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Prolonged cannabinoid exposure alters GABA_A receptor mediated synaptic function in cultured hippocampal neurons

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Abstract

Developing cannabinoid based medication along with marijuana's recreational use makes it important to investigate molecular adaptations the endocannabinoid system undergoes following prolonged use and withdrawal. Repeated cannabinoid administration results in development of tolerance and produces withdrawal symptoms that may include seizures. Here we employed electrophysiological and immunochemical techniques to investigate the effects of prolonged CB1 receptor agonist exposure on cultured hippocampal neurons. Approximately 60% of CB1 receptors colocalize to GABAergic terminals in hippocampal cultures. Prolonged treatment with the cannabinamimetic WIN 55,212-2 (+WIN, 1μM, 24-h) caused profound CB1 receptor downregulation accompanied by neuronal hyperexcitability. Furthermore, prolonged +WIN treatment resulted in increased GABA release as indicated by increased mIPSC frequency, a diminished GABAergic inhibition as indicated by reduction in mIPSC amplitude and a reduction in GABA_A channel number. Additionally, surface staining for the GABA_A β_{2/3} receptor subunits was decreased, while no changes in staining for the presynaptic vesicular GABA transporter were observed, indicating that GABAergic terminals remained intact. These findings demonstrate that agonist-induced downregulation of the CB1 receptor in hippocampal cultures results in neuronal hyperexcitability that may be attributed, in part, to alterations in both presynaptic GABA release mechanisms and postsynaptic GABA_A receptor function demonstrating a novel role for cannabinoid-dependent presynaptic control of neuronal transmission.

Keywords

cannabinoid withdrawal; hyperexcitability; GABA_A; CB1; receptor downregulation; WIN 55212-2

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Introduction

Cannabinoid type 1 (CB1) receptors play a critical role in modulating neuronal excitability and synaptic plasticity (Deshpande, et al., 2007, Monory, et al., 2006, Wallace, et al., 2003). The $G_{i/o}$ coupled CB1 receptor is the most abundant G-protein coupled receptor in the central nervous system, localized at both inhibitory and excitatory terminals and regulates a multitude of neurophysiological processes owing to its widespread expression throughout the brain with particularly high expression in the hippocampus (Tsou, et al., 1998). Studies have shown that postsynaptic neuronal depolarization results in “on demand” synthesis and release of endocannabinoids that activates presynaptic CB1 receptors in a retrograde fashion and modulate neurotransmitter release [reviewed in: (Kano, et al., 2009)]. Dependent on what class of terminals the CB1 receptor is present, this regulatory mechanism is termed depolarization-induced suppression of inhibition (DSI- when GABA is involved) or excitation (DSE- when glutamate is involved) (Katona and Freund, 2008). Thus, the CB1 receptor plays an important role in regulation of both inhibitory and excitatory synaptic transmission and has become an attractive therapeutic target in various pathophysiological conditions including seizures. Previous studies have demonstrated increased endocannabinoid release following seizures (Wallace, et al., 2003), CB1 receptor dependent anticonvulsant activity (Blair, et al., 2006, Deshpande, et al., 2007, Wallace, et al., 2002, Wallace, et al., 2001) and pro-convulsant effects following CB1 receptor antagonism (Deshpande, et al., 2007, Wallace, et al., 2003) in models of epilepsy.

There is a growing interest in targeting components of the endocannabinoid system for potential therapeutic use (Blair, et al., 2009, Howlett, et al., 2002, Mackie, 2006), which along with marijuana’s recreational use makes it important to identify molecular adaptations this system undergoes following prolonged use and withdrawal. Repeated exposure to CB1 agonists results in the development of tolerance to their predominantly inhibitory physiological effects [reviewed in: (Abood and Martin, 1992)] and upon withdrawal, irritability, restlessness, anxiety (Hasin, et al., 2008) and in some cases seizures (Wade, et al., 2006) have been observed. Recently, we reported that prolonged exposure of epileptic neuronal cultures to varying concentrations (10-1000 nM) of the CB1 receptor agonist WIN 55212-2 produced tolerance to its anticonvulsant effect as indicated by a concentration-dependent increase in epileptiform seizure discharge frequency and decrease/downregulation of CB1 receptor expression (Blair, et al., 2009). However, mechanisms underlying the increase in neuronal excitability following CB1 receptor downregulation are not fully understood.

To address this issue, we pharmacologically-induced CB1 receptor downregulation in hippocampal neuronal cultures and employed electrophysiological and immunochemical techniques to investigate its effects on GABAergic synaptic transmission. Previous findings from our laboratory demonstrate that in our hippocampal neuronal culture preparation, a large percentage ($\approx 60\%$) of the CB1 receptor colocalizes at VGAT+ (GABAergic) terminals, almost twice that ($\approx 36\%$) colocalized at VGLUT1+ (glutamatergic) terminals (Blair, et al., 2009). Thus, we chose to initially evaluate GABAergic synaptic function following pharmacologically-induced CB1 receptor downregulation in hippocampal neuronal cultures. This model provides an ideal preparation to study these synaptic events in a controlled environment. Our results indicate that immediately following withdrawal of the cannabimimetic WIN 55,212-2 (+WIN) after a prolonged exposure (24-h), a massive neuronal hyperexcitability ensues. Additionally, CB1 receptor downregulation was accompanied by an increased GABA release from presynaptic terminals as indicated by increased frequency for miniature inhibitory post synaptic current events (mIPSC). Furthermore, we observed a diminished GABAergic synaptic inhibition as indicated by a reduction in mIPSC amplitude and also a reduction in GABA_A channel number without an

effect on number of GABAergic synapses. This study demonstrates that the neuronal hyperexcitability observed following agonist-induced downregulation of the CB1 receptor may result from alterations in both presynaptic GABA release and postsynaptic GABA_A receptor function thus demonstrating a novel role of cannabinoid-dependent presynaptic control of neuronal transmission.

Materials and methods

All the drugs and reagents were purchased from Sigma Chemical Co (St. Louis, MO) unless otherwise noted. Sodium pyruvate, minimum essential media containing Earle's salts (MEM), fetal bovine serum and horse serum were obtained from Gibco-BRL (Invitrogen Corp., Carlsbad, CA). Stocks (1 mM) of S(-)-[2,3-Dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate (-WIN) and R(+)-[2,3-Dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate (+WIN) were made up in dimethyl sulfoxide (DMSO) that were then diluted at a minimum of 1:1000 to a final working concentration in the physiological bath recording solution (pBRS; see composition below).

Hippocampal neuronal cultures

All animal use procedures were in strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by Virginia Commonwealth University's Institutional Animal Care and Use Committee. Studies were conducted on primary mixed hippocampal neuronal cultures prepared as described previously with slight modifications (Blair, et al., 2009). Briefly, hippocampal cells were prepared from 2-day postnatal Sprague-Dawley rats (Harlan, Frederick, MD) and plated at a density of 2.0×10^4 cells/cm² onto a glial support layer previously plated onto poly-L-lysine (0.05 mg/ml) coated Lab-Tek two-well cover glass chambers or 35 mm cell culture dishes (Nunc, Naperville, IL). Cultures were maintained at 37°C in a 5% CO₂/95% air atmosphere and fed twice weekly with MEM enriched with N3 supplement containing 25 mM HEPES buffer (pH 7.4), 2 mM L-glutamine, 3 mM glucose, 100 mg/ml transferrin, 5 mg/ml insulin, 100 mM putrescine, 3 nM sodium selenite, 200 nM progesterone, 1 mM sodium pyruvate, 0.1% ovalbumin, 0.2 ng/ml triiodothyroxine, 0.4 ng/ml corticosterone and supplemented with a glial bed-condition media (20%).

Experimental protocol for cannabinoid treatment

Hippocampal neuronal cultures (DIV 14-16) were exposed to either the active enantiomer (+WIN, 1 μ M) or inactive enantiomer (-WIN, 1 μ M) or vehicle control (0.01% DMSO) in the maintenance medium for 24-h. At the end of this period, culture plates were washed with pBRS and immediately utilized for electrophysiology or immunocytochemistry experiments described below. Thus, WIN treatment refers to a prolonged (24-h) exposure to cannabinamimetic (1 μ M) and its subsequent removal (withdrawal). WIN was not present in the recording medium when experiments were conducted.

Electrophysiology

Whole cell current clamp recordings were performed using previously established procedures (Blair, et al., 2009). Briefly, a cell culture dish was mounted on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan) continuously perfused (1 ml/min) with pBRS containing (in mM): 145 NaCl, 2.5 KCl, 10 HEPES, 2 CaCl₂, 10 glucose, and 0.002 glycine, pH adjusted to 7.3 with NaOH, and osmolarity adjusted to 325 ± 5 mOsm with sucrose. Patch electrodes were pulled on a Brown-Flaming P-80C electrode puller (Sutter Instruments, Novato, CA), fire-polished and had a resistance of 3 to 5 M Ω when filled with

a solution containing (in mM): 140 K⁺ gluconate, 1.1 EGTA, 1 MgCl₂, and 10 Na-HEPES, pH 7.2, osmolarity adjusted to 290 ± 10 mOsm with sucrose. Neurons were recorded for up to 60 min.

Whole-cell voltage clamp configuration was employed to obtain mIPSCs. Patch electrodes were filled with an internal solution containing (in mM): 153.3 CsCl, 1.0 MgCl₂, 10.0 HEPES, 5.0 EGTA, with a pH of 7.30 (with sterile filtered CsOH) and osmolarity of 280-295 mOsm. The external solution composition was the same as described above in the current-clamp section. For mIPSC recordings, glutamate receptor-mediated synaptic currents were blocked by adding 50 μM D(-)-2-amino-5-phosphonopivalic acid (D-APV) and 20 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX) to the external solution. Action potentials were blocked by adding 1 μM tetrodotoxin (TTX) to the external solution. Voltage-clamp recordings were performed at a holding potential of -60 mV. Five-minute epochs of synaptic activity were recorded. Recording was terminated if the series resistance exceeded 20 MΩ. Signals were amplified using a Multiclamp 700B amplifier (Molecular Devices, Foster City, CA), low-pass filtered at 2 kHz, sampled at 10 kHz and recorded for off-line analysis using the Pclamp software (ver 10) using a Digidata 1440A (Molecular Devices, Foster City, CA).

Analyses of mIPSC

Off-line analyses of mIPSC were performed using MiniAnalysis software ver 6.0.7 (Synaptosoft, Decatur, GA). Offline sorting of the mIPSCs for further analysis used the following criteria: 1) the threshold for mIPSC detection was set at three times the root mean square of baseline noise, 2) to minimize the analysis of events that are dendritically filtered, all mIPSCs analyzed had a rise time of < 2 ms and half-width > 4 ms, 3) to attempt to minimize cases of inadequate space clamp, neurons in which series resistance increased >20% were discarded (Brünig, et al., 2001, Goodkin, et al., 2005, Rajasekaran, et al., 2007). In addition, event amplitudes were plotted against rise times and examined for a possible correlation. A significant correlation ($r^2 > 0.5$) was assumed to signify inadequate space clamp and neurons in which this occurred (<0.2%) were discarded (Cohen, et al., 2000). The accuracy of detection was visually confirmed. The amplitude of all mIPSCs greater than the detection criteria was included in the amplitude analysis. Because the distribution of mIPSC amplitude is skewed, median mIPSC amplitude was calculated for each neuron, and the mean of the medians is reported (Edwards, et al., 1990). The 10-90% rise time, the time interval between the last data point with a value of 10% and the first data point with a value of 90% of the peak amplitude, was determined for all selected events during the recording period. The decay phase was calculated by fitting individual mIPSCs with a 10-90% rise time <2 ms to a two-exponential function characterized by two time constants (τ_1 and τ_2) and accepted if $r^2 > 0.70$ (Goodkin, et al., 2005). For each neuron, the decay phase was based on at least 25 current traces selected randomly that met these criteria. Weighted decay (τ_w) was calculated using the formula:

$$\tau_w = [(\tau_1 \times A_1) + (\tau_2 \times A_2)] / [A_1 + A_2]$$

where τ_1 and τ_2 represent the fast and slow decay times and A_1 and A_2 represent the amplitude of the fast and slow components, respectively. Decay phase, 10-90% rise time, and frequency values are reported as mean ± SEM. Frequencies were compared between groups using the nonparametric Kolmogorov-Smirnov (K-S) test, and mIPSC amplitudes and their kinetics were compared with an unpaired t-test.

Peak scaled nonstationary noise analysis (NSNA) of mIPSCs (De Koninck and Mody, 1994, Otis, et al., 1994, Traynelis, et al., 1993) was conducted using the Mini analysis software

functionality by averaging 50 events to form a single mean ensemble mIPSC time course for a given neuron. The ensemble average was scaled up or down to the size of each original trace and then subtracted. Subtraction of the average ensemble current left a noise trace which fluctuated around the zero current level. The variance (σ^2) of the current was calculated for 20 amplitude bins. Then the values of σ^2 were plotted against the mean current (I) and data plotted in this manner were fitted by a parabolic curve with the equation:

$$\sigma^2 = iI - I^2 / NP_0$$

where i is the unitary current and N is the number of open channels activated during the mIPSC. The single-channel conductance (γ) was derived by dividing i by the driving force for GABA_A-mediated currents, determined from the Goldman-Hodgkin-Katz equation to be -60 mV ($E_{\text{holding}} - E_{\text{GABA}}$) in our solutions. The basal recording noise was subtracted prior to conducting NSNA (Cohen, et al., 2000, Hartman, et al., 2006, Kilman, et al., 2002).

Immunocytochemistry

Hippocampal cultures treated with WIN (1 μ M, 24 h, followed by washout) were evaluated immunocytochemically for CB1 receptor staining in association with staining for either the vesicular GABA transporter (VGAT) marker for inhibitory terminals or the GABA_A- $\beta_{2/3}$ receptor subunit using previously established procedures (Blair, et al., 2009, Blair, et al., 2004). Colocalization analysis for the CB1 receptor at VGAT positive inhibitory terminals was carried out using a rabbit antiserum to the C-terminal tail of CB1 (generous gift of Dr. Maurice Elphick) (Egertová and Elphick, 2000) followed by staining with rabbit antiserum to the vesicular GABA transporter (VGAT: 2 μ g/ml in SBBT, 16h 4°C; Millipore, Billerica, MA). Staining with the two rabbit primary antibodies was carried out using conditions to block any false host species cross-reactivity utilizing methodology previously published from our laboratory (Blair, et al., 2009). Briefly, fixed cultures (4% PF, 10 min) were blocked and permeabilized in SuperBlock® blocking buffer (Pierce, Rockford IL) containing 0.2% Triton X-100 for 60 min at room temperature, followed by a 3 h incubation with rabbit antiserum to the C-terminal tail of CB1 (1:5000) in SuperBlock® blocking buffer containing 0.1% Triton X-100 (SBBT). Labeled cultures were washed and incubated with a monovalent Fab fragment secondary antibody (biotin-SP-AffiniPure Fab fragment goat-anti-rabbit IgG; 1:100 in SBBT, 1h). Following wash, cultures were stained for CB1 with FITC-streptavidin (5 μ g/ml in SBBT, 1h). Stained cultures were then incubated in biotin (0.05% in PBST, 1h) to saturate all free sites on the FITC-streptavidin complex. Following wash, CB1 stained cultures were then incubated in the second primary antibody (rabbit anti-VGAT; 2 μ g/ml in SBBT, 16h 4°C), washed and incubated in biotin-SP-AffiniPure goat-anti-rabbit IgG (1:100 in SBBT, 1h). Following wash, labeled cultures were incubated in Texas red-streptavidin (5 μ g/ml in SBBT, 1h). All biotin conjugated secondary antibodies and streptavidin conjugates were purchased from Jackson ImmunoResearch (West Grove, PA). Appropriate no primary antibody controls were carried out to confirm no cross-reactivity between first and second rabbit antisera. For double-immunofluorescent staining of surface CB1 and GABA_A receptors, viable neuronal cultures were brought to 4°C in ice-cold pBRS and then incubated with rabbit antisera against the N-terminus CB1 receptor (1:1000; generously donated by Dr. Ken Mackie) (Tsou, et al., 1998) in combination with mouse antisera against the GABA_A- $\beta_{2/3}$ receptor subunits (20 μ g/ml, clone BD-17; Millipore, Billerica, MA) in Superblock for 90 min at 4°C. Following wash in ice-cold pBRS, labeled cultures were then fixed (4% PF, 10 min), washed in PBS and then incubated with Alexa Fluor® 488 (anti-rabbit) and 594 (anti-mouse) conjugated secondary antibodies (Invitrogen Corp., Carlsbad, CA). All stained cultures were covered with ProLong® Gold antifade reagent (Invitrogen) and cover slipped.

Confocal Microscopy

Immunofluorescent stained cultures were evaluated using a Leica TCS-SP2 confocal laser scanning microscope with a 63X/1.4 n.a. oil objective in sequential scan mode acquisition (Leica Microsystems Inc., Bannockburn, IL). Analysis of CB1 and VGAT colocalization was carried out on 16-bit gray scale confocal scans from each channel using ImageJ (NIH, public domain; colocalization threshold plug-in: authors Tony Collins and Wayne Rasband) to calculate Mander's colocalization coefficient (above threshold) for each channel (tM1: CB1; tM2: VGAT) and Pearson's correlation coefficient (above threshold) following automatic image background correction and threshold determination. Intensity analysis of CB1 and GABA_A- $\beta_{2/3}$ receptor staining was carried out on eight consecutive 16-bit gray scale confocal z-scans from each field evaluated. Confocal scan images underwent threshold adjustment and were then analyzed for whole field integrated density limited to threshold using ImageJ. Values for integrated density from each field evaluated were averaged from the eight consecutive confocal z-scans and a minimum of three fields per sample were analyzed.

Western blot analysis

Following experimental treatment, hippocampal neuronal cultures were rinsed with ice cold PBS (2 $\times$ 2.0 ml wash/35 mm plate) and then scraped into ice cold lysis buffer containing 50 mM Tris-HCL (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40 and a cocktail of protease inhibitors containing 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatinA, E-64, bestatin, leupeptin, and aprotinin (Sigma-Aldrich, St. Louis, MO). Three 35 mm neuronal culture plates were pooled into 300 μ L of lysis buffer per sample to acquire a sufficient concentration of protein for immunoblot analysis. Samples were vortexed and underwent freeze-thaw prior to resolving on SDS-PAGE. A maximum amount of sample was prepared into LDS sample buffer containing a reducing agent (NuPAGE®, Invitrogen, Carlsbad, CA) and heated at 70°C for 10 minutes. Fifteen microliters of each sample were resolved on a 4-12% Bis-Tris 15-well mini gel (NuPAGE®, Invitrogen). In addition, dual color Precision Plus Protein™ standards (Biorad, Hercules, CA) were used for molecular weight determination. Resolved samples were then transferred onto PVDF membrane using NuPAGE® transfer buffer containing 10% methanol. Following transfer, immunoblots were briefly washed in PBS containing 0.05% Tween-20 (PBST) and blocked overnight in PBST containing 3% blotting-grade blocker (Biorad). Immunoblots were then incubated in PBST with blocker containing either a rabbit antiserum to the C-terminal tail of CB1 (1:1000, 3 hours; generous gift of Dr. Maurice Elphick) (Egertová & Elphick 2000) or a mouse antisera against the GABA_A- $\beta_{2/3}$ receptor subunits (2 μ g/ml, 16h at 4°C; clone BD-17; Millipore, Billerica, MA). For protein loading control, immunoblots were incubated with mouse antisera against β -Actin (1:4000; clone AC-15, Sigma-Aldrich, St. Louis, MO). Following wash in PBST, immunoblots were incubated with PBST with blocker containing either goat-anti-rabbit-IgG-HRP or goat-anti-mouse-IgG-HRP (1:2000, 1h; Santa Cruz Biotech., Inc., Santa Cruz, CA), washed in PBST and incubated in chemiluminescent substrate (SuperSignal® West Pico; Thermo Scientific, Rockford, IL). Stained immunoblots were exposed to imaging film (Kodak X-OMAT™ Blue XB; Carestream Health Inc., Rochester, NY). Developed films were digitized using a high dynamic range transparency scanner (Epson Expression 1680; Epson U.S., Long Beach, CA). Digitized images were analyzed using ImageJ software to determine integrated density that was within threshold above background for each protein band. Values were normalized against their respective β -Actin integrated densities to correct for protein loading.

Data analysis

Data were analyzed using SigmaStat 2.0 and graphs were generated using SigmaPlot analysis software 11 (Systat Software Inc., San Jose, CA). All data are represented as mean

± SEM. Immunocytochemical intensity analysis is presented as a percent of control. A value of $p < 0.05$ was considered statistically significant. Image processing for representative figures was carried out with Adobe Photoshop software (CS3, Adobe Systems Inc., San Jose, CA).

Results

Prolonged +WIN treatment causes neuronal hyperexcitability

Neuronal activity in 14-day old cultured hippocampal neurons was assessed using the whole-cell current clamp configuration. Recordings from -WIN (control) treated neurons (1 μ M, 24-h, followed by washout) showed occasional spontaneous action potentials ($n = 10$, Fig. 1A). However, recordings obtained from +WIN treated neurons (1 μ M, 24-h, followed by washout) demonstrated high frequency spiking approaching 3 Hz in some neurons ($n = 10$, Fig. 1B). This continuous spike discharge activity was sustained throughout the recording period. No significant differences in resting membrane potential or input resistance were observed between the -WIN and +WIN treated neurons. -WIN treated neurons exhibited a mean membrane potential of -63.2 ± 2.5 mV and a mean input resistance of 125.8 ± 8.2 M Ω , whereas the +WIN treated neurons demonstrated a mean membrane potential of -61.8 ± 2.1 mV and a mean input resistance of 120.9 ± 9.4 M Ω ($n = 7$, $p > 0.05$, t -test). Control untreated neurons displayed electrophysiological characteristics that were not significantly different from -WIN treated neurons (data not shown). Hence data from -WIN treated neurons has been used as a control for comparing effects of prolonged +WIN exposure.

Prolonged +WIN treatment results in decreased inhibitory synaptic transmission

In order to investigate the effect of +WIN withdrawal on inhibitory synaptic transmission, we recorded mIPSCs from -WIN and +WIN (1 μ M, 24-h, followed by washout) treated neurons. Neurons were voltage clamped at -60 mV and mIPSCs were recorded as an inward current (Fig. 2A) in an isotonic chloride environment in the presence of 1 μ M TTX, 50 μ M D-APV and 20 μ M DNQX (see: materials and methods), to isolate miniature inhibitory currents. The presence of these agents also terminated the high frequency spiking observed following prolonged +WIN treatment and the addition of bicuculline (20 μ M) fully abolished the miniature currents, confirming that these events **were mediated by GABA_A receptors** (data not shown).

There were distinct differences between mIPSCs recorded from +WIN treated neurons compared to -WIN control neurons. The +WIN treated neurons had smaller amplitude and higher frequency of mIPSCs compared to -WIN treated neurons (Fig. 2A). The average amplitude of mIPSCs decreased to 40% of -WIN values (Fig. 2B). Because the distribution of individual mIPSC amplitudes tends to be skewed by larger-amplitude mIPSCs, the median value is reported since it is more representative of the distribution (Edwards, et al., 1990). The amplitude distribution histograms depicted a leftward shift towards smaller amplitude values in +WIN treated neurons (Fig. 2C). A population mean of the median values for the -WIN neurons was 66.6 ± 4.6 pA, while that for the +WIN treated neurons was 40.8 ± 3.2 pA ($n = 6$, $p < 0.01$, t -test).

In contrast to a decrease in amplitude, prolonged +WIN treatment resulted in a small but significant increase in the frequency of mIPSCs. In +WIN treated neurons, mIPSCs occurred at a frequency of 0.36 ± 0.06 Hz, which was significantly higher than mIPSC frequency in -WIN treated neurons of 0.18 ± 0.05 Hz ($n = 10$, $p < 0.01$, K-S test). The cumulative distribution graph for frequency indicates a slight leftward shift towards higher frequency values and decreased inter-event interval following prolonged +WIN treatment (Fig. 2D).

Analyses of mIPSC kinetics

We next compared the decay phases of mIPSCs. Compared to the –WIN treated neurons, the decay phase of mIPSCs was prolonged in +WIN treated neurons. The population means for both +WIN and –WIN groups was obtained by averaging the mean decay constants for each neuron recorded. The population means of mIPSCs decay constants recorded from the –WIN treated neurons were $\tau_1 = 21.77 \pm 2.71$ and $\tau_2 = 31.13 \pm 8.25$, and those for the +WIN treated neurons were $\tau_1 = 29.58 \pm 5.26$ and $\tau_2 = 90.69 \pm 10.5$. While the faster component τ_1 remained unchanged, the τ_2 component was significantly delayed in +WIN treated neurons ($n = 6$, $p = 0.02$, t -test). In addition, the mean weighted decay (τ_w) was also significantly slower in +WIN treated neurons ($\tau_w = 50.09 \pm 3.47$) compared to –WIN treated neurons ($\tau_w = 26.15 \pm 3.88$) ($n = 5$, $p < 0.01$, t -test).

The mean rise time in –WIN treated neuron (4.31 ± 0.17 ms) was not significantly different from the rise time in +WIN treated neuron (4.19 ± 0.24 ms) ($n = 6$, $p = 0.8$, t -test). The pooled rise times were binned at 1 ms intervals into three subpopulations: <3 ms, $3\text{--}5$ ms, and >5 ms. This analysis showed no significant shift in the proportion of events in each subpopulation of rise times for mIPSCs when comparing –WIN and +WIN treated neurons (Fig. 2E).

Charge transfer as represented by area of currents ($\text{pA} \times \text{ms}$) was then calculated as a measure of mIPSC inhibition. Total charge transfer was significantly smaller in +WIN treated neurons compared to –WIN treated neurons. The mean charge transfer for –WIN and +WIN treated neurons was 1295.15 ± 159.9 $\text{pA} \times \text{ms}$ and 850.97 ± 23.5 $\text{pA} \times \text{ms}$ respectively ($n = 5$, $p = 0.05$, t -test).

Taken together, our observation that mIPSC amplitude and charge transfer decreased significantly following +WIN treatment indicates a reduction in GABA mediated synaptic inhibition in these neurons. Additionally, the decreased mIPSC amplitude occurred without significant changes in the rise-times suggesting that the reduced inhibition was not related to loss of GABAergic synapses proximal to soma (also see immunohistochemistry data below).

Non stationary noise analysis indicates that average numbers of GABA_A channels but not their single-channel conductance is reduced from prolonged +WIN exposure

The reduction in mIPSC amplitude could be attributable to a decrease in the single-channel conductance (γ) of GABA_A receptors or to a reduction in the number of open channels (N) or the open probability of an activated channel (P_o). We performed nonstationary noise analysis on the mIPSC currents (De Koninck and Mody, 1994, Otis, et al., 1994, Traynelis, et al., 1993) to distinguish between these possibilities. For each neuron, individual mIPSCs were peak scaled to the average mIPSC amplitude (Fig. 3A and B) to give the mean versus variance curve that were fitted by a parabolic function (Fig. 3C and D, also see materials and methods). The mean single channel conductance (γ) from the +WIN group was 35.96 ± 1.5 pS, which was not significantly different from the γ in –WIN treated neurons (39.18 ± 1.6 pS) ($n = 5$, t -test, Fig. 3E). These findings for mean single channel conductance are in agreement to other previously reported values of 30–40 pS derived from noise analysis and single-channel recordings (Otis, et al., 1994, Traynelis, et al., 1993).

The peak amplitude is a function of both single channel conductance γ and number of activated channels NP_o . Since γ didn't decrease in +WIN treated neurons, we surmised that N should be affected. Indeed, the number of activated channels decreased significantly in +WIN treated neurons compared to –WIN treated neurons ($N = 19.04 \pm 2.25$ Vs. $N = 34.18 \pm 3.51$ respectively, $n = 6$, $p < 0.01$, t -test, Fig. 3F). These data indicate that the number of activated GABA_A channels at the peak of mIPSC amplitude are reduced in +WIN treated

neurons. This reduced inhibition could underlie the hyperexcitability observed in these neurons.

Prolonged +WIN exposure results in downregulation of the CB1 receptor at inhibitory GABAergic terminals

To evaluate the effect of +WIN treatment (1 μ M, 24-h, followed by washout) on CB1 receptor expression at GABAergic inhibitory terminals, colocalization analysis of hippocampal cultures double-labeled with two rabbit antibodies against the CB1 receptor and the vesicular GABA transporter (VGAT) was carried out using previously published methods to block any false host species cross-reactivity (Blair, et al., 2009). Control hippocampal cultures treated with –WIN showed distinct punctate CB1 receptor staining (green) **throughout the processes, some of which** also stained positive for VGAT (red) (Fig. 4). Prolonged exposure to +WIN resulted in a dramatic downregulation of CB1 receptor levels, while no effect on VGAT expression was observed (Fig 4). Additionally, intensity analysis of confocal scans revealed no significant change in VGAT expression between –WIN ($100 \pm 14.2\%$) and +WIN-treated ($110.9 \pm 4.5\%$) cultures ($n = 4$, $p = 0.493$, t -test). Overlay images for CB1 (green) and VGAT (red) demonstrate a high degree of colocalization (yellow) in the –WIN treated cultures while a near total loss in colocalization was observed in +WIN treated cultures (Fig 4, bottom panels). Additionally, the presence of exclusive CB1 (green) staining in the overlay image of the –WIN treated cultures (bottom left panel) indicates correct staining conditions for blocking any false host species cross-reactivity between the two rabbit antibodies. Colocalization analysis of confocal scans of double antibody stains from –WIN treated cultures indicate that staining for CB1 receptor and VGAT colocalize on inhibitory terminals with a Pearson's correlation coefficient of 0.825 ± 0.019 and Mander's overlap coefficients of $tM1 = 0.576 \pm 0.067$ and $tM2 = 0.484 \pm 0.031$ for CB1 and VGAT respectively ($n = 4$). This is in agreement with previous findings of CB1/VGAT colocalization in this culture preparation (Blair, et al., 2009). Analysis of confocal scans of double antibody stains from +WIN treated cultures indicated the absence of colocalization between CB1 and VGAT. Thus, prolonged exposure of hippocampal neuronal cultures to +WIN results in a dramatic downregulation of CB1 receptor expression on neuronal processes of which approximately 60% ($tM1 = 0.576 \pm 0.067$) are VGAT-positive inhibitory terminals.

Prolonged +WIN exposure decreases both CB1 and GABA_A receptor surface expression

To evaluate the effect that prolonged +WIN treatment (1 μ M, 24-h, followed by washout) has on surface expression for both CB1 and GABA_A receptors, immunocytochemical analysis was carried out by double-labeling live cells at 4° C with antibodies against the N-terminus of CB1 and the extracellular chain of the GABA_A $\beta_{2/3}$ receptor subunits. Following extensive washing in ice-cold pBRS, labeled cells were then fixed, stained immunofluorescently and analyzed by confocal microscopy. Surface CB1 receptor expression in –WIN treated hippocampal cultures demonstrated punctate staining (green) throughout the neuronal processes (Fig. 5A, top-left). Following prolonged treatment with +WIN, a dramatic loss of surface CB1 receptor staining was observed and was comparable to the loss of total cell receptor staining seen using the C-terminus specific antibody (Fig. 5A, **top-right**). Intensity analysis of surface CB1 receptor staining revealed a significant decrease in +WIN treated cultures of $87.7 \pm 1.52\%$ ($p < 0.001$, $n = 6$) in integrated density when compared to –WIN treated cultures (Fig. 5B).

Surface GABA_A $\beta_{2/3}$ receptor subunit staining was utilized as an indicator for overall surface levels of the GABA_A receptor since the majority of hippocampal receptors contain at least one of these two β isotypes (Wisden, et al., 1992). In –WIN treated hippocampal cultures, staining for the GABA_A $\beta_{2/3}$ receptor subunits (red) was demonstrated as intense

staining across the cell membrane visualized as distinct punctate staining representative of clusters of GABA_A receptors containing the $\beta_{2/3}$ subunits (Fig. 5A, bottom-left) in agreement with previous findings in this culture preparation (Blair, et al., 2004). Following exposure to +WIN, a decrease in staining intensity for the GABA_A $\beta_{2/3}$ receptor subunits was observed (Fig. 5A, bottom-right). Intensity analysis of surface GABA_A $\beta_{2/3}$ receptor subunit staining revealed a significant decrease in +WIN treated cultures of $25.0 \pm 6.86\%$ ($p \leq 0.04$, $n = 6$) in integrated density when compared to -WIN treated cultures (Fig. 5C).

To further analyze the effect that prolonged +WIN exposure to hippocampal neuronal cultures has on levels of both CB1 and GABA_A receptor protein levels, quantitative immunoblot analysis of total cellular lysates from -WIN control and +WIN treated neuronal cultures was carried out. CB1 receptor staining of immunoblots of hippocampal neuronal culture total cell lysates revealed a prominent band with a molecular mass of ≈ 45 kDa in the -WIN control group (Fig. 6A, *lane 1*). These findings are in agreement with a previous study characterizing the antibody directed to the C-terminal tail of the CB1 receptor used in the present study (Egertová & Elphick 2000). Prolonged exposure to +WIN resulted in a total loss of CB1 receptor staining (Fig. 6A, *lane 2*). Quantitative analysis revealed that prolonged +WIN exposure resulted in a significant and total loss in staining for the CB1 receptor when compared to -WIN controls ($100 \pm 24.6\%$) ($p \leq 0.01$, $n = 3$, Fig. 6B).

In -WIN control treated neuronal cultures, immunoblot analysis of total cellular lysates for GABA_A $\beta_{2/3}$ receptor subunit revealed a single protein band with a molecular mass of ≈ 55 kDa (Fig. 6C, *lane 1*). Prolonged exposure to +WIN resulted in an observed decrease in staining for the GABA_A $\beta_{2/3}$ receptor subunit (Fig. 6C, *lane 2*) which reflected a significant reduction of $40.8 \pm 8.6\%$ ($p \leq 0.04$, $n = 3$, -WIN; $n = 5$, +WIN) from -WIN treated controls ($100 \pm 15.6\%$, Fig. 6D).

Discussion

The present study demonstrates that prolonged +WIN exposure reduces GABAergic synaptic function in cultured hippocampal neurons. Massive neuronal hyperexcitability was observed immediately upon removal of +WIN following a 24-h exposure. Prolonged +WIN exposure resulted in profound downregulation of CB1 receptors, which was accompanied by a modification in GABAergic synaptic transmission as evidenced by decreased mIPSC amplitude and a reduction in number of postsynaptic GABA_A receptor channel number. However, there were no changes in number of GABAergic synapses or VGAT positive terminals between -WIN and +WIN-treated cultures. The findings from this study are the first to indicate that chronic exposure of CB1 receptors to agonists can modulate excitability by affecting both changes in presynaptic neurotransmitter release and postsynaptic GABA_A receptor channel number.

Miniature inhibitory post synaptic potentials (mIPSPs) are GABA_A receptor mediated synaptic events in which each **mIPSP** represents the release of single quantum of neurotransmitter. The frequency of these events is thought to represent presynaptic release machinery whereas the amplitude is indicative of post-synaptic receptor function (Frerking, et al., 1997). It has been reported that acute applications of CB1 agonists suppress both miniature inhibitory and excitatory post synaptic currents (mIPSC and mEPSC) in hippocampus (Basavarajappa, et al., 2008, Hájos and Freund, 2002, Ohno-Shosaku, et al., 2002). In light of the considerable role of CB1 receptor-dependent modulation of both excitatory and inhibitory neurotransmission, it is important to evaluate how long term cannabinoid use may affect presynaptic CB1 receptor-dependent mechanisms and how the subsequent withdrawal may affect neuronal behavior and synaptic strength.

Our electrophysiological studies showed a 40% reduction in mIPSC amplitude following prolonged +WIN exposure. There are a number of possibilities that could underlie the change in mIPSC amplitude (Barberis, et al., 2004). It could result from a reduction in number of postsynaptic GABA_A receptors (Nusser, et al., 1998), or a reduced packaging of GABA into synaptic vesicles (Frerking, et al., 1995, Frerking, et al., 1997), or a change in subunit composition of the receptors that alters single-channel properties (Cherubini and Conti, 2001). Nonstationary fluctuation analysis indicated no significant change in GABA_A receptor single-channel conductance, nor were there any major changes in mIPSC kinetics that might indicate differences in subunit composition. These data suggest that GABA_A receptor channel properties were not affected following prolonged cannabinoid exposure. However, we did observe a significant reduction in the number of channels open at the peak of the mIPSC. There are limitations to the peak-scaled fluctuation analysis. It cannot determine whether a change in the number of open channels is attributable to a change in channel open probability or a change in GABA concentration in the synaptic cleft, or a reduction in the number of channels clustered at postsynaptic sites (De Koninck and Mody, 1994). Our immunocytochemical and immunoblot analysis of +WIN treated cultures revealed both a reduction in the intensity of staining for surface GABA_A- $\beta_{2/3}$ and a significant decrease in total cellular GABA_A- $\beta_{2/3}$ subunit expression in parallel with reduced mIPSC amplitude suggesting that the reduction in number of GABA_A receptor open channels is, at least in part, due to a reduction in the number of synaptically localized GABA_A receptors containing the $\beta_{2/3}$ subunit.

The vesicular GABA transporter (VGAT) is a marker for inhibitory terminals. Analysis of double immune staining for VGAT and CB1 in –WIN treated hippocampal cultures revealed a high degree of colocalization which agrees with earlier findings in this preparation (Blair, et al., 2009). We observed no change in the staining intensity of VGAT suggesting that GABA synthesizing infrastructure of inhibitory terminals remained intact following prolonged +WIN treatment. However, it did affect the level of expression of CB1 receptor as demonstrated by an 87% reduction in staining intensity and a loss in CB1 colocalizing with VGAT. Downregulation of the CB1 receptor would result in a loss of its regulatory control over presynaptic GABA release (Ferraro, et al., 2001, Kofalvi, et al., 2005). Prolonged exposure to +WIN resulted in a 2-fold increase in mIPSC frequency **compared to** –WIN treated cultures, indicating increased GABA release in association with CB1 receptor downregulation.

It has been established that repeated administration of cannabinoids results in development of tolerance to hypothermia, anti-nociception and hypolocomotion (Abood and Martin, 1992). Our previous findings have demonstrated that prolonged exposure to the CB1 agonist +WIN produces tolerance to its anticonvulsant effect in an in vitro preparation of epileptiform activity (Blair, et al., 2009). Studies in humans have shown that cannabis withdrawal following prolonged exposure is prevalent and is characterized by a number of clinically significant symptoms some of which include psychomotor retardation, anxiety, restlessness, irritability, depression, insomnia and in small percentage of abusers, seizures (Hasin, et al., 2008). Recently it was reported that a small subset of multiple sclerosis patients undergoing a long-term oral cannabis treatment regimen to control spasticity, reported seizures while on the drug (Wade, et al., 2006). Additionally, reports have suggested that marijuana use and/or withdrawal could potentially trigger seizures in epileptic patients (Gordon and Devinsky, 2001). Animal studies have shown that after a single exposure to THC, there was enhanced CNS excitability during the withdrawal phase, resulting in increased susceptibility to electrical-induced convulsions (Karler, et al., 1986, Karler and Turkanis, 1980). In agreement with these studies, we observed significant hyperexcitability in our neuronal preparation upon withdrawal of +WIN after a prolonged

exposure. However, the precise molecular mechanism underlying this withdrawal related neuronal hyperexcitability is unknown.

Recent studies have shown that the endocannabinoid anandamide is a tonic activator of CB1 receptors on GABAergic terminals in hippocampal slice cultures (Kim and Alger, 2010). Our research has also shown that endocannabinoids block status epilepticus-like activity in cultured hippocampal neurons (Deshpande, et al., 2007). It will be insightful to study the contribution of long-term presence of endocannabinoids (anandamide or 2-AG) by using selective inhibitors of endocannabinoid degradation/reuptake and comparing it to long-term exogenous cannabinoid administration on GABAergic neurotransmission. While it has been shown that CB1 receptors colocalize with both GABAergic and glutamatergic terminals, we have shown that CB1 receptor colocalization on GABAergic terminals predominates over CB1 receptor colocalization on glutamatergic terminals in cultured hippocampal neurons (Blair, et al., 2009). Hence we chose to initially study the GABAergic system in this paper. However, several elegant studies have demonstrated an essential role of CB1-dependent regulation of glutamatergic transmission in models of neuronal hyperexcitability (Monory, et al., 2006, Marsicano, et al., 2003). Thus, future studies to evaluate the effect of agonist-induced downregulation of CB1 on glutamatergic transmission in our model system are warranted.

It has been demonstrated that the presynaptic CB1 receptor functions to regulate synaptic transmission through suppression of neurotransmitter release (Ferraro, et al., 2001, Katona and Freund, 2008, Kofalvi, et al., 2005). We hypothesize that downregulation of CB1 receptors following prolonged agonist exposure as observed in this study and reported earlier (Blair, et al., 2009, Coutts, et al., 2001) would remove this modulatory effect and cause an increased neurotransmitter release (de Fonseca, et al., 1991, Hoffman, et al., 2007), in this case GABA. Increased synaptic availability of GABA would result in a prolonged occupancy of postsynaptic GABA_A receptors, which in turn may cause a maladaptive response reflected by decreased receptor density at the cell surface (Alicke and Schwartz-Bloom, 1995, Barnes, 1996, Tehrani and Barnes, 1991). In agreement with this hypothesis, our electrophysiological and immunocytochemical studies show that prolonged +WIN exposure significantly reduced GABA_A receptor channel number. Together these effects could underlie the neuronal hyperexcitability immediately following cannabinoid withdrawal observed in this study. Indeed other studies have shown that prolonged neuronal firing following status epilepticus (Blair, et al., 2004, Goodkin, et al., 2005) or extended activity deprivation (Hartman, et al., 2006, Kilman, et al., 2002) in cultured neurons is associated with decreased GABA_A receptor number and function. Our study demonstrates a novel role of cannabinoid-dependent presynaptic control of neuronal transmission in that CB1 receptor downregulation following prolonged agonist exposure is associated with a reduction in postsynaptic GABAergic function.

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Abbreviations

CB1

Cannabinoid type 1

+WIN/ –WIN	WIN 55212-2/ 3
mIPSC	miniature inhibitory postsynaptic current
VGAT	vesicular GABA transporter

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Figure 1. Neuronal hyperexcitability following prolonged +WIN exposure in cultured hippocampal neurons

A. Representative recording from a **WIN 55212-3** (–WIN, control) treated neuron displaying intrinsic baseline activity consisting of occasional spontaneous action potentials.

B. Whole cell current clamp recording from a neuron displaying high frequency spiking upon removal of **WIN 55212-2** (+WIN, 1μM, 24-h). This data was representative of 10 experiments.

Figure 2. Diminished GABAergic transmission following prolonged +WIN exposure

A. Representative mIPSCs from $-WIN$ (control) and $+WIN$ ($1\ \mu M$, 24-h) treated neurons recorded as inward currents from a holding potential of $-60\ mV$. **B.** Averaged mIPSC traces from a $-WIN$ and $+WIN$ ($1\ \mu M$, 24-h) treated neuron. Removal of $+WIN$ produced neuronal hyperexcitability as shown in Fig. 1B, which was suppressed by $1\ \mu M$ TTX, $50\ \mu M$ D-APV and $20\ \mu M$ DNQX cocktail used for recording mIPSCs. **C.** Representative cumulative probability plot of amplitudes reveals significant leftward shifts toward smaller amplitudes in $+WIN$ ($1\ \mu M$, 24-h) neurons. **D.** Representative cumulative probability plot of frequencies reveals significant rightward shifts toward increased frequency in prolonged $+WIN$ treated neurons. **E.** Rise time distribution of mIPSCs from prolonged $-WIN$ and $+WIN$ treated neurons when binned at 1-ms intervals showing no significant difference ($n=6$, $p>0.05$) in the proportion of events in different rise time subpopulations.

Figure 3. Nonstationary noise analysis suggested a decrease in the number of open GABA_A receptor channels per quantum of transmitter released after a prolonged +WIN exposure
A, B. 20 consecutive mIPSCs were scaled to average and superimposed (blue), with average trace (red) and variance (green) shown from -WIN (control) and +WIN (1 μ M, 24-h) treated neurons respectively. **C, D.** Plot of variance in mIPSC about the mean current (with baseline variance subtracted) for prolonged -WIN and +WIN treated neurons respectively. Line shows parabolic fit of the relationship. **E, F.** Estimates for average single channel conductance (γ) was not significantly different ($n=5$, $p>0.05$), but the number of open channels (N) was significantly reduced ($n=6$, $p<0.01$) in prolonged +WIN neurons compared to -WIN neurons.

Figure 4. Loss of CB1 receptor expression at GABAergic (VGAT +) terminals after a prolonged +WIN exposure

Double immuno-fluorescent staining to evaluate colocalization of CB1 (*green*) with VGAT (GABAergic) (*red*) positive terminals in HNCs. In prolonged –WIN (control) treated cultures, staining revealed punctate colocalization of CB1 receptor with VGAT as indicated by *yellow* in the merged channel images (*white arrowheads*). Following prolonged +WIN exposure, downregulation of CB1 receptor resulted in loss of colocalization with VGAT at GABAergic terminals (scale= 20 microns).

Figure 5. Decrease in surface GABA_A-β_{2/3} receptor subunit and CB1 receptor expression following prolonged +WIN exposure

Following experimental manipulation, viable cultures were membrane-surfaced labeled with antisera against CB1 and GABA_A-β_{2/3} receptor subunits at 4°C. Cultures were fixed and further processed for double-immunofluorescent staining. **A.** Representative images of CB1/ GABA_A-β_{2/3} staining from prolonged -WIN (control) and +WIN treated cultures. Surface staining for CB1 (*green*) using antisera against the N-terminus of the receptor demonstrated punctate staining throughout the neuronal processes in -WIN cultures, while downregulation of surface CB1 with near total loss of staining was evident following +WIN treatment. Staining for surface GABA_A receptors (*red*) using antisera against the β_{2/3} subunits revealed a distinct staining pattern throughout the somato-dendritic region and neuronal processes in -WIN cultures. Prolonged exposure to +WIN resulted in a decrease in staining intensity for GABA_A-β_{2/3} throughout all neuronal cellular regions. **B.** Intensity analysis of CB1 staining demonstrated a pronounced and significant decrease from -WIN in surface receptor expression following prolonged exposure to +WIN ($p < 0.001$, $n = 6$). **C.** Intensity analysis of surface GABA_A-β_{2/3} receptor subunit staining following prolonged +WIN exposure revealed a significant decrease ($p \leq 0.04$, $n = 6$) (scale= 20 microns).

Figure 6. Western blot analyses of total cell lysates from hippocampal neuronal cultures following prolonged exposure to either –WIN control (lane 1) or +WIN (lane 2) using antibodies against CB1 and the GABA_A-β_{2/3} receptor subunits

Protein samples were resolved on SDS-PAGE and transferred to PVDF membrane for antibody staining using the ECL technique. **A.** Representative staining for CB1 revealed a prominent band with molecular masses of ≈45 kDa. Prolonged exposure to +WIN (lane 2) resulted in a total loss of immunostaining of the CB1 receptor 45 kDa band as compared to the –WIN control (lane 1). **B.** Quantitative analyses of the 45 kDa CB1 band demonstrated that prolonged +WIN exposure resulted in a total loss of the 45 kDa CB1 band which was significantly decreased from control –WIN (**p<0.01, n= 3; –WIN = 100±24.6%). **C.** Representative staining with the antisera against the GABA_A-β_{2/3} receptor subunits revealed a single band with a molecular mass of ≈55 kDa. Compared to the –WIN control (lane 1), prolonged exposure of hippocampal neuronal cultures to +WIN (lane 2) resulted in decreased immunostaining for the GABA_A-β_{2/3} receptor subunits. **D.** Quantitative analyses revealed a significant decrease in GABA_A-β_{2/3} receptor subunit staining with +WIN exposure when compared to control (* p<0.04; n= 3, –WIN = 100±15.6%; n=5, +WIN = 59.2±8.6%; results pooled from two separate experiments). For quantitative analyses of Western blots, all experimental values were normalized against their respective β-actin levels to correct for protein sample loading and expressed as a percent of control (–WIN) ±SEM.