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Characteristics of tolerance in the guinea pig ileum produced by chronic *in vivo* exposure to opioid versus cannabinoid agonists

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ABSTRACT

Few studies have compared the nature of tolerance that develops following chronic opioid treatment with that which develops after chronic cannabinoid exposure in the same tissue and species. The degree and character of tolerance induced by 7 twice daily injections of morphine or 5 daily injections of the cannabinoid receptor agonist, WIN-55,212-2, was examined by comparing the ability of DAMGO, 2-chloroadenosine (CADO) and WIN-55,212-2 to inhibit neurogenic contractions of the longitudinal muscle/myenteric plexus preparation (LM/MP) and the ability of nicotine to elicit contractions in the LM/MP. Chronic morphine treatment resulted in subsensitivity to all inhibitory agonists (rightward shift in IC_{50} values of 4–5-fold) and an increased responsiveness to the excitatory effect of nicotine while chronic WIN-55,212-2 exposure resulted in subsensitivity only to WIN-55,212-2 and a reduction in maximum response to both WIN-55,212-2 and DAMGO but no change in responsiveness to CADO. Chronic WIN-55,212-2 treatment significantly reduced CB_1 but not MOR receptor protein abundance while chronic morphine treatment did not change either. Assessment of the distribution of MOR and CB_1 receptors in myenteric neurons revealed distinct individual receptor expression as well as co-localization which was unaffected by either cannabinoid or opioid treatment. Thus, in contrast to the heterologous tolerance that develops after opioid treatment, tolerance in the LM/MP following chronic *in vivo* WIN-55,212-2 exposure appears to be homologous in character and is accompanied by a selective decrease in CB_1 receptor protein abundance. The data suggest that the cellular basis of tolerance differs between the two systems.

1. Introduction

While opioids have been used clinically for centuries, the therapeutic utility of cannabinoids and related agents has only just begun to emerge. The use of both cannabinoids and opioids is associated with the emergence of tolerance [1,2] to common physiological effects like analgesia, hypomotility, hypothermia and inhibition of gastrointestinal motility [3,4]. Tolerance to the gastrointestinal effects of opioids following chronic exposure has been well studied, but little information exists regarding the development of tolerance to the acute gastrointestinal effects of cannabinoids [5,6]. Guagnini et al. [7] have, however, reported the development of tolerance in human intestinal tissue following *ex vivo* exposure to cannabinoids.

There is increasing emphasis on the common anatomical distribution and shared cell signaling pathways between opioid and cannabinoid receptor systems as potential overlapping sites for producing the physiological effects of each. Common cellular effects include inhibition of adenylyl cyclase [8], inactivation of Ca^{2+} channels [9,10], activation of the MAPK pathway [11], and activation of G protein-activated inwardly rectifying potassium (GIRK) channels [12]. Similar distribution of opioid and cannabinoid receptors in the central nervous system [13–15] and their cellular co-localization [16] may contribute to the comparable physiological and clinical effects [17] including dependence and tolerance [18].

Chronic exposure to opioids and cannabinoids has been shown to induce adaptive alterations in the responsiveness to agonists at other receptors coupled through $G_{i/o}$ proteins [19–21] as well as agonists that appear to act through different mechanisms [22]. Processes proposed to be involved in the development of homologous or heterologous tolerance include uncoupling of receptors from cognate G protein, receptor proteins, phosphorylation by G protein-coupled receptor kinase (GRK), changes in the adenylyl cyclase pathway and beta-arrestin-facilitated receptor internalization and downregulation. Our laboratory and others

Abbreviations: DAMGO, Tyr-D-Ala-Gly-N-methyl-Phe-Gly-ol; CADO, 2-chloroadenosine; LM/MP, longitudinal muscle/myenteric plexus; MOR, mu-opioid receptor; WIN-55,212-2, R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone; CB_1, cannabinoid receptor subtype 1.

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have studied the heterologous tolerance in the LM/MP following chronic exposure to morphine [23,24] and have proposed that it was due to membrane depolarization of the cell [22,25] which accounted for both increased sensitivity to excitatory agents and reduced responsiveness to inhibitory agents [23,26]. Later studies tied the depolarization to reduced expression of the alpha$_3$ subunit isoform of the Na^+/K^+-ATPase [27,28]. However, few studies have explored the character of the tolerance expressed in the longitudinal muscle/myenteric plexus (LM/MP) following chronic *in vivo* cannabinoid exposure.

The guinea pig LM/MP model is a reliable, robust *in vitro* model for evaluating the development of both tolerance and dependence [29–31]. Activation of both cannabinoid (CB_1) and opioid (kappa- and mu-) receptors reduce ileal intestinal peristaltic activity. CB_1 and mu-opioid receptors (MOR) are located on the soma and reduce transmitter release by hyperpolarization-mediated reduction in excitability while kappa opioid receptors, located on the axon terminals, decrease acetylcholine release by inhibiting calcium influx into the nerve terminal [32,33]. The present study investigated the changes in LM/MP sensitivity to inhibitory agents (WIN-55,212-2 [non-selective cannabinoid receptor agonist], DAMGO [selective MOR agonist] and 2-chloroadenosine (CADO) [adenosine receptor agonist]) and an excitatory agent (nicotine) following chronic *in vivo* treatment with morphine or WIN-55,212-2. CADO was included because previous studies suggested that it hyperpolarized 'S' neurons and produced inhibition of neurogenic contractions through a mechanism different from that of morphine [22]. Assessment and comparison of the effect of chronic *in vivo* opioid versus cannabinoid exposure on LM/MP sensitivity to nicotine and CADO would also provide insight into the possible mechanisms that may contribute to the development of tolerance.

Studies using *in vitro* exposure of the LM/MP preparation to opioid and cannabinoid agonists have reported the development of heterologous tolerance extending to both cannabinoid and opioid agonists [5–7] but no studies have assessed whether this type of change in response occurs following *in vivo* exposure or whether *in vivo* exposure to either agonist alters CB_1 and/or MOR protein abundance in the LM/MP. Based upon *in vitro* exposure data, we hypothesized that qualitatively similar tolerance will develop following *in vivo* drug exposure. Since previous studies have demonstrated that CB_1 activation results in a significant degree of CB_1 receptor internalization [34,35] whereas morphine displays cellular desensitization with low MOR internalization [36], we further hypothesize that the tolerance induced by CB_1 activation will be associated with receptor level changes whereas that induced by morphine will not be characterized by changes in receptor protein levels. In light of the controversies, conflicts and limited data comparing the development of tolerance following chronic *in vivo* opioid or cannabinoid exposure, we set out to determine whether chronic *in vivo* exposure to opioids or cannabinoids would: (1) *result in the development of heterologous tolerance*; (2) *lead to an increase in responsiveness to nicotine*; (3) *produce changes in CB_1 or MOR protein abundance; and/or* (4) *modify the spatial distribution of MOR and CB_1 positive neurons including those displaying receptor co-localization.*

2. Materials and methodology

2.1. Drugs and chemicals

Morphine (morphine sulfate pentahydrate salt), WIN-55,212-2 [(R)-(+)-[2,3-dihydro-5-methyl- 3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone], CADO (2-chloroadenosine), DAMGO ([D-Ala^2, N-Me-Phe^4, Gly^5-ol]-Enkephalin acetate) and nicotine (Nicotine hydrogen tartrate) were procured from Sigma–Aldrich Co. (St. Louis, MO). For organ bath studies, solutions of DAMGO, morphine and CADO were made by dissolving their respective salts in distilled water while the lipid soluble agent, WIN-55,212-2, was first dissolved in a vehicle containing DMSO/normal saline (1:9) from which serial dilutions were made using normal saline. Parenterally administered morphine was dissolved in normal saline whereas WIN-55,212-2 was dissolved in a vehicle consisting of normal saline and 10% DMSO (v:v).

2.2. Subjects

Dunkin-Hartley guinea pigs (Charles-River labs; Raleigh, NC) of either sex weighing 200–450 g were used in the study. The animals were housed two per cage with access to food and water *ad libitum*. The guinea pigs were kept in the animal facility for one week to permit acclimation prior to initiation of the treatment. All experimental procedures employing animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Brody School of Medicine at East Carolina University and were conducted in accordance with the guidelines for the humane use of animals in research (NIH "Public Health Service Policy on Humane Care and Use of Laboratory Animals" [revised 2002]). Every effort was made to reduce the use of animals to the minimum number required to achieve sufficient statistical power.

2.3. Procedures

2.3.1. Chronic drug injection schedules

The following dosing regimen was used for the morphine s.c. 7-day exposure: day 1, 10 mg/kg b.i.d.; days 2 and 3, 20 mg/kg b.i.d.; days 4–6, 40 mg/kg b.i.d.; and day 7, 80 mg/kg b.i.d. This protocol was based on one used in guinea pigs to produce tolerance [28] and dependence [37]. WIN-55,212-2 was administered at a dose of 6 mg/kg body weight once daily for 5 consecutive days. This drug regimen was adopted from a previously used regimen [38] and was modified following preliminary experiments performed to determine when tolerance is induced. Morphine was injected 12-hourly (10:00 a.m. and 10:00 p.m.) and WIN-55,212-2 was administered intraperitoneally once daily (10:00 a.m.) for 5 days. The animals were euthanized between 9 and 10 a.m. the following day after the last dose. The animals were weighed daily prior to dosing and were also examined daily for signs discomfort. The control groups were injected with the drug-free vehicle in a similar dosing schedule.

2.3.2. Longitudinal smooth muscle/myenteric plexus (LM/MP) preparations

The LM/MP from treated animals was removed and isolated as previously described by Taylor et al. [24]. Segments of the ileum were obtained from animals sacrificed by decapitation following isoflurane anesthesia. The abdomen was opened to expose the cecum. The 10 cm section of ileum closest to the cecum was removed and discarded, and 2–4 cm segments of ilea from the adjacent 10 cm of ileum were used to set the LM/MP preparation. The segments of ilea were threaded onto a glass rod and, using a cotton swab moistened with Krebs solution, the LM/MP carefully stripped tangentially from the point of mesenteric attachment until the muscle-nerve preparation was detached from the total area of the ileum.

The resulting sheet of LM/MP was tied at each end with a fine thread, passed through platinum-ring electrodes and placed in a 10 ml organ bath containing Krebs buffer solution. One thread was tied to a PowerLab force transducer and the other fixed to a tissue holder. A basal tension of 1.0 g was set and isometric tension generated by the muscle was recorded using the PowerLab/Chart 5 computer program (AD Instruments, Colorado Springs, CO)

through a 4 channel power lab system using a 4 channel Quad bridge converter interface (AD instruments, Colorado Springs, CO).

The tissues were maintained at 37 °C in a physiological Krebs buffer solution bubbled continuously with a mixture of 95% O_2/5% CO_2 consisting of the following (in mM): NaCl (117), KCl (4.7), $CaCl_2$ (2.5), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $NaHCO_3$ (25) and Dextrose (11.5). Neurogenic contractions were elicited via electrical stimulation using supramaximal voltage delivered to the tissue through platinum-ring electrodes using a stimulation system consisting of a Grass S48 stimulator connected to the electrodes by a Med Lab Attenuator and Stimu-splitter. To ensure only nerve endings were stimulated, the following parameters were used: voltage (50 V); impulse duration (<1 ms); delay setting (zero); and frequency (0.1 Hz).

During the initial 1 h equilibration period, the buffer solution was replaced at 15 min intervals. Following equilibration, the tissues were exposed to cumulatively increasing concentrations of the inhibitory drugs (final concentrations in the organ bath ranging between 1 nM and 10 μM). Three 5 min washes followed by three 15 min washes with drug-free Krebs solution performed between concentration–response curves of different drugs permitted full recovery of the amplitude of neurogenic contractions. In each experiment, two LM/MP preparations from each test group of animals were studied simultaneously and the responses of the tissues from the same animal averaged. The effect of each agonist on the amplitude of the neurogenic contractions was calculated as percent inhibition from the original amplitude. Due to the highly lipophilic nature of the cannabinoid agonist WIN-55,212-2, concentration–response curves for this agonist were always constructed last as neurogenic contractions were not recovered following exposure to this agent. Each of the other agonists was alternated in sequence to reduce the impact of the sequence upon the calculated IC_{50} value. Geometric mean IC_{50} values were calculated and compared among treatment groups as previously described [24].

The procedure outlined above was also used for experiments using nicotine with the only difference being the absence of electrical stimulation since nicotine elicited contractions by ganglionic stimulation of acetylcholine release. Nicotine (final bath concentrations ranging between 0.3 and 100 μM) was added in a non-cumulative manner with at least three 5-min washes performed before the next drug concentration was added. Responses were calculated in grams of tension or percent of the maximum contraction for that tissue. The values were used to determine the EC_{50} (i.e. concentration required to produce 50% of the maximum response) and to calculate the maximum tension produced by nicotine. Computer assisted analysis of each concentration–response curve using Sigmaplot® software (SPSS Inc.) was employed to determine the IC_{50} or EC_{50}.

2.3.3. Receptor protein analysis

Western blotting was used to determine the MOR and CB_1 receptor protein levels in the LM/MP. The LM/MP tissue prepared as outlined earlier was immediately snap frozen in liquid nitrogen then stored at −80 °C pending homogenization at which time the tissue was thawed, weighed and placed in ice-cold protease inhibitor buffer (0.25 M sucrose, 10.0 mM EDTA, 4.08 mM phenylmethylsulfonyl fluoride, 1 mM 4-aminobenzamidine and 1 mg/ml bacitracin) and homogenized using a glass homogenizer (PowerGen 125; Fisher Scientific, Pittsburgh, PA). The homogenate was centrifuged at 14,000 rpm for 5 s and the supernatant used for analysis.

Spectrophotometric protein determination was performed using a Pierce® BCA protein assay kit (Pierce Biotechnology, Rockford, Illinois). Tissue homogenates or standard samples were mixed with Pierce® BCA reagent A/B solution and absorbance was measured in triplicate using the Synergy HT spectrophotometer (BiTek®) at a wavelength of 562 nm. The sample absorbance was calculated and protein concentrations extrapolated from a standard curve generated using pre-determined standard samples of bovine serum albumin.

The Western blotting procedure is similar to that previously described [27]. Homogenates containing 10 μg total protein were loaded on 10% precast Tris–HCl Ready gels (Bio-Rad Laboratories, Inc., Hercules, CA) and size fractionated via electrophoresis at 110 V using a Mini-PROTEAN II Cell (Bio-Rad Laboratories, Inc., Hercules, CA). The proteins were then transferred to nitrocellulose membranes presoaked in transfer buffer using a Trans-Blot SD semi-dry transfer cell (Bio-Rad Laboratories, Inc., Hercules, CA). After allowing the membrane to completely dry, it was prehybridized in pre-made Odyssey® blocking buffer (Li-Cor Biosciences, Lincoln, NE) for 3 h. The membrane was washed with phosphate buffered saline (PBS: 137 mM NaCl; 2.7 mM KCl; 10.1 mM Na_2HPO_4 and 1.8 mM KH_2PO_4) containing Tween 20 (0.1%) three times for 15 min, and incubated overnight with the primary antibody. The following primary antibodies were used; mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:60,000 – Millipore, Burlington, MA), rabbit anti-MOR (1:2000 – Millipore, Burlington, MA) and rabbit anti-CB_1 (1:200 – Cayman, Ann Arbor, MI). Following incubation, the blots were washed three times with PBS-T and incubated for 1 h with the appropriate secondary antibody obtained from Li-Cor® Biosciences (Lincoln, NE). The final blot was washed an additional three times in PBS to remove any excess secondary antibody before detection using the Odyssey® near-infrared imaging system (Li-Cor® Biosciences).

2.3.4. Immunofluorescence procedures

2.3.4.1. Tissue preparation. Ileum segments (1 cm) excised from the terminal ileum were placed in cold PBS solution where they were cut longitudinally along the mesenteric border. The sheet of tissue was then pinned on a dissecting plate containing Sylgard® with the mucosal side facing up. The tissues were incubated and fixed overnight in 4% paraformaldehyde. Following the overnight incubation, the paraformaldehyde was removed by rinsing the tissue with PBS. The mucosal and circular layers were removed using fine forceps under a microscope and the LM/MP immersed and stored in PBS containing 0.1% sodium azide at 4 °C pending immunofluorescence probing.

2.3.4.2. Immunostaining procedure. The CB_1 receptor was localized using a rabbit primary polyclonal antibody (Cayman, Ann Arbor, MI) directed against the C-terminal (1:50) and the secondary antibody was a donkey anti-rabbit FITC-conjugate (1:100 – Jackson Immunoresearch, Westgrove, PA). MOR was detected using a goat primary polyclonal antibody (Santa Cruz, Santa Cruz, CA) directed against the C-terminal (1:50) and the secondary antibody was a donkey anti-goat Cy5-conjugate (1:100 – Jackson Immunoresearch, Westgrove, PA). Prior to primary antibody incubation, the LM/MP tissues were blocked with donkey serum (10%) for 45 min. Dual-labeling experiments were performed with simultaneous incubation of antibodies targeting both MOR and CB_1 populations. The tissues were incubated overnight using the primary antibodies at 4 °C. Following the overnight incubation, the tissues are washed with phosphate buffered saline (PBS) and then incubated for 4 h at room temperature in a mixture containing both FITC- and Cy5-conjugate secondary antibodies. Negative control experiments excluded the primary antibodies and these revealed negligible faint labeling due to non-specific binding of the secondary antibodies.

2.3.4.3. Image acquisition and processing. A Zeiss® LSM 510 laser scanning confocal microscope and imaging system was used for image acquisition and processing. The donkey anti-goat Cy5-

conjugate was excited at 633 nm, whereas the donkey anti-rabbit FITC-conjugate was excited at 488 nm. In dual-labeling experiments, a composite image targeting the FITC- and Cy5- conjugates was scanned simultaneously and could be separately analyzed offline.

2.3.4.4. Assessment of MOR and CB_1-immunopositive cells. Qualitative analysis was used to determine the distribution pattern and extent of co-localization of MOR and CB_1 positive neurons. Cells were considered to be immunopositive if they expressed visually detectable labeling. Immunopositive neurons with bright to faint labeling were analyzed, because the faint labeling could represent low protein expression in positively labeled cells. Zeiss® LSM software was used to capture the images and Image J® software was used to analyze the distribution and possible co-localization of MOR and CB_1-immunopositive neurons, and was also employed to determine the density of immunopositive neurons. The density of immunopositive neurons was assessed by counting the number of immunopositive neurons in a manually circumscribed region of the myenteric ganglia (i.e. number of neurons/area of circumscribed region). The area of the circumscribed region was computed using Image J® software whereas the number of visually detectable immunopositive neurons was counted manually. Neurons expressing both receptor proteins were counted individually and converted to a density based upon the circumscribed area evaluated. The relative expression of co-localized was expressed as a percentage of the total population of neurons possessing both receptor proteins versus those neurons expressing either CB_1 or MOR receptor proteins only.

2.3.5. Data analysis

For the LM/MP organ bath experiments, the sensitivity of a group of tissues to an inhibitory agonist was determined by calculating the mean negative log of the concentration producing 50% inhibition of the electrically induced contraction ($IC_{50} \pm$ SEM). Percent inhibition was calculated using the mean contraction height at the maximum inhibition following addition of a given concentration of agonist, divided by the average contraction height 1 min before exposure to the initial dose of that agonist. Differences in sensitivity to a given agonist between two groups of tissues were determined by comparing the geometric mean IC_{50} values and the mean ratio of geometric mean IC_{50} values (calculated as the mean antilog of the difference in IC_{50} values between the two groups). For nicotine stimulation experiments, the EC_{50} (i.e. the concentration of drug required to produce a contraction magnitude equal to 50% of the maximum contraction obtained in that tissue) and the maximal isometric tension developed were determined and compared. Analysis of the immunoblotting results was performed by comparing the receptor protein to GAPDH protein intensity ratios (i.e. MOR or CB_1/GAPDH densitometric units) between the control and the test groups. Immunofluorescence images were analyzed qualitatively to determine co-localization of immunopositive neurons expressing both MOR and CB_1. Significant differences between the test and control groups were determined using unpaired Student's "*t*" test. Comparison of mean values between three or more groups was performed using one-way ANOVA followed by the appropriate post hoc test, usually Tukey's test, with the probability level of ≤ 0.05 accepted as significantly different.

3. Results

3.1. LM/MP organ bath assay

Treatment for 7-days with morphine resulted in the development of subsensitivity to 2-chloroadenosine (CADO), DAMGO and WIN-55,212-2, suggesting that the heterologous tolerance previously reported extends to cannabinoid agonists as well. As illustrated in Fig. 1, concentration–response curves showed a significant rightward shift for all agonists and comparison of the calculated IC_{50} values of the morphine and vehicle treated groups

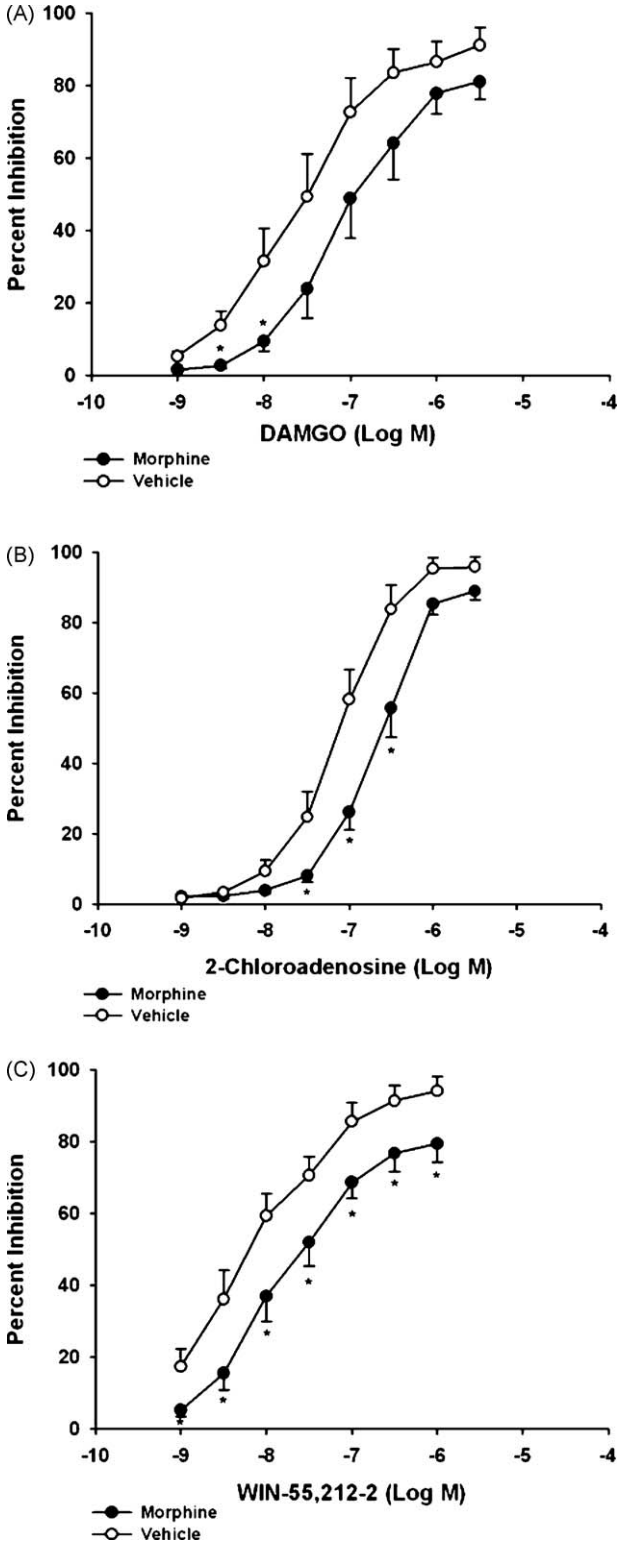

Fig. 1. Mean concentration–response curves for DAMGO (A), CADO (B) and WIN-55,212-2 (C) in LM/MP from control and guinea pigs chronically treated with morphine. A significant rightward shift of the concentration–response curve was observed for DAMGO, CADO and WIN-55,212-2. The maximum response obtained to WIN-55,212-2 was significantly reduced Statistically significant differences ($p \leq 0.05$) are identified by *. The *N* value for each experimental set is 8.

revealed a significant reduction in responsiveness as indicated by the significantly lower IC_{50} values: DAMGO, 6.8 vs. 7.5 ($p = 0.04$); CADO, 6.6 vs. 7.1 ($p < 0.01$); WIN-55,212-2, 7.5 vs. 8.2 ($p < 0.01$). The calculated magnitude of rightward shift (i.e. ratio of mean IC_{50} values) or loss of sensitivity of the treated compared to the control group was 4.8-fold for DAMGO, 3.5-fold for CADO, and 5.2-fold for WIN-55,212-2. The calculated mean ratio of IC_{50} values (based on the determined geometric mean IC_{50} values for each group) for tissues obtained from animals that received morphine compared to the control group was not significantly different among the three agonists hence the degree of tolerance development was quantitatively comparable (F (2, 5) = 1.07, $p = 0.37$). In addition, analysis of the maximum inhibitory effect for each of the agonists between the control and morphine-treated groups did not show any statistically significant difference in the magnitude of the maximum inhibitory response for DAMGO ($p = 0.17$) and CADO ($p = 0.06$). However, chronic treatment with morphine did lead to a significant reduction (23%; $p = 0.02$) in the maximum response to WIN-55,212-2.

As illustrated in the concentration–response curves presented in Fig. 2, 5-day treatment with WIN-55,212-2 resulted in a reduction in sensitivity to WIN-55,212-2 only as shown by the IC_{50} values for the WIN-55,212-2 and vehicle groups: DAMGO, 7.67 vs. 7.73 ($p = 0.52$); CADO, 7.2 vs. 7.3 ($p = 0.13$); WIN-55,212-2, 7.3 vs. 8.3 ($p < 0.001$). Data calculated from Fig. 2 revealed that the magnitude of rightward shift in the concentration–response curve (i.e. ratio of mean IC_{50} values) was 1.3-fold for CADO, 1.1-fold for DAMGO and 9.8-fold for WIN-55,212-2. The rightward shift of the IC_{50} values was only statistically significantly different for WIN-55,212-2, suggesting that homologous tolerance had developed in the LM/MP preparation following chronic *in vivo* cannabinoid treatment. Analysis of the maximum inhibitory effect revealed a statistically significant reduction (25%; $p < 0.001$) in the maximum inhibition of neurogenic contractions produced by WIN-55,212-2 in tissues obtained from animals chronically treated with WIN-55,212-2. Interestingly, a small but statistically significant reduction (11%; $p = 0.03$) in the maximum inhibitory response to DAMGO was also observed in those same tissues obtained from animals chronically treated with cannabinoid agonist. In contrast, chronic cannabinoid treatment produced no significant change in the maximum inhibitory response obtained to CADO in the same tissues. The impact of chronic drug treatment on the response of the LM/MP to nicotine, illustrated in Fig. 3, revealed no statistically significant difference in the nicotine EC_{50} values following either chronic morphine (treated, 5.32 vs. vehicle, 5.45; $p = 0.15$) or WIN-55,212-2 treatment (treated, 4.98 vs. vehicle, 5.34; $p = 0.24$). However, chronic morphine treatment (treated, 3.7 g vs. vehicle, 2.44 g; $p = 0.04$) but not WIN-55,212-2 treatment (treated, 2.73 g vs. vehicle, 2.68 g; $p = 0.89$) produced a significant increase the magnitude of the maximum contractions produced by nicotine (Fig. 3C).

3.2. Assessment of MOR and CB_1 protein abundance

In light of the fact that chronic agonist exposure often results in receptor downregulation, we assessed whether the treatments had any effect on receptor protein levels. Fig. 4 shows results of Western blot analyses performed to assess possible changes in the MOR and CB_1 receptor protein abundance following chronic opioid or cannabinoid exposure. Quantitative analysis involved comparison of the specific receptor protein to GAPDH intensity ratios (MOR or CB_1/GAPDH densitometric units) between the control and the test groups. CB_1 receptor protein analysis showed a statistically significant reduction in receptor protein abundance in homogenates from tissues obtained from the WIN-55,212-2 treated group compared to the control (Fig. 4A) as indicated by a 32% ($p = 0.04$) lower CB_1/GAPDH intensity ratio. In contrast, no statistically significant difference was observed in the MOR/GAPDH intensity ratios between the control and WIN-55,212-2 treated groups ($p = 0.29$). As illustrated in Fig. 4B, 7-day morphine

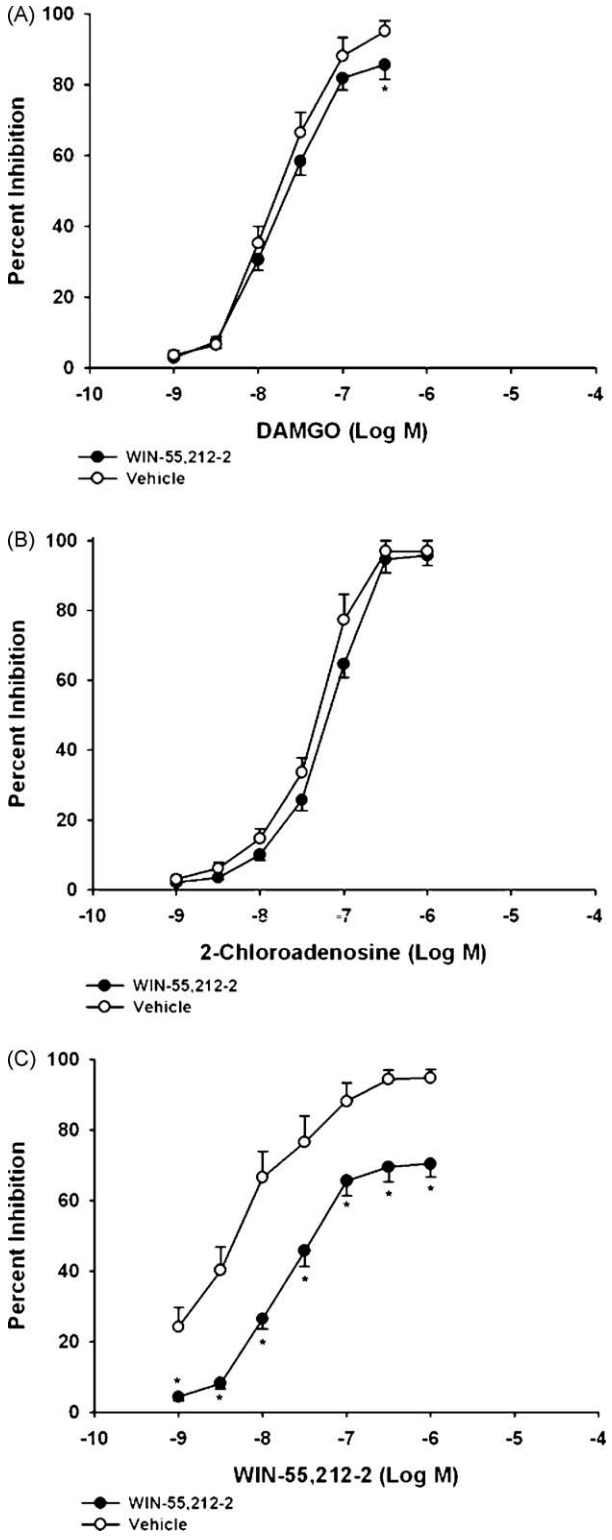

Fig. 2. Mean concentration–response curves for DAMGO (A), CADO (B) and WIN-55,212-2 (C) in LM/MP from control and guinea pigs chronically treated with WIN-55,212-2. A significant rightward shift of the curve was observed for WIN-55,212-2 only. No significant rightward shift observed for DAMGO and CADO. Significant reduction in the maximum response was observed to both WIN-55,212-2 and DAMGO. Statistically significant differences ($p \leq 0.05$) are identified by *. The N values for the experimental sets are between 6 and 13.

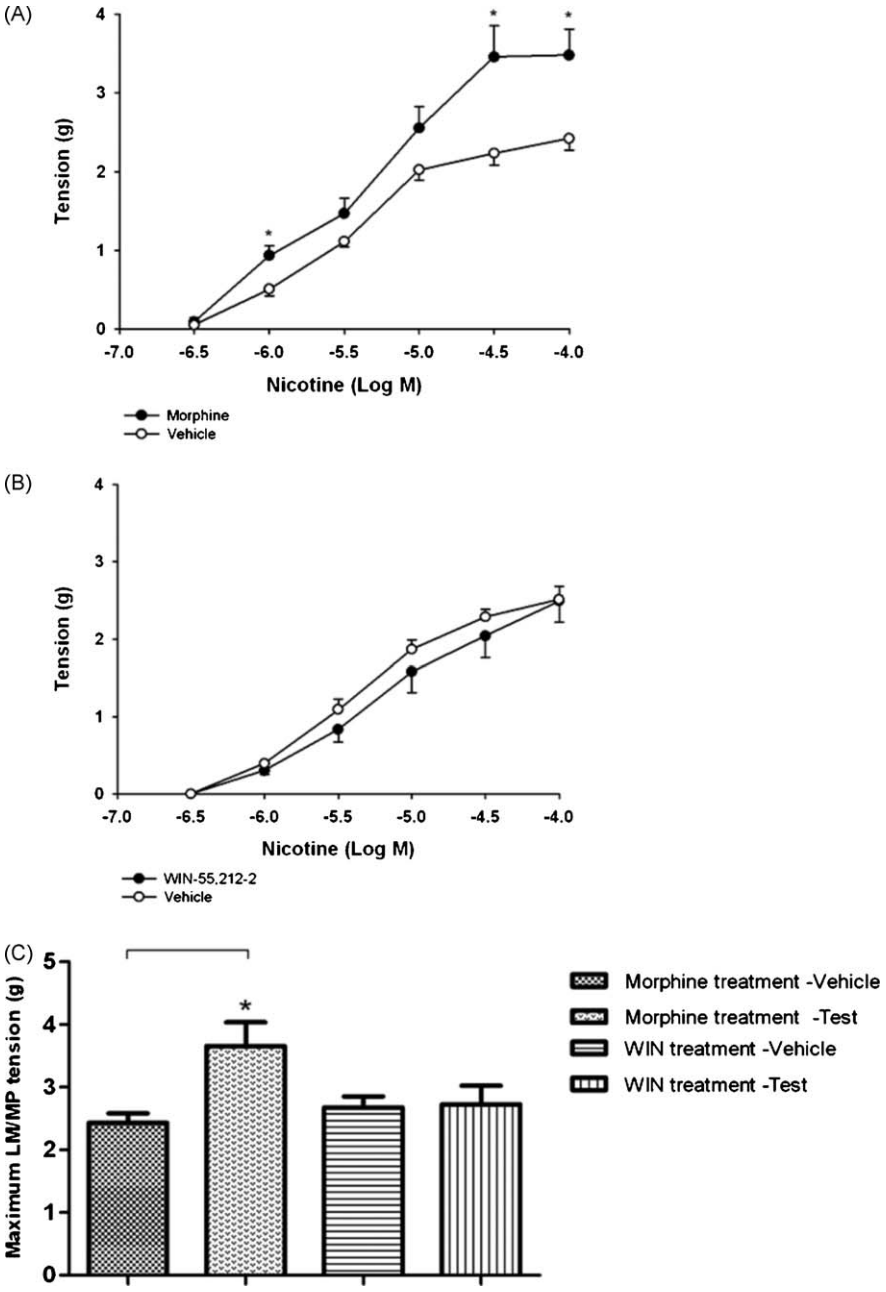

Fig. 3. Mean concentration–response curves for nicotine in LM/MP preparations obtained from morphine or WIN-55,212-2 pretreated guinea pigs and their respective controls. No significant change in the EC_{50} values for nicotine was observed following chronic treatment with morphine (A) or WIN-55,212-2 (B). (C) A bar graph comparing the maximal tension values attained by nicotine stimulation in the control and test groups. A significant increase in nicotine maximal effect is observed in morphine-treated animals. Statistically significant differences ($p \leq 0.05$) are identified by *. The *N* value for each experimental set is 4.

treatment did not alter either MOR ($p = 0.59$) or CB_1 receptor protein abundance ($p = 0.79$). For both treatments, no statistically significant difference was observed in the GAPDH levels between control and drug-treated tissue homogenates thus implying that the total cellular protein levels remained relatively constant.

3.3. Immunofluorescence assessment of CB_1 and MOR localization

Representative images from whole mounts of the guinea pig LM/MP showing CB_1 receptor and MOR-immunopositive neurons in the myenteric plexus are provided in Fig. 5. The red immunofluorescence (Fig. 5A) indicates MOR-expressing neurons while the green immunofluorescence (Fig. 5B) depicts CB_1 receptor-expressing neurons. Neurons expressing both receptor populations appear orange-yellow (Fig. 5C) in color. There are a number of important characteristics observed from these tissues. As indicated in Fig. 5A and B which depict MOR and CB_1 receptor immunopositive neurons, respectively, it appears that a significant proportion of myenteric neurons actually express both receptor populations simultaneously. It is also interesting to note that neurons exclusively expressing MOR protein seem to reside predominately in the periphery of the myenteric ganglia whereas neurons that exclusively express CB_1 receptors appear to congregate in the central part of the ganglia; this spatial distribution pattern was consistent in drug-naïve, vehicle, WIN-55,212-2 or morphine-treated animals. Finally, there appears to be some degree of co-localization as indicated by the high intensity of the orange-yellow color in Fig. 5C. The co-localization observed

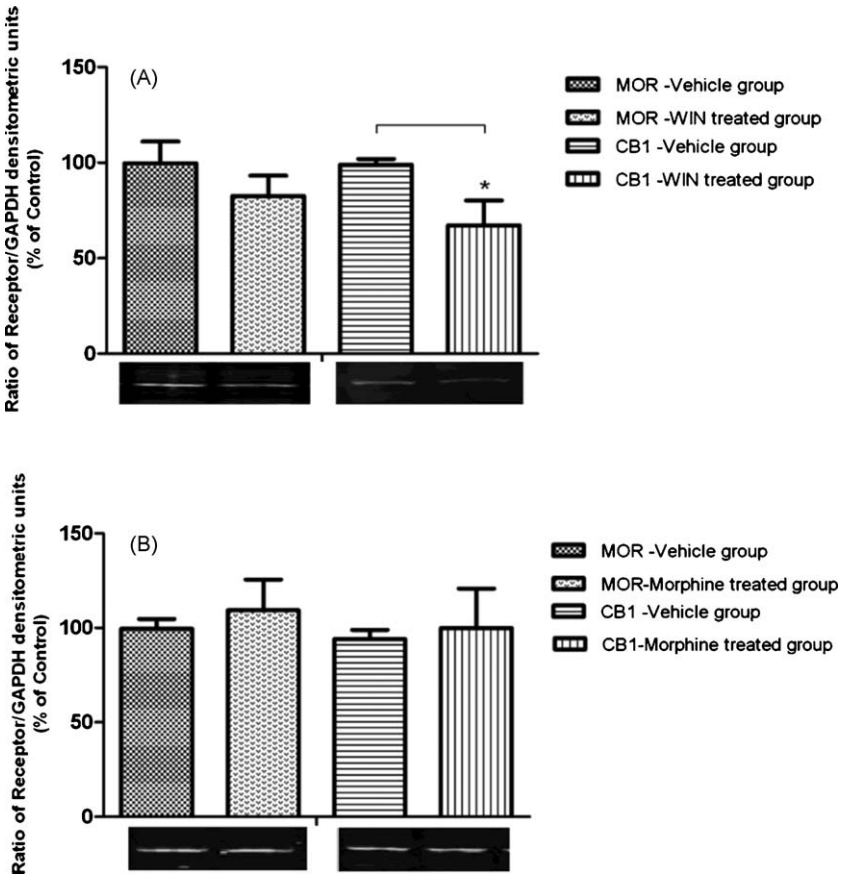

Fig. 4. Comparison of MOR and CB_1 receptor protein levels following chronic morphine or WIN-55,212-2. (A) The ratio of the CB_1 receptor or MOR protein intensity to the GAPDH intensity from animals treated chronically with WIN-55,212-2. (B) The ratio of the CB_1 or MOR protein intensity to the GAPDH intensity from animals treated chronically with morphine. Respective prototypical images of the immunoblot membrane showing the density of the MOR and CB_1 protein are shown below the bar graphs. $^*p < 0.05$ considered to be statistically significant. The *N* values for the experimental sets are between 4 and 6.

(Fig. 5C) between the MOR and CB_1 receptor positive neurons further suggests that the two receptors are co-expressed in some but not all myenteric neurons. The results in Fig. 5D show a similar relative density of neurons expressing MOR or CB_1 in the LM/MP as assessed using Image J® software (16.7 neurons/μm^2 vs. 14.3 neurons/μm^2 respectively; $p = 0.28$). The density of neurons co-expressing both CB_1 and MOR receptors in drug-naïve animals was 2.9 neurons/μm^2 which translates to 17% and 20% of the total MOR or CB_1 receptor populations, respectively.

Comparison of vehicle or WIN-55,212-2 treated groups (Fig. 6A) shows no statistically significant difference the density of MOR-immunopositive ($p = 0.86$), CB_1-immunopositive neurons ($p = 0.79$) or neurons co-expressing both receptors ($p = 0.14$). Similarly, chronic morphine treatment did not alter the relative density of MOR ($p = 0.23$), CB_1-immunopositive neurons ($p = 0.11$) or neurons co-expressing both receptors ($p = 0.09$). In tissues obtained from animals chronically treated with morphine (Fig. 6A) or WIN-55,212-2 (Fig. 6B) or respective drug-free vehicle solutions, the relative density of neurons expressing either CB_1 receptors ($F(4, 3) = 1.05$, $p = 0.45$) or MOR ($F(4, 3) = 1.43$, $p = 0.33$) was similar in magnitude to that observed in naïve animals (Fig. 5D). Interestingly, in the preparations from vehicle and drug-treated animals, the relative percentage of neurons co-expressing both families of receptors appears to slightly increase from the 20% level seen in naïve animals to around 50% in the preparations from treated animals. Negative controls showed faintly labeled neurons, possibly due to non-specific binding, but the signal was not as nearly as intense as that observed in the non-control test groups.

4. Discussion

Based upon the similarity of cellular, physiological and pharmacological effects of cannabinoids and opioids, and the conflicting results regarding the character of tolerance that develops following chronic exposure, we conducted experiments to determine whether parenteral *in vivo* exposure to either opioids or cannabinoids produced tolerance that was qualitatively similar. In order to assess potential cellular mechanisms, we also sought to determine the LM/MP sensitivity and responsiveness to excitatory agents like nicotine following chronic drug treatment. Since we also hypothesized that chronic *in vivo* treatment with cannabinoids but not opioids would evoke adaptive responsiveness associated with changes in receptor protein levels, we investigated the impact of chronic opioid or cannabinoid exposure on both the CB_1 receptor and MOR protein abundance. The present study found important qualitative similarities of the tolerance that develops following chronic 7-day morphine exposure with that previously reported in both *in vivo* and *in vitro* exposure studies [6,24,28]. However, the qualitative nature of the change in sensitivity to a limited number of agents (i.e. homologous tolerance) observed following chronic WIN-55,212-2 treatment appears to contrast with that previously reported in morphine tolerant animals which exhibit subsensitivity to a variety LM/MP inhibitory agents and increased responsiveness to excitatory agents like nicotine [23,26]. This difference in the character of the subsensitivity was not predicted but could have important implications for defining the potential mechanisms that underlie the development of the

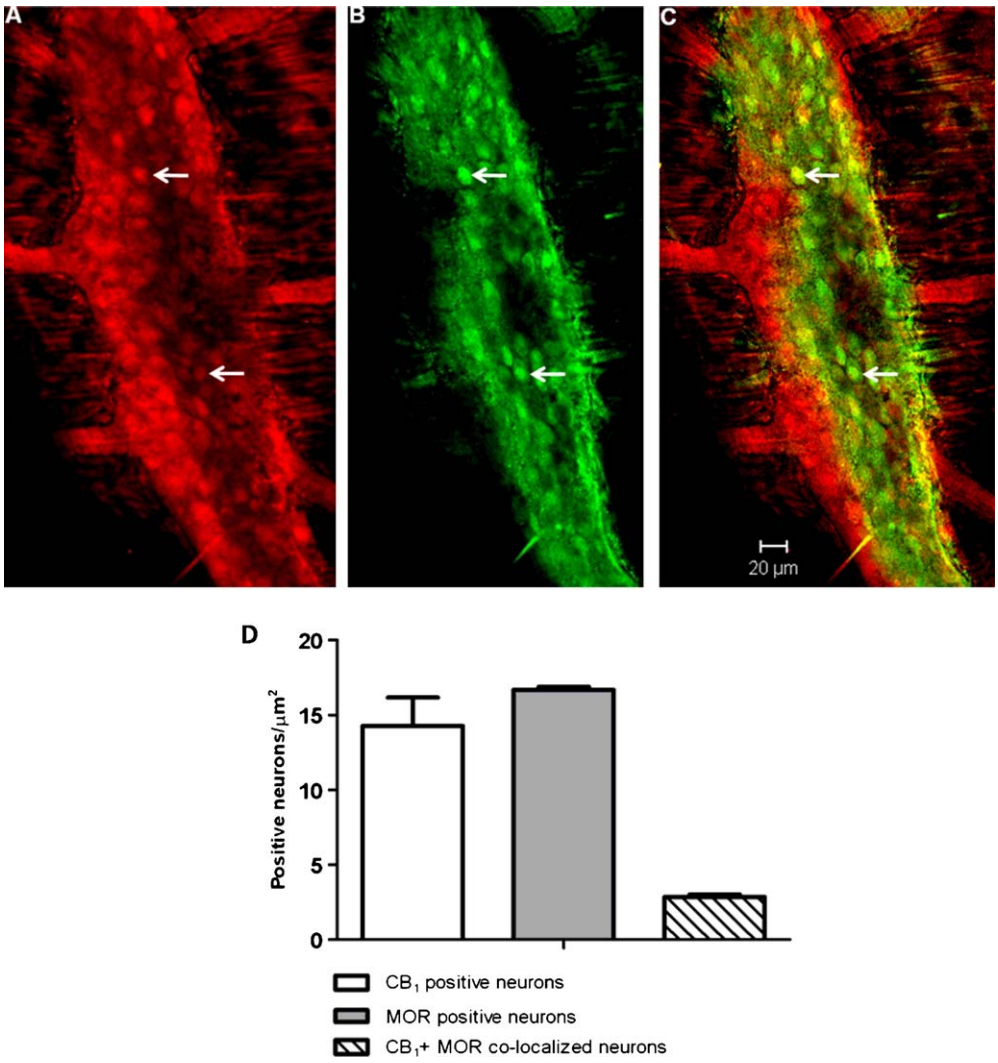

Fig. 5. Representative images from whole mounts of the LM/MP of drug-naïve animals showing immunofluorescence in neurons expressing MOR and CB_1 receptors in myenteric ganglia. (A) MOR-immunopositive neurons (red) only whereas (B) illustrates CB_1 receptor immunopositive neurons (green). (C) A merged image depicting neurons in the myenteric plexus immunopositive for both MOR and CB_1 receptors. (D) A comparison of the density of MOR and CB_1 receptor immunopositive neurons (number of immunopositive neurons per unit area) in the myenteric plexus of drug-naïve animals. The graph also illustrates the density of neurons co-expressing MOR and CB_1 receptors. Each bar represents the mean ± S.E.M. of measurement from three guinea pigs. $^*p < 0.05$ considered to be statistically significant.

phenomenon in the guinea pig LM/MP and could provide an avenue to explore the combinatorial use of these agents therapeutically.

The development of heterologous tolerance following chronic *in vivo* exposure to morphine is consistent with previously reported data [24,28] that have shown tolerance to develop between 1 and 4 days of exposure, become maximal by day 7 and remain intact for several days beyond that [25,28]. However, these are the first studies in the LM/MP model to examine the question of whether the heterologous subsensitivity following chronic *in vivo* opioid exposure extended to the cannabinoid receptor agonist, WIN-55,212-2. The observed subsensitivity to WIN-55,212-2 substantiates previous studies using the LM/MP that revealed heterologous tolerance following chronic *in vitro* exposure to opioids [6]. However, the change in responsiveness was accompanied by a significant reduction in the maximum response suggesting that there may also be a change in efficacy [39]. Since opioids and cannabinoids employ similar and, perhaps, overlapping signaling pathways, the shared components of these pathways offer some sites at which adaptive changes could occur that impact upon both systems. However, the basis for the difference in efficacy could also reside in the receptor reserve that is present for each receptor family. As demonstrated in these studies, chronic morphine treatment did not alter the abundance of either MOR or CB_1 receptor protein; this is consistent with the maintenance of the maximal inhibitory effect of DAMGO, and suggests an adaptive mechanism independent of receptor level changes to be, at least in part, responsible for the development of tolerance. Furthermore, the inability of chronic morphine exposure to reduce MOR protein or the maximal effect of DAMGO is consistent with previous studies showing that morphine has a low capacity to induce MOR internalization and downregulation [36] and supports the fact that the MOR reserve approaches ~90% the LM/MP [40]. We have proposed that the cellular plasticity underlying heterologous tolerance involves a partial depolarization of the resting membrane potential [22] secondary to a reduction in the level of functional $alpha_3$ subunit of the Na^+/K^+-ATPase [27,28,41]. The alteration in membrane potential ultimately accounts for the reduced sensitivity to inhibitory agents (e.g. DAMGO, CADO and WIN-55,212-2) and the enhanced responsiveness to excitatory agents like nicotine. The reduction in WIN-55,212-2 maximal response could have been caused by a non-

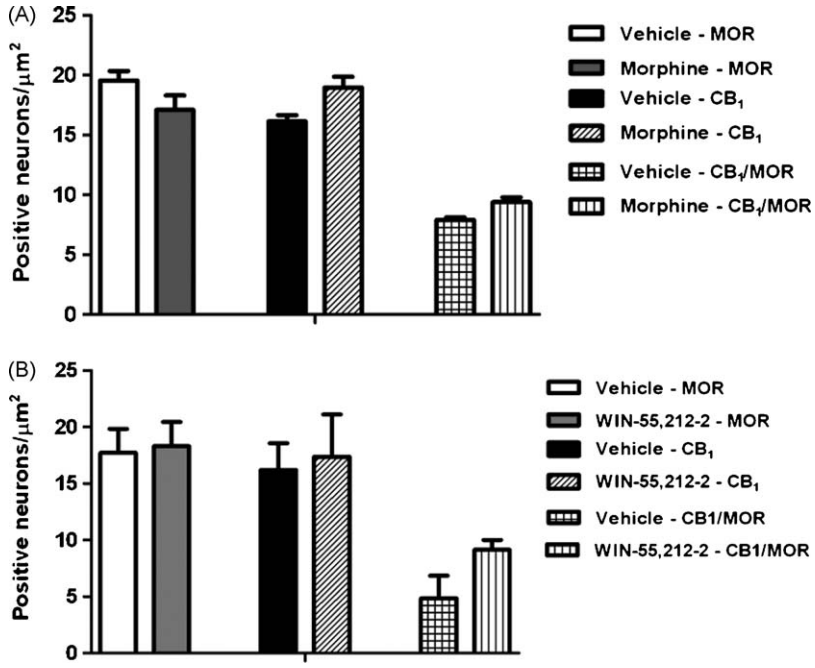

Fig. 6. Comparison of the density of MOR and CB_1 receptor immunopositive neurons in the myenteric plexus in vehicle and drug-treated animals. (A) A comparison of the density of neurons expressing MOR or CB_1 receptors, and the density of neurons co-expressing both MOR and CB_1 receptors in the myenteric plexus in vehicle and morphine-treated animals. (B) Compares the same parameters between vehicle and WIN-55,212-2 treated animals. Each bar represents the mean ± S.E.M. of measurement from three guinea pigs. $^*p < 0.05$ considered to be statistically significant.

receptor mediated change since the cannabinoid system in the LM/MP appears to have a low functional reserve that could be susceptible to the non-specific adaptive changes induced by chronic morphine exposure [6,7]. The fact that CADO appears to inhibit neurogenic activity through a cellular mechanism different from that of morphine [22] and WIN-55,212-2 [32] suggests that the adaptation events precipitated by chronic morphine exposure must involve some alteration in basic cellular function of LM/MP neurons.

In contrast to chronic opioid exposure, chronic *in vivo* cannabinoid exposure resulted in the development of homologous tolerance that was expressed as a loss of sensitivity selectively to WIN-55,212-2 as evidenced by a 9.8-fold rightward shift of the concentration–response curve. Interestingly, the maximum responses to both WIN-55,212-2 and DAMGO but not CADO were also significantly decreased suggesting that the change in the maximal effect may extend to agents that share common signaling partners. However, the reduction in maximal effect was significantly greater for WIN-55,212-2 than DAMGO and was associated with a selective reduction in the CB_1 receptor protein abundance that would account in part for the large decrease in maximum. The fact that the percent reduction in CB_1 receptor protein levels was relatively proportionate to the reduction in maximal effect of WIN-55,212-2 (32% vs. 25%, respectively) further reinforces the idea that the CB_1 receptor system in the LM/MP may possess a low functional receptor reserve as observed following *in vitro* exposure [6,7]. The reduction in CB_1 receptor protein abundance is consistent with previous studies which demonstrated robust cannabinoid receptor downregulation that occurs through beta-arrestin mediated desensitization, internalization and ultimately degradation [42]. The tolerance induced by chronic *in vivo* cannabinoid exposure mediated by CB_1 receptor activation may involve at least two components; one related exclusively to CB_1 receptor downregulation (receptor-dependent and specific) and another possibly through dual internalization of CB_1 receptors and MOR as heterodimers (receptor-dependent but non-specific). Studies using cell expression systems have reported heterodimerization of CB_1 receptors and MOR resulting in signaling through common G proteins [43]; co-internalization of MOR and other G protein-coupled receptors as heterodimers has also been reported to result in receptor desensitization [44]. Internalization or uncoupling of CB_1/MOR heterodimers following chronic CB_1 receptor activation would also result in a small reduction in the apparent functional efficacy of MOR based on the proportion that the heterodimers contributed to the total receptor population and response. Since no statistically significant reduction in relative abundance of the MOR protein (17%, $p = 0.29$) was observed following chronic WIN-55,212-2 treatment, the CB_1/MOR heterodimer could have been uncoupled from common intracellular G proteins or internalized without significant degradation even though functional signaling would have been lost. The possibility also exists that the lack of correlation between the change in responsiveness and receptor protein abundance may not be physiologically significant or relevant.

Our immunofluorescence data also seem to support the possibility of a heterodimeric interaction of these receptors since up to 50% of CB_1-immunopositive neurons also expressed MOR in preparations from treated animals. However further studies would need to be performed to verify direct physical interaction between the receptor populations. The observed reduction in CB_1 receptor protein following WIN-55-212-2 does not seem to alter the relative spatial distribution of CB_1-, MOR- or CB_1/MOR co-expressing neurons and is further reinforced by the similar pattern of distribution observed in drug-naïve, control and morphine groups. The absence of changes in CB_1 receptor immunopositive neuron distribution following WIN-55,212-2 treatment in the presence of a reduction in total CB_1 receptor protein could be due to a modest but uniform reduction in CB_1 protein that does not totally eliminate the receptor from individual neurons hence would not alter the qualitative neuron spatial distribution. Further studies would be required to determine whether the mean integrated CB_1 immunofluorescence intensity levels per neuron were decreased

and whether they correspond to the observed reduction in CB_1 receptor protein levels. While the fact that the expression of both receptors on the same neuron does not automatically translate into heterodimers, the observed CB_1/MOR co-localization suggests a common neuronal expression that may facilitate convergence of the two receptor signaling pathways involved in regulating acetylcholine release. Approximately 15% of the myenteric 'S' motor neurons (Dogiel type I) are reported to be cholinergic neurons; over 98% of these cholinergic neurons in the guinea pig have been reported to express CB_1 [45] whereas 43% (mostly Dogiel type 1) express MOR [46]. It is therefore reasonable to assume that co-localization of MOR and CB_1 is likely as evidenced by these studies which show up to 50% of the MOR and CB_1 neurons co-expressing both receptors. The larger relative density of co-localized neurons in vehicle and drug-treated animals compared to drug-naïve animals could represent the impact of treatment or could reflect different samples or different areas of evaluation of the stretch preparations.

The absence of supersensitivity to the excitatory effect of nicotine after chronic cannabinoid exposure contrasts to the supersensitivity observed in morphine tolerant animals [23,26] and the enhanced maximum response observed in these studies. Since opioid-induced supersensitivity is thought to be associated with a partial cell membrane potential depolarization [22], this could imply that chronic cannabinoid treatment does not alter the cell resting membrane potential of neurons in the LM/MP model. Furthermore, it could signify that cannabinoid exposure may not alter Na^+/K^+ ATPase isoform expression or function, a proposed key factor in the maintenance of the resting membrane potential, as observed following chronic opioid exposure [27]. Previous studies assessing the development of tolerance have reported conflicting data on the interactions between opioids and cannabinoids suggesting that the diverse interplay between the two receptor systems could be influenced by the cell/tissue/model system employed and the parameter(s) assessed. The differential results may be due to different levels of enzymes or isoforms involved in the adaptive desensitization; namely adenylyl cyclase, beta-arrestin, G protein-coupled receptor kinase (GRK), PKA, PKC and other kinases [47–51].

We conclude that the type and nature of tolerance exhibited in the guinea pig LM/MP model following chronic *in vivo* WIN-55,212-2 treatment is qualitatively and mechanistically different from that observed following chronic morphine exposure despite the large number of similarities that exist between the two systems. The data presented in this study provide support for the concept that the development of tolerance is a function of several converging influences and is subject to considerable variation that may define which cellular processes are employed to elicit the adaptive response. The fact that cannabinoid treatment produces tolerance that is primarily homologous in nature compared to the heterologous form of tolerance associated with chronic opioid exposure provides an important foundation upon which to develop future mechanistic studies. Furthermore, the data raise the possibility that such differences in adaptive responses to these agents could be advantageously employed to lead to modification in the therapeutic management of patients with these agents.

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