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Working memory- and anxiety-related behavioral effects of repeated nicotine as a stressor: the role of cannabinoid receptors

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Abstract

Background: Like emotional symptoms such as anxiety, modulations in working memory are among the frequently-reported but controversial psychiatric symptoms associated with nicotine (NC) administration. In the present study, repeated NC-induced modulations in working memory, along with concurrently-observed anxiety-related behavioral alterations, were investigated in mice, and compared with the effects of a typical cognition-impairing stressor, immobilization stress (IM). Furthermore, considering the structural and functional contributions of brain cannabinoid (CB) receptors in NC-induced psychiatric symptoms including emotional symptoms, the interactive effects of brain CB receptor ligands (CB ligands) and NC and/or IM on the working memory- and anxiety-related behaviors were examined.

Results: Statistically significant working memory impairment-like behavioral alterations in the Y-maze test and anxiety-like behavioral alterations in the elevated plus-maze (EPM) test were observed in the groups of mice treated with 0.8 mg/kg NC (subcutaneous (s.c.) 0.8 mg/kg treatment, 4 days) and/or IM (10 min treatment, 4 days). In the group of mice treated with NC plus IM (NC-IM group), an enhancement of the behavioral alterations was observed. Among the CB type 1 (CB1) antagonist AM 251 (AM), the non-selective CB agonist CP 55,940 (CP), and the CB1 partial agonist/antagonist virodhamine (VD), significant recovering effects were provided by AM (0.2-2.5 mg/kg) and VD (5 mg/kg) against the working memory impairment-like behaviors, whereas significant anxiolytic-like effects (recoveries from both attenuated percentage of entries into open arms and attenuated percentage of time spent on open arms) were provided by VD (1–10 mg/kg) and CP (2 mg/kg) against the anxiety-like behaviors.

Conclusions: Although working memory impairment- and anxiety-like behavioral alterations were commonly induced in the NC, IM, and NC-IM groups and the therapeutic involvement of CB receptors was shown, there were discrepancies in the types of effective CB ligands between the working memory- and anxiety-related behaviors. The differential involvements of CB receptor subtypes and indirectly activated neurotransmitter systems may contribute to these discrepancies.

Keywords: Nicotine, Immobilization stress, Working memory, Anxiety, Cannabinoid, AM 251, CP 55,940, virodhamine

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Background

Nicotine (NC) is the substance which sustains the addictive use of tobacco, and tobacco results in numerous harmful health effects and continues to be the leading cause of preventable death [1,2]. It has been reported that addicted tobacco users suffer from NC-induced cognitive impairments in some conditions of smoking, as well as modulated moods such as anxiety- and depression-related symptoms [3-5]. Cognitive impairments including deficits in working memory, a process for maintaining temporary active information [6], have been regarded as being among the representative symptoms of NC withdrawal observed in NC-dependent human and rodent models [3,7,8]. Furthermore, the direct neurotoxic effects of NC have also been reported depending on the treatment conditions such as dose, period and paradigm, and this neurotoxicity has been suggested to induce memory impairments, particularly at earlier periods in development [9-12]. However, in some clinical and experimental animal studies, cognitive improvements or absence of any effects have been demonstrated [13-17]. Negative, positive or no effects of NC have also been reported against the anxiety-related behaviors [18-20].

Working memory impairments have been reported for various stressors such as restraint stress (immobilization stress) in both humans and rodent models [21-23]. Like certain NC treatments, such stressors also induce and exacerbate the anxiety-like behavioral responses in rodent models [24,25]. Furthermore, it has been suggested that brain regions such as the medial prefrontal cortex, for which NC-induced modulations have been demonstrated [26,27], are concurrently involved in the development of stress-induced working memory impairments and anxiety [28-31]. However, there are only a few studies investigating the characteristic effects of NC as a stressor, particularly those on cognitive function [32,33].

A considerable number of studies have implicated the relationship between NC and stress. For example, in some rodent models, repeated or acute stress has been shown to aggravate the behavioral and neuronal effects of NC [34-36]. Recent human studies have shown some directly-exacerbated mood symptoms induced by stress in smokers [37,38]. However, against the behavioral and neuronal impairments caused by stress, antagonistic effects of subsequently administered NC have been shown in some rodent models [39-41]. With respect to cognitive function, NC has also been reported to block stress-induced impairments in several experimental conditions in rodents [41,42]. Nevertheless, the above-mentioned neurotoxic effects of NC which could lead to cognitive dysfunction [10-12] may be correlated with the possibility that NC and stress augment each other's unfavorable effects on cognitive function.

In previous studies, a strong involvement of brain cannabinoid (CB) receptors, typically CB type 1 (CB1) receptors, was reported in the representative emotion-related behaviors (anxiety- and depression-like behaviors) induced by NC [18,43,44] and stress [45] in rodents. This is consistent with the prominent behavioral alterations induced by NC in CB1 knockout mice [46], and the overlapping distribution of CB1 receptors and nicotinic acetylcholine receptors (nAChRs) in some brain regions which supports functional interactions between these receptors [47,48]. Furthermore, recent reviews suggest that CB1 receptors contribute to deficits in memory including working memory by demonstrating that CB1 agonists impair memory formation and CB1 antagonists reverse these impairments [49,50]. However, there have been a limited number of studies on the direct contribution of brain CB receptors to the memory-related effects of NC [51,52]. The participation of CB1 receptors has also been reported in anxiety processes, but the roles of CB1 agonist are contradictory in that both anxiolytic-like and anxiogenic-like effects have been induced depending on the treatment conditions [53,54]. Against the NC-induced anxiety-related behaviors, inconsistent and contradictory effects of CB1 agonists and other CB ligands have also been demonstrated [18,43].

In the present study, using behavioral tests in mice (Y-maze and elevated plus-maze (EPM) test), the working memory- and anxiety-related behavioral alterations caused by NC were assessed and compared with those caused by immobilization stress (IM), a typical stressor. The interactions between the NC- and IM-induced behavioral effects were also examined. Furthermore, considering the possible involvement of brain CB receptors in these NC- and/or IM-induced memory- and anxiety-related behavioral alterations, the effects of selected CB ligands (the CB1 antagonist AM 251, the non-selective CB agonist CP 55,940, and the CB1 partial agonist/antagonist virodhamine) were evaluated against these behavioral alterations, as described in previous studies [43,51,52].

Methods

Animals

Based on previous studies on NC and stressor treatments [43,55], male ICR mice (80 ± 10 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 h/12 h light/dark cycle. 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. The experiments described in this report were conducted in accordance with the "Guidelines for Animal Experiments" of the institution (updated in

2007) [56], which are based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and any pain experienced by the mice was minimized. These guidelines were approved by the institutional ethics committee for animal experiments [56]. All of the observations and evaluations were performed by a trained observer who was blinded to the treatment conditions and was not informed of the treatment conditions in advance. Each experimental group contained 10 mice.

Drug and stressor treatments

The protocols for the NC and stressor treatments were determined based on preliminary experiments and previous studies [43,55,57]. With respect to NC, repeated subcutaneous (s.c.) doses of NC which caused the emotional behaviors (anxiety- and depression-like behaviors) effectively in mice [43] were selected: single s.c. doses of 0.3 or 0.8 mg/kg were administered daily for 4 days. NC (Nacalai Tesque, Inc., Kyoto, Japan) was supplied in free-base form at 95% purity, and was freshly dissolved in saline to a volume of 5 ml/kg immediately before each administration. With respect to the stressor, treatments using IM, which have also been demonstrated to cause these emotional behaviors in rodents [44,58], were used. In the present experiments, repeated IM treatments in which the effects were almost equivalent to the peak effects of the NC treatments in preliminary experiments were selected: 10 min of IM, which was induced by placing the mouse in a narrow space (diameter about 12 cm) in a vinyl bag with some breathing holes, was performed once per day for 4 days. Furthermore, to investigate the interactions between NC and IM, the behavioral alterations were examined in the NC plus IM group (NC-IM group) which received the above s.c. dose of NC 10 min before the IM treatment once per day for 4 days, according to a previous study [59].

The CB ligands AM 251 (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) (AM), CP 55,940 ((−)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl) cyclohexanol) (CP), and virodhamine (O-(2-aminoethyl)-5Z,8Z,11Z,14Z-eicosatetraenoate) (VD) were purchased from Tocris Cookson Inc. (Ellisville, Missouri, USA), and the doses were selected based on previous studies and preliminary experiments [43,51,52]. For each drug, the data were collected and shown for those intraperitoneal (i.p.) doses which induced no toxic behavioral alterations by themselves at the prescribed time point: 0.2, 1 and 2.5 mg/kg for AM, 0.5, 2 and 5 mg/kg for CP, and 1, 5 and 10 mg/kg for VD. The CB ligands were dissolved and diluted using a mixed solution of dimethylsulphoxide (DMSO) plus distilled water, and were administered in a volume of 5 ml/kg, 60 min before each NC, IM or NC-IM treatment, considering the previously examined time course of the effects of CB ligands against the NC- and/or IM-induced working memory- and anxiety-related behaviors [43,58]. Since VD was provided in an ethanol solution (Tocris Cookson Inc.), the ethanol was evaporated immediately before use under nitrogen gas, and the residue was re-suspended in the same mixed DMSO/distilled water solution. In the NC- and IM-only groups, a mixed vehicle solution of DMSO and distilled water at the same ratio as the CB ligand solutions was injected instead of the CB ligands. In the CB ligand-only groups, the same volume of saline vehicle was injected instead of the NC or IM treatment. In the control group without any drug or stressor treatment (control group), the mixed vehicle solution of DMSO and distilled water was injected instead of the CB ligands, 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 h of the light cycle.

Y-maze test

Based on previous studies [28,60,61], alterations in working memory-related behaviors were examined in the Y-maze test using a cardboard apparatus that consisted of three enclosed arms 30 × 5 × 15 cm (length, width, and height) which converged on an equilateral triangular center platform (5 × 5 × 5 cm). After the number of spontaneous alteration performance (SAP), which was defined as the number of successive triplet entry performances into each of the three arms without any repeated entries [28,60,61], and the total number of entries into arms were evaluated (8 min test periods), the rate of spontaneous alteration performance (SAP rate) (%) was calculated as a parameter for the working memory-related behaviors. The total number of entries into arms was assessed as a parameter representing locomotor activity [60,61]. Considering the previous data [58], the evaluations of these parameters were performed at the 2 h time point after the last NC, IM or NC-IM treatment. At the beginning of each experimental session, each mouse was placed in the center platform of the maze, facing all three arms immediately before the session [58].

Elevated plus-maze (EPM) test

Based on previous studies [18,43,62-64], alterations in anxiety-related behaviors were examined in the EPM test using a cardboard apparatus that consisted of two opposite open arms 50 × 10 cm (length and width) and two enclosed arms 50 × 10 × 30 cm (length, width, and height), positioned 50 cm from the floor. After the number of entries into open arms, the time spent on open arms (sec), and the total number of entries into arms were evaluated (5 min test periods), the percentage of

entries into open arms and the percentage of time spent on open arms were calculated as parameters for the anxiety-related behaviors. The total number of entries into arms was assessed as a parameter representing locomotor activity [63]. Considering the previous data [43], the evaluations of these parameters were performed at the 2 h time point after the last NC, IM or NC-IM treatment. At the beginning of each experimental session, each mouse was placed diagonally in the center platform of the maze, facing both the open and enclosed arms [43].

Statistical analysis

The data were subjected to two-way analysis of variance (ANOVA) for both effects of NC and/or IM and effects of the CB ligands [65]. With respect to the experiments examining the effects of NC and/or IM, a 3 (0.3 mg/kg NC, 0.8 mg/kg NC versus vehicle) × 2 (IM versus vehicle) factorial design was used for the factors NC × IM treatment. With respect to the experiments examining the effects of the CB ligands, a 4 (NC, IM, NC-IM versus vehicle) × 4 (three doses of each CB ligand versus vehicle) factorial design was used for the factors NC and/or IM treatment × treatment using each CB ligand. For pairwise comparisons, Bonferroni post-hoc tests were performed [65]. All of the comparisons were performed using a statistical software package ("Excel Statistics" from Social Survey Research Information Co. Ltd. Inc., Tokyo, Japan). P values less than 0.05 were considered to be statistically significant.

Results

NC- and/or IM-induced working memory-related behavioral alterations in the Y-maze test

In the 0.8 mg/kg NC, IM and NC-IM groups, at the 2 h time point, behavioral alterations indicating working memory impairments, i.e. statistically significantly attenuated SAP rates (Figure 1), in spite of the absence of significant changes in the total numbers of entries into arms (Table 1), were observed in the Y-maze test. This is consistent with the results of the ANOVA revealing statistically significant main effects of NC ($F(2, 54)=11.02$, $P<0.001$) and IM ($F(1, 54)=34.03$, $P<0.001$). For the NC-IM groups, the SAP rates were significantly attenuated as compared to the NC and/or IM groups.

Table 1 Total number of entries into arms in experiments examining the effects of NC and/or IM (experiments shown in Figures 1 and 2)

	Y-maze test (Figure 1)	EPM test (Figure 2)
Control group	47.1 ± 8.3	63.2 ± 12.4
NC 0.3 group	51.2 ± 8.4	59.1 ± 12.3
NC 0.8 group	43.7 ± 8.5	58.5 ± 11.9
IM group	44.5 ± 8.2	60.8 ± 12.1
NC 0.3-IM group	49.0 ± 8.6	56.5 ± 12.0
NC 0.8-IM group	43.2 ± 8.6	55.0 ± 11.7

NC- and/or IM-induced anxiety-related behavioral alterations in the EPM test

In the NC, IM and NC-IM groups, at the 2 h time point, anxiety-like behavioral alterations, i.e. statistically significantly attenuated percentage of entries into open arms (Figure 2a) and significantly attenuated percentage of time spent on open arms (Figure 2b), in spite of the absence of significant changes in the total numbers of entries into arms (Table 1), were observed in the EPM test. This is consistent with the results of the ANOVA revealing statistically significant main effects of NC ($F(2, 54)=195.21$, $P<0.001$ for the percentage of entries into open arms and $F(2, 54)=70.18$, $P<0.001$ for the percentage of time spent on open arms) and IM ($F(1, 54)=104.38$, $P<0.001$ for the percentage of entries into open arms and $F(1, 54)=46.89$, $P<0.001$ for the percentage of time spent on open arms). For the NC-IM groups, the parameter values were significantly attenuated as compared to the IM group, which is consistent with the results of the ANOVA revealing significant interactions between the NC and IM treatments for each parameter value ($F(2, 54)=91.17$, $P<0.001$ for the percentage of entries into open arms and $F(2, 54)=18.90$, $P<0.001$ for the percentage of time spent on open arms).

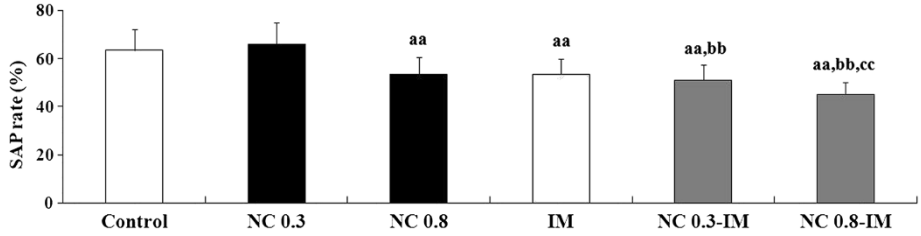

Figure 1 Working memory-related behavioral alterations (SAP rate (%)) caused by repeated nicotine (NC) and/or immobilization stress (IM) in the Y-maze test. The values at the 2 h time point after the last NC (0.3 or 0.8 mg/kg, s.c.) or IM treatment are shown as means ± S.D. (n=10 for each group). aa ($p<0.01$): significant attenuation as compared to the control group; bb ($p<0.01$): significant attenuation as compared to the NC group; cc ($p<0.01$): significant attenuation as compared to the IM group.

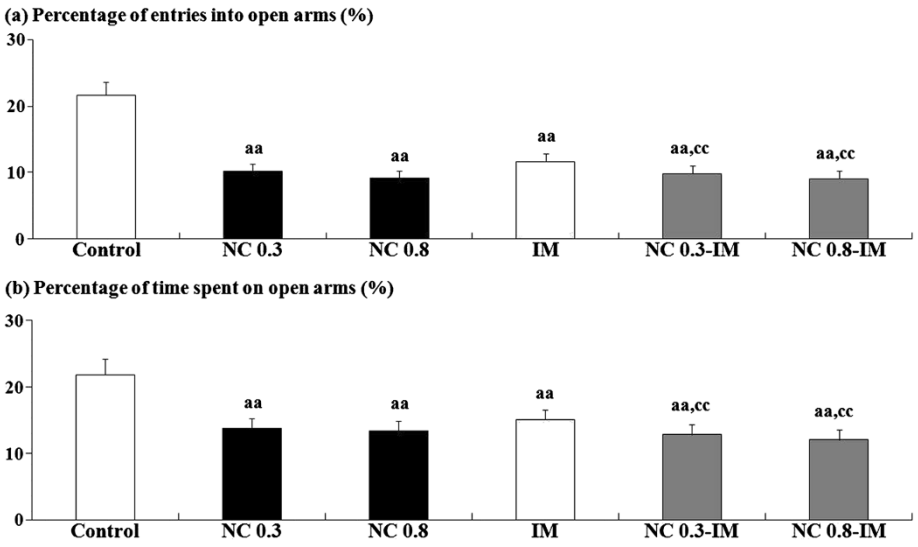

Figure 2 Anxiety-related behavioral alterations caused by repeated nicotine (NC) and/or immobilization stress (IM) in the elevated plus-maze (EPM) test. Data are presented for percentage of entries into open arms (**a**) and percentage of time spent on open arms (**b**). The values at the 2 h time point after the last NC (0.3 or 0.8 mg/kg, s.c.) or IM treatment are shown as means ± S.D. (n=10 for each group). aa ($p<0.01$): significant attenuation as compared to the control group; cc ($p<0.01$): significant attenuation as compared to the IM group.

Effects of CB ligands against NC (0.8 mg/kg)- and/or IM-induced working memory-related behavioral alterations in the Y-maze test

For the 0.8 mg/kg NC, IM and 0.8 mg/kg NC-IM groups, at the 2 h time point, statistically significant recoveries from the impairments in working memory-related behavioral alterations, i.e. recoveries from the attenuated SAP rates, in spite of the absence of significant changes in the total numbers of entries into arms (Table 2), were observed in the groups co-treated with AM (Figure 3a). This is consistent with the results of the ANOVA revealing statistically significant main effects of AM ($F(3, 144)= 11.20$, $P<0.001$) for the SAP rate. Furthermore, in the groups co-treated with 5 mg/kg VD, significant recoveries from the behavioral alterations were also observed (Figure 3c), which is consistent with the results of the ANOVA revealing statistically significant main effects of VD ($F(3, 144)=11.74$, $P<0.001$) for the SAP rate. In each CB ligand-only group, no significant alterations as compared to the control group were observed for each parameter value under the present experimental conditions.

Table 2 Total number of entries into arms in experiments examining the effects of CB ligands in the Y-maze test (experiments shown in Figure 3)

(a) AM group	Control	NC	IM	NC-IM
Control group	47.1 ± 8.3	43.7 ± 8.5	44.5 ± 8.2	43.2 ± 8.6
AM 0.2 group	46.9 ± 8.3	44.0 ± 8.6	45.1 ± 8.2	43.7 ± 8.6
AM 1 group	46.6 ± 8.3	44.5 ± 8.5	45.5 ± 8.3	44.1 ± 8.6
AM 2.5 group	46.2 ± 8.3	45.0 ± 8.6	46.0 ± 8.3	44.6 ± 8.6
(b) CP group	**Control**	**NC**	**IM**	**NC-IM**
Control group	47.1 ± 8.3	43.7 ± 8.5	44.5 ± 8.2	43.2 ± 8.6
CP 0.5 group	46.8 ± 8.3	43.5 ± 8.6	44.2 ± 8.4	43.0 ± 8.7
CP 2 group	46.5 ± 8.4	43.4 ± 8.6	44.0 ± 8.5	42.8 ± 8.8
CP 5 group	46.1 ± 8.4	43.2 ± 8.7	43.7 ± 8.5	42.6 ± 8.9
(c) VD group	**Control**	**NC**	**IM**	**NC-IM**
Control group	47.1 ± 8.3	43.7 ± 8.5	44.5 ± 8.2	43.2 ± 8.6
VD 1 group	46.8 ± 8.4	43.9 ± 8.6	44.8 ± 8.3	43.5 ± 8.7
VD 5 group	46.5 ± 8.4	44.2 ± 8.7	45.1 ± 8.4	43.7 ± 8.8
VD 10 group	46.2 ± 8.5	44.4 ± 8.8	45.4 ± 8.5	43.9 ± 8.9

Effects of CB ligands against NC (0.8 mg/kg)- and/or IM-induced anxiety-related behavioral alterations in the EPM test

For the 0.8 mg/kg NC, IM and 0.8 mg/kg NC-IM groups, at the 2 h time point, statistically significant recoveries from the anxiety-like behavioral alterations, i.e. recoveries from both attenuated percentage of entries into open arms and attenuated percentage of time spent on open arms, in spite of the absence of significant changes in the total numbers of entries into arms (Table 3), were observed in the groups co-treated with VD (1–10 mg/kg) (Figure 4c). This is consistent with the results of the ANOVA revealing statistically significant main effects of VD ($F(3, 144)= 205.84$, $P<0.001$ for the percentage of entries into open arms and $F(3, 144)=58.29$, $P<0.001$ for the percentage of time spent on open arms) and significant interactions of the VD versus NC and/or IM treatments ($F(9, 144)=18.88$, $P<0.001$ for the percentage of entries into open arms and $F(9, 144)=5.58$, $P<0.001$ for the percentage of time spent

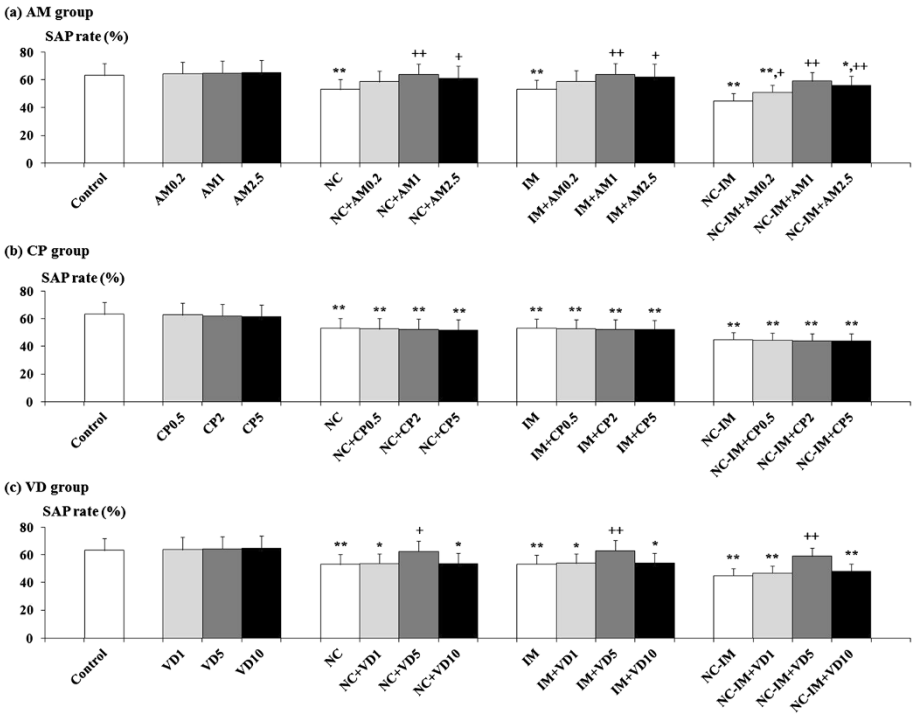

Figure 3 Effects of cannabinoid receptor ligands (CB ligands) on the working memory-related behavioral alterations (SAP rate (%)) caused by repeated nicotine (NC) and/or immobilization stress (IM) in the Y-maze test. Data of SAP rate are presented for groups of mice co-treated with AM (**a**), CP (**b**) and VD (**c**). The values at the 2 h time point after the last NC (0.8 mg/kg, s.c.) or IM treatment are shown as means ± S.D. (n=10 for each group). The abbreviations of the co-administered CB ligands with each i.p. dose (mg/kg) are noted in the text. The data for the control, NC, IM, and NC plus IM (NC-IM) groups without any CB ligand co-treatments, as well as the CB ligand-only groups, are also shown. * (p<0.05), ** (p<0.01): significant attenuation as compared to the control group; + (p<0.05), ++ (p<0.01): significant increase as compared to the NC, IM, or NC plus IM (NC-IM) group without any CB ligand co-treatments.

Table 3 Total number of entries into arms in experiments examining the effects of CB ligands in the EPM test (experiments shown in Figure 4)

(a) AM group	Control	NC	IM	NC-IM
Control group	63.2 ± 12.4	58.5 ± 11.9	60.8 ± 12.1	55.0 ± 11.7
AM 0.2 group	62.7 ± 12.6	58.9 ± 12.0	61.1 ± 12.3	55.5 ± 12.1
AM 1 group	62.4 ± 12.9	59.4 ± 12.2	61.5 ± 12.4	56.2 ± 12.4
AM 2.5 group	61.9 ± 13.1	59.9 ± 12.6	61.7 ± 12.7	57.5 ± 12.7
(b) CP group	**Control**	**NC**	**IM**	**NC-IM**
Control group	63.2 ± 12.4	58.5 ± 11.9	60.8 ± 12.1	55.0 ± 11.7
CP 0.5 group	62.6 ± 12.7	58.3 ± 12.0	60.6 ± 12.3	54.8 ± 12.1
CP 2 group	62.1 ± 12.8	58.0 ± 12.1	60.3 ± 12.4	54.7 ± 12.3
CP 5 group	61.5 ± 13.0	57.8 ± 12.2	60.0 ± 12.5	54.6 ± 12.5
(c) VD group	**Control**	**NC**	**IM**	**NC-IM**
Control group	63.2 ± 12.4	58.5 ± 11.9	60.8 ± 12.1	55.0 ± 11.7
VD 1 group	62.6 ± 12.6	58.8 ± 12.1	61.0 ± 12.4	54.9 ± 12.2
VD 5 group	62.3 ± 12.8	59.2 ± 12.2	61.3 ± 12.4	55.9 ± 12.4
VD 10 group	61.7 ± 13.0	59.5 ± 12.6	61.5 ± 12.7	56.9 ± 12.7

on open arms). Furthermore, in the groups co-treated with 2 mg/kg CP, significant recoveries in both entries into and time spent on open arms were also observed (Figure 4b), which is consistent with the results of the ANOVA revealing statistically significant main effects of CP ($F(3, 144)=206.08$, $P<0.001$ for the percentage of entries into open arms and $F(3, 144)=47.32$, $P<0.001$ for the percentage of time spent on open arms) and significant interactions of the CP versus NC and/or IM treatments ($F(9, 144)=20.61$, $P<0.001$ for the percentage of entries into open arms and $F(9, 144)=4.50$, $P<0.001$ for the percentage of time spent on open arms). In each CB ligand-only group, no significant alterations as compared to the control group were observed for each parameter value under the present experimental conditions.

Discussion

NC- and/or IM-induced working memory- and anxiety-related behavioral alterations

In the NC group using repeated treatments of 0.8 mg/kg NC, as well as in the IM group, behavioral alterations suggestive of working memory impairments were observed in the Y-maze test (attenuated SAP rate) (Figure 1). However,

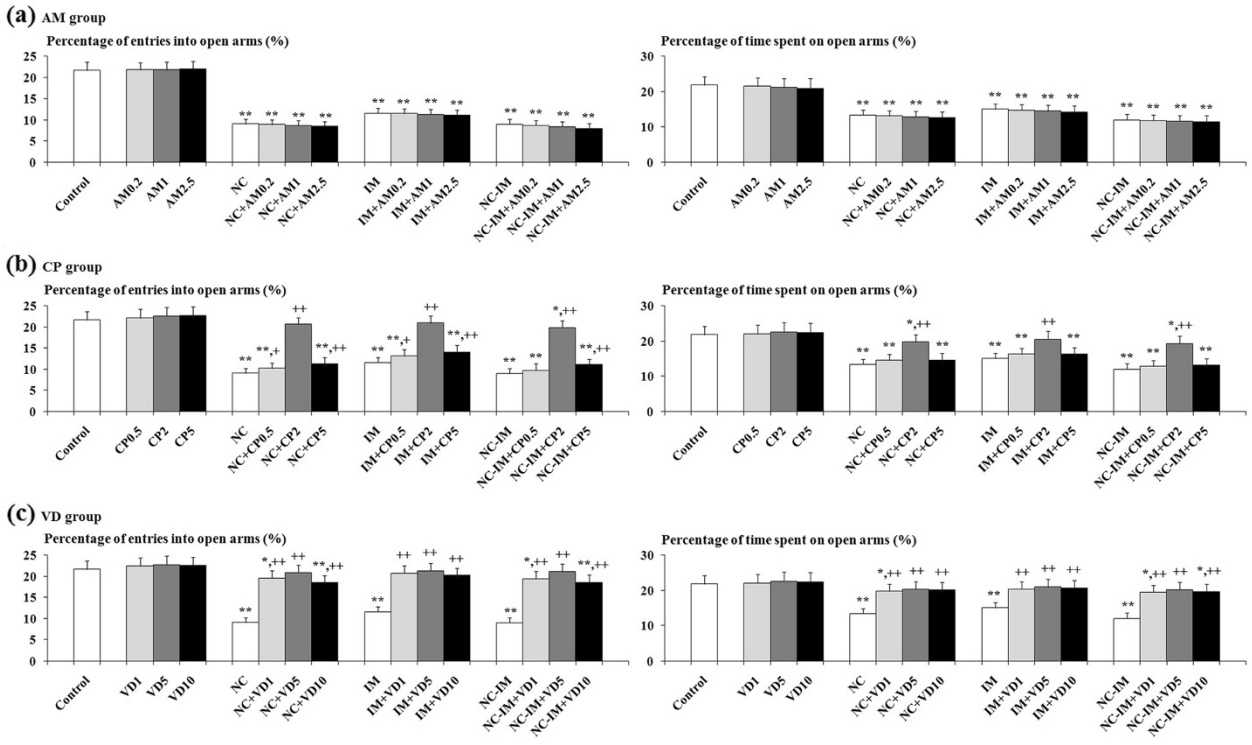

Figure 4 Effects of cannabinoid receptor ligands (CB ligands) on the anxiety-related behavioral alterations caused by repeated nicotine (NC) and/or immobilization stress (IM) in the elevated plus-maze (EPM) test. Data of percentages of entries into open arms and time spent on open arms are presented for groups of mice co-treated with AM (**a**), CP (**b**) and VD (**c**). The values at the 2 h time point after the last NC (0.8 mg/kg, s.c.) or IM treatment are shown as means ± S.D. (n=10 for each group). The abbreviations of the co-administered CB ligands with each i.p. dose (mg/kg) are noted in the text. The data for the control, NC, IM, and NC plus IM (NC-IM) groups without any CB ligand co-treatments, as well as the CB ligand-only groups, are also shown. * ($p<0.05$), ** ($p<0.01$): significant attenuation as compared to the control group; + ($p<0.05$), ++ ($p<0.01$): significant increase as compared to the NC, IM, or NC plus IM (NC-IM) group without any CB ligand co-treatments.

in the NC group using repeated treatments of 0.3 mg/kg NC, in spite of the appearance of anxiety-like behavioral alterations similar to the IM group (Figure 2), the absence of overt working memory-related behavioral alterations (a slightly increased SAP rate) was observed in the Y-maze test (Figure 1). Previous studies have shown that brain serotonergic and cholinergic systems play crucial roles in mediating anxiety-related behavioral responses [66-68]. In addition to these systems, several neuroendocrine responses (e.g. secretion of corticosterone, norepinephrine, etc.) have been reported to participate in the control of anxiety-like behaviors [69,70]. Similar modifications in these responses were observed between NC and IM [71], and may contribute to their anxiogenic-like effects in the present study (Figure 2). On the other hand, the NC-induced impairments in working memory, unlike the IM-induced impairments, occurred with a limited range of doses (Figure 1). It has been reported that modifications in working memory (both ameliorations and impairments) can occur due to even minuscule changes in prefrontal dopamine (DA) levels [72]. Therefore, the working memory-related behaviors in the NC groups may be correlated with characteristic but subtle alterations in nAChR-mediated prefrontal DA release, which was controlled by specific nAChR subtypes (e.g. alpha7 nAChRs) [73]. In addition to DA release, the release of other neurotransmitters such as glutamate has been implicated in the NC-induced working memory processes [74].

With respect to the interactions between NC and IM in the NC-IM group, both NC and IM enhanced each other's effects on the working memory- and anxiety-related behavioral alterations in the Y-maze and EPM tests. Although the relationship between stressors such as IM and NC remains controversial as mentioned above (i.e. "antistress" effects of NC have also been reported depending on the conditions), synergistic effects like those observed in previous studies [34-36] were provided by the NC plus IM treatment in the present experimental model. An augmented increase in secreted hypothalamic-pituitary-adrenal (HPA) hormones and/or immediate early gene expression was demonstrated in those studies [35,36]. Furthermore, the involvement of these molecular changes has also been reported for working memory- and anxiety-related behaviors [75-78]. However, further analyses are needed to elucidate the

mechanisms underlying these complex interactions between NC and IM.

Effects of cannabinoid (CB) ligands

Consistent with the previous studies described above [18,43,49,50,52], the NC- and/or IM-induced working memory impairment-like behaviors were antagonized by the CB1 antagonist AM (Figure 3a), and the anxiety-like behaviors were antagonized by the CB agonist CP (Figure 4b). Furthermore, VD, a mixed CB1 ligand with partial agonist plus antagonist activities [79], provided recovering effects against the impaired behaviors related to both working memory and anxiety (Figure 3c, 4c). Similarities in some neuronal responses such as prefrontal DA responses have been observed in the immunohistochemical studies between the effects of NC, stressors, and CB1 agonists [21,80-82]. These similarities may result in the CB1 antagonist-induced recoveries in the working memory-related behavioral alterations. On the other hand, against the NC- and/or IM-induced anxiety-related behavioral alterations, only the CB1 ligands acting at least partially as agonists exerted any anxiolytic-like effects. Although the mechanisms underlying these discrepancies in the effects of CB ligands have not been elucidated, anxiolytic-like effects of CB agonists have been shown under several different conditions [83,84]. Furthermore, certain doses of CB1 agonists have been reported to be able to activate the neurotransmission systems related to anti-anxiety (e.g. GABAergic and serotonergic systems) at the molecular level [53,83,84]. Therefore, it could be predicted that the antagonistic effects against the anxiety-like behaviors were provided at least indirectly by way of agonistic activity on CB1 receptors.

Against both working memory- and anxiety-related behavioral alterations, the CB1 partial agonist/antagonist VD exerted some recovering effects (Figure 3c, 4c). These recovering effects were observed equally in the NC, IM and NC-IM groups. For the behavioral alterations related to working memory impairments, the recovering effects of VD did not exceed those of the CB1 antagonist AM, which could be predicted considering the partial CB1 agonistic effects of VD. On the other hand, for the behavioral alterations related to anxiety, the recovering effects of VD exceeded those of the CB agonist CP. These results could not be predicted considering the above-mentioned anxiolytic-like effects of the CB1 agonists without any antagonistic effects. However, contrary to the present results, there are experimental data showing anxiogenic-like or anti-anxiolytic effects provided by high doses of CB agonists including CP [53,54,85]. The involvement of an abnormal release of anxiety-related neurotransmitters has been reported for those effects [53,54]. Unlike CP, VD possesses CB1 antagonistic potential for counteracting anti-anxiolytic effects as an agonist (high doses), and thus may function as an effective anxiolytic-like ligand. Furthermore, recent studies suggest the possibility that CB2 receptors, the CB receptors initially defined as peripheral receptors, may contribute to anti-anxiolytic effects [86,87]. CB2 receptors are distributed over several regions of the central nervous system [87] and the non-selective CB agonist CP seems to provide agonistic effects as well as against CB1 receptors. With respect to the effects of AM and VD, agonistic effects on the GPR55 receptor subtype, a newly-identified G protein coupled CB receptor subtype, have also been reported [88-91]. Although the behavioral roles of GPR55 receptors have not been investigated, it is possible that GPR55 distributed in the brain [89] may participate in cognitive processes such as working memory. Furthermore, in vitro studies demonstrated that VD acted as a partial agonist on GPR55 receptors and provided antagonistic effects at high concentrations [91], whereas only full agonistic effects have been reported for AM [88-90]. On the other hand, on CB1 receptors, both VD and AM provided antagonistic effects at high concentrations [79,89]. These characteristic effects of the partial agonist/antagonist VD as a GPR55 antagonist at high concentrations may be correlated with its limited and non-dose-dependent ameliorating effects against working memory impairments, i.e. only 5 mg/kg VD, but not a lower (1 mg/kg) or higher (10 mg/kg) dose, was effective. In addition to GPR55, the existence of other yet-to-be-cloned CB receptors has been suggested in memory-related brain regions such as the hippocampus [92]. There may be some contributions of these receptors to the AM- and VD-derived attenuating effects against the NC- and/or IM-induced working memory impairments.

Conclusions

The present study demonstrated working memory impairment- and anxiety-like behaviors induced by NC, IM and NC-IM treatments in mice. Mutual synergistic effects for NC plus IM were observed for both types of behavioral alterations. In the present study, the involvement of endocannabinoid system was also shown in the processes of working memory and anxiety. However, between the working memory- and anxiety-related behavioral alterations, discrepancies in the types of effective CB ligands were observed: the CB1 antagonist AM was the most effective against the working memory impairment-related behaviors, whereas the CB1 partial agonist/antagonist VD was the most effective against the anxiety-related behaviors. Since the presence of new CB receptor subtypes such as GPR55 receptors has been clarified recently and the interactions with each CB ligand have been suggested, further research

into the therapeutic contributions of each CB receptor subtype is expected.

Competing interests
The author declares that there are no potential competing interests.

Authors' contributions
TH designed the study, carried out all experiments and statistical analyses, and prepared the manuscript.

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