and excited with a blue (488 nm) laser to fluorescence at intensity correlated with intracellular calcium concentrations. Fig. 1A displays the measurement of calcium-dependent fluorescence as a function of NMDA exposure. The photomicrographs illustrate the peak fluorescence in response to NMDA and are color-coded to depict relative differences in intensity of cellular fluorescence for each of the conditions indicated. Fig. 1B shows a portion of the field in Fig. 1A to illustrate detection of cytosolic calcium in soma and dendrites by the Calcium Green fluorescence label. Calcium Green fluorescence is further quantified as the ratio of change in intensity relative to the baseline fluorescence ($\Delta E/E_0$) recorded for the 3.0 min prior to NMDA