

Changes in Opioid and Cannabinoid Receptor Protein following Short-Term Combination Treatment with Δ^9 -Tetrahydrocannabinol and Morphine

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ABSTRACT

Recent studies in our laboratory have shown that in mice, low doses of morphine in combination with Δ^9 -tetrahydrocannabinol (Δ^9 -THC) have a similar antinociceptive effect to high doses of morphine alone. After short-term administration of this combination, there is no behavioral tolerance to the opioid. Previous binding studies and Western analyses following chronic morphine exposure in rodent models indicate significant μ -receptor down-regulation, as well as decreased levels of receptor protein, in both brain and spinal cord regions. We hypothesized that combination-treated animals would show no receptor protein down-regulation. The levels of opioid (μ , δ , κ) and cannabinoid (CB1) receptor protein were evaluated in mouse models of short-term exposure to Δ^9 -THC, morphine, or both drugs in combination. Western blot analysis revealed that all three types

of opioid receptor protein are significantly decreased in morphine-tolerant mouse midbrain. This down-regulation was not seen in combination-treated animals. In the spinal cord, there was an up-regulation of μ -, δ -, and κ -opioid receptor protein in combination-treated mice when compared with morphine-tolerant mice. There were no apparent changes in levels of CB1 receptor protein in midbrain regions, and there was an up-regulation of CB1 protein in the spinal cord. The data presented here indicate that there is a correlation between morphine tolerance and receptor protein regulation. A combination of Δ^9 -THC and morphine retains high antinociceptive effect without causing changes in receptor protein that may contribute to tolerance.

With long-term use of opioids, tolerance can develop to many of the drug effects, such as analgesia. In a tolerant state, an increase in dosage is required to maintain the same degree of analgesia. In such cases, high doses of opioids such as morphine can have unpleasant and often harmful side effects such as respiratory depression, constipation, and nausea (Ellison, 1993; de Stoutz et al., 1995). Changes that occur in the cell upon chronic μ -receptor activation include protein kinase A activation, L-type calcium channel activity, and a decrease in overall μ -receptor number (Nestler and Tallman, 1988; Mestek et al., 1995; Bernstein and Welch, 1998).

In mice treated chronically for 4 days with 75-mg morphine pellets plus supplemental injections of 20 mg/kg morphine s.c. twice daily, there was a robust tolerance to morphine as determined by the tail-flick test for antinociception (Bernstein and Welch, 1998). There was also a decrease in μ -receptor protein by almost 50%. Other studies confirm these findings and demonstrate a dose-dependent down-regulation of the μ -opioid receptor in chronically treated mice or rats (Tao et al., 1987; Yoburn et al., 1993). However, some studies

report contradictory data, failing to show any change in μ -opioid binding site number (Klee and Streaty, 1974; Yoburn et al., 1990). It has been suggested that homogenization and processing of the membranes may affect the number of opioid binding sites, resulting in a lack of consistent data. Down-regulation of other opioid receptors, including the δ - and κ -receptors, is also found to occur with tolerance to opioids (Tao et al., 1988; Trapaidze et al., 2000).

A combination of low doses of Δ^9 -THC and morphine has been shown to yield a high antinociceptive effect similar to that found with a high dose of morphine alone (Smith et al., 1998; Cichewicz et al., 1999). Chronic morphine administration via the pellet implantation method yields a significant tolerance to morphine antinociception and, furthermore, is accompanied by receptor down-regulation as mentioned previously (Bernstein and Welch, 1998). However, chronic treatment with the low-dose combination of Δ^9 -THC and morphine avoids the development of morphine tolerance while maintaining high antinociceptive effect (Cichewicz and Welch, 1999). Since this combination is equally efficacious without inducing tolerance to morphine, we hypothesized that there would be no evidence of receptor down-regulation as seen in morphine tolerance.

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ABBREVIATIONS: Δ^9 -THC, Δ^9 -tetrahydrocannabinol; CB1, cannabinoid receptor 1; O.D., optical density.

This study involves the examination of opioid and CB1 receptor protein levels via Western immunoassay in tolerant and nontolerant mice. We also evaluated similar receptor proteins in animals treated with a combination of Δ^9 -THC and morphine to determine whether the drug combination-induced alteration in receptor proteins differed from that with each drug alone.

Materials and Methods

Animals. Male ICR mice (Harlan Laboratories, Indianapolis, IN) weighing 25 to 30 g were housed four per cage in an animal care facility maintained at $22 \pm 2^\circ\text{C}$ on a 12-h light/dark cycle. Food and water were available ad libitum. The mice were brought to the test room 24 h before testing to allow acclimation and recovery from transport and handling.

Drug Administration Protocol. Mice were rendered tolerant to the various drugs in the following manner. Tolerance to morphine was attained via subcutaneous pellets as described earlier (Bernstein and Welch, 1998). The animals were anesthetized briefly under ether and implanted with a 75-mg morphine pellet. The wound was closed with surgical staples. Beginning 12 h postsurgery, the mice received supplemental injections of 20 mg/kg morphine s.c. twice daily at 9:00 AM and 5:00 PM for 5 days. A challenge dose of 70 mg/kg morphine p.o. was given 24 h after the last injection, and the mice were tested 30 min later using the tail-flick test for antinociception (D'Amour and Smith, 1941) to confirm morphine tolerance. Antinociception was quantified as the percentage of maximum effect (% MPE) as developed by Harris and Pierson (1964).

Tolerance to Δ^9 -THC was achieved by administration of 20 mg/kg Δ^9 -THC p.o. twice daily at 9:00 AM and 5:00 PM for 6.5 days. A challenge dose of 150 mg/kg Δ^9 -THC p.o. was administered 24 h after the last injection, and the mice were tested 30 min later using the tail-flick test to confirm Δ^9 -THC tolerance.

Short-term treatment of the combination of morphine and Δ^9 -THC consisted of a 7-day protocol, with mice receiving 20 mg/kg Δ^9 -THC p.o. followed 15 min later by 20 mg/kg morphine p.o. twice daily at 9:00 AM and 5:00 PM. The mice were challenged 24 h after the last injection with either 70 mg/kg morphine p.o., 150 mg/kg Δ^9 -THC p.o., or the combination of 20 mg/kg Δ^9 -THC p.o. and 20 mg/kg morphine p.o. Thirty minutes later the mice were evaluated for tolerance to either or both of these drugs in the tail-flick test.

For each drug administration protocol, a corresponding group of animals was treated for similar times with the appropriate vehicle (placebo pellet; 1:1:18 ethanol, Emulphor, saline; or 1:1:18 followed 15 min later by distilled water).

Tissue Preparation. Following the tail-flick test procedure, mice were sacrificed by cervical dislocation and decapitated. Spinal cords and midbrains (six animals pooled per sample) were removed and quickly frozen on dry ice. All tissue was stored at -80°C until the day of tissue preparation. Frozen tissue was allowed to thaw and then homogenized in 10 ml of suspension buffer [10 mM EGTA, 20 mM EDTA, 10 mM Tris base, 20 mM β -glycerophosphate, 50 mM sodium fluoride, 50 mM sodium pyrophosphate, 1 mM *p*-nitrophenylphosphate, pH 7.4, 200 μM microcystin LR (Sigma Chemical Co., St. Louis, MO), and protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN)]. A P2 membrane preparation was made by a 10-min 3500g centrifugation, followed by a 30-min spin of the supernatant at 20,000g at 4°C . The second pellet was resuspended in suspension buffer plus detergents (1% Triton X-100 and 0.5% Igepal). The pellet was slowly homogenized for 30 s and then placed on a rocking platform for 1 h at 4°C . The samples were then centrifuged for 20 min at 12,000g to remove insoluble material. Protein concentrations were determined by the Bio-Rad (Hercules, CA) protein assay. Tissue was aliquoted and stored at -80°C .

Western Immunoassays. Electrophoresis was performed using a standard Laemmli method. Samples were diluted 1:1 with $2\times$ sam-

ple buffer and loaded in a 1.5-mm 8% SDS-polyacrylamide gel. The amount of protein loaded into each gel varied with the receptor being studied as follows: μ in midbrain, 25 μg ; μ in cord, 40 μg ; δ in midbrain, 2.5 μg ; δ in cord, 2.5 μg ; κ in midbrain, 5 μg ; κ in cord, 5 μg ; CB1 in midbrain, 15 μg ; CB1 in cord, 25 μg . These protein amounts were determined from concentration-effect control gels run for each antibody used (for example, see Fig. 1). There was a high correlation between protein loading and O.D. readings in these control gels (data not shown). The protein amounts selected from the control gels to load into our test gels reflect the lowest possible amounts that still yielded a detectable, quantifiable band. Following protein separation, transfer onto Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA) was performed by the tank method. Blots were reversibly stained with Ponceau solution (Sigma Chemical Co.) and then blocked at least 1.5 h in 5% nonfat dry milk in Tris-buffered saline plus 0.5% Tween 20 (TBST). The blots were incubated in appropriate rabbit anti-rat opioid receptor antibody concentrations overnight at room temperature (μ , 1:500 in 5% nonfat dry milk in TBST; δ , 1:8000 in casein; κ , 1:1000 in casein). After washing three times for 5 min in TBST, blots were incubated in horseradish peroxidase-conjugated goat anti-rabbit IgG antiserum (Sigma Chemical Co.) at a 1:20,000 (μ , κ) or 1:50,000 (δ) dilution in casein for 3 h at room temperature. Again, blots were washed three times for 5 min in TBST and then incubated for 5 min in SuperSignal CL-HRP chemiluminescence (Pierce, Rockford, IL). Blots were then exposed on XAR-2 film (Eastman Kodak, Rochester, NY). Bands were quantified using scanning densitometry, and comparisons of the optical density values were done by Student's unpaired *t* test. As a control for gel loading accuracy, some blots were stripped and reprobed with anti-actin antibody.

For the CB1 receptor studies, the gels were transferred, stained, and blocked 1 h at room temperature in casein. The blots were incubated overnight in rabbit anti-rat CB1 antibody (1:100) in casein at room temperature. After washing three times for 5 min in TBST, blots were incubated in biotinylated goat anti-rabbit IgG antiserum (Life Technologies, Grand Island, NY) at a 1:30,000 dilution in casein for 2 h at room temperature. After washing three times for 5 min in TBST, blots were incubated in ^{125}I -streptavidin (specific activity 704-1480 kBq/ μg , 20-40 $\mu\text{Ci}/\mu\text{g}$; Amersham Pharmacia Biotech, Piscataway, NJ) at a 1:1000 dilution for 1 h. Blots were again washed three times for 5 min in TBST, wrapped in plastic wrap, and placed on a mounted phosphor screen cassette (Molecular Dynamics, Sunnyvale, CA) overnight. Blots were analyzed using a PhosphorImager computer (Molecular Dynamics), and the integrated volumes were compared using the Student's unpaired *t* test.

Drugs and Antibodies. Morphine pellets were obtained from the National Institute on Drug Abuse (NIDA, Rockville, MD). Δ^9 -THC and morphine sulfate were also obtained from NIDA. Anti- μ -antibody (anti-acetyl-EAETAPLP-amide, C-terminal) was obtained from Research and Diagnostic Antibodies (Berkley, CA). Anti- δ - (anti-acetyl-CGRQEPGSLRRPRQA-amide, C-terminal), anti- κ - (anti-acetyl-SREK-DRNLRRIT KL-amide, C-terminal), and anti-CB1 antibody (anti-

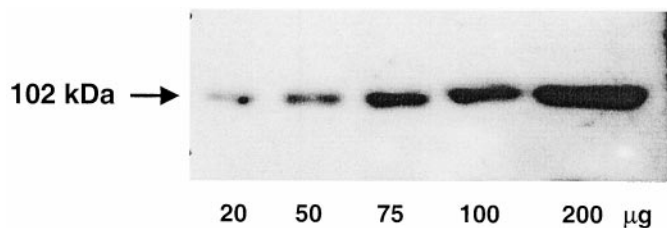


Fig. 1. Example of a concentration-effect Western blot control gel. Various amounts of mouse midbrain protein were loaded into the gel and analyzed for μ -receptor protein levels. This type of control gel was generated for each region studied (midbrain and spinal cord) and each antibody used (μ , δ , κ , CB1) (data not shown). The amount of protein selected to load into subsequent gels was the lowest quantifiable amount, as listed under *Materials and Methods*.

acetyl-NKLSLSSFKENEENIQC-amide, N-terminal) antibodies were obtained from Biosource International (formerly Quality Controlled Biochemicals, Camarillo, CA).

Results

μ -Receptor Protein Levels. Western blot analyses of the μ -receptor protein in mouse midbrain and spinal cord revealed a band at approximately 102 kDa (Figs. 2 and 3). This size is somewhat higher than reported in the recent literature (Roy and Loh, 1987; Ueda et al., 1988). The size difference may be accounted for by post-translational modifications such as glycosylation. It has been reported that a μ -receptor detected at 105 kDa was reduced to 43 kDa after treatment with endoglycosidase-F (Garzon et al., 1995). Furthermore, Bero et al. (1988) suggested that glycosylated opioid receptors may form dimers of 100 to 114 kDa. The specificity of the anti- μ -antibody used here was previously verified in the laboratory (Bernstein and Welch, 1998).

No significant changes in quantity of μ -receptor protein were detected in the midbrains of mice treated for 6.5 days with Δ^9 -THC (Fig. 2A). However, μ -receptor protein was significantly decreased in morphine-tolerant mice (Fig. 2B) as determined by comparison of optical density readings shown in Table 1 ($p < 0.05$). This down-regulation was not evident in mice treated with the low-dose combination of Δ^9 -THC and morphine (Fig. 2C). In spinal cord studies (Fig. 3), there were no significant changes in μ -receptor protein levels in either Δ^9 -THC- or morphine-tolerant mice (Fig. 3, A

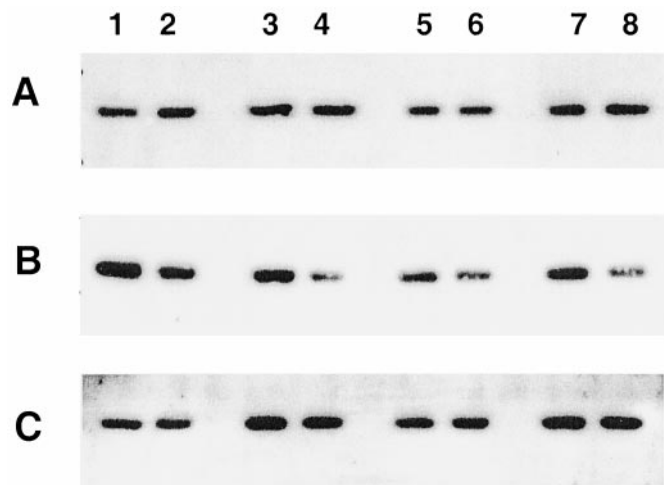


Fig. 2. Western immunoassay of μ -receptor protein from midbrains of ICR mice after short-term treatment with either Δ^9 -THC, morphine, or both. Odd-numbered lanes indicate animals treated with the appropriate vehicle, while even-numbered lanes indicate animals treated with the drug(s). All wells were loaded with 25 μ g of protein. Each lane represents a pooled sample of six mice. Blots were stripped and reprobed with anti-actin to ensure accurate protein transfer. A, analysis of μ -receptor protein in Δ^9 -THC-tolerant mice compared with nontolerant mice. Vehicle animals were treated for 6.5 days with 1:1:18, and Δ^9 -THC-tolerant animals were treated for the same time with 20 mg/kg Δ^9 -THC p.o. twice daily. B, analysis of μ -receptor protein in morphine-tolerant and nontolerant mice. Vehicle animals were treated for 5 days with a placebo pellet and supplemental injections of distilled water, while morphine-tolerant animals were treated for 5 days with a 75-mg morphine pellet s.c. plus supplemental injections of 20 mg/kg morphine s.c. twice daily. C, analysis of μ -receptor protein in combination-treated animals. Vehicle animals were treated for 6.5 days with a combination of 1:1:18 and water vehicles twice daily, while combination-treated animals were treated for 6.5 days with a combination of 20 mg/kg Δ^9 -THC p.o. and 20 mg/kg morphine p.o. twice daily.

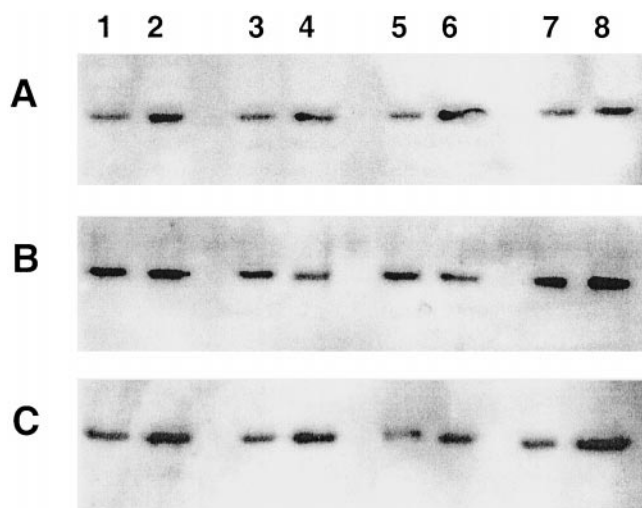


Fig. 3. Western immunoassay of μ -receptor protein from spinal cords of ICR mice after short-term treatment with either Δ^9 -THC, morphine, or both. Odd-numbered lanes indicate animals treated with the appropriate vehicle, while even-numbered lanes indicate animals treated with the drug(s). All wells were loaded with 40 μ g of protein. See Fig. 2 legend for treatments (A, B, C). Each lane represents a pooled sample of six mice. Blots were stripped and reprobed with anti-actin to ensure accurate protein transfer.

and B), but in combination-treated animals there was an up-regulation of the receptor protein (Fig. 3C) ($p < 0.05$).

δ -Receptor Protein Levels. Western blot analyses of the δ -receptor protein in mouse midbrain and spinal cord revealed a band at approximately 43 kDa (Figs. 4 and 5). This size is consistent with findings in the literature, which describes molecular masses for the δ -receptor in rodents of 45 to 65 kDa (DeMoliou-Mason and Barnard, 1984; Gomathi and Sharma, 1993; Anand and Oommen, 1995).

Although there were no changes in δ -receptor protein in midbrains of Δ^9 -THC-tolerant mice (Fig. 4A), morphine-tolerant mice showed a significant 3-fold down-regulation of receptor protein (Fig. 4B) as determined by the optical density readings ($p < 0.05$) (Table 2). There was no down-regulation of the δ -receptor protein in combination-treated animals (Fig. 4C), similar to results found with the μ -opioid receptor above. Figure 5 illustrates the changes in the receptor protein levels in mouse spinal cord. There were no significant changes in the δ -receptor protein levels in either Δ^9 -THC- or morphine-tolerant mice (Fig. 5, A and B), but in combination-treated animals there was a 2-fold up-regulation of the receptor protein ($p < 0.05$) (Fig. 5C). These findings are also consistent with the μ -receptor spinal cord data reported above.

κ -Receptor Protein Levels. Western blot analyses of the κ -receptor protein in mouse midbrain and spinal cord revealed a band at approximately 57 kDa (Figs. 6 and 7). This size is consistent with findings in the recent literature (Chow and Zukin, 1983; Simon et al., 1987). The κ -receptor appears as a doublet, which indicates the presence of two subtypes of κ -receptor protein. The upper band of the doublet was used for quantification.

Figure 6 shows the κ -receptor protein as seen in Western blots of mouse midbrains. There was no difference in levels of receptor protein in Δ^9 -THC-tolerant mice (Fig. 6A). Morphine-tolerant mice exhibited a significant reduction in receptor protein ($p < 0.05$) (Fig. 6B), while combination treat-

TABLE 1

Levels of μ -receptor protein in Δ^9 -THC-treated, morphine-treated, and combination-treated mice. Western blots were quantified using scanning densitometry, and an average of the four trials was taken.

Treatment	Midbrain O.D.		Spinal Cord O.D.	
	Vehicle	Drug	Vehicle	Drug
Δ^9 -THC	0.35 $\pm$ 0.02	0.37 $\pm$ 0.02	0.20 $\pm$ 0.02	0.28 $\pm$ 0.03
Morphine	0.36 $\pm$ 0.03	0.19 $\pm$ 0.03*	0.12 $\pm$ 0.01	0.13 $\pm$ 0.04
Δ^9 -THC + morphine	0.56 $\pm$ 0.02	0.54 $\pm$ 0.04	0.14 $\pm$ 0.01	0.23 $\pm$ 0.03*

* Significantly different from vehicle-treated, nontolerant control. $p < 0.05$, by Student's t test.

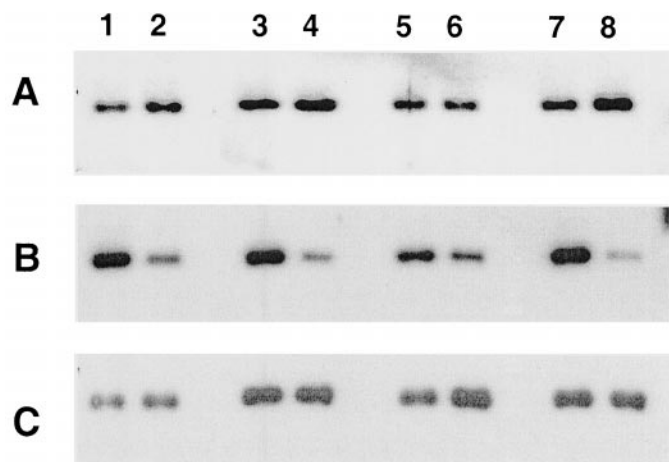


Fig. 4. Western immunoassay of δ -receptor protein from midbrains of ICR mice after short-term treatment with either Δ^9 -THC, morphine, or both. Odd-numbered lanes indicate animals treated with the appropriate vehicle, while even-numbered lanes indicate animals treated with the drug(s). All wells were loaded with 2.5 μ g of protein. See Fig. 2 legend for treatments (A, B, C). Each lane represents a pooled sample of six mice. Blots were stripped and reprobed with anti-actin to ensure accurate protein transfer.

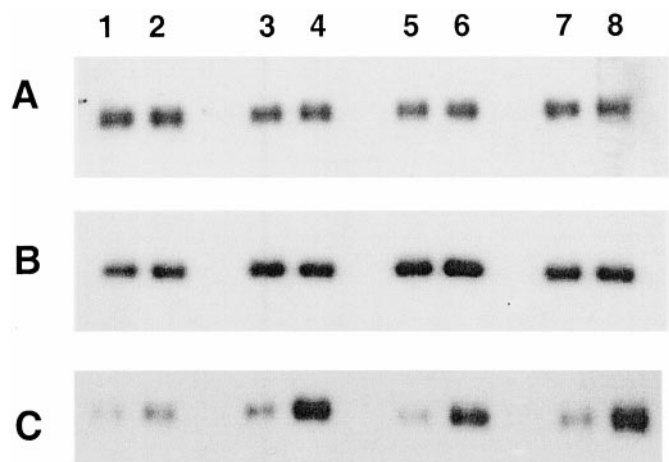


Fig. 5. Western immunoassay of δ -receptor protein from spinal cords of ICR mice after short-term treatment with either Δ^9 -THC, morphine, or both. Odd-numbered lanes indicate animals treated with the appropriate vehicle, while even-numbered lanes indicate animals treated with the drug(s). All wells were loaded with 2.5 μ g of protein. See Fig. 2 legend for treatments (A, B, C). Each lane represents a pooled sample of six mice. Blots were stripped and reprobed with anti-actin to ensure accurate protein transfer.

ment (Fig. 6C) showed no down-regulation. These results are similar to the other opioid receptor studies mentioned above. Upon examination of the spinal cord, we found no significant changes in κ -receptor protein in either Δ^9 -THC- or morphine-tolerant mice (Fig. 7, A and B). However, mice treated with a

combination of Δ^9 -THC and morphine showed a 2-fold up-regulation of the κ -receptor protein ($p < 0.05$) (Fig. 7C), similar to results found in the spinal cord with the μ - and δ -receptors.

CB1 Receptor Protein Levels. Western blot analyses of the CB1 receptor protein in mouse midbrain and spinal cord revealed a band at approximately 74 kDa (Figs. 8 and 9). This size is very close to that reported in the previous literature (Song and Howlett, 1995). The CB1 receptor has also been purported to undergo glycosylation at three potential sites on the N terminus (Matsuda et al., 1990), which could account for size variations.

In all drug treatment groups in the midbrain, there were no differences in the levels of CB1 receptor protein (Fig. 8). Thus, there was no down-regulation of the CB1 receptor protein in tolerant animals, contrary to what was seen with the opioid receptor proteins in midbrain regions. However, in the spinal cord, there was a significant up-regulation of the receptor protein in combination-treated animals ($p < 0.05$) (Fig. 9C), similar to the up-regulation seen in previous Western blots with opioid receptors. Tables 1 through 4 summarize these findings by reporting the optical density values or integrated volume values for each receptor type.

Discussion

The goal of this study was 2-fold. First, we wanted to confirm whether various receptor proteins were down-regulated in a state of opioid tolerance. Many groups have examined μ -, δ -, and κ -receptors in animals tolerant to morphine, etorphine, and other opioids (Klee and Streaty, 1974; Tao et al., 1987; Roy et al., 1988; Yoburn et al., 1990, 1993), with varied results. We have successfully shown here that in mouse midbrain, all three of the opioid receptor protein subtypes were down-regulated in morphine tolerance. This is in agreement to data previously published (Bhargava and Gulati, 1990). However, in the spinal cord, there was no detectable down-regulation of any of the opioid receptor proteins in tolerant animals. We can conclude that tolerance to morphine is accompanied by a decrease in not only μ - but also δ - and κ -receptor proteins in the midbrain, indicating an alteration in cellular opioid receptor protein synthesis or degradation in tolerant animals. These decreases in receptor protein levels may or may not be related to function of the receptors since Selley et al. (1997) found that chronic morphine treatment decreased inhibitory G-protein activity in rat locus coeruleus without producing any detectable desensitization. Thus it is possible that mechanisms other than decreased receptor protein are causing a reduction in agonist efficacy.

The role of the CB1 receptor in cannabinoid tolerance has also been extensively studied, with varied results. Several

TABLE 2

Levels of δ -receptor protein in Δ^9 -THC-treated, morphine-treated, and combination-treated mice
Western blots were quantified using scanning densitometry, and an average of the four trials was taken.

Treatment	Midbrain O.D.		Spinal Cord O.D.	
	Vehicle	Drug	Vehicle	Drug
Δ^9 -THC	0.27 $\pm$ 0.05	0.34 $\pm$ 0.05	0.30 $\pm$ 0.03	0.32 $\pm$ 0.02
Morphine	0.47 $\pm$ 0.04	0.13 $\pm$ 0.03*	0.41 $\pm$ 0.05	0.45 $\pm$ 0.05
Δ^9 -THC + morphine	0.42 $\pm$ 0.07	0.46 $\pm$ 0.06	0.22 $\pm$ 0.04	0.55 $\pm$ 0.08*

* Significantly different from vehicle-treated, nontolerant control. $p < 0.05$, by Student's t test.

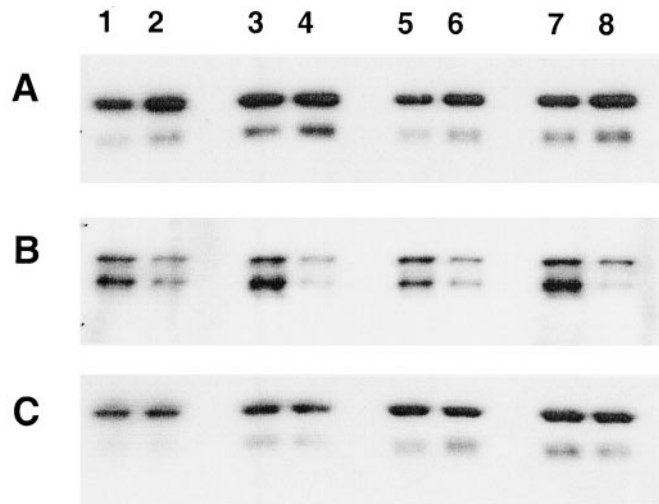


Fig. 6. Western immunoassay of κ -receptor protein from midbrains of ICR mice after short-term treatment with either Δ^9 -THC, morphine, or both. Odd-numbered lanes indicate animals treated with the appropriate vehicle, while even-numbered lanes indicate animals treated with the drug(s). All wells were loaded with 5 μ g of protein. See Fig. 2 legend for treatments (A, B, C). Each lane represents a pooled sample of six mice. Blots were stripped and reprobed with anti-actin to ensure accurate protein transfer.

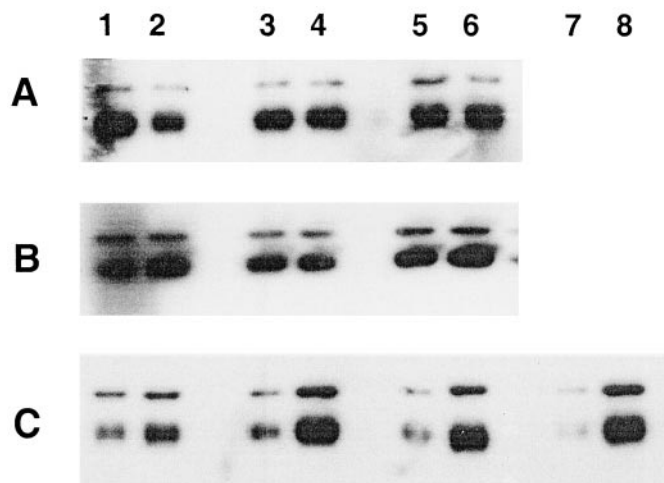


Fig. 7. Western immunoassay of κ -receptor protein from spinal cords of ICR mice after short-term treatment with either Δ^9 -THC, morphine, or both. Odd-numbered lanes indicate animals treated with the appropriate vehicle, while even-numbered lanes indicate animals treated with the drug(s). All wells were loaded with 5 μ g of protein except for combination treatment, which was loaded with 2.5 μ g. See Fig. 2 legend for treatments (A, B, C). Each lane represents a pooled sample of six mice. Blots were stripped and reprobed with anti-actin to ensure accurate protein transfer.

groups have indicated that there is a down-regulation of the receptor in tolerant rats (e.g., Fan et al., 1996), while others report no change (e.g., Abood et al., 1993). In examining the

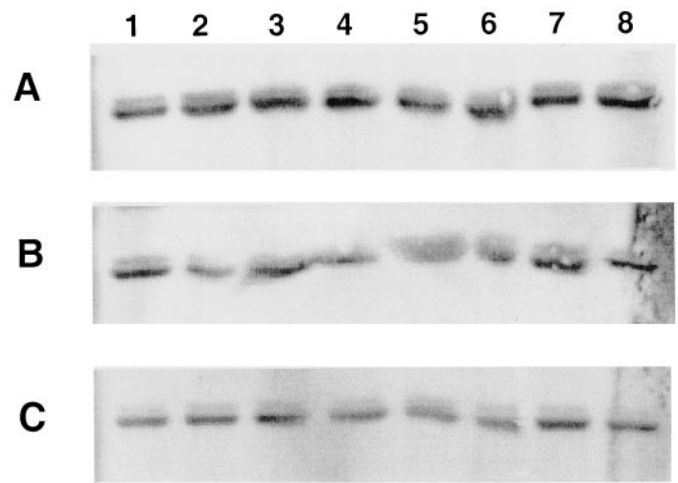


Fig. 8. Western immunoassay of CB1 receptor protein from midbrains of ICR mice after short-term treatment with either Δ^9 -THC, morphine, or both. Odd-numbered lanes indicate animals treated with the appropriate vehicle, while even-numbered lanes indicate animals treated with the drug(s). All wells were loaded with 15 μ g of protein. See Fig. 2 legend for treatments (A, B, C). Each lane represents a pooled sample of six mice. Blots were stripped and reprobed with anti-actin to ensure accurate protein transfer.

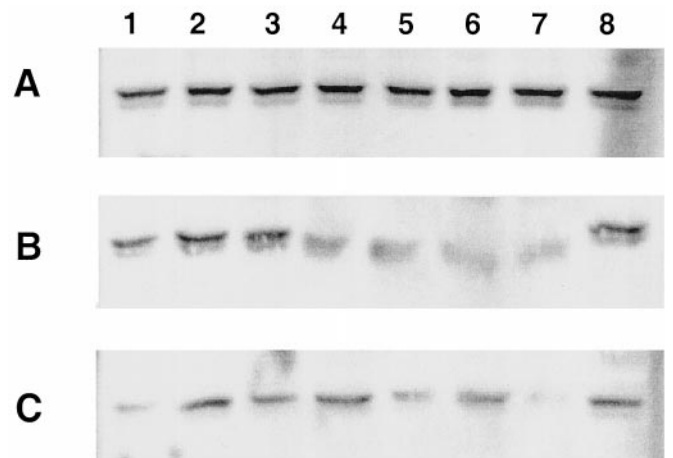


Fig. 9. Western immunoassay of CB1 receptor protein from spinal cords of ICR mice after short-term treatment with either Δ^9 -THC, morphine, or both. Odd-numbered lanes indicate animals treated with the appropriate vehicle, while even-numbered lanes indicate animals treated with the drug(s). All wells were loaded with 25 μ g of protein. See Fig. 2 legend for treatments (A, B, C). Each lane represents a pooled sample of six mice. Blots were stripped and reprobed with anti-actin to ensure accurate protein transfer.

levels of CB1 mRNA in cannabinoid-tolerant animals, both an increase and a decrease of mRNA has been shown (Rubino et al., 1994; Fan et al., 1996) in various brain regions. Our data agree with previous reports in that we saw no change in

TABLE 3

Levels of κ -receptor protein in Δ^9 -THC-treated, morphine-treated, and combination-treated mice. Western blots were quantified using scanning densitometry, and an average of the four trials was taken.

Treatment	Midbrain O.D.		Spinal Cord O.D.	
	Vehicle	Drug	Vehicle	Drug
Δ^9 -THC	0.53 $\pm$ 0.02	0.60 $\pm$ 0.01	0.46 $\pm$ 0.06	0.36 $\pm$ 0.02
Morphine	0.52 $\pm$ 0.01	0.40 $\pm$ 0.02*	0.54 $\pm$ 0.04	0.54 $\pm$ 0.04
Δ^9 -THC + morphine	1.50 $\pm$ 0.37	1.31 $\pm$ 0.31	0.26 $\pm$ 0.01	0.42 $\pm$ 0.04*

* Significantly different from vehicle-treated, nontolerant control. $p < 0.05$, by Student's t test.

TABLE 4

Levels of CB1 receptor protein in Δ^9 -THC-treated, morphine-treated, and combination-treated mice. Western blots were quantified using phosphorimaging, and an average of the four trials was taken.

Treatment	Midbrain Int. Vol.		Spinal Cord Int. Vol.	
	Vehicle	Drug	Vehicle	Drug
Δ^9 -THC	3.2 $\pm$ 0.69 ^a	5.1 $\pm$ 0.79	3.6 $\pm$ 0.30	4.2 $\pm$ 0.33
Morphine	2.5 $\pm$ 0.42	1.6 $\pm$ 0.12	1.7 $\pm$ 0.43	2.2 $\pm$ 0.72
Δ^9 -THC + morphine	1.4 $\pm$ 0.48	1.6 $\pm$ 0.09	0.34 $\pm$ 0.09	0.89 $\pm$ 0.06*

Int. Vol., integrated volume.

^a All integrated volumes are presented as table value $\times 10^5$.

* Significantly different from vehicle-treated, nontolerant control. $p < 0.05$, by Student's t test.

CB1 receptor protein levels in Δ^9 -THC-tolerant mice. There are few reports that address how the CB1 receptor is regulated in conditions of opioid tolerance. Here we show that CB1 protein levels were unchanged in animals tolerant to morphine.

Our second aim was to determine whether these receptor protein changes were abolished by the prevention of morphine tolerance with Δ^9 -THC. We have shown previously that a nonantinociceptive oral dose of Δ^9 -THC (20 mg/kg) can enhance the potency of an acute dose of morphine (Cichewicz et al., 1999). Similarly, we observed that after short-term treatment in mice with low doses of Δ^9 -THC and morphine in combination, there is an avoidance of tolerance to the opioid without compromising antinociceptive effect (Cichewicz and Welch, 1999). Therefore, it seemed likely that by avoiding the antinociceptive tolerance to morphine by introducing Δ^9 -THC, we should also avoid the effects on receptor protein levels that normally accompany morphine tolerance in areas of the body where pain responses are processed. In fact, we were able to show this lack of receptor protein down-regulation with all three opioid receptors in the midbrain. Thus, a combination treatment preserves antinociceptive potency while eliminating protein down-regulation, suggesting a beneficial alternative to chronic morphine therapy. Surprisingly, there was an up-regulation of opioid and CB1 receptor proteins in the spinal cord after combination treatment. This seems to indicate two distinct mechanisms for expression of tolerance, supraspinal and spinal. In the spinal cord, there may be a compensatory increase in receptor protein level to preserve antinociceptive potency in that region.

It has been suggested that μ -receptors are desensitized upon chronic morphine exposure, resulting in phosphorylation of the receptors followed by internalization and degradation. This process yields a decreased number of μ -receptors available on the membrane to bind to morphine. Loh et al. (1988) hypothesized that after chronic opioid exposure, there is a two-step process that takes place: first, the opioid receptors uncouple from their respective G-protein ($G_{i/o}$), which results in reduced affinity for the agonist (desensitization); and second, the inactive receptors are internalized, and thus

there is a loss of opioid receptor binding sites (down-regulation). Our studies focused on receptor protein quantity, and thus we have not measured the affinity of the receptors. Evaluation of receptor binding would give us insight as to whether the desensitization step occurs simultaneously with the down-regulation we observed.

A dissociation of desensitization and down-regulation was also observed by Nishino et al. (1990). They reported no opioid receptor down-regulation in the brain after chronic morphine treatment, even though tolerance did develop. Thus it seems that receptor down-regulation may be a consequence, rather than a cause, of morphine tolerance. The time course of this subsequent receptor down-regulation after development of tolerance is yet unknown. However, it is generally thought that opioid receptor down-regulation is not a necessary condition for the development of tolerance, suggesting an intracellular locus for tolerance development (Yoburn et al., 1993). In examining the levels of receptor protein in nontolerant and tolerant mice, we have effectively been able to use down-regulation as a marker for tolerance, and this marker is conspicuously absent in combination-treated animals. The use of opioid receptor regulation as a marker related to tolerance and dependence has previously been suggested by Rothman et al. (1991).

Conversely, it appears that CB1 receptor protein cannot be used as a marker for Δ^9 -THC tolerance. We saw no difference when comparing Δ^9 -THC-tolerant midbrain to combination-treated midbrain. However, this may be because the dose of Δ^9 -THC used in the combination is sufficient to cause tolerance itself; thus the combination-treated animals were also tolerant to Δ^9 -THC. In the spinal cord, however, we did see an up-regulation of CB1 receptor protein in combination-treated animals. This may be due to the compensatory mechanisms discussed earlier. An up-regulation of the CB1 receptor protein may be needed to maintain high antinociceptive effect in the spinal cord.

In examining the differences between receptor protein regulation in midbrain and spinal cord regions, it is important to consider a synergistic interaction between supraspinal and spinal sites of analgesia. Several groups have shown that

morphine coadministered to brain and spinal sites results in a multiplicative interaction (Yeung and Rudy, 1980; Roerig et al., 1984). This suggests that systemic morphine administration acts not only on the spinal cord but inhibits the opposing supraspinal influence by acting on the brain (Roerig and Fujimoto, 1988). In our p.o.-treated combination animals, there may be a similar regulation occurring. Opioid and cannabinoid receptors have been shown to be colocalized in areas of the dorsal horn (Welch and Stevens, 1992). Thus the spinal blockade of pain transmission becomes greater than additive as both of these receptor types are activated. Simultaneous activation of brain receptors would then disinhibit interneurons regulating endogenous opioid release, further contributing to the antinociceptive effect. Up-regulation of opioid receptor protein in the spinal cord that we observed in combination-treated animals may underlie the retention of efficacy of the drug combination.

It is important to note that although the Δ^9 -THC and the combination treatments were administered orally, the method to develop morphine tolerance was by subcutaneous administration of pellets. This method was chosen due to the many reports that confirm it as a reliable procedure to obtain a mouse model of significant morphine tolerance. In these experiments, we wanted to have definitive models of tolerance established with which we could compare our combination-treated groups. In a related experiment, we attempted to create a model of oral morphine tolerance by administering 70 mg/kg morphine p.o. twice daily for 7 days; however, even at this high dose of morphine, significant tolerance on the test day could not be achieved. Future work to evolve an oral morphine tolerance model in the mouse using varied administration times and dosages would yield an even better comparison for our oral combination studies. In summary, we have been able to show that μ -, δ -, and κ -receptor protein levels are decreased in morphine-tolerant mice. However, this down-regulation is circumvented by administration of a combination of low doses of Δ^9 -THC and morphine. Thus, we have shown a correlation between the absence of tolerance and prevention of receptor protein level changes in neurons involved in pain transmission. These findings may lead to a better understanding of the mechanisms of tolerance and point to the reasons why combination treatment may be clinically beneficial.

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