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Activation of the CB1 receptor mediates the anticonvulsant properties of cannabinoids in the hippocampal neuronal culture models of acquired epilepsy and status epilepticus

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Running Title**:** CB1 receptor mediates anticonvulsant effects of cannabinoids

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Abbreviations:

AE: acquired epilepsy
AMPA: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CB1: cannabinoid type-1 receptor
EPSPs: excitatory post-synaptic potentials
HNC: hippocampal neuronal culture
IPSPs: inhibitory post-synaptic potentials
KA: kainic acid
MES: maximal electroshock
NMDA: *N*-methyl-*D*-aspartate
PDSs: paroxysmal depolarization shifts
pBRS: physiological bath recording solution
SE: status epilepticus
SR141716A: N-(piperidin-1-yl-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-
        methyl-1H-pyrazole-3-carboxamidehydrochloride
SREDs: spontaneous recurrent epileptiform discharges.
THC: Δ^9-tetrahydrocannabinol
WCC: Whole-cell current-clamp
WIN55,212-2: *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolol[1,2,3
        de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone
WIN55,212-3: *S*(-)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolol[1,2,3
        de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone

Section: Neuropharmacology

ABSTRACT

Cannabinoids have been shown to have anticonvulsant properties, but no studies have evaluated the effects of cannabinoids in the hippocampal neuronal culture models of acquired epilepsy (AE) and status epilepticus (SE). This study investigated the anticonvulsant properties of the cannabinoid receptor agonist WIN 55,212-2 in primary hippocampal neuronal culture models of both AE and SE. WIN 55,212-2 produced dose-dependent anticonvulsant effects against both spontaneous recurrent epileptiform discharges (SREDs) (EC$_{50}$ = 0.85 µM) and SE (EC$_{50}$ = 1.51 µM), with total suppression of seizure activity at 3 µM and of SE activity at 5 µM. The anticonvulsant properties of WIN 55,212-2 in these preparations were both stereospecific and blocked by the CB1 receptor antagonist, SR141716A (1 µM), demonstrating a CB1 receptor-dependent pathway. The inhibitory effect of WIN 55,212-2 against low-Mg^{2+}-induced SE is the first observation in this model of total suppression of SE by a selective pharmacological agent. The clinically used anticonvulsants phenytoin and phenobarbital were not able to abolish low-Mg^{2+}-induced SE at concentrations up to 150 µM. The results from this study demonstrate CB1 receptor-mediated anticonvulsant effects of the cannabimimetic WIN 55,212-2 against both SREDs and low-Mg^{2+}-induced SE in primary hippocampal neuronal cultures and demonstrate that these *in vitro* models of AE and SE may represent powerful tools to investigate the molecular mechanisms mediating the effects of cannabinoids on neuronal excitability.

INTRODUCTION

Since the isolation and purification of the psychotropically active constituent Δ^9-tetrahydrocannabinol (THC) from *Cannabis* in the 1960s (Reviewed in: Mechoulam, 2000), a number of studies have demonstrated the anticonvulsant effects of cannabinoids in a variety of experimentally induced seizure models that include maximal electroshock induced convulsions, electrical kindling, chemoconvulsants, audiogenic and photogenic seizures (Corcoran et al., 1973; Karler et al., 1974; Wada et al., 1975; Consroe and Wolkin, 1977; Chiu et al., 1979; Wallace et al., 2001; Wallace et al., 2002; Shafaroodi et al., 2004). In addition, several reports have been published on the clinical use of cannabinoids as antiepileptics in man (reviewed in: Consroe, 1998). Thus, it is important to elucidate the molecular mechanisms mediating the anticonvulsant effects of cannabinoids**.**

A major advance in the field of cannabinoid research came with the discovery and cloning of receptor proteins that bind cannabinoids with high affinity (Reviewed in: Mechoulam, 2000). Within the central nervous system (CNS), the $G_{i/o}$-protein coupled cannabinoid type-1 (CB1) receptor is widely distributed and is the primary mediator of the physiological and psychotopic effects of cannabinoids in the brain (Devane et al., 1988; Matsuda et al., 1990; Herkenham et al., 1991; Howlett, 1995). In recent years, a better understanding of the endocannabinoid system has led to the development of highly specific synthetic compounds, which have been instrumental in the pharmacological evaluation of cannabinoid receptor-mediated regulation of synaptic transmission

(Howlett et al., 2004). Utilizing the maximal electroshock (MES) induced seizure model, studies from this laboratory provided the first evidence that both cannabinoid and endocannabinoid compounds produced anticonvulsant effects through activation of the CB1 receptor (Wallace et al., 2001; Wallace et al., 2002). Additionally, the active cannabimimetic compound WIN,55,212-2 was shown to totally suppress spontaneous recurrent epileptiform discharges (SREDs, seizures) via CB1 receptor activation in the rat pilocarpine model of acquired epilepsy (AE) (Wallace et al., 2003). This study further demonstrated that endocannabinoids, acting through the CB1 receptor, were essential for maintaining tonic inhibition of seizure frequency and duration in this *in vivo* model of AE (Wallace et al., 2003). Although these *in vivo* models of acute seizure and AE are useful for studying the anticonvulsant effects of CB1 receptor activation on intact systems (Wallace et al., 2001; Wallace et al., 2002; Wallace et al., 2003), they are limited in their ability to carry out sophisticated molecular techniques needed to study underlying cellular mechanisms. Thus, it is important to utilize well-established *in vitro* neuronal preparations that are more amenable to sophisticated electrophysiological and molecular biological procedures to evaluate the cellular mechanisms underlying the anticonvulsant properties of cannabinoids. The hippocampal neuronal culture (HNC) model of AE is a well established model that exhibits SREDs for the life of the neurons in culture (Sombati and DeLorenzo, 1995) and has been shown by our laboratory and others to manifest many of the electrophysiological and molecular properties of intact animal models of AE (Kim and Rhim, 2004; Delorenzo et al., 2005). It is

also important to evaluate the anticonvulsant effects of cannabinoids against continuous seizure activity, status epilepticus (SE), a major neurological emergency that is often resistant to conventional anticonvulsant treatments (Delorenzo et al., 2005). The well-established HNC model of SE (Sombati and DeLorenzo, 1995) has been widely used to evaluate the effects of SE on neuronal cell physiology and molecular changes (Pal et al., 1999; Blair et al., 2004; Mangan and Kapur, 2004; Delorenzo et al., 2005). Thus, the HNC models of AE and SE may serve as valuable tools for elucidating the cellular mechanisms underlying the anticonvulsant properties of cannabinoids, since these *in vitro* models are amenable to experimental manipulation and allow for direct analysis of neurons undergoing SREDs and SE in culture (Sombati and DeLorenzo, 1995; Churn et al., 2000; Blair et al., 2004; Delorenzo et al., 2005).

In the current study, we set out to investigate the effects of the cannabimimetic WIN55,212-2 on seizure activity in the *in vitro* HNC models of AE and SE (Sombati and DeLorenzo, 1995). Whole-cell current-clamp analysis was utilized to directly evaluate the effect of WIN55,212-2 on SREDs and SE, and to determine if a CB1 receptor-dependent pathway was involved. The results demonstrate that WIN55,212-2 was effective in terminating SREDs and SE in a stereoselective manner. In addition, these anticonvulsant effects of WIN55,212-2 were mediated through activation of the CB1 receptor. This study demonstrates that the HNC models of AE and SE provide powerful tools to further elucidate the cellular mechanisms underlying the effect of cannabinoids on seizure activity and neuronal excitability.

METHODS

Materials: WIN55,212-2 (*R*(+)-[2,3-dihydro-5-methyl-3-

[(morpholinyl)methyl]pyrrolol[1,2,3 de]-1,4-benzoxazinyl]-(1-

naphthalenyl)methanone), WIN55,212-3 (*S*(-)-[2,3-dihydro-5-methyl-3-

[(morpholinyl)methyl]pyrrolol[1,2,3 de]-1,4-benzoxazinyl]-(1-

naphthalenyl)methanone), phenytoin (5,5-Diphenyl-2,4-imidazolidinedione) and

phenobarbital (5-Ethyl-5-phenyl-2,4,6-trioxohexahydropyrimidine) were

purchased from Sigma Chemical (St. Louis, MO). SR141716A (N-(piperidin-1-yl-

5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-

carboxamidehydrochloride) was supplied through the NIDA Chemical Synthesis

and Drug Supply Program. Stocks of WIN55,212-3, WIN55,212-2 and

SR141716A were made up in a vehicle stock solution of absolute ethanol,

Emulphor-620 (Rhone-Poulenc, Inc., Princeton, NJ) and 0.9% saline at a ratio of

1:1:18 that was then diluted at a minimum of 1:500 to a final working

concentration in the physiological bath recording solution.

Hippocampal neuronal culture: Primary mixed hippocampal cultures were

prepared as described previously by our laboratory with slight modifications

(Sombati and DeLorenzo, 1995). Briefly, hippocampal cells were prepared from

2-day postnatal Sprague-Dawley rats (Harlan, Frederick, MD) and plated at a

density of 2.5 x 10^4 cells/cm^2 onto a glial support layer previously plated onto

poly-L-lysine (0.05 mg/ml) coated 35 mm grid cell culture dishes (Nunc,

Naperville, IL). Cultures were maintained at 37°C in a 5% CO_2/95% air

atmosphere and fed twice weekly with NeuroBasal-A Medium supplemented with

B-27 (Invitrogen Corp., San Diego, CA) containing 0.5 mM L-glutamine. Unless

otherwise noted, reagents were purchased from Sigma-Aldrich Co. (St. Louis,

MO).

Induction of SE and SREDs by Low-Mg^{2+} treatment of hippocampal

neuronal cultures: After two weeks, cultures were utilized for experimentation.

Maintenance media was replaced with physiological bath recording solution

(pBRS) with or without MgCl$_2$ containing (in mM): 145 NaCl, 2.5 KCl, 10 HEPES,

2 CaCl$_2$, 10 glucose, 0.002 glycine, pH 7.3, and osmolarity adjusted to 325mOsm

with sucrose. Thus, low-Mg^{2+} treatment was carried out with pBRS without

added MgCl$_2$, while sham controls were treated with pBRS containing 1 mM

MgCl$_2$. Unless indicated as low-Mg^{2+} treatment, experimental protocols in this

study utilized pBRS containing 1 mM MgCl$_2$.

HNC Model of SE: Continuous epileptiform high-frequency bursts (SE) were

induced by exposing neuronal cultures to pBRS without added MgCl$_2$ (low-Mg^{2+}).

The SE continued until pBRS containing 1 mM MgCl$_2$ was added back to the

cultures. This represents the HNC model of SE used in this study that has been

well characterized as an *in vitro* model of SE manifesting essentially identical

electrographic feature of SE observed with *in vivo* animal models and in human

SE (Sombati and DeLorenzo, 1995; Mangan and Kapur, 2004; Delorenzo et al.,

2005). Thus, the *in vitro* HNC model of SE is well suited to study the effects of

cannabinoids on SE.

HNC Model of AE: A 3 h exposure of SE was employed to induce AE with

SREDs in the HNC model using established procedures (Sombati and

DeLorenzo, 1995). In this model of AE it has been shown that the brief 3 h

exposure and injury from SE produces SREDs for the life of the neurons in

culture (Sombati and DeLorenzo, 1995; DeLorenzo et al., 1998; Kim and Rhim,

2004).   We used neurons 1 d after exposure to SE in this study. The 1 d after

SE time point represents the chronic phase of AE in this model where the

neurons manifest SREDs with the same frequency as neurons sampled from

cultures at days 3, 5 and 7 after SE injury. Thus, the HNC model of AE is ideally

suited for the pharmacological studies on the effects of cannabinoids on SREDs.

Briefly, after removal of maintenance media, cells were washed gently with 3 x

1.5 ml of pBRS ($\pm$1 mM $MgCl_2$) and then allowed to incubate in this solution at

37°C under 5% CO_2 /95% air. For the HNC model of AE, at the end of treatment

with either sham control (1 mM $MgCl_2$) or low Mg^{2+} (without added $MgCl_2$)

conditions for 3 hours, cultures were restored to the physiological concentration

(1mM) of $MgCl_2$ by washing gently with 3 x 1.5 ml of MEM at 37°C, returned to

maintenance feed and incubated at 37°C under 5% CO_2 /95% air. The neurons

exposed to this 3 h treatment with low Mg^{2+} pBRS manifested SREDs for the life

of the neurons in culture.

Whole-Cell Current-Clamp recordings in low-Mg^{2+} treated hippocampal

neuronal cultures: Whole-cell current-clamp (WCC) recordings were performed

using previously established procedures in our laboratory (Sombati and

DeLorenzo, 1995; Blair et al., 2004). Briefly, cell culture media was replaced with

pBRS, mounted on the stage of an inverted microscope (Nikon Diaphot, Japan),

continuously perfused with pBRS and then studied using the WCC recording

procedure. Patch electrodes with a resistance of 2-4 MΩ were pulled on a

Brown-Flaming P-80C electrode puller (Sutter Instruments, Novato, CA), fire

polished and filled with a solution containing (in mM) 140 K^+gluconate, 1 $MgCl_2$

and 10 Na-HEPES, pH 7.2, osmolarity adjusted to 310 ± 5 mOsm with sucrose.

Data was digitized and transferred to videotape using a PCM device

(Neurocorder, New York, NY) and then played back on a DC-500 Hz chart

recorder (Astro-Med Dash II, Warwick, RI).   Intracellular recordings were carried

out using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) in

whole-cell current-clamp mode.

Data Analysis: For concentration response analysis, suppression of SREDs

and SE was determined as a percent decrease in frequency over increasing

concentrations of WIN55,212-2. Analysis of SREDs frequency for each recorded

neuron was carried out over 60 min and determined by counting individual

epileptiform events that had discreet onset and termination and consisted of

multiple individual paroxysmal depolarization shifts (PDSs). SE frequency was

determined by counting individual epileptiform bursts over a recording duration of

5 min for each neuron analyzed. For both SREDs and SE, WCC frequency

analysis was carried out on multiple hippocampal cultured neurons at each

concentration of WIN55,212-2. Mean frequencies at each concentration of

WIN55,212-2 were then represented as a percent inhibition from control

frequency (SREDs or SE frequency in the absence of WIN55,212-2). Least

squares linear regression analysis was used to calculate the EC_{50} (effective

concentration that produced 50% of maximal effect) of WIN55,212-2 for

suppression of both SREDs and SE burst discharges. Data was plotted using

SigmaPlot analysis software 8.02 (SPSS Inc., Chicago, Ill).

RESULTS

The anticonvulsant effects of WIN55,212-2 are stereospecific in the HNC model of AE

To evaluate the anticonvulsant activity of the optically active cannabimimetic compound WIN55,212-2 against AE, this study employed the *in vitro* HNC model of AE, previously developed in our laboratory (Sombati and DeLorenzo, 1995). This *in vitro* model of AE has been established as a model of AE with many of the electrographic and biochemical features of *in vivo* models of AE (Sombati and DeLorenzo, 1995; DeLorenzo et al., 1998; Kim and Rhim, 2004). To induce AE in cultured neurons, two-week-old hippocampal neuronal cultures were exposed to Mg^{2+}-free media for three hours and then returned to a maintenance media containing Mg^{2+}. Hippocampal neurons develop SREDs for the life of the culture following this treatment (Sombati and DeLorenzo, 1995). Whole-cell current-clamp recordings of age matched control neurons revealed "normal" baseline recordings, displaying spontaneously occurring action potentials and not manifesting SREDs (Fig. 1A). One day following 3 hr of low-Mg^{2+} treatment, WCC recordings of a neuron demonstrated an "epileptic" phenotype as evident by the presence of SREDs or "seizure" events (Fig 1B). Expansion of one of the SREDs (Fig 1B:a) revealed the presence of individual paroxysmal depolarization shifts (PDSs), a pathophysiological characteristic similar to those observed in epilepsy (Lothman et al., 1991). The SREDs were comprised of multiple PDSs and occurred intermittently for the life of the neurons in culture (Sombati and DeLorenzo, 1995).

WIN55,212 inhibited SREDs stereoselectively in the HNC model of AE. WIN55,212-2 (3 µM), the active enantiomer at the CB1 receptor, fully inhibited the expression of SREDs (Fig 2C) in comparison to the non-treated "epileptic" cultures (Fig 2A). Conversely, the inactive stereoisomer WIN55,212-3 (3 µM) had no effect on the expression or frequency of SREDs in "epileptic" cultures (Fig 2B). These results demonstrate that only the active enantiomer of the cannabimimetic WIN55,212-2 had anticonvulsant effects.

We carried out a concentration-response analysis of inhibition of SREDs with WIN55,212-2 and found that it inhibited SREDs in a concentration-dependent manner with an $EC_{50} = 0.85$ µM as determined by least square linear regression analysis (Fig 3A). The anticonvulsant effects of WIN55,212-2 were observed with concentrations as low as 500 nM and were maximal at 3 µM (Fig 3A). The total inhibition of SREDs by WIN55,212-2 in a concentration dependent and stereospecific manner indicates that this cannabimimetic compound is functioning as a full CB1 receptor agonist.

The anticonvulsant effects of WIN55,212-2 are mediated by the CB1 receptor in the HNC model of AE

Since CB1 receptor antagonists have been shown to be effective in demonstrating the role of CB1 receptor activation in mediating anticonvulsant effects (Wallace et al., 2001; Wallace et al., 2002; Wallace et al., 2003), it is important to determine if the anticonvulsant effects of WIN55,212-2 in the HNC model of AE, described above, are also mediated by CB1 receptor activation. To evaluate the role of CB1 receptor activation, we employed the CB1 receptor

antagonist SR141617A to evaluate the effect of CB1 receptor blockade on the

ability of WIN55,212-2 to inhibit SREDs in the HNC model of AE. SR141617A (1

µM) totally blocked the ability of WIN55,212-2 to suppress SREDs in the HNC

model of AE (Fig 2D). Thus, both the stereospecificity of WIN55,212-2 to fully

inhibit SREDs and the antagonism of this effect by SR141617A confirm that this

anticonvulsant effect of WIN55,212-2 occurs via a CB1 receptor mediated

mechanism*.*

**The anticonvulsant effects of WIN55,212-2 against SE are stereospecific in
the HNC model of SE**

The anticonvulsant properties of the cannabimimetic WIN55,212-2 were

further evaluated in the *in vitro* HNC model of electrographic SE to determine its

effectiveness in suppressing continuous epileptiform high-frequency bursts

induced by low-Mg^{2+} treatment.   The HNC model of SE shares many of the

same electrophysiological and biochemical changes observed in the *in vivo*

models of SE and in human SE (Sombati and DeLorenzo, 1995; Mangan and

Kapur, 2004; Delorenzo et al., 2005). The continuous epileptiform activity in this

HNC model of SE is characterized by 3.0-20 Hz high-frequency epileptiform

bursts during the low-Mg^{2+} exposure and has been shown to be dependent on

interneuronal networking (Sombati and DeLorenzo, 1995). WCC recordings from

age-matched control neurons showed baseline activity with the occasional

spontaneous action potentials (Fig 4A). Removal of Mg^{2+} (low-Mg^{2+}) from the

recording solution resulted in continuous tonic high-frequency epileptiform bursts

(Fig 4B). This hyper excitable state consisted of repetitive individual burst-

discharges (Fig. 4B:a) of which each burst is comprised of multiple spikes that

overlay a depolarization shift (Fig. 4B:b and c).

WIN55,212-2 inhibited the continuous tonic high-frequency epileptiform

bursts stereospecificaly in the HNC model of SE. Addition of the active

enantiomer WIN55,212-2 (5 µM) during the low-Mg^{2+} treatment totally inhibited

expression of high-frequency epileptiform bursts (Fig 5C) when compared to low-

Mg^{2+} treatment alone (Fig 5A). The inactive stereoisomer WIN55,212-3 (5 µM)

had no effect on low-Mg^{2+} induced SE (Fig 5B).

To evaluate the effectiveness of WIN 55,212-2 at suppressing SE,

inhibition of burst frequency was determined over a concentration-response

curve (see Methods: Data Analysis). WIN55,212-2 inhibited low-Mg^{2+}-induced

SE in a concentration-dependent manner with an EC_{50} = 1.51 µM, as determined

by least square linear regression analysis (Methods). Suppression of SE was

observed at concentrations of WIN55,212-2 ranging from partial inhibition at 500

nM to full inhibition at 5 µM, demonstrating that it is functioning as a full agonist

(Fig 3B).

The anticonvulsant affects of WIN55,212-2 on SE are mediated by the CB1

receptor

To determine if WIN55,212-2 is anticonvulsant via a CB1-receptor

dependent mechanism, analysis was carried out in the presence of the CB1

receptor antagonist SR141716A. The total suppression of low-Mg^{2+}-induced SE

by WIN55,212-2 (5 µM) was blocked by addition of SR141716A (1 µM) (Fig. 5D).

Thus, the anticonvulsant properties of WIN55,212-2 with low-Mg^{2+}-induced SE in

hippocampal cultures were both stereospecific and blocked by SR141716A,

demonstrating a CB1 receptor-dependent mechanism of SE suppression.

Comparison of the cannabinoid anticonvulsant effects against SE with

other anticonvulsant agents

Both of the antiepileptic agents phenobarbital and phenytoin, used at very

high concentrations up to 150 µM, had no effect at reducing low-Mg^{2+}-induced

high-frequency epileptiform bursts in the HNC model of SE (Fig. 6). Thus, while

agents known to be effective anticonvulsants had no effects on SE expression in

this preparation, the cannabimimetic compound WIN55,212-2 (5 µM) fully

suppressed low-Mg^{2+}-induced SE in a stereospecific manner (Fig. 5). This is the

first evidence of a selective pharmacological agent to fully suppress high-

frequency epileptiform bursts activity in the HNC model of SE.

DISCUSSION

In the current study, the synthetic cannabinoid WIN55,212-2 was anticonvulsant in a stereospecific and concentration-dependent manner against SREDs and acute low-Mg^{2+}-induced high-frequency epileptiform bursts (SE) in the HNC models of AE and SE (Sombati and DeLorenzo, 1995). The anticonvulsant properties of WIN55,212-2 for SE and SREDs were both stereospecific and blocked by the CB1 receptor antagonist SR141716A (1 μM), demonstrating CB1 receptor-dependent mechanisms. The antiepileptic effect of WIN55,212-2, demonstrating total suppression of SREDs at 3 μM, was as efficacious as the clinically established agents phenytoin (10 μM) and phenobarbital (100 μM) previously characterized in this HNC model of AE (Sombati and DeLorenzo, 1995). WIN55,212-2 (5 μM) produced a total suppression of high-frequency epileptiform bursts (SE) during low-Mg^{2+} treatment. Previous studies have demonstrated that SE in this model was not significantly blocked by inhibiting NMDA, AMPA, KA and metabotropic glutamate receptors or L-type Ca^{2+} channels (DeLorenzo et al., 1998). Furthermore, total suppression of SE in this model was not produced by the clinically established anticonvulsants, phenobarbital or phenytoin, even at very high concentrations of these anticonvulsants up to 150 μM. SE is often refractory to treatment and the ability of cannabinoids to abolish SE discharges in this model is an important observation that may have clinical implications for considering the use of cannabinoids in the treatment of refractory SE. In addition, the demonstration that cannabinoids are effective in totally preventing SREDs and SE in these HNC

in vitro models offers a major advance over *in vivo* models in the ability to

investigate the molecular mechanisms involved in the CB1 receptor-dependent

regulation of seizure frequency and duration. It is hoped that the use of these

models to study the effects of cannabinoids on seizure activity and SE *in vitro* will

lead to new advances in understanding the molecular mechanisms regulating the

actions of cannabinoids in modulating neuronal excitability.

Since the discovery and cloning of the CB1 receptor, a number of

synthetic compounds have been developed allowing for the study of this

receptor's role in modulating seizure activity. Earlier studies from this laboratory

have demonstrated that the cannabimimetic compound WIN55,212-2 had CB1

receptor-dependent anticonvulsant activity in both the MES model of acute

seizures and in the rat pilocarpine model of AE (Wallace et al., 2001; Wallace et

al., 2003). Furthermore, work from our laboratory demonstrated that blockade of

CB1 receptor function with the selective antagonist SR141716A reduced seizure

threshold in the MES model and increased epileptic seizure frequency and

duration in the pilocarpine model of temporal lobe epilepsy (Wallace et al., 2002;

Wallace et al., 2003). These earlier studies suggested a role for the

endocannabinoid system in regulating neuronal seizure discharge in a CB1

receptor-dependent manner. Other studies have demonstrated a protective role

of CB1 receptor activation in different models of neuronal excitotoxicity which

include glutamate receptor-dependent induced neuronal cell death (Abood et al.,

2001), kainic acid induced seizures in conditional mutant mice null for CB1

receptor on principle forebrain neurons (Marsicano et al., 2003), low Mg^{2+}-

induced epileptiform activity in hippocampal slice (Ameri and Simmet, 2000) and

repetitive Ca^{2+} spiking-dependent neuronal death in hippocampal cultures (Shen

and Thayer, 1998). The current findings that the anticonvulsant effects of

cannabinoids in the HNC models of AE and SE are mediated by CB1 receptor

activation underscore the potential utilization of these *in vitro* models as powerful

tools for future studies on the molecular mechanisms underlying CB1 receptor-

mediated regulation of seizure activity.

To date, two endogenous ligands for the CB1 receptor in brain have been

isolated and termed arachidonylethanolamine (AEA) and 2-arachidonylglycerol

(2-AG) (Devane et al., 1992; Mechoulam et al., 1995), and the highest amount of

AEA has been found in the hippocampus (Felder et al., 1996). These

endocannabinoids are synthesized "on demand" in response to neuronal

depolarisation and a subsequent increase in intracellular calcium (Stella et al.,

1997). The CB1 receptor is primarily localized presynaptically on nerve terminals

and, upon activation, has been shown to suppress neurotransmitter release.

Thus, cannabinoids can act to significantly modulate neuronal synaptic

transmission through their actions at the CB1 receptor.

Considerable research has explored the mechanisms mediating the

effects of endogenous CB1 receptor activation. Activation of presynaptic CB1

receptors result in decreased N and P/Q type voltage-gated Ca^{2+} currents and

increased type-A K^+ conductance via a direct interaction with the receptor-

coupled $G_{i/o}$ proteins (reviewed in: Howlett et al., 2004). A physiological outcome

of presynaptic CB1 receptor activation following postsynaptic depolarization-

induced synthesis and release of the endocannabinoids AEA or 2-AG, is the

attenuation of Ca^{2+}-dependent neurotransmitter release from nerve terminals.

This phenomenon has been termed either depolarization-induced suppression of

inhibition (DSI) or excitation (DSE), dependent on its action at GABAergic or

glutamatergic neuronal synaptic terminals respectively (Kreitzer and Regehr,

2001; Wilson et al., 2001). Although the presence of CB1 receptor dependent

DSI and DSE have been observed *in vitro*, studies have demonstrated that DSE

is less likely to take place under physiological conditions (Ohno-Shosaku et al.,

2002). In such a scenario, DSI would predominate and would be expected to

confer a proconvulsant effect of CB1 receptor activation. However, hippocampal

DSE has been shown to be induced by prolonged postsynaptic membrane

depolarization (Ohno-Shosaku et al., 2002), a characteristic event that occurs

during seizure discharge. Thus, the total suppression of both SE and SREDs by

WIN55,212-2 in the current study may result from increasing CB1 receptor-

dependent suppression of excitation, a mechanism that may predominate during

the seizure discharge in this hippocampal preparation. Such a hypothesis has

been recently posed in the literature (Hajos and Freund, 2002).

It has been suggested that the anticonvulsant effects of antiepileptic

agents result in the attenuation of hyperexcitability that results from excessive

glutamatergic transmission, a phenomenon that is a characteristic occurrence

with seizure discharge (Lothman et al., 1991). Utilizing a hippocampal neuronal

culture preparation, Thayer and colleagues observed a CB1 receptor-dependent

suppression of glutamatergic transmission as demonstrated by WIN 55,212-2

inhibition of both a low-Mg^{2+}-induced increase in intracellular Ca^{2+} concentration

spikes and the amplitude of presynaptically evoked EPSPs (Shen and Thayer,

1999), while having no effect on GABA-mediated IPSC amplitudes (Shen et al.,

1996). Additionally, in a hippocampal brain slice preparation, WIN 55,212-2

blocked stimulus-evoked field EPSPs in CA1 pyramidal neurons and decreased

low-Mg^{2+}/high K^+-induced epileptiform burst discharge frequency in hippocampal

CA3 neurons (Ameri and Simmet, 2000). In light of these studies, a plausible

mechanism for the anticonvulsant properties of WIN 55,212-2 in our model may

involve a CB1 receptor-dependent suppression of glutamatergic transmission.

The results of this study demonstrate for the first time that the

cannabimimetic compound WIN55,212-2 is anticonvulsant against SE and AE via

a CB1 receptor-dependent mechanism using the *in vitro* hippocampal neuronal

culture models of AE and SE. These *in vitro* hippocampal culture models of

electrographic seizure activity are well suited to carry out biochemical (Blair et al.,

2004; Delorenzo et al., 2005), electrophysiological (Sombati and DeLorenzo, 1995;

DeLorenzo et al., 1998) and molecular investigations (Churn et al., 2000). Future

research efforts employing the HNC models of AE and SE towards elucidating both

presynaptic and postsynaptic neuronal mechanisms involved in the anticonvulsant

effects of CB1 receptor activation may allow for the development of more

efficacious treatment strategies for epilepsy and SE.

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Footnotes

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FIGURE LEGENDS:

Fig. 1. Induction of SREDs following a 3 h exposure of hippocampal neuronal cultures to low-Mg^{2+}. Whole-cell current-clamp recordings were obtained from hippocampal pyramidal neurons before and 1 day after a 3 h exposure to Mg^{2+}-free solution. **A.** A recording from a representative control neuron displaying intermittent spontaneous action potentials that are consistently observed during basal activity in this hippocampal culture preparation. **B.** A continuous 45 min recording from a representative "epileptic" neuron 1 day following exposure to 3h of Mg^{2+}-free media. The pathophysiological state of SREDs in this *in vitro* preparation is evident by the presence of three independent spontaneous seizure episodes. The lower panel recording (**a**) is an expansion of the corresponding segment in the trace above (B) to discriminate the high-frequency burst discharges that overlie individual paroxysmal depolarisation shifts (PDSs), a pathophysiological characteristic observed in epilepsy, and the numerous spikes associated with each SRED.

Fig. 2. CB1-dependent suppression of SRED activity. **A.** Whole-cell current-clamp recordings were obtained from hippocampal pyramidal neurons displaying SRED activity 1 day following a 3 h exposure to Mg^{2+}-free solution. **B.** Addition of the inactive stereoisomer WIN 55,212-3 (-WIN)(3 µM) had no effect on the expression of SREDs in comparison to A. **C.** The active cannabimimetic WIN 55,212-2 (+WIN) (3 µM) fully inhibited the expression of SREDs in "epileptic" hippocampal cultures. This anticonvulsant effect was prolonged and was

observed as far out as 60 min following addition of WIN 55,212-2 (data not

shown). **D.** The anticonvulsant effect of WIN 55,212-2 (3 µM) in suppressing the

expression of SREDs was completely blocked by addition of the CB1 receptor

antagonist SR141716A (SR) (1 µM).

Fig. 3. Log concentration response curves for WIN 55,212-2 in the HNC models

of low-Mg^{2+}-induced AE and SE. **A.** Concentration-dependent inhibition of

SRED frequency by WIN 55,212-2 (EC_{50} = 0.85 µM as determined by least

square linear regression analysis, Methods). Frequency of individual SRED

events (see Fig. 1B) in the presence of varying concentrations of WIN 55,212-2

were measured over a 60 min period and then plotted as a percent change

(inhibition) from control (absence of WIN 55,212-2) SRED frequencies. **B.**

Concentration-dependent inhibition of high-frequency epileptiform burst (SE)

frequency by WIN 55,212-2 (EC_{50} = 1.51 µM as determined by least square

linear regression analysis, Methods). Frequency of epileptiform bursts (see Fig.

4B, b-c) in the presence of varying concentrations of WIN 55,212-2 were

measured over a 5 min period and then plotted as a percent change (inhibition)

from control (absence of WIN 55,212-2) SE frequencies. Each data point

represents percent change from control ± SEM (n = 3-7 neurons/concentration).

Fig. 4. Induction of tonic high-frequency epileptiform bursts (SE) in cultured

hippocampal neurons during exposure to low-Mg^{2+}. **A.** Representative

intracellular recording from a control neuron showing occasional spontaneous

action potentials. The resting potential of individual neurons ranged from 65 to

50 mV. **B.** Representative intracellular recordings during low-Mg^{2+} treatment

demonstrating continuous seizure activity (SE) with burst discharge frequencies

of 3-20 Hz. **(a)** Expansion of a segment from (B) shows the presence of

continuous burst discharges. **(b-c)** Further expansion of two segments from (a)

revealing the individual epileptiform bursts, each consisting of depolarization

shifts overlaid with multiple spike activity. Response data in Fig 3B was

determined by frequency analysis of epileptiform bursts.

Fig. 5. CB1 receptor-dependent suppression of low-Mg^{2+}-induced SE activity.

A. Exposure of hippocampal cultures to Mg^{2+}-free media (low-Mg^{2+}) induces

tonic high-frequency epileptiform bursts (SE). **B.** The presence of the inactive

isomer WIN 55,212-3 (-WIN) (5 µM) during exposure to low-Mg^{2+} had no effect

on high-frequency epileptiform burst activity. **C.** WIN 55,212-2 (+WIN) (5 µM),

the active cannabimimetic, totally suppressed low-Mg^{2+}-induced high-frequency

epileptiform bursts (SE). **D.** Addition of the CB1 receptor antagonist SR141716A

(1 µM) completely blocked the anticonvulsant effects of WIN 55,212-2 (5 µM)

against low-Mg^{2+}-induced SE.

Fig. 6. Evaluation of anticonvulsant agents on low-Mg^{2+}-induced SE activity in

hippocampal neuronal cultures. **A.** Following addition of the cannabimimetic

WIN 55,212-2 (+WIN) (5 µM), the low-Mg^{2+}-induced high-frequency epileptiform

burst activity was fully suppressed. Addition of the established anticonvulsant

drugs phenytoin (PHT) (50 µM) **(B)** or phenobarbital (PB) (50 µM) **(C)** during low-

Mg^{2+} treatment at high therapeutic concentrations had no inhibitory effect on SE

activity in this preparation. In addition, PHT and PB up to concentrations as high

as 150 µM were not able to abolish SE as shown in **(A)** for WIN 55,212-2 at 5
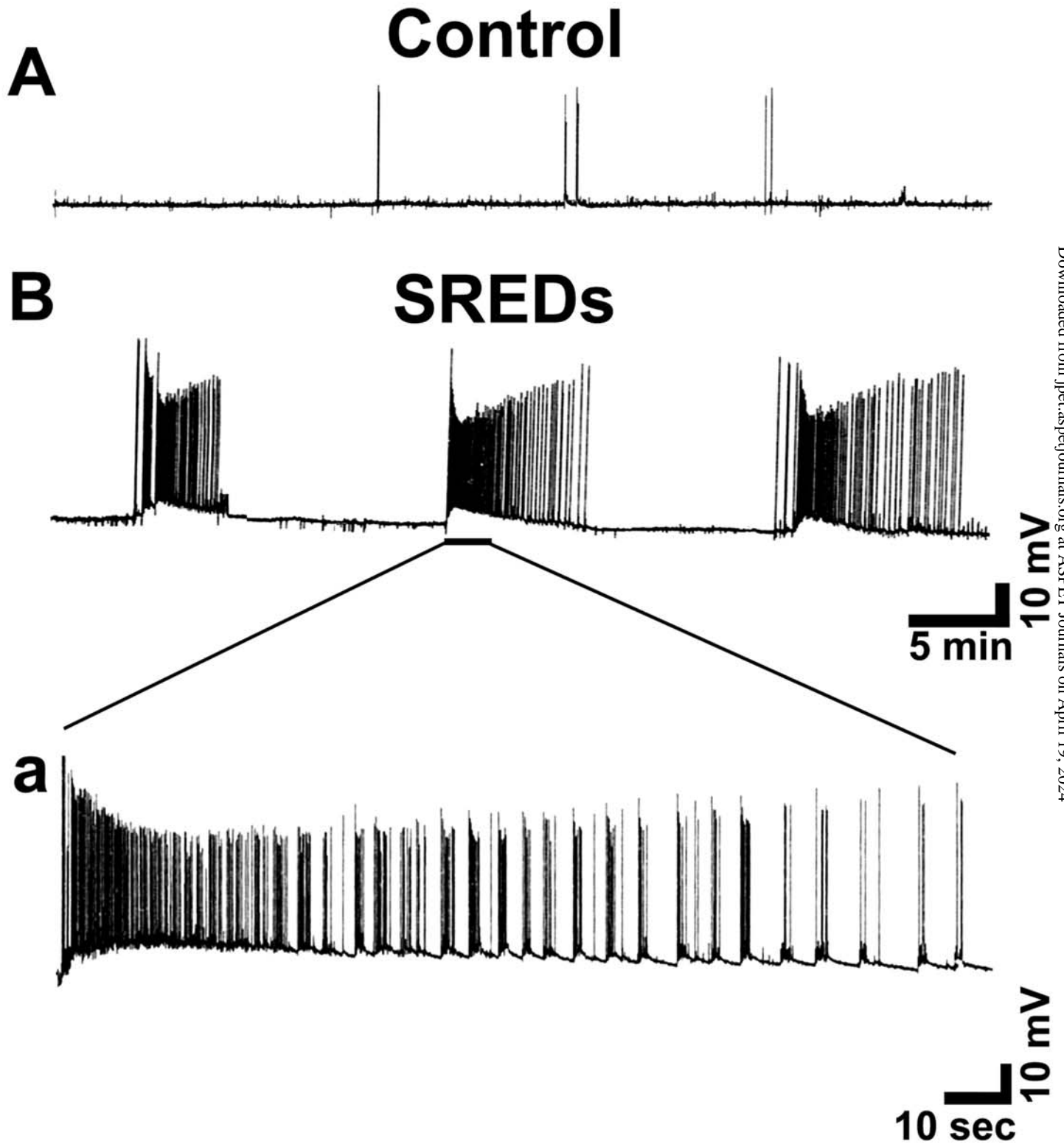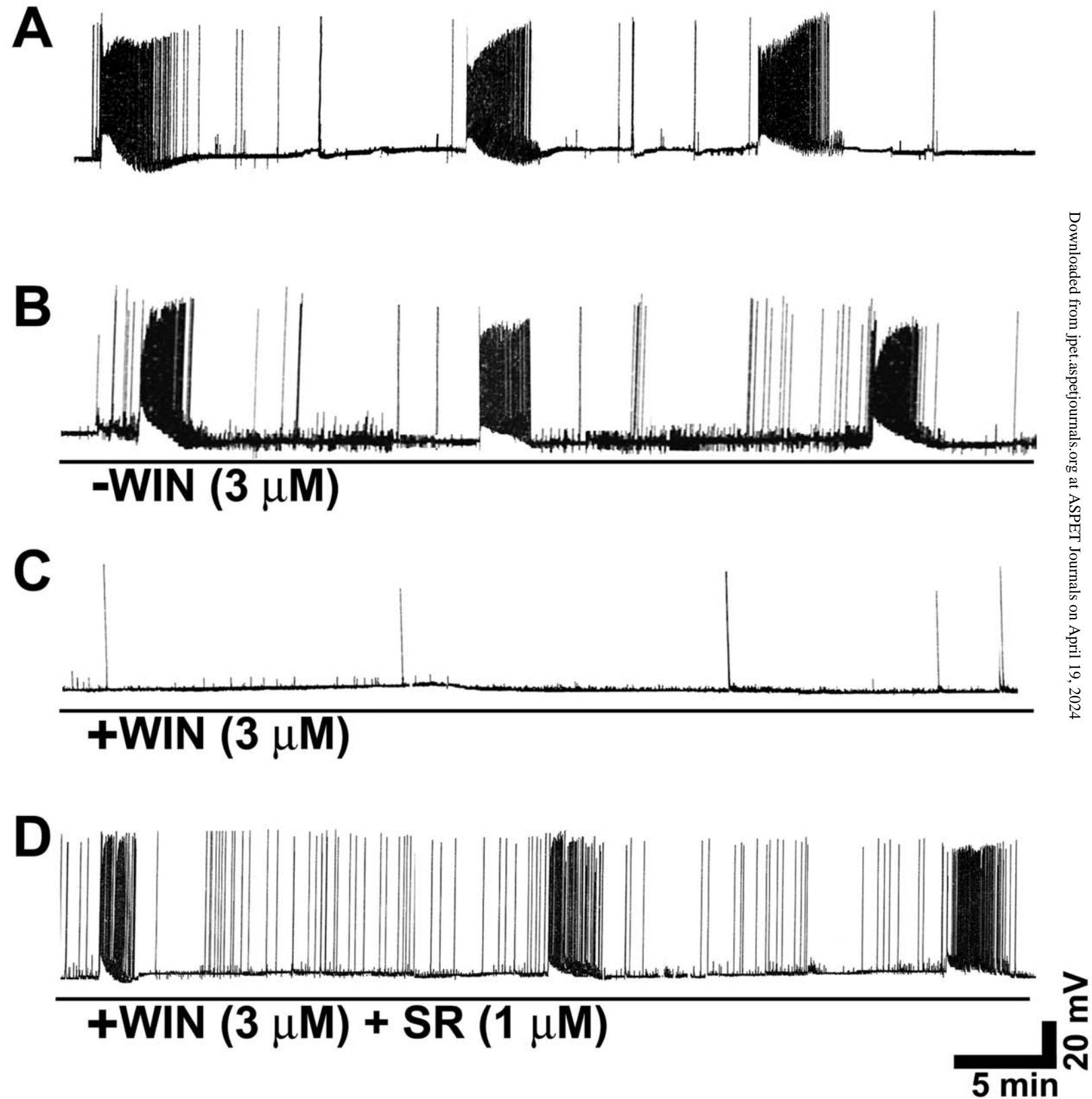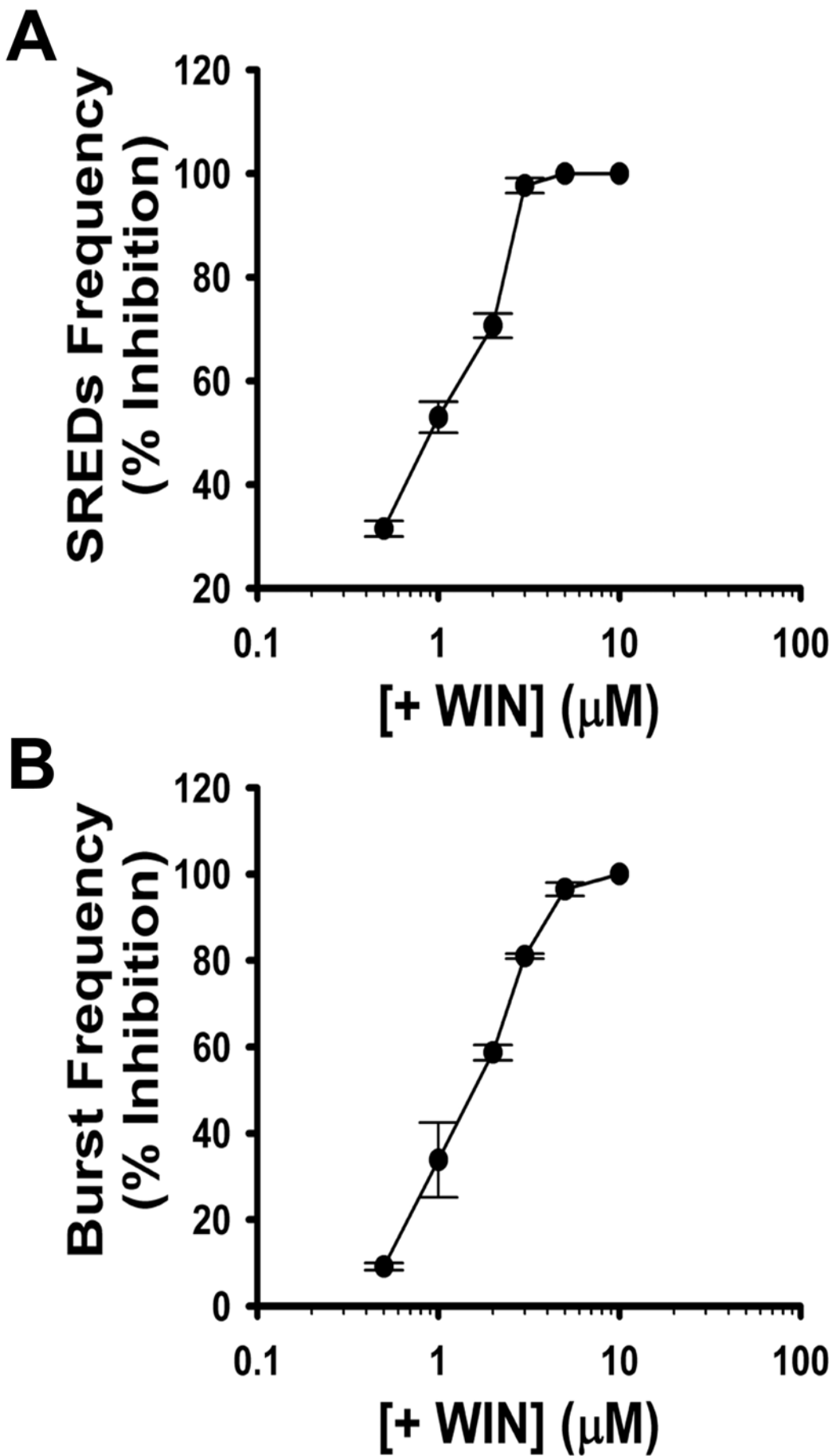
µM.

Figure 1

Figure 2

SREDs

A

B

-WIN (3 μM)

C

+WIN (3 μM)

D

+WIN (3 μM) + SR (1 μM)

20 mV

5 min

Figure 3

Figure 4

A   Control
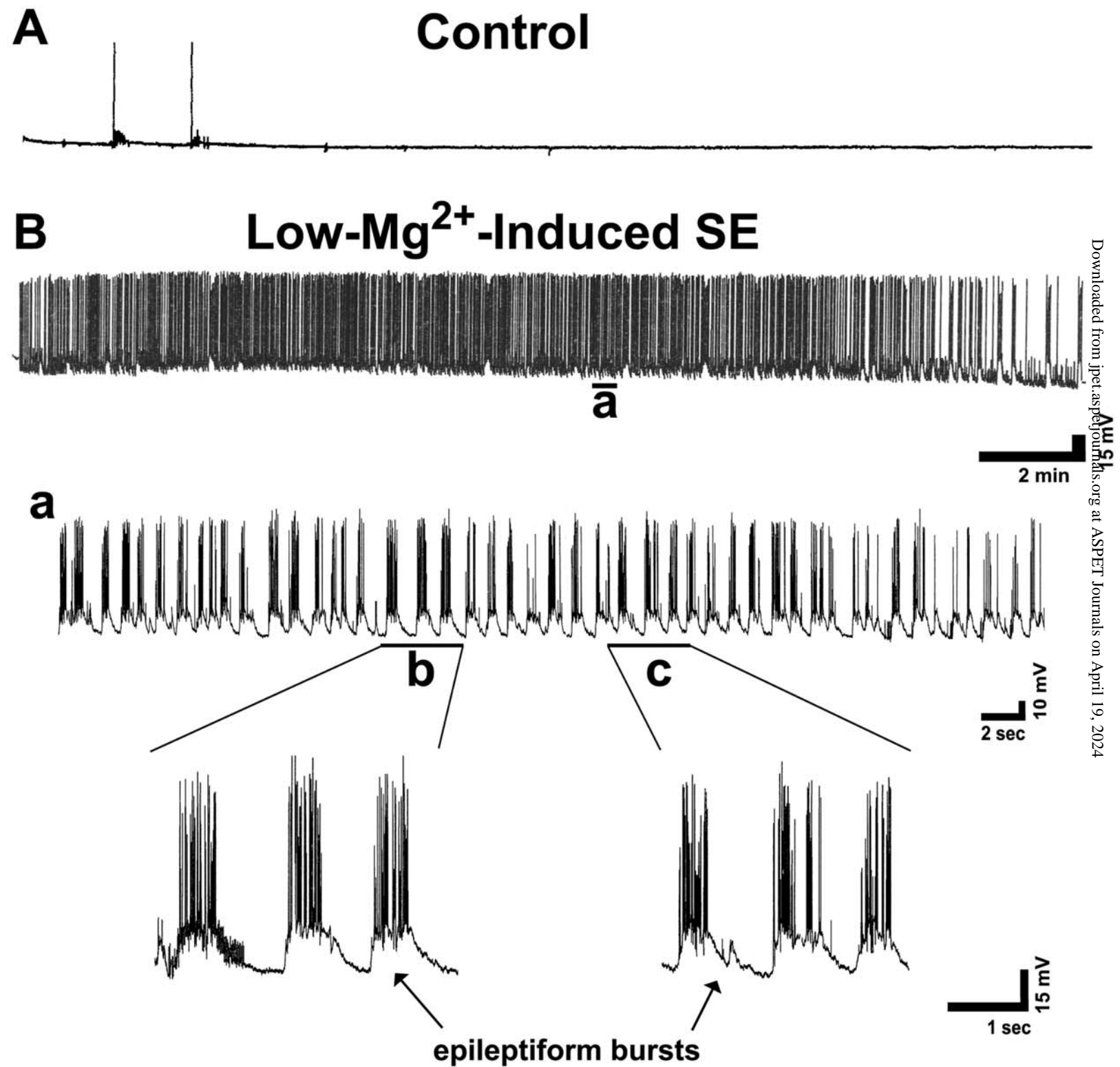
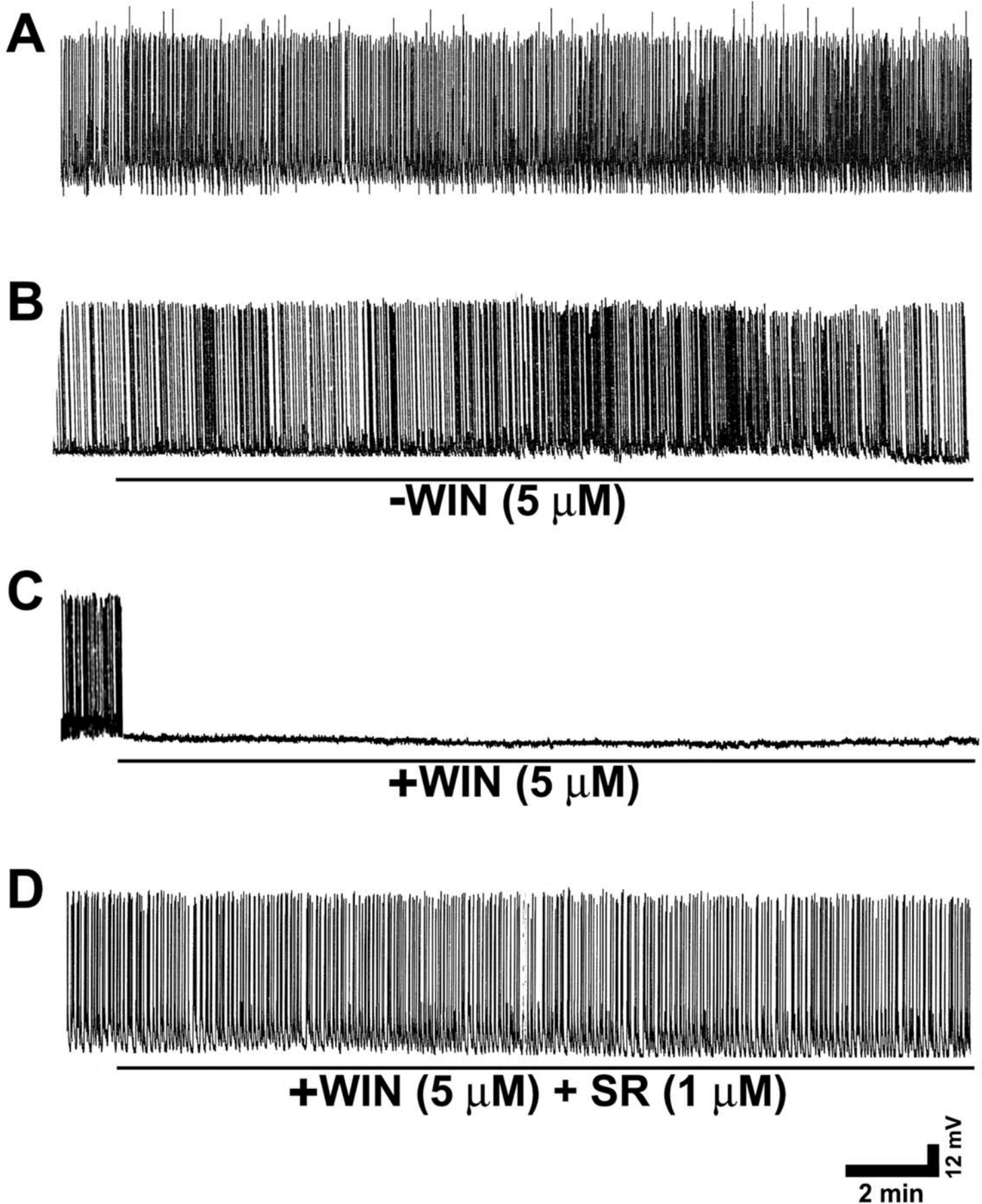## B   Low-Mg^{2+}-Induced SE

epileptiform bursts

Figure 5

Low-Mg²⁺-Induced SE

Figure 6

Low-Mg²⁺-Induced SE

A

+WIN (5 μM)

B

PHT (50 μM)

C

PB (50 μM)

12 mV

2 min