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Changes in Rat Spleen Cannabinoid Receptors after Chronic CP-55,940: An Autoradiographic Study

PAOLA MASSI, GABRIELA PATRINI, TIZIANA RUBINO
DOMENICA FUZIO AND DANIELA PAROLARO

Institute of Pharmacology, Faculty of Sciences, University of Milan,
Via Vanvitelli 32/a, 20129, Milan, Italy

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MASSI, P., G. PATRINI, T. RUBINO, D. FUZIO AND D. PAROLARO. *Changes in rat spleen cannabinoid binding sites after chronic exposure to synthetic cannabinoid CP-55,940: An autoradiographic study.* PHARMACOL BIOCHEM BEHAV **58**(1) 73–78, 1997.—We examined whether cannabinoid receptor density changes in the rat spleen after in vivo chronic exposure to cannabinoids. Rats received daily injections of 0.4 mg/kg IP of the synthetic cannabinoid receptor ligand CP-55,940 for 11 days. One h after the last injection on day 11, the rats were killed and spleen coronal sections were processed for receptor binding autoradiography with 10 nM of [^{3}H]CP-55,940 in the absence or presence of unlabeled CP-55,940 (10 μM). Densitometric analysis of the autoradiograms showed significant loss of [^{3}H]CP-55,940 binding of about 42% in chronic cannabinoid-treated, tolerant rats. Our findings indicate that cannabinoid receptors basically present in immune spleen cells are down-regulated by chronic exposure to cannabinoids, suggesting a role in immune modulation and in the impairment of immune function. © 1997 Elsevier Science Inc.

Cannabinoids CP-55,940 Chronic treatment Spleen Cannabinoid receptor Down regulation

CANNABINOIDS reportedly impair immune function. Cell culture models, human data and experimental animals have served to demonstrate that cannabimimetic agents profoundly inhibit humoral and cell-mediated immune responses (11). Marijuana users may have signs of impaired cellular immunity (12) and treatment of experimental animals with high doses of cannabinoids reduced immune responses (13). In vitro cannabinoids reduced the proliferative responses of T lymphocytes (8), cytotoxic T-cell activity and antibody synthesis (7), and the microbicidal activity of macrophages (1) and increase the immune suppression induced by murine retrovirus infection (18).

The mechanisms of these effects on the immune system are still questioned. The recent identification of functional cannabinoid receptors on immune cells suggests that the immune effects are at least partly mediated through a highly specific receptor-associated mechanism. This is supported by the expression of the CB1 receptor on lymphocytes (2), and the cloning of a second G-protein coupled cannabinoid receptor (CB2) which is expressed in spleen but not in the brain (10). Thus the immune modulatory effects of cannabinoids could be receptor-mediated.

We still lack evidence of the functional modulation of spleen cannabinoid receptors under acute and chronic treatment with cannabimimetic drugs. Therefore the question addressed in the present work was whether chronic exposure to CP-55,940, a potent synthetic cannabinoid compound, alters cannabinoid receptor binding in rat spleen under conditions where rats develop tolerance to some behavioural effects. Recently, we reported that chronic treatment with CP55,940 in rats (0.4 mg/kg, IP for 11 days) induced tolerance to analgesia and to a reduction in spontaneous locomotor activity that can be related to the strong reduction in CB1 receptor density and expression (17). Upon the same experimental condition, we examine in the present work whether alterations in cannabinoid binding sites occur also at the peripheral cannabinoid receptor performing in vitro autoradiographic receptor binding on spleen coronal sections from rats treated either acutely or chronically with CP-55,940.

Requests for reprints should be addressed to Daniela Parolaro, Institute of Pharmacology, Fac. of Sciences, Univ. of Milan, Via Vanvitelli 32/a, 20129 Milan, Italy, Tel: +39-2-7385568, Fax: +39-2-70002270, E-mail: Gori@imiucca.csi.unimi.it

METHOD

Animals and Drug Administration

Adult male Sprague–Dawley rats (Charles River, Calco, Italy), weighing 175–200 g at the beginning of the experiment were used. The animals were housed three per cage in standard conditions. Rats received one daily IP injection of CP-55,940 (0.4 mg/kg, 20% ethanol in distilled water, a generous gift from Dr. D. Casilli, Pfizer Italiana S.p.A., Rome, Italy) or vehicle (20% ethanol in distilled water) or distilled water for 11 days. This schedule is in line with our earlier study (17) demonstrating development of tolerance to the motor impairment and analgesia caused by CP-55,940.

Autoradiographic Binding Studies

One h after the last injection on day 11, the tolerant rats were killed and their spleens were rapidly removed and frozen by being gently lowered into liquid nitrogen. They were stored at −80°C until processing. Spleens were brought to −16°C in a cryostat and a series of 16 μm-thick serial sections were collected on gelatin-coated slides. The sections were briefly dried at 30°C and stored at −80°C until they were processed for receptor binding autoradiography. A series of adjacent sections was fixed in formaldehyde vapors, delipidated, and stained with haematoxylin/eosin.

Binding was carried out as described previously (9). The slides were brought briefly to room temperature, then incubated for 2.5 h at 37°C with 10 nM [^{3}H]CP-55,940 (Du Pont, Milan, Italy, custom-labeled, 129.1 Ci/mmol) in binding buffer (50 mM Tris-HCl, pH 7.4, 5% BSA). Adjacent spleen sections were incubated in parallel with 10 μM CP-55,940 to assess nonspecific binding. Sections were washed for 4 h at 0°C in 50 mM Tris-HCl (pH 7.4)/1% BSA buffer twice. After washing, sections were dipped in 50 mM Tris-HCl buffer (pH 7.4)/0.5% formaldehyde at room temperature (5 min) to remove excess BSA and preserve tissue integrity, dipped briefly in water (5 s) and dried under a cool stream of air. Autoradiograms were made by exposing the dried sections in X-ray cassettes for 10 days to tritium-sensitive film Hyperfilm-^{3}H (Amersham). Autoradiograms were developed with Kodak D 19 developer (25°C, 4 min), fixed in Kodak Unifix (8 min) and rinsed with water (15 min).

Quantification of Binding

The intensity of the receptor binding signal was assessed by measuring the grey levels of the autoradiographic films with an image analysis system consisting of a solid-state video camera (Hamamatsu, Japan) connected to an Apple Macintosh II personal computer. The public domain Image 1.47 software was used (NIH, Bethesda, MD,USA). Each spleen section was traced with mouse cursor control and the light transmittance was determined as grey level. The grey level of densitometric measurements calculated after subtraction of the film background density was established within the linear range, determined using tritium standards ([^{3}H]Microscales, Amersham).

Densitometric analysis was done in various sections taken at different antero-posterior levels of the spleen with no less than six levels for each binding experiment. The mean light transmittance values were obtained by averaging the measurements from autoradiograms of the spleen sections from at least five rats. Statistical differences were evaluated using Student's *t*-test.

RESULTS

Naive Rats

Figure 1 shows representative autoradiograms of control rat spleen coronal sections incubated with [^{3}H]CP-55,940 10 nM in the absence (A) or presence (B) of an excess of CP-55,940 (10 μM). In autoradiograms from naive rats [^{3}H]CP-55,940 binding sites were distributed with the same pattern reported previously (9). Within the whole spleen, receptor binding was heterogeneous, with the highest amount mainly in the white pulp marginal zone and the mantle of white pulp follicles; it was low in the periarteriolar lymphatic sheaths around central arteries and moderate in the red pulp. Co-incubation with an excess of cold CP55,940 (10 μM, Fig. 1B) strongly reduced [^{3}H]CP-55,940 binding. Densitometric analysis indicated a high degree of specific binding ranging from 56 to 82% of total binding, with a mean of 70.3 ± 2.5%. When average light transmittance was converted to femtomoles bound per milligram of wet weight of tissue using the conversion values of tritium standards, the specific bound level in the whole spleen was 11.3 ± 2.2 fmol/mg tissue.

Acute Treatment

The effect of a single dose of CP-55,940 (0.4 mg/kg IP), its vehicle (20% ethanol in distilled water) or distilled water (control) on binding of [^{3}H]CP-55,940 in rat spleen sections is shown in Fig. 2. Neither acute treatment with synthetic cannabinoid nor the vehicle changed cannabinoid receptor density, as demonstrated by the quantitative densitometric assessment of the [^{3}H]CP-55,940 specific binding in acutely treated rats (Fig.2, bottom right-hand panel).

Chronic Treatment

Figure 3 shows the autoradiograms of rat spleen coronal sections and the quantitative assessment of [^{3}H]CP-55,940 binding in tolerant rats. Eleven days of treatment with distilled water (control) or vehicle (20% ethanol in distilled water) had no effect on cannabinoid receptor density, whereas chronic treatment with CP-55,940 (0.4 mg/kg IP for 11 days) markedly down-regulated cannabinoid receptor sites in spleen. Densitometric analysis of the autoradiograms showed a significant decrease of [^{3}H]CP-55,940 specific binding, ranging from 29% to 75% compared to control depending on the experiment, with a mean reduction of 41.88 ± 13.5% (Fig.3, bottom-right hand panel). When light transmittance values were converted to femtomoles bound per milligram of tissue (wet weight) using the standard curve generated from ^{3}H standards, the mean reduction was 39 ± 10.3%.

DISCUSSION

There are many reports of cannabinoids exerting suppressive effects on the immune system but the structural basis for these effects and their physiological significance is still unclear. There is no evidence that cannabinoid receptors in the immune system can be influenced by acute or chronic exposure to their agonists.

The basal binding in the whole spleen we found in this study is strictly in accordance with the pattern of distribution reported previously (9). The highest amount was mainly in the white pulp, i.e. the mantle layers and the marginal zone. These regions are highly enriched with lymphocytes and mapping studies of lymphocyte subtypes in the rat (5) show they contain mostly B lymphocytes. Macrophages cannot be ruled out as a possible binding target in the spleen because high-binding areas also contain these cells.

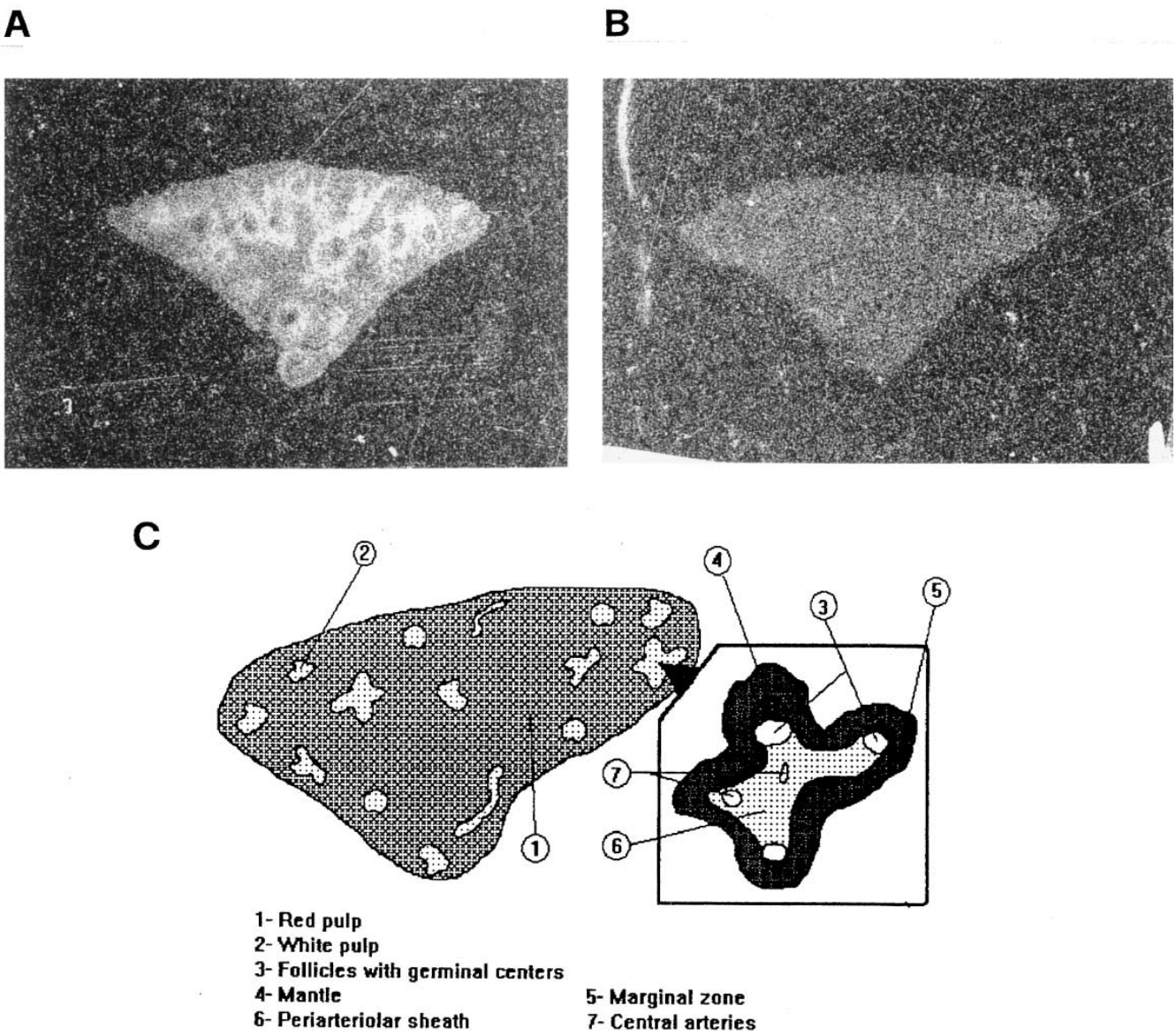

FIG. 1. Autoradiograms of representative coronal sections of rat spleen after binding with [^{3}H]CP-55,940 (10 nM). Binding was done on parallel sections in the absence (A) or presence (B) of an excess of unlabeled CP-55,940 (10 μM). (C) Diagrammatic representation of spleen is shown for reference.

We found that cannabinoid receptors in rat spleen cells could be profoundly down-regulated (40% loss in binding capacity) after chronic treatment with CP-55,940, at doses inducing behavioural tolerance (17). These results indicate that splenic cannabinoid receptors parallel to the cannabinoid receptors are present in the central nervous system, that are also decreased and modulated in conditions of tolerance (14,15).

One inherent problem in binding studies of tissue from treated animals is the possibility that residual drug may compete with binding of labeled compound. Concerning the acute treatment, we found in our experiments the same binding parameters in CP-55,940-treated rats vs control rats, thus indicating that at the time of sacrifice we chose (1 h after treatment) no residual drug was present at the receptor level. For this reason we used the same interval of time for sacrifice also in chronic treatment although one possibility not completely ruled out by our data is that circulating CP-55,940 could accumulate over time at the receptor level and make the receptor unavailable for [^{3}H]CP-55,940 binding , resulting in the appearance of down regulation. The same problem for CB1 receptor was previously addressed by many authors (3,14,16) which performed several strategies for eliminating the contribution of prebound drug but all the technical approaches used, did not preserve the affinity state, capacity and normal distribution of the receptor. In our opinion, studies performing a time dependent assessment of the receptor down regulation, employing longer survival times of rats after the last injection, could be useful to clarify the problem.

It has been hypothized that behavioral tolerance for cannabinoids is pharmacodynamic in nature being due to a great loss

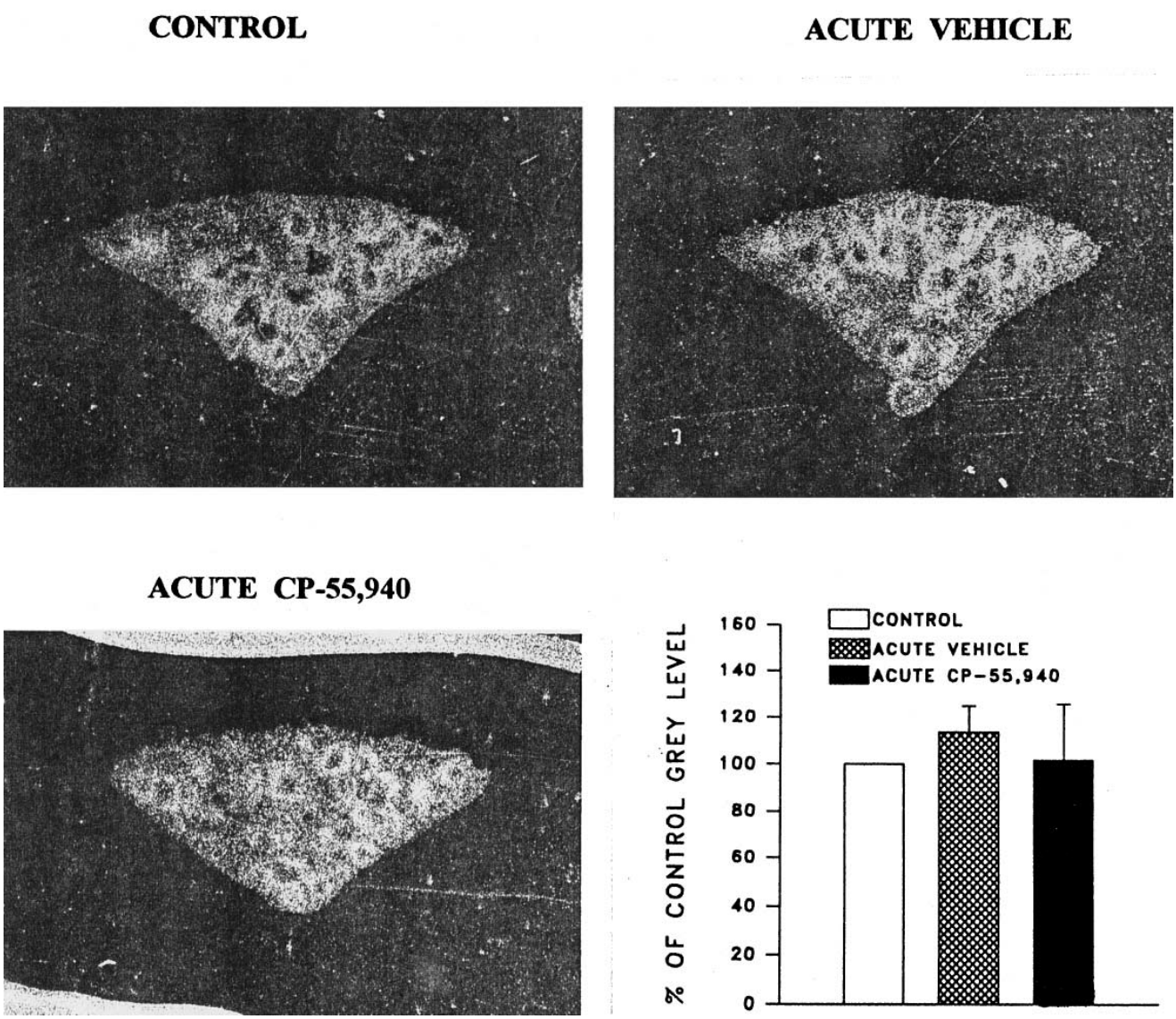

FIG. 2. Autoradiograms of representative coronal sections of rat spleen showing the effect of acute in vivo treatment with CP-55,940 (0.4 mg/kg IP), CP vehicle (20% ethanol in distilled water) and distilled water (control) on [^{3}H]CP-55,940 binding (10 nM) in rat spleen. The bottom right-hand panel shows the quantitative densitometric assessment of [^{3}H]CP-55,940 specific binding in the acute condition. The results are the mean ± SEM of five rats for each group.

of functional binding sites in the rat brain (3,14,15). Presently it is very difficult to establish the same parallel in the spleen given the lack of some important informations about the development of tolerance to the immunosuppressive effects of cannabinoids. In fact data available in the literature refer mostly to the immunosuppressive effects of cannabinoids after in vitro exposure to the drug, lacking an appropriate animal model which describes the kinetic of immunoparameters in animals chronically treated with the drug.

Moreover, establishing a direct link between receptor modifications and functional changes, such as production of antibody, proliferation, modulation of cellular activity, which in many cases require several days, is difficult, since we still have no complete view of the pathway correlating receptor stimulation with immunological action. However, recently evidence (6) suggests that inhibition of lymphocyte proliferation and sheep erythrocyte IgM antibody-forming cell response by cannabinoids are accompanied by a marked inhibition of adenylate cyclase activity in spleen cells, supporting the role of adenylate cyclase inhibition at least in some aspects of immunosuppression by cannabinoids. In this line our results could represent the first step in establishing a link between cannabinoid receptor modulation, intracellular adaptations and immune function.

Another important observation arising from our data is that the down regulation of CB receptor can be attributed to CB2 receptor subtype. In fact, although the cannabinoid receptor ligand we used exibits similar binding affinity for CB2 as for CB1 receptor (10), the lack of CB1 receptor in rat spleen (4) and the specific anatomical localization of the signal

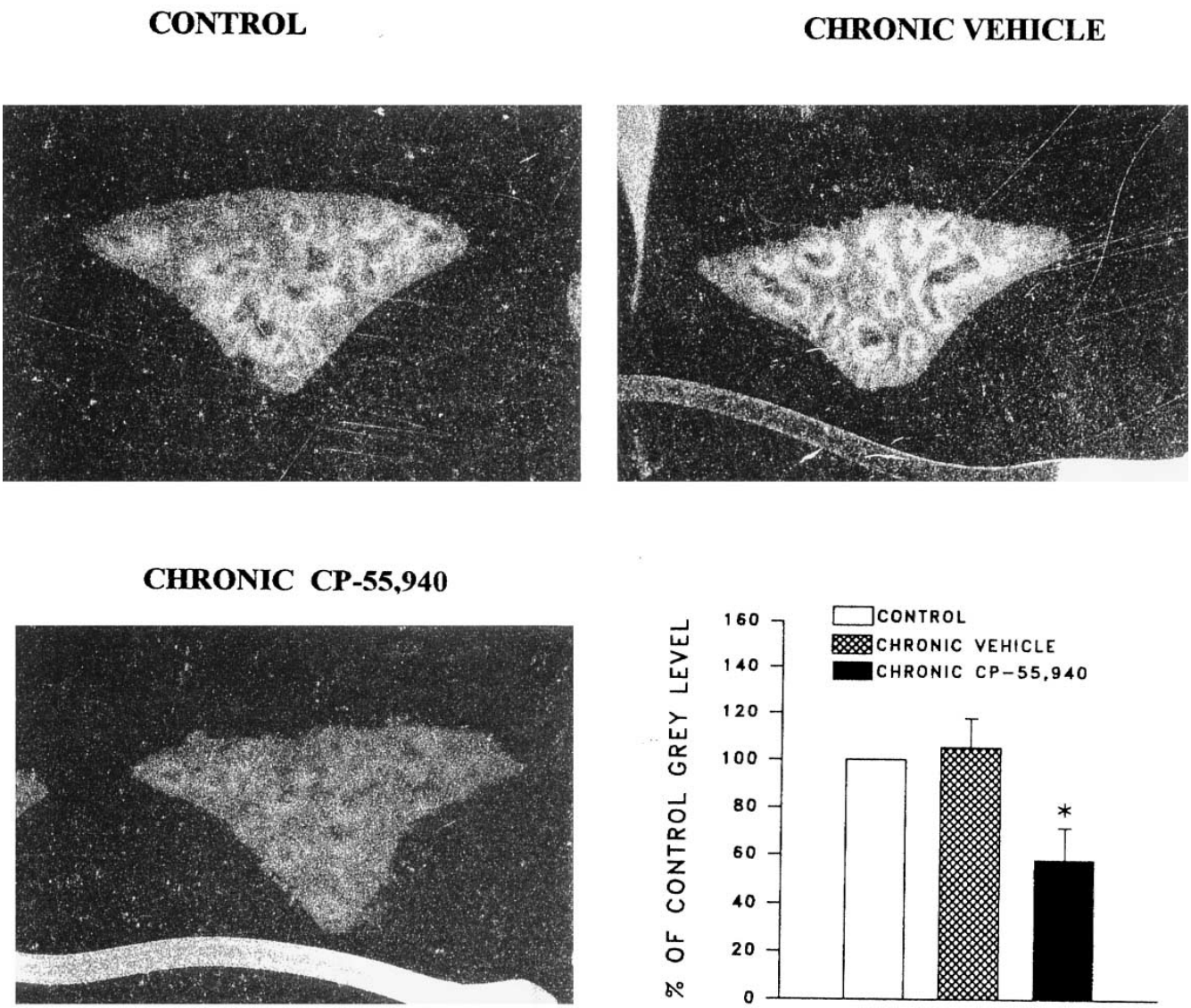

FIG. 3. Autoradiograms of representative coronal sections of rat spleen showing the effect of chronic in vivo treatment with CP-55,940 (0.4 mg/kg IP for 11 days), CP vehicle (20% ethanol in distilled water) and distilled water (control) on [^{3}H]CP-55,940 binding (10 nM) in rat spleen. The bottom right-hand panel shows the quantitative densitometric assessment of [^{3}H]CP-55,940 specific binding in the chronic condition. The results are the mean ± SEM of five rats for each group.
*$p < 0.05$ vs control, Student's t-test.

in the marginal zone of the white pulp strongly suggest that the down regulation affects the CB2 receptor.

In conclusion, the present findings indicate that CB2 receptors in the spleen are down-regulated by chronic exposure to cannabinoids and that this event might be involved in immune modulation by cannabinoid compounds. The intriguing potential physiological, therapeutic and pathogenic significance of cannabinoid receptor modulation awaits further investigation.

ACKNOWLEDGEMENTS

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