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Inverse relationship of cannabimimetic (R+)WIN 55, 212 on behavior and seizure threshold during the juvenile period

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ABSTRACT

Cannabinoids have anti-convulsant effects in both in vivo and in vitro models of status epilepticus. Since the development of spontaneous seizures and neuronal vulnerability are age-dependent, we hypothesized that the anti-convulsant effects of cannabimimetics are also age-dependent. We administered a single injection of varied doses of (R+)WIN 55,212 (0.5, 1, 5 mg/kg) to postnatal (P) day 20 rats 90 min prior to induction of kainate (KA)-induced status epilepticus. The highest dose of (R+)WIN 55,212 (5 mg/kg) resulted in rapid onset of behavioral stupor, loss of balance, stiffening and immobility while standing on hind legs or laying flat in prone position; lower doses had minimal or no behavioral effect. After KA administration, seizure scores and electroencephalography (EEG) recordings were inversely related to (R+)WIN 55,212 dosage whereby higher doses were associated with high seizures scores and synchronous epileptiform activity and low doses with low seizure scores and diminished spiking in the EEG. Immunohistochemistry revealed a dose-dependent reduction in CB1 receptor expression with increasing concentrations of (R+)WIN 55,212 in presence or absence of KA seizures. Nissl and NeuN staining showed hippocampal injury was attenuated only when seizures were mild following low doses of WIN 55,212 (0.5, 1 mg/kg), consistent with the level of CB1 expression. Since low doses abolished seizures without psychotropic side-effects further study may facilitate a groundbreaking cannabamimetic therapeutic strategy to treat early-life seizures. Higher doses had adverse effects on behavior and failed to prevent seizures and protect CA1 neurons possibly due to inactivation or loss of CB1 receptors.

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1. Introduction

Marijuana usage is highest during adolescence when the brain is still developing which may subsequently lead to beneficial and non-beneficial effects. For instance, the active psychotropic constituent of marijuana, Δ^9-tetrahydrocannabinol, was reported to impair short-term memory and learning, cause neuronal cell death of the hippocampus after chronic exposures, and increase the risk of developing psychotic disorders with maturity (Scallet, 1991; Lichtman and Martin, 1996; Chan et al., 1998; Pattij et al., 2008; Ranganathan and D'Souza, 2006; Bossong and Niesink, 2010). In addition, retrospective cohort studies of women smoking one or more marijuana cigarettes a day during the third trimester of pregnancy documented prolonged startle responses, disturbances in sleep cycling, and increased motility in the offspring (Fried, 1982; Scher et al., 1988). Experimentally, 15 days of daily prenatal treatment of the synthetic cannabimimetic, [(R+)WIN 55,212 [4,5-dihydro-2-methyl-4(4-morpholinylmethyl)-1-(1-napthylenylcarbonyl)-6H-pyrrolol[3,2,1-1]quinolin-6-one] to pregnant rats (0.5 mg/kg) caused in the offspring transient impairment of a number of cognitive functions as well as altered glutamatergic transmission (Ferraro et al., 2009). On the other hand, cannabinoids also have a long history of medicinal effects. They have been used in various forms to treat a variety of disorders such as cancer, multiple sclerosis, spinal cord injuries, glaucoma, ischemia, and epilepsy due to their analgesic, appetite stimulant, antiemetic, antioxidant, anti-inflammatory and anti-convulsant properties (reviews: Consroe, 1998; Hampson et al., 2003; Amar, 2006). Interestingly, cannabinoids have also been proposed to improve memory and cognition that may be useful to treat Alzheimer's disease (Grotenhermen, 2005).

In experimental epilepsy models in adult rats or in primary hippocampal cultures, synthetic and endogenous cannabinoids were shown to attenuate neuronal excitation and epileptic activity (Shen et al., 1996; Wallace et al., 2003; Blair et al., 2006; Deshpande et al., 2006; 2007). Accordingly, stimulation of CB1 receptors with endocannabinoids, anandamide or 2-arachidonylglycerol(2-AG), attenuates epileptic seizures and the associated neuronal cell loss (Deshpande et al., 2006; 2007). Anticonvulsant effects in a number of structures were attributed to reducing presynaptic release of glutamate via activation of presynaptic G protein-coupled cannabinoid type-1 (CB1) receptors, a negative feedback mechanism (Shen and Thayer, 1999; Ameri and Simmet, 2000;

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Wallace et al., 2001, 2002; Freund et al., 2003; Marsicano et al., 2003; Blair et al., 2006; Deshpande et al., 2007; Haj-Dahmane and Shen, 2009; Zarrindast et al., 2009).

In adult rats, the expression of the CB1 receptor is ubiquitously expressed throughout the hippocampus, frontal cortex, basal ganglia and cerebellum, regions responsible for seizure susceptibility, higher cognitive function, and motor coordination (Mailleux and Vanderhaeghen, 1992; Köfalvi, et al., 2005; Mackie, 2005). In early life, robust expression of CB1 receptors also occurs and these receptors were described to play critical roles in normal brain development and maturation (Harkany et al., 2007). However, there is currently a paucity of research on the anticonvulsant effect of cannabinoids in development, particularly during the adolescent period when the brain first becomes vulnerable to seizure-induced brain damage (Albala et al., 1984; Nitecka et al., 1984: Holmes and Thompson, 1988; Sankar et al., 1999; Liu et al., 2006). One study showed WIN 55,212 had anticonvulsant activity in young mice (20–28 days old) treated with pilocarpine (Wallace et al., 2001; 2002). In immature rat pups (P10), another study showed seizures produce long-lasting delayed increases in CB1 receptor expression within the hippocampus as determined by Western blotting (Schuchmann et al., 2006). In contrast, increases and decreases in expression have been observed in adults depending on the time and area examined (Falenski et al., 2007; 2009). We hypothesized that the cannabimimetic (+R)WIN 55,212 would have anticonvulsant activity and protect the juvenile brain from kainate (KA)-induced seizures as observed in adults but that these effects may be age and dose-dependent which may be critical to potential clinical outcomes. Since synthetically derived cannabinoids or endogenous up-regulation of endocannabinoids in humans with epilepsy have attractive therapeutic potential, we tested various doses of (+R)WIN 55,212 on the seizure threshold, injury, and CB1 receptor expression distribution of the hippocampus at the end of the 3rd postnatal week of development in a juvenile rat model of status epilepticus.

2. Materials and methods

2.1. Drug administration of CB1 receptor agonist R(+)WIN 55,212

Male Sprague–Dawley rats from postnatal (P) day P20 (40–50 g) were used in accordance with NIH guidelines. Animals were housed in single cages with their lactating mother until sacrifice (P23), given food and water ad libitum, and kept on a 12-h light/dark cycle at room temperature (55% humidity) in our own accredited animal facility. Animals were divided into control and experimental groups. A single injection of (R+)WIN 55,212 (0.5, 1 or 5 mg/kg, i.p., n = 10, n = 7, n = 15, respectively) (Tocris, Bioscience, Ellisville, MO) was administered to P20 rats 1.5 h prior to administration of kainate (KA), used to induce status epilepticus. A (R+)WIN 55,212 stock was suspended in 50% DMSO then diluted according to dosage used. Final concentration of DMSO was <5%. A separate group was injected only with KA (n = 12). Age-matched control animals (n = 8) were given equal volumes of vehicle containing the diluted DMSO. The animals were placed into clean and comfortable cages and their behavior was observed for 90 min. Changes in behavior were both noted and videoed with a SONY 4 mega pixel camera. Seizures were subsequently induced with kainic acid (KA) (i.p., 9–10 mg/kg) (Ascent Scientific, Princeton, NJ) in presence and absence of several doses of (R+)WIN 55,212 (5, 1, 0.5 mg/kg) (see Fig. 1). Following the KA injection, epileptic behavior was monitored every 5 min for an additional 2 h. After the seizure recording observation period, juvenile control and experimental animals were returned to their lactating mother until sacrifice at 72 h.

2.2. Seizure scoring

In order to determine seizure threshold and severity, seizure behavior was scored from the freely moving juvenile rats. Seizure rating was determined using our modified Racine method as previously reported (Liu et al., 2006). Briefly, seizure scores were recorded every 5 min and were representative of the behavior observed during that time period. The seizure rating scale consisted of the following: 1 = scratching, chewing, and grooming; 2 = head nods, tail wagging, and chirping; 3 = wet dog shakes, standing tonus, and occasional loss of posture; and 4 = four limb clonus, frothing salivation, and loss of postural control with side tonus. The number scored was subsequently multiplied by the number of 5 min intervals recorded over the 2 h recording period so that the total raw score was equal to the sum of these scored numbers divided by the number of intervals. Rated scores in the presence and absence of (R+)WIN 55,212 were averaged and subjected to statistics.

2.3. EEG and digital video recordings

To obtain EEG recordings, juvenile control and experimental rats were first anesthetized with a mixture of 70 mg/kg ketamine and 6 mg/kg of xylazine and then bipolar electrodes were steriotaxically implanted into the right hippocampus as described previously (Friedman and Velísková, 1998) (coordinates in mm with respect to bregma of P20 rats: AP: −3.2; L: 2.6; D: −2.8; incisor bar at −3.5) (Paxinos and Watson, 1986). The electrodes were perpendicular, angled at 0° from the vertical plane. After surgery, dental acrylic was used to close the wound and hold the electrode assembly in place. Rats recovered from anesthesia and became active 1–2 h following the surgery. Animals were kept warm at 30 °C in a clean cage box under an incandescent lamp then returned to their lactating mother until sacrifice. In order to obtain EEG recordings, KA and (R+)WIN 55,212 + KA treated animals were paired, placed in an insulated chamber, and connected to the recording set up through flexible low noise leads (Plastics One), which permitted free movement (Nickel and Szelenyi 1989). Baseline EEG recordings were obtained before and after drug administrations. For off-line analysis, Data Wave software was used to quantify wave frequency, amplitude, spike number, and burst activity in the EEG traces. The program uses digital filtering with a Butterworth 3 pole filter and a 6 dB per octave roll-off. EEG traces were filtered prior to being subjected to burst, Fourier, and spectral analyses to calculate changes in oscillation frequency and duration. Experimental traces were compared from rats treated with (R+) WIN 55,212 ± KA. PBS/DMSO injected control rats were also used for baseline comparison (n = 3). Electrode placement into the CA1 was verified histologically with thionin staining. Histological cell counting from these animals was only from hippocampus on the side contralateral to the EEG electrode. Digital recordings with a 6 Mega pixel Sony digital camera were taken for 30 min following 5 mg/kg administration of WIN 55,212 to monitor alterations in behavior at this dosage. Still photographs were downloaded from the camera video to illustrate stumbling and catatonic-like behaviors.

2.4. Immunohistochemistry

To examine the expression of CB1 receptor protein at the level of the hippocampus following KA induced seizures with or without (R+)WIN 55,212 pretreatment, both chromogen based and fluorescence immunohistochemistry were performed with a CB1 specific antibody as described (Laurén et al., 2010). This antibody was previously tested for specificity in a number of applications, such as Western blotting (60 kDa), immunocytochemistry, and immunohistochemistry and can also be blocked with the CB receptor 1 peptide (ab50542) (Abcam, Cambridge, MA). P20 rats were first anesthetized with a lethal dose of sodium pentobarbital (50 mg/kg) and perfused intra-aortically with ice cold 0.9% saline followed by 200–300 ml 4% PFA prepared in PBS. Brains were removed and post-fixed for 15 h at 4 °C. Free-floating vibratome sections (40 μm) were subsequently prepared from control and experimental groups. Sections were washed in PBS (2×) then incubated with 0.5% H_2O_2 for 30 min to remove endogenous peroxides. Brain sections were then washed (4× for 10 min each) followed by immersion in blocking

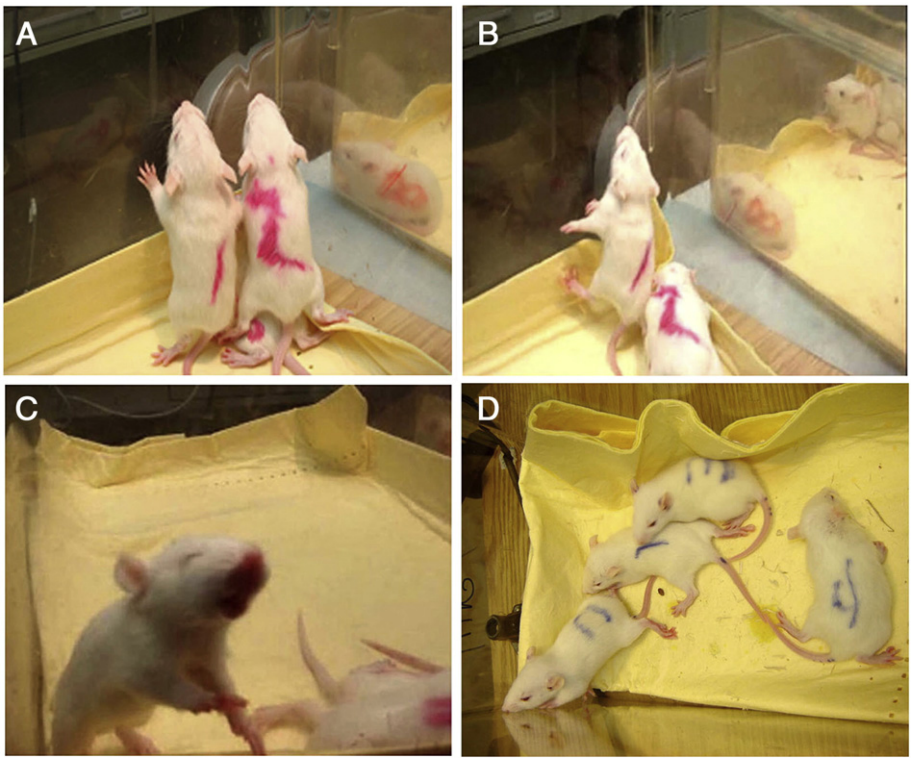

Fig. 1. Still photographs were downloaded from the video taken of P20 rats following (5 mg/kg) of (R+)WIN 55,212 administration. A, Example of two juvenile rats (#1 and #2) that stood on their hind legs and leaned still and stiff on the cage wall for extended periods. Animals remained stiff and immobile in a catatonic-like state for approximately 90 min. B, The same two animals shown in A also display stumbling and falling over drunken-like behavior. Notice animal #8 in 2nd cage on right that had a low dose and was unaffected, standing on four paws in prone position akin to a naïve control. C, Front view of high dose rat with stupor and face leaning into the cage with eyes closed. D, Another group of high (R+)WIN 55,212 treated animals showing still, drunken-like behavior while laying flat with loss of hind leg control.

solution (5% horse serum/0.5% BSA-PBS) for 1 h at room temperature. The CB1 primary antibody was diluted (1:50) and added to brain sections for 48 h at 4 °C. Tissue sections were subsequently washed (3× for 10 min each) in PBS to remove the primary antibody. Secondary biotinylated anti-rabbit IgG (diluted 1:200) was added (Vector Laboratories, Burlingame, CA) and incubated for 2 h at room temperature. After three washes with PBS, ABC solution (Vector Laboratories, Burlingame, CA) was added for 1 h at room temperature. For visualization, the sections were reacted with 3′,3-diaminobenzidine tetrahydrochloride (DAB) (5 mg/ml and 0.001% H_2O_2) (Sigma, Milwaukee, WI) and then washed, mounted, dehydrated, cleared, and coverslipped and viewed under bright-field optics and phase contrast according to the atlas of Paxinos and Watson Rat Brain Atlas (1986). For fluorescent labeling of CB1 receptors, sections were incubated with primary antibody overnight followed by a 2 h incubation of secondary FITC or Texas Red antibodies, washed, air dried in the dark and coverslipped with histomount (Zymed, CA). Specimens were viewed and scanned with an Olympus BX51 microscope equipped with selective excitation/emission filters for FITC and Texas Red.

2.5. Immunodensity measurements

Hippocampal sections were scanned with a digital spot camera attached to an Olympus BX51 microscope interfaced with a Pentium IV DELL computer. All camera settings were held constant during image capturing. To enable quantification of CB1 immunohistochemistry, two complimentary procedures with NIH-Scion image software were used. For DAB developed sections, bilateral optical densitometry (O.D.) pixel measurements, in gray scale, were taken from selected areas of interest (pyramidal cell layers of subiculum and CA1 subregions of the hippocampus). CA3 and dentate gyrus (DG) were also quantified for comparison. Characteristic bands of punctuate CB1 dendritic labeling were observed surrounding the CA1 pyramidal cell body layer ($CA1_{pyr}$), therefore, the stratum oriens (SO) and stratum radiatum (SR) layers were further subdivided and referred to as SO_{band}, SO_{layer}, SR_{band}, and SR_{layer} in order to estimate differences within the dendritic layers (see Fig. 4). The DG was also subdivided into classic divisions, granule cell layer (GCL), outer molecular layer (OM), and inner molecular layer (IM). Estimating the level of CB1 distribution of DAB stained sections was accomplished by evaluating the number of pixels within the separate laminated dendritic sublayers. NIH image point to point area tool was used to accurately outline the layers in one area sweep per subarea as illustrated in Fig. 4A–B. The cell body layer of the CA1 ($CA1_{pyr}$) was calculated as the density between the two bands by moving the area tool between the bands and obtaining the O.D. values in gray scale. For the subiculum and CA1, immunodensity measurements were averaged from the three sublayers and the SR_{layer} O.D. values were averaged, used as background, and subtracted. To asses specific immunodensity differences within the neuropilar layers, SO_{band}, SO_{layer}, SR_{band}, and SR_{layer} the CC and no tissue background measurements were used (see schematic, Fig. 4A–B). Immunodensity density values of CB1 protein expression were averaged from both hippocampal hemispheres. Mean values from individual animals were then averaged to obtain total immunodensity means for each experimental condition. Since laminated expression of the CA1 was lost in animals treated with high (R+)WIN 55,212 in presence or absence of KA, estimating the level of CB1 distribution in these groups was accomplished by using the Scion Image area tool to draw a section surrounding all layers the subiculum including the CA1 pyramidal ($CA1_{pyr}$) cell body layer, SO_{band}, and SR_{band} (see Fig. 4A–B). Since sections may label non-uniformly due to variability in perfusions, at least 4 slides with 3–4 serial sections per slide were prepared from all animals and several areas were used for subtracting averaged background signaling, the neuropil, corpus collosum (CC), and glass slide with no tissue. Values were normalized against the control group. Thus, variations in the immunodensity of

experimental groups were also represented in percentage relative to the control group.

For immunofluorescence, sections were scanned under a 4× and 40× objective lenses with selective excitation/emission filters for FITC (475 nm) and Texas Red (559 nm). Segments of the subiculum and CA1 images were analyzed with Scion image software and files were then subjected to the intensity threshold tool followed by the analytical tool. These values estimated the total number of CB1 fluorescent particles. The number of particles was averaged from several segments along the CA1 (three sectors of 300 μm^2) to estimate fluorescence intensities under all of the conditions tested (see Fig. 5).

2.6. Western blotting

Western blot analysis was used for the quantification of CB1 receptor protein from the dissected CA1 hippocampal subfield after status epilepticus, as previously described (Friedman et al., 2007). Brains were immediately removed, placed in ice-cold Ringer solution (120 mM NaCl, 1.5 mM KCl, 1.3 mM $MgSO_4$, 2.4 mM $CaCl_2$, 1.25 mM KH_2PO_4, 26.2 mM $NaHCO_3$, 11.0 mM D-glucose). After each brain was sectioned into 1 mm thick slices, the CA1 was dissected away from CA3 and DG subfields, collected in microcentrifuge tubes on dry ice, and stored at −80 °C. For analysis, rat brains were thawed on ice and homogenized with pellet pestle and tube (Kimble-Kontes, Vineland, NJ). 50 µl of 4× SDS-PAGE loading buffer (250 mM Tris–HCl [pH 6.8], 9.2% [w/v] SDS, 50% [w/v] glycerol, 0.05% [w/v] bromophenol blue) plus 100 mM dithiothreitol were directly added to 20–30 mg of the homogenized tissue samples and heated to 100 °C for 5 min while agitated. Cell lysates were cleared by centrifugation (20,000 *g*, 5 min, 4 °C) and supernatants applied to 4–15% denaturing SDS-polyacrylamide gradient gel electrophoresis followed by wet transfer onto nitrocellulose membranes at 125 mA/gel for 90 min. Protein transfer was assured with reversible Ponceau-S staining (0.1% [w/v] Ponceau-S in 5% [v/v] acetic acid). Membranes were probed overnight at 4 °C 1:200 with either of the following primary antibodies: Rabbit polyclonal anti-cannabinoid receptor I antibody (CBI; ab23703; Abcam, Cambridge, MA) or rabbit polyclonal anti-actin (H-300; sc-10731; Santa Cruz Biotechnology, Santa Cruz, CA). Then, membranes were treated 1:1500 with goat anti-rabbit horseradish peroxidase-coupled secondary antibody (Thermo Fisher Scientific, Rockford, IL), developed and recorded on X-ray film. For signal analysis and documentation, autoradiographs were scanned and images imported into Adobe Photoshop CS3 (Adobe Systems Incorporated, Mountain View, CA) and the optical density of the bands was quantified by computer densitometry to measure the quantity of the receptor subunit protein. Protein standards, obtained from Bio Rad in kDal include: myosin, galactosidase, phosphorylase B, BSA, and ovalbumin.

2.7. Histology

Hematoxylin/eosin (H&E) staining was carried out on fixed serial air-dried sections (40 µm) from brains processed for CB1 immunohistochemistry in order to monitor eosinophilia and/or cell loss from the same animals. NeuN immunohistochemistry was simultaneously performed to estimate the number of CA1 neurons from three averaged 1 mm sectors per condition in serial sections. Mounted sections were processed through graded ethanols and cleared with three changes of xylene for 15 min each then viewed and photographed under phase contrast microscopy.

2.8. Cell counting

To estimate survival of surface neurons along the CA1 axis, eosinophillic cells and NeuN labeled cells were counted under phase contrast microscopy in three consecutive 0.3 mm^2 sectors with a 40× lens objective and calibrated grid reticule (10×100 μm^2 boxed sectors) that was aligned along the CA1 region as previously described (Kaur et al., 2007). A manual cell counter was used by two students blind to the experimental conditions. Counts were averaged from 2 to 3 sections from 4 dorsal hippocampal levels [every 5th and 15th section between −2.4 and −4.6 mm from Bregma] (Paxinos and Watson, 1986). NeuN labeled cells were counted regardless if they had regular or irregular appearance. Raw 2-D numbers were averaged from a total area (2.99 ± 0.51 mm^3) and plotted. Averaged results from all groups were quantified and subjected to statistical analysis for comparison.

2.9. Statistics

Results are given as means for each animal group. Significant differences were determined from numbers assessed from injured cell counting and immunodensity measurements for animals in all groups. Values were compared using Sigma Stat software. One-way analysis of variance (ANOVAs) with pairwise multiple comparisons and Holme–Sidak method were performed to account for differences in neuronal cell death and immunodensity as a function of (R+)WIN 55,212 pretreatment dosage and seizure history. Student's *t*-test was used for single comparisons. Pearson's correlation coefficient (r) was calculated to compare the relationship between injury and seizure severity and level of CB1 expression from gray scale thresh holding of luminescence values. Significance was set at $p<0.05$ for all tests.

3. Results

3.1. Dose dependent behavior induced by cannabinoid

In order to investigate whether a selective CB1 agonist is neuroprotective and an effective anticonvulsant against KA seizures in the juvenile period, (R+)WIN 55,212 was administered to P20 rats at three doses (0.5, 1 and 5 m g/kg) 90 min before induction of seizures with KA. Based on animals studies conducted in adult rats or juvenile mice (Wallace et al., 2002; 2003) no significant behavioral changes were expected, however, pronounced behavioral manifestations were observed in juvenile rats receiving the highest dose of (R+) WIN 55,212 (5 mg/kg). Within 5 min of the higher dose injection, rats became sluggish, wandered about the cage with drunken, stumbling mannerisms then stood up on their hind legs leaning on the cage. They were stiff and immobile and remained in a catatonic-like state for approximately 90 min (Fig. 1). Three behaviors were assessed, standing stiff on hind legs (Fig. 1A), drunken-like falling over (Fig. 1B–C), and laying flat in prone position with hind legs sprayed out with head down (Fig. 1D and Table 1). None of these behaviors were observed in control animals or at the lowest dosage of (R+)WIN 55, 212. Mild or no behavioral change was observed at the middle dosage (1 mg/kg).

After 90 min, when behavioral symptoms subsided, all pretreated animals were subsequently injected with KA to test the seizure threshold. Seizure behavior was monitored and scored for an additional 2 h. Seizure scores were inversely related to (R+)WIN 55,212 dosage whereby higher doses were associated with high seizure scores and

Table 1
Behavior scores were quantified for each dosage of WIN 55,212. The number of behavioral events was recorded for 90 min. Naïve controls and the lowest dose did not produce any of the recorded symptoms. Note: standing on hind legs typically lasted about 10–15 min at a time. One way ANOVA was used to test for significance.

Treatment	Standing on hind legs	Falling over	Laying flat/head down
Win 0.5 mg (n = 10)	0	0	0
Win 1.0 mg (n = 6)	0	1.8 ± 0.9	2 ± 0.6
Win 5.0 mg (n = 15)	$6.7 \pm 1.8^{**}$	$7.2 \pm 2^{**}$	$6 \pm 1.8^{**}$

** $p<0.01$.

low doses with low seizure scores. Animals that were only treated with KA in the absence of (R+)WIN 55,212 had the strongest seizures as indicated by obtaining the highest seizure score of (Table 2). In contrast to our expectation, 80% (12 of 15) of the animals that received the highest dose of (R+)WIN 55,212 (5 mg/kg) prior to the KA injection also exhibited strong behavioral seizures, similar to the KA treated group. Animals that received lower doses of (R+)WIN 55,212 (0.5 or 1 mg/kg) had significantly lower seizure scores; the lowest dose treatment also resulted in little or no seizure behavior in 80% of the animals (8 of 10) (Table 2). The onset to mild seizure behaviors and mild to moderate seizure activity at 1 and 0.5 mg/kg were unchanged, however behavior manifestations such as fore limb clonus FLC, which is associated with delayed neurodegeneration, were absent (one-way ANOVA, $^{*}p<0.05$; $^{**}p<0.01$).

3.2. Electrographic activity is inversely related to (R+)WIN 55,212 dosage in presence of KA

Unilateral EEG recordings with a bipolar/cannula electrode assembly inserted into the CA1 were carried out after KA administration and at the three doses of (R+)WIN 55,212 ± KA (Fig. 2). The effects of (R+)WIN 55,212 in the absence of KA on EEG activity were concentration dependent. The highest dose (5 mg/kg) led to long lasting changes in baseline EEG oscillations. Within 10 min of injection, periods of rhythmic low amplitude oscillations (~0.2 mV) were observed and continued to appear 30–60 min after the KA injection. Fourier analysis revealed typical 20–40 Hz and atypical ~70 Hz frequencies (Fig. 2A, *3rd and 4th traces*). After the KA injection in the absence of (R+)WIN 55, 212, single spikes in the EEG traces could occasionally be detected of varied magnitude (0.2–10.0 mV) (15–20 min), whereas the onset to seizure behavior (scratching and head bobbing) occurred approximately after 30 min consistent with our previous recordings at this age (Liu et al., 2006). Onset to spiking burst activity occurred after ~60 min, and correlated with the onset to rearing, salivation, and fore limb clonus, behavior parameters that were positively correlated with high seizure scores and CA1 injury ($r=0.90$, $p<0.05$) (Table 2). High frequency rhythmic oscillations and rhythmic burst activity with high amplitude spikes (0.5–20 mV, 20–40 Hz and ≥100 Hz) were commonly observed at this time (Fig. 2B, *top trace*).

Lower doses of (R+)WIN 55,212 (0.5–1 mg/kg) conferred anticonvulsant activity in the EEG and did not alter baseline oscillations (Fig. 2B, *2nd and 3rd traces*). The number of bursting events were either absent or significantly attenuated ($F=4.34$, $p<0.015$) (Table 2). However, if low amplitude bursts appeared at the low dose then their duration time was similar to the high amplitude bursting indicating occasional seizure activity was present but with little behavioral change (Table 2). Neither of these lower doses led to FLC or muscle tonus (Table 2). In contrast, the higher dosage of (R+)WIN 55,212 (5 mg/kg) had either a proconvulsant effect where animals could not resume postural control and displayed rapid bursting in the EEG or a similar effect as KA alone with advanced motor symptoms such rearing, salivation, forelimb clonus (FLC) and muscle tonus and bursting in the EEG (Fig. 2B and Table 2). Pearson's correlation coefficient was determined to reflect that the inverse dose-dependent effects of (R+)WIN 55,212 on burst activity corresponded to extent of neuronal injury ($r=0.90$).

3.3. Neuronal cell injury is inversely related to (R+)WIN 55,212 dosage in presence of KA

In order to determine whether the dose-dependent effects of (R+)WIN 55,212 on CB1 distributions would correspond to amount of neuronal injury, brain sections from all groups were processed for H&E staining (Fig. 3C, F, I, L, O). The number of injured (eosinophillic) neurons of the subiculum and CA1 areas, the hippocampal regions most sensitive to injury at this juvenile age, was counted under bright field microscopy. In accordance with seizure scoring, dose-dependent injury was observed. Histological evaluation demonstrated a considerably large number of injured eosinophilic neurons after KA without pretreatment of (R+)WIN 55,212 (Table 2). Those animals that received the highest dose of (R+)WIN 55,212 (5 mg/kg) prior to their KA injection also demonstrated highly noticeable injury, with numerous eosinophilic cells in the CA1 mimicking the KA treated group (Table 2). Animals that were pretreated with the lowest dose of (R+)WIN 55,212 (0.5 mg/kg) before KA seizure induction illustrated minimal injury, and those with 1 mg/kg of (R+)WIN 55,212 showed only slight injury (Table 2). Seizure scores and the number of injured CA1 neurons were highly correlated ($r=0.903$; $p<0.001$). In animals treated only with (R+)WIN 55,212 (5 mg/kg), pyramidal cells of the hippocampus were healthy and regular in appearance exhibiting minimal or no injury and were indistinguishable from control animals.

3.4. Dose-dependent alterations in CB1 distribution

To examine the expression of CB1 receptor protein distribution following KA-induced status epilepticus with or without (R+)WIN 55,212 pretreatment during the juvenile period. Serial sections from control and experimental animals at the level of the hippocampus were analyzed with a CB1 specific antibody and then developed with either a DAB chromogen or secondary antibody conjugated to Texas Red. Fluorescent labeling allowed for intensity luminescence analysis whereas DAB staining gave optical density measurements (Figs. 3–5). In age-matched control animals, the distribution of CB1 receptor immunoreactivity with DAB was intense surrounding the CA1 pyramidal cell bodies (Pyr). Rich punctuate labeling also surrounded each cell body but label was most pronounced in areas juxtaposed to the cell bodies of the CA1 ($CA1_{Pyr}$) named the SR_{band} and SO_{band} (Figs. 3–4). CB1 immunolabel was comparatively much weaker but relatively uniform throughout the overlying and underlying hippocampal neuropil, labeled as SO_{layer} and SR_{layer} (Fig. 3). This laminated pattern was similar to the control pattern reported by others in adult and juvenile rats (Tsou et al., 1998; Falenski et al., 2007: Laurén et al., 2010). Since there were two narrow

Table 2
Seizure latency, severity and burst activity in EEG.

Treatment	Seizure score	Behavior onset (min)	FLC/Tonus onset (min)	Burst frequency (#/s)	Burst duration (s)	Spike amplitude (mV)	# Injured cells
Win 0.5 mg + KA	1.86 ± 0.35**	29.75 ± 1.1	0	0.08 ± 0.02**	0.68 ± 0.12	0.25 ± 0.38**	82.02 ± 5.68**
Win 1.0 mg + KA	2.84 ± 0.29	30.3 ± 1.1	0	0.193 ± 0.08	0.26 ± 0.04	0.85 ± 0.41	194.50 ± 3.82*
Win 5.0 mg + KA	3.39 ± 0.12	33.3 ± 2.1	66.7 ± 4.4	1.05 ± 0.25	0.856 ± 0.23	1.95 ± 0.92	481.90 ± 124
KA	3.60 ± 0.14	28.54 ± 2.1	58.5 ± 3.4	1.23 ± 0.27	0.533 ± 0.13	3.75 ± 1.11	418.67 ± 111

Quantification of the number of spikes and high synchronous burst events showed lower but not higher doses of (R+)WIN55, 212 decreased burst activity and/or prevented KA-induced rhythmic oscillations and reduced the amplitude of spikes in the EEG. Attenuation of burst number was significant, however low amplitude bursting at the low dose were of similar duration. The onset to seizure behaviors and to spike activities was unchanged in animals exhibiting seizure activity, however certain behavior manifestations were absent, such as fore limb clonus (FLC). Due to a wide range of spike amplitudes EEG amplitude was measured within a range to exclude signals below 0.1 mV, or above 18 mV. Bars are means ± SEM of 60 min of tracing per animal per group. One-way ANOVA.

** $p<0.01$.

* $p<0.05$.

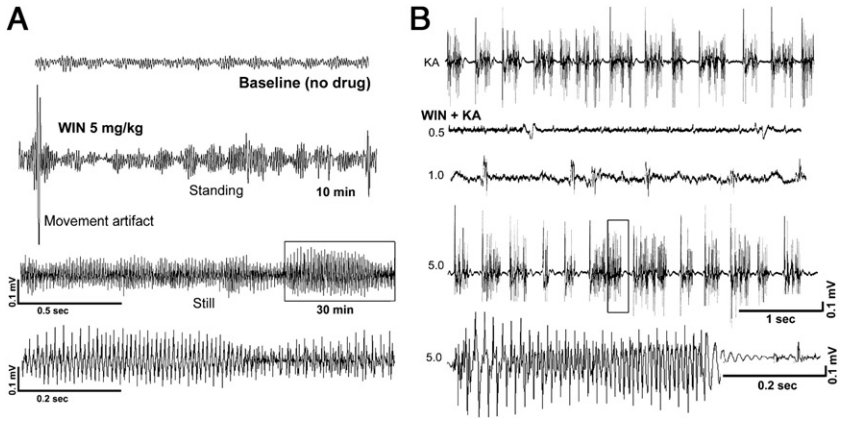

Fig. 2. Representative intrahippocampal EEG recordings from juvenile rats exposed to varied doses of WIN 55,212 in presence and absence of KA. A, Top trace is of the baseline EEG showing low amplitude, arrhythmic oscillations. Second trace: high doses of (R+)WIN 55,212. (5 mg/kg) led to periods of rhythmic low amplitude oscillations within 10 min of injection; oscillations fluctuated between 20 and 40 Hz; larger amplitude oscillations were intermingled in the range of 25 Hz (0.2–0.3 mV). Third trace, within 30 min occasional clusters of these high frequency low amplitude oscillations appeared 20–30 min after the injection ranging from 40 to 75 Hz. The boxed part of the third trace is expanded in the 4th trace to illustrate the rhythmic oscillations possibly due to disinhibition. B, Top trace, KA-induced status epilepticus in the absence of WIN 55,212 showed high frequency rhythmic oscillations and rhythmic burst activity. After (R+)WIN 55,212, inverse dose dependent activity was observed. Recordings at low doses at 0.5 mg/kg + KA were relatively flat with minimal number of low amplitude spikes in most of the animals tested. After (R+)WIN 55,212 (1 mg/kg) + KA, single spikes and occasional bursting were observed. After higher doses of (R+)WIN 55,212 (5 mg/kg) + KA, high-synchronous activity with high magnitude spiking and frequent burst activity of long duration were observed, similar to KA treated rats.

bands of punctuate CB1 immunolabeling observed just above and below the $CA1_{Pyr}$ (SO_{band} and SR_{band}) particularly visible with DAB staining, changes in expression in these dendritic sub-layers were assessed individually. A schematic color-coded representation of the layer-specific distribution is illustrated to reference the layers analyzed (Fig. 4A–B). Densitometry measurements of the control hippocampus confirmed significant lamination ($F=4.2$, $p<0.01$). Comparison analysis confirmed that the SO_{band} and SR_{band} had the highest optical intensities (Fig. 4E) ($n=6$, $p<0.005$). CB1 expression was also particularly intense within the inner molecular layer (IM) of the DG (Figs. 3A–B and 4F). Sub-layer analysis of the dentate gyrus showed that the immunoreactivity of the IM was greater than that measured in the outer molecular layer (OM) which was greater than the granule cell layer (GCL) (IM > OM > GCL) ($F=4.9$, $p<0.01$). The CA3 immunolabeling was more uniform and of similar intensity as the $CA1_{Pyr}$ (Figs. 3–4). The laminated pattern of the DAB stain observed could be distinguished with Scion image software in gray scale (Fig. 4C–F).

After KA induced-status epilepticus, significant alterations in the distribution of CB1 receptor expression were observed. CB1 immunoreactivity was significantly distinct within specific sub-layers such that the CA1 SR_{band} exhibited the highest intensity and appeared to cover a wider area relative to the other sub-layers ($F=10.9$, $p<0.001$) (Figs. 3–4). Significant punctuate increases were also noted in the SO_{band} relative to controls ($t=-2.48$, $p<0.05$). In contrast, when all three sub-layers of the CA1 were averaged as a whole, the increased expression of the CA1 did not reach statistical significance relative to controls confirming a layer-specific effect (O.D. C: 44.6 3.37 vs. KA: 54.37 4.4). In the DG, distinction between the layers became greater than the laminated patterns observed in control animals. The IM became consistently more intense after KA treatment ($F=17.9$, $p<0.001$). Comparative analysis illustrated that the increased CB1 expression in the DG was significant in the IM and OM but not in the GCL ($t=-2.5$, $p<0.05$). The CA3 expression remained intense and uniform but was not significantly different from control CA3 labeling.

In the presence of varied concentrations of (R+)WIN 55,212 and KA seizures, a dose-dependent reduction in CB1 DAB immunostaining was observed throughout the subiculum and most of the CA1 (Fig. 3). Animals with the highest dose of (R+)WIN 55,212 + KA had a striking depletion in CB1 immunoreactivity throughout the subiculum and $CA1_{Pyr,}$ SO_{band} and SR_{band} (Figs. 3J–K and 4). This decrease in CB1 protein was also associated with the most CA1 injury and higher seizure scores (Fig. 3 L and Table 2). Similarly, animals treated only with the highest dose of (R+)WIN 55,212 demonstrated a reduction in CB1 protein expression in subiculum and CA1 subregions but to a lesser degree than animals treated with both KA and (R+)WIN 55,212 relative to controls (Figs. 3–5). Pairwise multiple comparison analysis of experimental groups expressed as percent optical density of control demonstrated that the greatest loss was in the subiculum and adjacent CA1 subregions for both groups, (R+)WIN 55,212 (5 mg/kg) + KA and (R+)WIN 55,212 (5 mg/kg), respectively (subiculum: 34.9 ± 3.56%; CA1: 53.2 ± 7.8% vs. subiculum: 55.6 ± 4.1%; CA1: 55.9 ± 4.1% ($F=12.97$, $p\leq0.001$) (Fig. 4). Pretreatment with lower doses of (R+)WIN 55,212 (0.5 mg/kg and 1 mg/kg) followed by KA-induced seizures also resulted in lower levels of CB1 expression; however, immunodensity measurements were only significantly different from animals treated with KA, the group with increased CB1 expression (Fig. 4A). Accordingly, histology of these groups was similar to controls (Fig. 3).

Fluorescent labeling with secondary antibody conjugated to Texas Red in controls was intense exhibiting a uniform laminated pattern that mimicked the DAB staining; however with less punctuate labeling within the neuropil (Fig. 5A–E). Control sections labeled with the NeuN antibody were uniform and without nuclear staining (Fig. 5F). After KA, CB1 immunofluorescence of the CA1/subiculum was steady or increased. In contrast, CB1 protein expression decreased after WIN 55,212 + KA (by 40.4 ± 8.2%, $n=4$). In one of two cases where 0.5 mg/kg of (R+)WIN55, 212 did not prevent status epilepticus (seizure score = 3.8) decreases in CB1 immunofluorescence were similar to the higher dosage plus KA treatment and considered as "unprotected". In this case, many NeuN+ neurons were dark, exhibited nuclear staining, and were dispersed within the layer (Fig. 5G). In contrast, in an animal with 0.5 mg/kg of WIN55, 212 "protected" from seizures had uniform CB1 immunolabeling that resembled the control. Occasional nuclear staining was observed (seizure score = 1.7′) (arrows) (Fig. 5H). Reduced CB1 immunofluorescence and altered morphology of NeuN+ in this animal confirms that downregulation of CB1 receptors depends on severity of KA seizures and the resulting injury and not the actual agonist dosage of (R+)WIN55, 212.

Western blotting with the CB1 antibody was carried out on equal amounts of protein from the dissected hippocampal CA1 region pooled from both hemispheres of single animals from four treatment groups (Fig. 5K). Typical 60 kDa bands were visualized and the films were assessed with densitometry. The westerns confirmed the immunohistochemical observations such that the CB1 subunit protein was significantly reduced by 68.3 ± 8.8% ($n=4$) in the subiculum/CA1 of animals pretreated with 5 mg/kg of WIN 55, 212 (Fig. 5L). Similar to

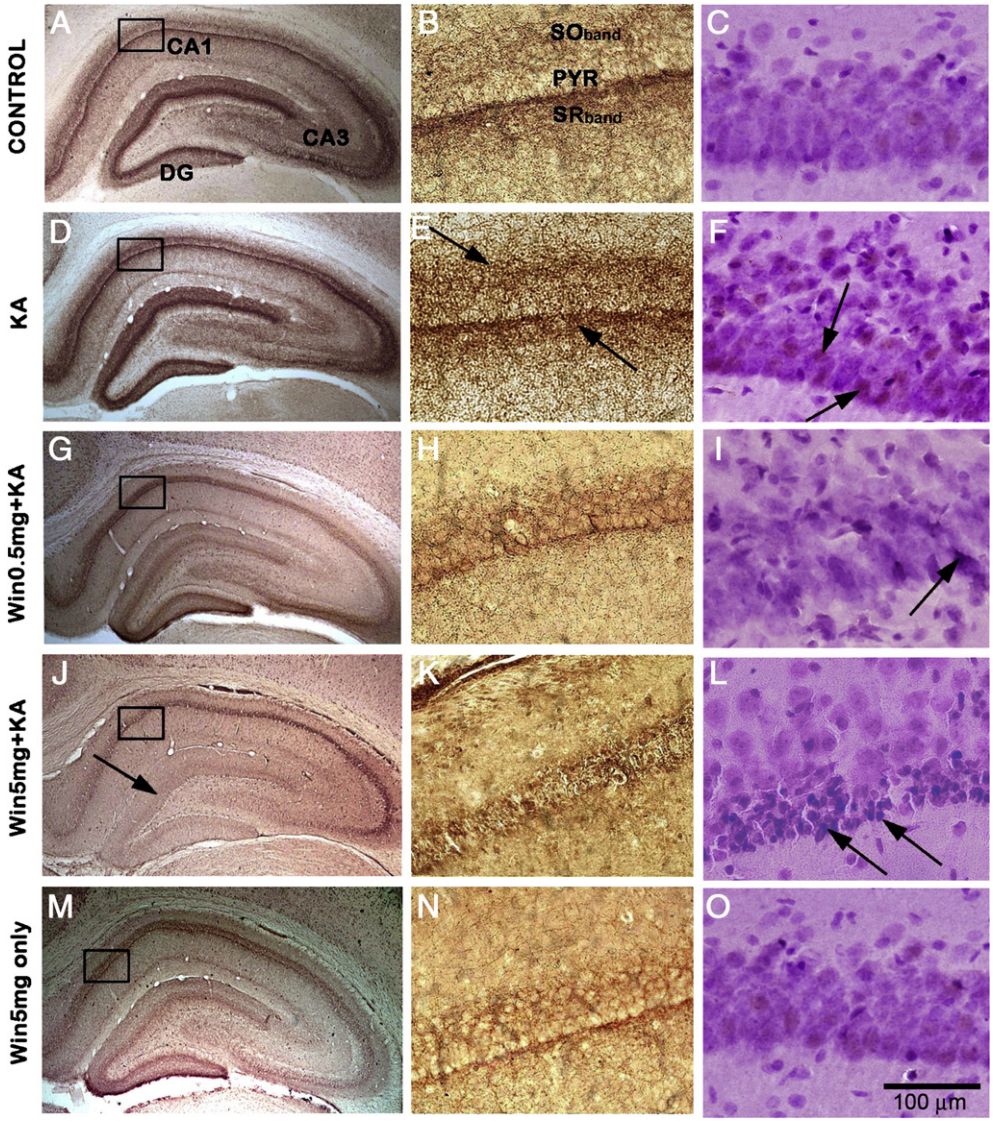

Fig. 3. Photomicrographs (40× and 400×) depicting CB1 receptor expression with DAB and Nissl staining in serial sections from controls and following a single seizure in the presence and absence of various doses of (R+)WIN55, 212 at P20. A–B, Control CB1 immunoreactivity was intense and had a laminated pattern. (C) H&E staining of control CA1 neurons was regular with round cell bodies. D–E, After KA, CB1 expression was significantly increased in SR_{band}, OM and IM. F, Histology after KA showed considerable number of injured, shrunken, and eosinophilic CA1 neurons (arrows). After 0.5 mg (R+)WIN55, 212 + KA (G–H), CB1 expression was relatively uniform with moderate staining that was not significantly different from control untreated rats. I, Histology showed minimal injury at the lower doses. (J–K), Following 5 mg/kg (R+)WIN55, 212 + KA, CB1 expression was significantly reduced throughout the hippocampus; the highest the decreases in the DG and subiculum. (L) Histology showed highly significant injury in the CA1, resembling the KA only treated animals. M–N, After 5 mg/kg (R+)WIN55, 212, CB1 expression was also reduced throughout most of the CA1 and subiculum. O, Histology was similar to controls.

the animal that was not protected from the KA-induced seizure processed for immunohistochemistry, a second "unprotected" animal also had a marked reduction in CB1 protein in the Western blot (Fig. 5K lane 8). Animals treated only with KA showed more variable expression where they exhibited either steady or reduced levels (Fig. 5K–L). Similar to the immunohistochemistry, reduced expression was correlated with seizure severity.

4. Discussion

Previously, we have illustrated age-dependent differences in gene and protein expression of glutamatergic systems as a result of status epilepticus supporting that seizures have different effects at different ages (Friedman, 2006). The present study tested the effects of the exogenous cannabinoid agonist, (R+)WIN 55,212, on behavior, seizure threshold, and CB1 distribution during a critical stage in development, an age when the brain first becomes sensitive to seizure-induced hippocampal injury. In contrast to our expectation, a moderate dosage of (R+)WIN 55,212 (5 mg/kg) produced rapid behavioral manifestations including drunken, catatonia-like symptoms and had poor seizure control. In contrast, a 10 fold lower dose had no behavioral side effects but served as a potent anticonvulsant and neuroprotectant. Results indicate that cannabinoid agonists are highly efficacious anticonvulsants in the juvenile brain at low concentrations but disturb psychomotor systems which may lower the seizure threshold at higher concentrations.

4.1. Age-dependent effect of cannabinoids

Age-dependent effects of cannabinoid agonist treatment in rodents have recently emerged and may in part explain our dose dependent observations during the juvenile period. In hippocampal slices, depolarization of CA1 neurons provokes an endocannabiniod-mediated inhibition of GABA release, called depolarized-induced suppression of inhibition (DSI) and involves Group I metabotropic receptors (Sheinin et al., 2008; Ohno-Shosaku et al., 2001; 2002). In development, acute suppression of $GABA_A$ receptor mediated responses and DSI were

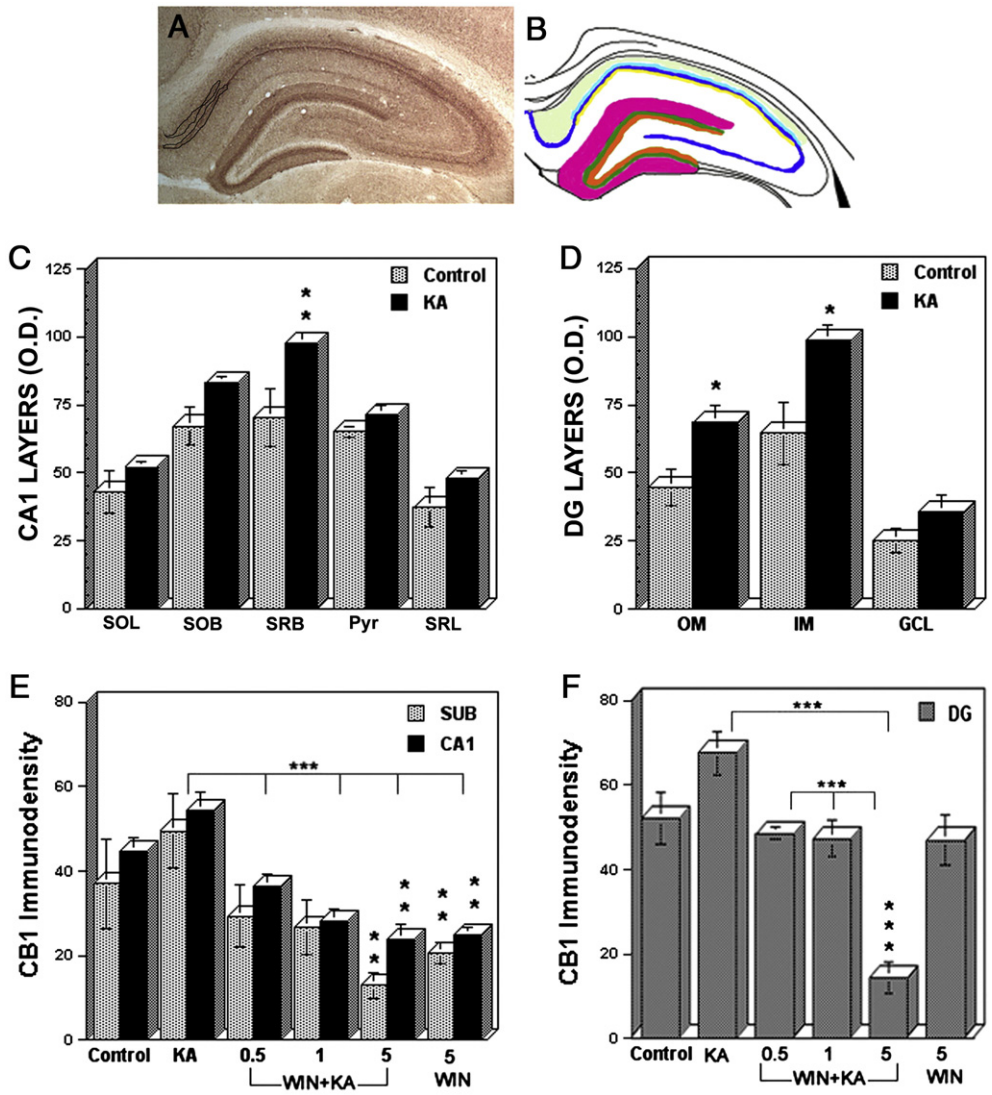

Fig. 4. A, Photomicrograph illustrating quantification of control CB1 distribution with DAB staining at the level of the hippocampus; laminated layers of the subiculum and CA1 were quantified separately as indicated (two loop sweeps). B, Schematic color coded representation of the laminated, layer-specific distribution of CB1 immunoreactivity. Hippocampal sub-layers: pale green — stratum oriens layer (SO_{layer}); light blue — stratum oriens band (SO_{band}); dark blue — pyramidal layer (Pyr); yellow — stratum radiatum band (SR_{band}); white — stratum radiatum layer (SR_{layer}); pink — DG outer molecular layer (OM), green — DG inner molecular layer (IM), orange — granule cell layer (GCL). C, Graphical illustration of CA1 layer-specific immunodensity measurements. After KA, there was a significant increase in CB1 optical density measurements within the SR_{band}. D, Quantification of the dentate gyrus showed the outer and inner molecular layers were significantly upregulated in CB1 expression after KA. E, In contrast, there was a linear decline in CB1 expression with increasing doses of (R+)WIN55, 212. The greatest decreases in CB1 expression were noted in the subiculum and at the highest dose [(5 mg/kg) of (R+)WIN55, 212 + KA0]. F, Decreased CB1 expression was also observed in the DG with increasing concentrations of (R+)WIN55, 212 + KA. Bars are means ± SEM of optical immunodensity measurements per group. One-way ANOVA, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

more pronounced in slices prepared from juvenile rats compared to adults whereas the suppression of excitatory transmission by (R+)WIN 55 212, was indistinguishable supporting that inhibitory synaptic function is more sensitive to cannabinoids in the immature brain (Kang-Park et al., 2007). In addition, acute Δ^9-tetrahydrocannabinol (Δ^9 THC) or (R+)WIN55, 212, 212 treatment markedly enhanced the proapoptotic properties of ethanol and the NMDA antagonist, MK801, during the first postnatal week which then declined thereafter as a function of age confirming the heightened sensitivity of cannabinoids in development (Hansen et al., 2008). Moreover, both acute and chronic treatment of (R+)WIN 55,212 induced more severe behavioral object/social recognition deficits in pubescent rats than in mature animals. Persistent disturbances in social behavior, social play and self-grooming were also reported (Schneider et al., 2008). Accordingly, chronic exposure of another CB1 receptor agonist (CP 55,940), led to increased anxiety and persistent memory dysfunction in adolescent but not adult rats (O'Shea et al., 2004). Even in adults chronic treatment of Δ^9 THC or (R+)WIN55, 212 can protect neurons and promote tolerance to acute psychotropic effects and to seizure-induced cognitive deficits. These were associated with reduced phophorylated-ERK (extracellular signal-regulated kinase) activation (Assaf et al., 2011; Romero et al., 1997). Similarly, lower doses of (R+)WIN 55,212 or anandamide provided the best protection from cerebral ischemia or oxygen deprivation to cultured neurons consistent with our observations (Nagayama et al., 1999).

In acute adult preparations, physiological studies also show that endocannabinoids differentially promote synaptic plasticity by suppressing inhibitory and excitatory neurotransmission of the hippocampus, cerebellum, and spinal cord (El Manira and Kyriakatos, 2010, review). In hippocampal slices, voltage clamped recordings illustrate that WIN 55,212 decreases stimulus-evoked $GABA_A$ receptor mediated IPSCs ($EC_{50} = 138$ nM) (Hoffman and Lupica, 2000). In the cerebellum,

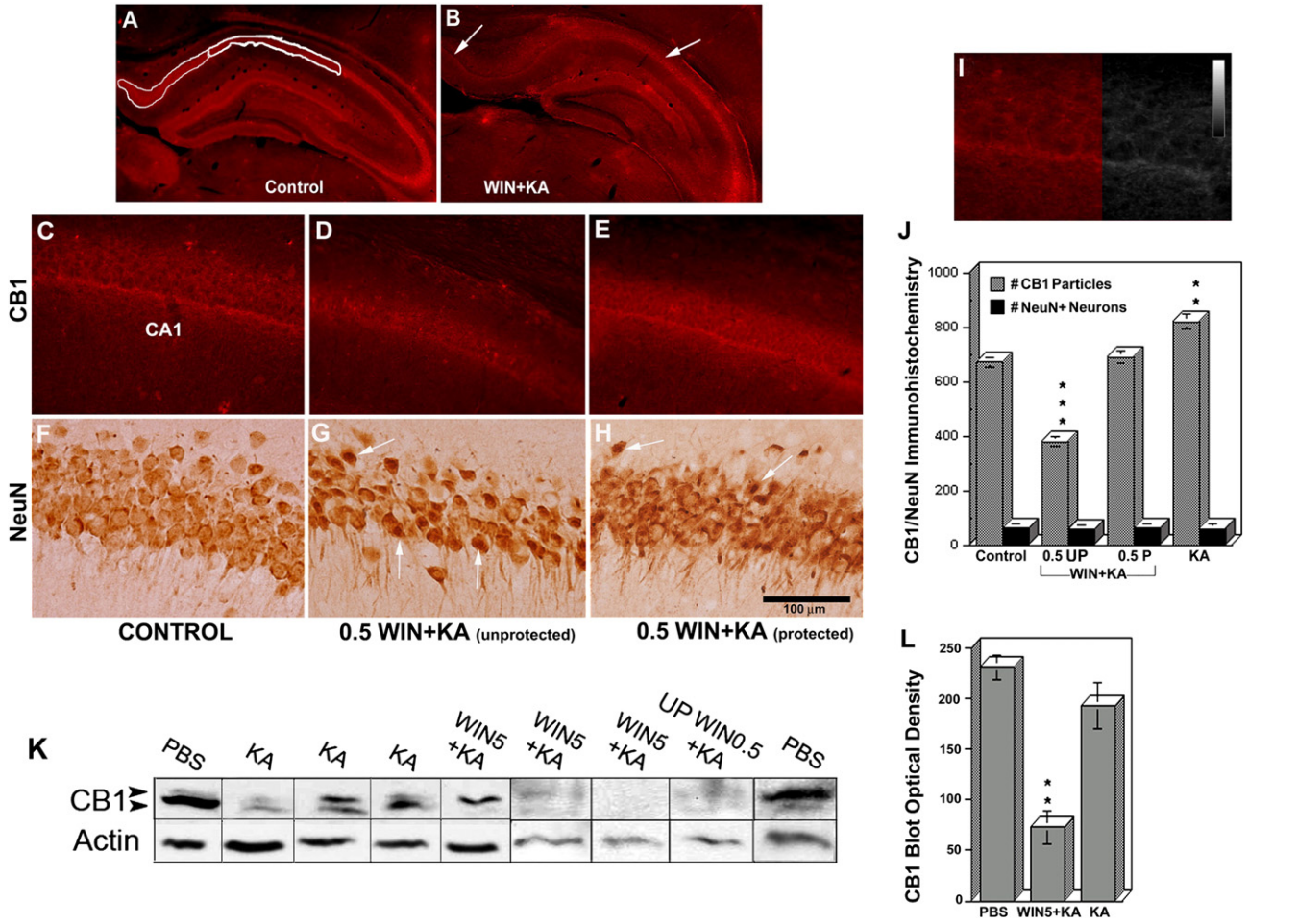

Fig. 5. A, Control immunofluorescence label showed uniform CB1 labeling conjugated to Texas Red (559 nm, 40× magnification). Laminated patterns were more difficult to distinguish therefore measurements were done in one sweep as indicated (one loop sweeps). B, Example at low magnification of low WIN + KA without anticonvulsant activity and loss of CB1 expression (40×). CB1 immunofluorescence (top panels) and NeuN immunostaining (bottom panels) of CA1 neurons in control (A, D) and 0.5 WIN + KA treated rats, one protected (C, F) and one unprotected (B, E) animals. C, Control CB1 immunofluorescence of the CA1 was intense and uniform; NeuN+ neurons labeled uniformly without nuclear staining (F). D, One of two cases where 0.5 mg/kg of WIN55, 212 did not prevent status epilepticus (seizure score = 3.8) causing decrease in CB1 immunofluorescence similar to higher doses and KA treatment; many NeuN+ neurons were dark and exhibited nuclear staining (arrows) and dispersion (G). E, Example of an animal with 0.5 mg/kg of WIN55, 212 and protection from seizures; CA1 neurons displayed intense uniform CB1 immunolabel resembling controls but with occasional nuclear staining in adjacent sections labeled with NeuN (seizure score = 1.7′) (arrows) (H). I, Luminescence quantification was performed in gray scale with Scion Image software. J, Quantification of CB1 immunofluorescence and the number of NeuN+ neurons revealed reduced CB1 expression in the presence of the cannabinoid agonist and increases after KA in its absence suggesting that downregulation depends on the severity of KA seizures and effective occupation of CB1 receptors but not the actual agonist dosage. J, Graphical analysis of immunodensity particles per group after thresholding with Scion image and counting of NeuN positive neurons. K, Western blotting of dissected subiculum/CA1 subregion of four conditions. L, Densitometry of the Western blot. Bars are means ± SEM. One-way ANOVA, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. Bar = 100 μm. UP = "unprotected"; P = "protected".

WIN 55,212 reduces mini IPSPs and increases Purkinje cell firing and inhibition which results in a change in the balance of motor network activity leading to disinhibition (Ma et al., 2008; Wang et al., 2011). These data are consistent with our observations where high WIN 55,212 led to motor manifestations and higher baseline frequency oscillations in the CA1 EEG recording. In the spinal cord, CB1 receptors are expressed in the dorsal horn but also on motor neurons, and were shown to contribute to normal locomotor patterns and play important roles in nociceptive information processing (Tsou et al., 1998; Hegyi et al., 2009; El Manira and Kyriakatos, 2010). High doses of WIN 55,212 likely competed with postsynaptically synthesized 2-AG to alter presynaptic release of GABA and glutamate and subsequent locomotor firing patterns of the spinal cord to contribute to the unusual standing and falling behaviors observed.

4.2. CB1 receptor distribution and neuronal vulnerability

The cellular and subcellular expression of CB1 type receptors to modulate ongoing activity was also theorized to critically contribute to the dose dependent effects of WIN 55,212 (Harkany et al., 2007; Herkenham et al., 1990). In adult rats after pilocarpine-induced seizures, specific decreases in CB1 immunoreactivity were observed in the pyramidal cell layer neuropil of the CA1 and dentate gyrus inner molecular layer; increases in staining were observed in the strata oriens and radiatum but were primarily in the CA2–CA3 subregions (Falenski et al., 2007). In our juvenile age group after KA, increases were observed predominantly in the strata oriens and radiatum layers surrounding the $CA1_{Pyr}$, (SO_{band}, and SR_{band}) not the CA3 and increases were also within both molecular layers of the dentate gyrus, consistent with increases observed in a Western blot study of the whole hippocampus when seizures were induced at a neonatal age (P10) (Schuchmann et al., 2006). However, our westerns of the isolated subiculum/CA1 subregion did not reveal an increase at 72 h post-treatment, probably due to dilution with the other sub-layers and during a time when injury can be present (Liu et al., 2006). The juvenile brain displayed a different pattern of CB1 receptor expression when compared with reports on adult rats suggesting that an age-dependent redistribution of CB1 receptors occurs after status

epilepticus. Since anatomical location of cannabinoid receptors regulates synaptic transmission this redistribution of CB1 receptors after an initial seizure episode may contribute to the higher seizure threshold of juvenile rats and help explain why they do not develop spontaneous seizures until after a certain age, and why serial KA-induced seizures at similar juvenile ages produce further resistance to seizures (Albala et al., 1984; Sarkisian et al., 1997). In keeping with this, animals with three KA-induced seizures (on P6, P9 and P20) also had high elevations in CB1 receptor expression (unpublished observation). In contrast, pre-exposure of (R+)WIN 55,212 at the higher dose (5 mg/kg) caused CB1 receptor expression to decline in the CA1 and DG when KA-induced seizures were severe. This is consistent with the pronounced and sustained downregulation of CB1 receptor expression in regions of the hippocampus reported 1–2 weeks post pilocarpine-induced status epilepticus (Falenski et al., 2009). Neuronal adaptations of the hippocampus have been attributed to dendritic re-organization that involving changes in MAP-2 (microtubule associate protein) immunostaining patterns in the absence of cell death (Lawston et al., 2000; Tagliaferro et al., 2006). Accordingly, induced loss of CB1 receptor number and activated G-proteins were previously proposed to reduce acute psychotropic and toxic effects (Breivogel et al., 1999). However, it must be taken into account that two animals that had the lower dosage of (R+)WIN 55,212 also had similar seizures, injury, and decline in CB1 expression akin to the 5 mg/kg treated animals (see Fig. 5) indicating that the severity of seizures was responsible for the level of hippocampal injury and CB1 receptor expression and not the actual concentration of WIN 55,212.

Other causes for protection at the lower doses aside from inhibition of glutamate release are anti-oxidation, reduced calcium influx and free radical scavenging (see review: Van der Stelt and Di Marzo, 2005). It should be also be noted that (R+)WIN55, 212 also can act upon CB2 type receptors in microglia that may be activated by KA seizures to reduce inflammation and neuronal cell loss (Van Sickle et al., 2005; Palazuelos et al., 2009). In addition, although not well understood, anandamide is a full agonist at vanilloid (type 1) receptors which may also contribute to the observed protection (Veldhuis et al., 2003). Developmental regulation of presynaptic calcium channels that are coupled to presynaptic CB1 receptors required to deactivate glutamatergic transmission may also contribute to the dose dependent effects on seizure threshold. Accordingly, seizures exert developmental differences on G-coupled metabotropic glutamate receptors (mGluR) whereby mGluR5 receptors are downregulated in the adult hippocampus (Kirschstein et al., 2007) but sustained in the immature hippocampus (Avallone et al., 2006). Moreover, perisynaptic mGluR5 receptors regulate the amount of glutamate spillover, so that loss of these receptors would impair the endocannabinoid retrograde signaling pathway in adult but not immature hippocampal neurons to contribute to the age-dependent seizure threshold. Furthermore, genetic inactivation of specific G-coupled metabotropic receptor proteins (G_q and G_{11}) in principal forebrain neurons caused age-dependent seizures and prevented the neuroprotective mechanisms produced by endocannabinoids due to their depletion in the mutants (Wettschureck et al., 2006). Thus, metabotropic receptors are key players in the balance between inhibition and excitation to regulate the seizure threshold as a function of age.

In summary, our study showed that a single low dose injection of (R+)WIN 55,212 served as a very potent and long-lasting antiepileptic in the juvenile brain. This implies that administration of an optimal dose of a cannabamimetic or inducing endogenous increases of postnatal cannabinoid synthesis may provide a groundbreaking therapeutic strategy for controlling seizures during certain stages in development without psychoactive or deleterious side-effects. We also found that seizures produce changes in the distribution of the CB1 receptor that may directly regulate the seizure threshold. Because CB2 and vanilloid type receptors represent an alternative nonpsychotropic site of action of endocannabinoids characterization with selective antagonists such as rimonabant are under investigation.

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