Introduction

Depression is one of the frequently-observed behavioral symptoms associated with nicotine (NC) use. In the present study, considering the unique effects of NC (e.g., antidepressant effects have also been reported), the time course of the NC-induced depressive behavioral alterations in a mouse model was compared with a typical depression-inducing stressor. Furthermore, based on the involvement of cannabinoid (CB) receptors in the behavioral effects of NC, the effects of antidepressants including CB ligands (CBs) against the NC-induced behavioral alterations were also investigated. Repeated subcutaneous NC treatments (0.3 mg/kg, 4 days), like repeated immobilization stress (IM) treatments (10 min, 4 days), caused prolonged depressive effects (increased immobility time) at both 2 hr and 1 day time points after the last treatment in the tail suspension test. However, in the NC group, depressive effects (suppressed swimming behaviors) were observed only at the 2 hr time point in the forced swimming test. The antidepressants amitriptyline, clomipramine, and fluvoxamine, the endogenous mixed CB agonist/antagonist virdhamine and the anandamide-like cannabimimetic O-2093 provided antagonistic effects against the depressive behaviors in the tail suspension test. However, in the forced swimming test, NC-induced depressive behaviors were antagonized only by the CBs virdhamine and O-2093. The present results demonstrated depressive effects of NC in two typical behavioral tests, which support the risk of repeated NC use. The shortened behavioral alterations in the forced swimming test, as compared to the IM group, seemed to reflect the neuronal modifications peculiar to NC, which are antagonized by some CBs.

Key words: Nicotine (NC), Cannabinoids (CBs), Immobilization stress, Depressive behaviors, Antidepressants
NC. Close functional interactions between nAChRs and monoamine neurons, the targets of clinically used antidepressants, have been demonstrated by experiments modifying nAChRs by inhibiting monoamine reuptake (Lopez-Valdés and Garcia-Colunga, 2001). Therefore, it is possible that clinically used antidepressants such as brain monoamine reuptake inhibitors are effective against these NC-induced depressive behavioral alterations. On the other hand, it is predicted that brain neurons other than nicotinic acetylcholine (nACh) neurons are also modified characteristically by NC, as suggested previously (Yu and Wecker, 1994), and contribute to behavioral effects of NC. Brain cannabinoid (CB) neurons are among the neurons interacting closely with NC; for example, the amount of endogenous CB ligands (cannabinoids (CBs)) was modified by NC exposure (González et al., 2002). Furthermore, NC-induced behavioral responses were altered immediately by direct modifications of CB receptors (Castañé et al., 2002). Antidepressant effects were also provided by the modifications of CB receptors or the altered amount of some endogenous CBs (Jiang et al., 2005; Gobbi et al., 2005; Bambico et al., 2007).

In the present experiment, using two behavioral tests in mice (forced swimming and tail suspension tests), NC-induced depression-related behavioral alterations were investigated and compared with the depressive behaviors caused by typical stressor immobilization stress (IM). Furthermore, the effects of antidepressants including CBs were examined.

**MATERIALS AND METHODS**

**Animals**

Male ICR mice (60-90 days old) (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) were housed in a forced-air facility, which was maintained at 23°C and 50% relative humidity, with a 12 hr/12 hr light/dark cycle (Boyer and Petersen, 1992; Hayase et al., 2000). The mice were kept separately in single transparent cages measuring 23.5 × 16.5 × 12 cm, and were allowed water and rodent chow ad libitum (Boyer and Petersen, 1992; Hayase et al., 2000). The experiments described in this report were conducted in accordance with the “Guidelines for Animal Experiments” of our institution (1988), which are based on the National Institutes of Health Guide for Care and Use of Laboratory Animals, and any pain experienced by the mice was minimized. For the mouse behavioral experiments, all of the observations and evaluations were performed by a trained observer who was blinded to the treatment conditions and had not been informed of the treatment conditions in advance. Each experimental group contained 7 mice.

**Drug and stressor treatments**

The protocols for the NC and stressor treatments were determined based on preliminary experiments and previous studies examining its behavioral effects (Armario et al., 1991; Hayase et al., 2000; Hayase, 2007). With respect to the NC treatment, repeated subcutaneous (s.c.) doses of NC which caused depressive behaviors most effectively were selected: a single s.c. dose of 0.3 mg/kg was administered daily for 4 days (Hayase, 2007). NC (N-acetyl-5,6,7,8-tetrahydro-1H-indole-3-carboxamide) (N-acetyltoxine), purchased from Banyu Pharmaceutical Co., Ltd. (Tokyo, Japan), clomipramine (clomipramine hydrochloride (CL)), purchased from Ciba-Geigy Ltd. (Takarazuka, Japan), and fluvoxamine (fluvoxamine maleate (FL)) purchased from Tocris Cookson Inc. (Ellisville, MO, USA). As CBs, those drugs which are predicted to act on brain CB receptors were administered. Therefore, the endogenous CBs anandamide (arachidonylethanolamide (AEA)) and vandohamine (VD), the synthetic agonist CP 55940 (((-)-cis-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol) (CP), the synthetic antagonist AM 251 (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) (AM), and synthetic AEA-like ligand O-2093 (N’-di-(3-chloro-4-hydroxy)benzyl-arachidonamide), all purchased from Tocris Cookson Inc., were selected (Devane et al., 1988; Felder et al., 1993; Di Marzo et al., 2002; Porter et al., 2002; McLaughlin et al., 2003).

With respect to the doses of antidepressants and CBs, the doses for the repeated administrations were selected after preliminary experiments examining a broad range
of concentrations. For each drug, data were collected and shown for a dose which induced no depression-related behavioral alterations by itself at the selected time point, but which provided the most effective antidepressant effects (if any). An intraperitoneal (i.p.) injection of the selected dose of each drug was then performed repeatedly before each NC or IM treatment. Except for the CBs, the antidepressants were administered 15 min before each NC or IM treatment, whereas the CBs were administered 60 min before each NC or IM, based on previous studies and preliminary experiments (Van Dijken et al., 1992; Romero et al., 1995).

Since the CBs are not soluble in water, they were initially dissolved in dimethylsulphoxide (DMSO) (Nacalai Tesque Inc.) to one-third of the total volume, and then diluted in distilled water to a volume of 5 ml/kg. Since AEA, VD and O-2093 were provided in ethanol solutions (Tocris Cookson Inc.), the ethanol was evaporated immediately before use under nitrogen gas, and the residues were initially dissolved in DMSO to one-third of the total volume, and then diluted in distilled water to a volume of 5 ml/kg (Compton and Martin, 1997; Costa et al., 2000). The other antidepressants were also dissolved or diluted in DMSO to one-third of the total volume, and then diluted in distilled water to a volume of 5 ml/kg. In the NC- and IM-only groups, even for the time course study of the NC-only treatment, a mixed vehicle solution of DMSO and distilled water at the same ratio as the solutions of antidepressants or CBs was injected instead of the i.p. injection of the antidepressants or CBs, 15 min before each NC or IM treatment. In the antidepressant- and CB-only groups, the same volumes of saline vehicle were injected instead of the s.c. injection of NC. In the control group without any drug or stressor treatment (control group), a control vehicle solution of DMSO and distilled water at the same ratio as the solutions of antidepressants or CBs was injected instead of the antidepressants or CBs, and then the same volume of saline vehicle was injected instead of the NC or IM treatment. The drug and stressor treatments and each experimental session were performed between 15 and 19 hr light cycle.

**Forced swimming test**

Based on previous studies (Porsolt et al., 1977; Hayase et al., 2000), a glass cylinder apparatus 33 cm in height and 18 cm in diameter containing 14 cm of water at 21-23°C was used for the forced swimming test. As parameters for the test, the time until immobility (the time after when only modest swimming behaviors necessary to avoid drowning were observed) and the activity counts for 10 min yielded by the swimming behaviors were evaluated. In the time course study, evaluations of these parameters were performed at the 2 hr and 1 day time points after NC or IM treatment, for different groups of mice at each time point. The activity was counted using the activity-measuring and recording system Supermex-CompACT AMS instrument (Muromachi Kikai Co. Ltd., Tokyo, Japan) by placing the sensor of the instrument over the cylinder at a distance of 20 cm from the water.

**Tail suspension test**

Based on previous studies (Steru et al., 1985), a card-board cube apparatus with one side of 35 cm was used for the tail suspension test. The front surface of the apparatus was open, and each mouse was suspended by fixing the tail in the center of the upper surface using a tail hanger and non-irritating adhesive tape. As a parameter for the test, the total duration of immobility (total immobility time) during the 6 min experimental period was calculated. In the time course study, evaluations of the parameters were performed at the 2 hr and 1 day time points after the NC or IM treatment, for different groups of mice at each time point.

**Statistical analysis**

The data obtained were subjected to two-way analysis of variance (ANOVA) (Alves et al., 2004; Hayase, 2007). In the experiments on the time course of the NC-induced behavioral alterations, a 3 (NC, IM versus vehicle) × 2 (2 hr versus 1 day) factorial design was used for the factors NC or IM treatment × test time. In the experiments on the effects of antidepressants or CBs, a 3 (NC, IM versus vehicle) × 9 (AT, CL, FL, AEA, VD, CP, AM, O-2093 versus vehicle) factorial design was used for the factors NC or IM treatment × treatment × treatment of each antidepressant or CB drug. The results from the ANOVA analyses are summarized in Table 1. For pairwise comparisons, post-hoc Bonferroni tests were performed (Alves et al., 2004; Hayase, 2007). All of the comparisons were performed using statistical software packages (“Excel Statistics” from Social Survey Research Information Co. Ltd. Inc., Tokyo, Japan). Unless otherwise noted, P values less than 0.05 were considered to be statistically significant.

**RESULTS**

**Time course of NC- and IM-induced depressive behavioral alterations in the forced swimming test**

In both NC and IM groups, at the 2 hr time point, depressive behavioral alterations in swimming behaviors, i.e. significantly attenuated times until immobility (Fig. 1a) and
attenuated activity counts (Fig. 1b), were observed for all parameters in the forced swimming test. In the NC group, all of the altered parameter values returned to the control levels at the 1 day time point. However, in the IM group, depressive alterations in the swimming behaviors were observed at both 2 hr and 1 day time points.

The ANOVA (Table 1a) revealed significant main effects of the NC or IM treatment for each parameter value. Effects of test time and interactions between the NC or IM treatment and the test time were also observed, which indicated some time-dependent changes.

Time course of NC- and IM-induced depressive behavioral alterations in the tail suspension test

In both NC and IM groups, at both 2 hr and 1 day time points, depressive behavioral alterations, i.e. significant increases in the total immobility time, were observed in the tail suspension test (Fig. 2).

The ANOVA (Table 1a) revealed significant main effects of the NC or IM treatment.

<table>
<thead>
<tr>
<th>Parameters in the time course of NC- or IM-induced depression-related behavioral alterations.</th>
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<tbody>
<tr>
<td>Forced swimming test</td>
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<tr>
<td>----------------------</td>
</tr>
<tr>
<td>Time until immobility (min)</td>
</tr>
<tr>
<td>NC or IM treatment F(2, 36)=10.32 ***</td>
</tr>
<tr>
<td>Test time F(1, 36)=2.16</td>
</tr>
<tr>
<td>NC or IM treatment × test time interaction F(2, 36)=1.26</td>
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</table>

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<tr>
<th>Parameters in effects of antidepressants or CBs on NC- or IM-induced depression-related behavioral alterations.</th>
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<tbody>
<tr>
<td>Forced swimming test</td>
</tr>
<tr>
<td>----------------------</td>
</tr>
<tr>
<td>Time until immobility (min)</td>
</tr>
<tr>
<td>NC or IM treatment F(2, 162)=28.73 ***</td>
</tr>
<tr>
<td>Antidepressants or CBs treatment F(8, 162)=1.91</td>
</tr>
<tr>
<td>NC or IM × antidepressants or CBs treatment interaction F(16, 162)=0.59</td>
</tr>
</tbody>
</table>

F values with degrees of freedom are shown. Significant effects and interactions are noted: *P < 0.05, **P < 0.01, ***P < 0.001.

Effects of antidepressants or CBs against NC- and IM-induced depressive behavioral alterations in the forced swimming test

The effects of the antidepressants or CBs were examined at the 2 hr time point after the last NC or IM treatment, and the data for the doses selected in the above-way (10 mg/kg for the drugs except for CP or 2.5 mg/kg for CP) are shown in Fig. 3. For the NC group, in those groups co-treated with the CBs, VD and O-2093, significant recoveries from the depressive behavioral alterations (i.e. recoveries from the attenuated times until immobility and attenuated activity counts) to the control levels were observed for each parameter. For the IM group, in those groups co-treated with the antidepressants AT, CL, and FL as well as the CBs, VD and O-2093, significant recoveries from the depressive behavioral alterations to the control levels were observed for each parameter. With respect to those groups co-treated with AT, CL, and FL, all values in the NC group tended to be attenuated compared to the IM group, and significant decreases were observed for the activity counts. No significantly different effects were observed between the monoamine reuptake inhibitors AT, CL, and FL, although the rank of selectivity for serotonin is FL > CL > AT...
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The ANOVA (Table 1b) revealed significant main effects of the NC or IM treatment for each parameter value. Furthermore, for the activity counts, significant main effects of the antidepressants or CBs treatment and interactions between NC or IM treatment and antidepressants or CBs treatment were also observed, which indicated some influence of the antidepressants and/or CBs on the NC- or IM-induced depression-related behavioral alterations.

**Effects of antidepressants or CBs against NC- and IM-induced depressive behavioral alterations in the tail suspension test**

The effects of the antidepressants or CBs were examined at the 2 hr time point after the last NC or IM treatment, and the data for the dose selected in the above-explained way (10 mg/kg for the drugs except for CP or 2.5 mg/kg for CP) are shown in Fig. 4. For both NC and IM treatment groups, in the groups co-treated with the antidepressants AT, CL and FL as well as the CBs, VD and O-2093, significant recoveries from the depressive behavioral alterations (i.e. recovery from the increased total immobility time) to the control levels were observed. No significantly different effects were observed between the monoamine reuptake inhibitors AT, CL and FL. In each antidepressant- or CB-only group, no significant alterations in each parameter value were observed (Table 2).

The present experiments demonstrated the occurrences and continuations of NC-induced “depressive” effects in the forced swimming and tail suspension tests. Like the groups of mice treated with IM, a typical stressor which induces depressive effects, both suppressed swimming behaviors in the forced swimming test and increased immobility time in the tail suspension test were observed in the NC group under the present conditions. However, antidepressant effects have also been demonstrated for NC in other animal models (Tizabi et al., 1999; Vazquez-Palacios et al., 2004; Slawecki et al., 2005). The investigation of those NC treatment animal models, in which antidepressant effects were predominantly observed, has revealed that the appearance of depressive versus antidepressant effects seemed to be controlled by the treatment

**DISCUSSION**

(Brocco et al., 2002). In each antidepressant- or CB-only group, no significant alterations in each parameter value were observed (Table 2).
conditions, such as the dose, number of administrations, time after use, previous exposure to NC, etc., which suggests a complicated mechanism underlying the effects of NC (Tizabi et al., 1999; Vazquez-Palacios et al., 2004; Slawecki et al., 2005). In previous studies, even repeated doses of subcutaneous NC (less than 0.5 mg/kg for less than 15 days), such as a single dose of NC, caused antidepressant effects at early time points (within 15 min) after the last NC treatment (Tizabi et al., 1999; Vazquez-Palacios et al., 2004; Slawecki et al., 2005). This is consistent with the preliminary unpublished data of the author. On the other hand, with respect to the NC-induced depressive behavioral alterations in mouse experimental models, withdrawal depressive behaviors were observed after the 15-day time point following NC treatments of more than 0.5 mg/kg per day for at least 15 days (Mannucci et al., 2006), but the detailed time course before the 1 day time point has not been studied. In the present mouse experimental model, depressive behaviors were observed before and/or at the 1 day time point after repeated NC treatments for 4 days. Although the mechanisms underlying the appearance of these dual contrary behavioral effects have not been elucidated sufficiently, complicated and immediate neuronal and endocrinial modifications (such as modified monoamine turnover, stimulation of the sympathoadrenal system, increased stress hormones, etc.) have been reported to accompany NC treatment, and seem to be affected by differing conditions such as the number of NC administrations (Kirch et al., 1987; Morse, 1989; Sun et al., 2003). Furthermore, the time course of those modifications seems to be correlated with the appearance of these dual behavioral effects.

Between the NC and IM groups, there were some differences in the time courses and in terms of the drugs which antagonized these “depressive” effects. One of the characteristics of the NC group is that, unlike the IM group, prolonged depressive effects at the 1 day time point were not detected in the forced swimming test (Fig. 1). On the other hand, in the tail suspension test, prolonged depressive behavioral symptoms were observed in both NC and IM groups. The absence of prolonged depressive effects for NC in the forced swimming test seems to be correlated with the mechanisms underlying the suppressed swimming behaviors. Depression-relat-

![Graph 1](image1.png)

![Graph 2](image2.png)

**Fig. 3.** Effects of antidepressants or CBs on NC- and IM-induced depressive behavioral alterations in the forced swimming test. The values for the time until immobility (a) and the activity counts (b) at the 2 hr time point after the last NC or IM treatment are shown as means ± S.D. (n = 7 for each group). The data for the selected doses of the antidepressants or CBs (10 mg/kg for the drugs except for CP or 2.5 mg/kg for CP), as well as the data in the control, and NC- and IM-only groups (NC/IM), are shown. a: significant (p < 0.05) decrease as compared to the control group; b: significant (p < 0.05) decrease as compared to the IM group. The abbreviations of the co-administered antidepressants or CBs captioned below the X-axis are noted in the text.
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### Table 2. Data for behavioral tests in antidepressant- and CB-only groups.

<table>
<thead>
<tr>
<th></th>
<th>Forced swimming test</th>
<th>Tail suspension test</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Time until immobility (min)</td>
<td>Activity counts (counts/10 min)</td>
</tr>
<tr>
<td>Control group</td>
<td>3.79 ± 0.59</td>
<td>3016 ± 321</td>
</tr>
<tr>
<td>AT group</td>
<td>3.86 ± 0.52</td>
<td>3041 ± 330</td>
</tr>
<tr>
<td>CL group</td>
<td>3.86 ± 0.69</td>
<td>3059 ± 333</td>
</tr>
<tr>
<td>FL group</td>
<td>3.93 ± 0.62</td>
<td>3069 ± 346</td>
</tr>
<tr>
<td>AEA group</td>
<td>3.79 ± 0.59</td>
<td>3019 ± 330</td>
</tr>
<tr>
<td>VD group</td>
<td>3.86 ± 0.69</td>
<td>3058 ± 349</td>
</tr>
<tr>
<td>CP group</td>
<td>3.79 ± 0.70</td>
<td>3024 ± 332</td>
</tr>
<tr>
<td>AM group</td>
<td>3.79 ± 0.70</td>
<td>3025 ± 336</td>
</tr>
<tr>
<td>O-2093 group</td>
<td>3.86 ± 0.69</td>
<td>3065 ± 349</td>
</tr>
</tbody>
</table>

Values represent means ± S.D. (n = 7 for each group).

Fig. 4. Effects of antidepressants or CBs on NC- and IM-induced depressive behavioral alterations in the tail suspension test. The values for the total immobility time at the 2 hr time point after the last NC or IM treatment are shown as means ± S.D. (n = 7 for each group). The data for the selected doses of the antidepressants or CBs (10 mg/kg for the drugs except for CP or 2.5 mg/kg for CP), as well as the data in the control, and NC- and IM-only groups (NC/IM), are shown. The symbols for the differences are the same as in other figures. The abbreviations of co-administered antidepressants or CBs captioned below the X-axis are noted in the text.

ed behavioral reactions seem to be mediated by a number of neuronal mechanisms activated by various stressors. Furthermore, regardless of the type of stressors, the relative contribution of each mechanism may be different between the forced swimming and tail suspension tests. Therefore, it was hypothesized that the NC-induced neuronal modifications correlated with the behavioral alterations in the forced swimming test, would continue for a shorter time as compared to the IM-induced neuronal modifications. Although the detailed neuronal modifications correlated with the NC-suppressed swimming behaviors have not been elucidated, nAChR-mediated immediate modifications in the monoamine system (e.g. alterations in dopamine levels, etc.), which can be caused characteristically by NC, have been demonstrated (Tani et al., 1997). Furthermore, even without any drug or stressor treatments, behavioral alterations during the forced swimming test, unlike the tail suspension test, have been reported to be accompanied immediately by rapid alterations in the brain monoamine levels (Renard et al., 2003). These characteristic alterations in the monoamine system in the forced swimming test, when modified by NC, may
give rise to the shorter duration of depressed swimming behaviors in the NC group as compared to the IM group.

Against the NC-induced depressive behavioral alterations, significant antagonistic effects were provided by some antidepressants which inhibit the reuptake of monoamines and several CBs. However, unlike the IM group, the antidepressant drugs which inhibit the reuptake of monoamines (AT, CL, and FL) were not significantly effective in the NC group in the forced swimming test (Fig. 3). This absence of significant antidepressant effects for these drugs could not be predicted based on the previous data and suggested a contribution of the monoamine system to the depression-related behavioral alterations in both forced swimming and tail suspension tests (Steru et al., 1985; Rénéric et al., 1998; Fujishiro et al., 2001). Nevertheless, considering the characteristic NC-induced immediate modifications in the monoamine system in the forced swimming test, which include the alterations in dopamine levels (Tani et al., 1997; Renard et al., 2003), there seems to be a possibility that the antidepressants such as AT, CL and FL, which inhibit mainly the reuptake of noradrenaline and 5-hydroxy-tryptamine (5-HT), cannot antagonize effectively the NC-induced depressive swimming behaviors, at least during the test time, as compared to the other “antidepressant” drugs.

Among the CBs, in both NC and IM groups, only the mixed agonistic/antagonistic endocannabinoid VD (Porter et al., 2002) and the AEA-like cannabimimetic O-2093 (Di Marzo et al., 2002) provided significant antidepressant effects. On the other hand, the other CBs, which have been reported to function as pure agonists (AEA, CP, etc.) or antagonists (AM etc.) on brain CB receptors (Devane et al., 1988; Felder et al., 1993; McLaughlin et al., 2003), did not provide any antidepressant effects. VD has been reported to provide agonistic effects on brain CB receptors when administered alone, but has also been reported to attenuate the effects of other agonistic drugs such as AEA (Porter et al., 2002). O-2093 has been reported to provide behavioral effects similar to AEA (e.g. hypolocomotion, antinociception, hypothermia, etc.), but has also been reported to exhibit very low affinity for known brain CB receptors which interact with AEA (Di Marzo et al., 2002). Based on the observed effects of VD, it can be hypothesized that the mixture of agonistic and antagonistic effects on brain CB receptors, but not pure agonistic effects, was favorable for blocking the depressive effects. However, based on the previous literature on O-2093 (Di Marzo et al., 2002) and the present results, the contribution of other unidentified brain receptors which interact with O-2093 is suspected. Furthermore, it is possible that these unidentified receptors may interact with VD. Although the CB-related mechanisms underlying the depressive effects of NC have not been elucidated sufficiently, the present results strongly support the presence of diverse neuronal mechanisms contributing to this phenomenon.

In conclusion, the present results demonstrated that the depressive effects of NC are similar to the effects of a typical depression-inducing stressor in two representative behavioral tests. In the tail suspension test, prolonged depressive effects which support the risk of repeated NC use were observed. The shorter duration of the behavioral alterations in the forced swimming test as compared to the IM group seemed to reflect neuronal modifications peculiar to NC, which were not antagonized by those antidepressants which inhibit the reuptake of monoamines. Some clinical studies have reported that NC induced a long-lasting depression for mainly withdrawal cases after repeated NC use (West et al., 1989; Lerman et al., 1996). The present study, using an animal model, adds more data on the NC-induced depressive behavioral alterations after a relatively small number of repeated NC administrations (4 days), which warns of the risk of NC use. Furthermore, considering the “antidepressant” effects of the atypical CBs, VD and O-2093 against the NC-induced depressive behaviors, one can predict that the characteristic effects as a mixed CB agonist/antagonist and/or the involvement of unidentified CB receptors contributed to their effects.

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