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Toxic cocaine- and convulsant-induced modification of forced swimming behaviors and their interaction with ethanol: comparison with immobilization stress

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Abstract

Background: Swimming behaviors in the forced swimming test have been reported to be depressed by stressors. Since toxic convulsion-inducing drugs related to dopamine [cocaine (COC)], benzodiazepine [methyl 6,7-dimethoxy-4-ethyl-β-carboline-carboxylate (DMCM)], γ-aminobutyric acid (GABA) [bicuculline (BIC)], and glutamate [N-methyl-D-aspartate (NMDA)] receptors can function as stressors, the present study compared their effects on the forced swimming behaviors with the effects of immobilization stress (IM) in rats. Their interactions with ethanol (EtOH), the most frequently coabused drug with COC which also induces convulsions as withdrawal symptoms but interferes with the convulsions caused by other drugs, were also investigated.

Results: Similar to the IM (10 min) group, depressed swimming behaviors (attenuated time until immobility and activity counts) were observed in the BIC (5 mg/kg IP) and DMCM (10 mg/kg IP) groups at the 5 h time point, after which no toxic behavioral symptoms were observed. However, they were normalized to the control levels at the 12 h point, with or without EtOH (1.5 g/kg IP). In the COC (60 mg/kg IP) and NMDA (200 mg/kg IP) groups, the depression occurred late (12 h point), and was normalized by the EtOH cotreatment. At the 5 h point, the COC treatment enhanced the swimming behaviors above the control level.

Conclusions: Although the physiological stress (IM), BIC, and DMCM also depressed the swimming behaviors, a delayed occurrence and EtOH-induced recovery of depressed swimming were observed only in the COC and NMDA groups. This might be correlated with the previously-reported delayed responses of DA and NMDA neurons rather than direct effects of the drugs, which could be suppressed by EtOH. Furthermore, the characteristic psychostimulant effects of COC seemed to be correlated with an early enhancement of swimming behaviors.

Background

Similarities between the effects of several convulsants and physiological stressors have been suggested, based on the close relationship between stressors and seizure-related

brain receptors such as γ-aminobutyric acid (GABA) (including benzodiazepine binding sites), and N-methyl-D-aspartate (NMDA) glutamate receptors [1–3]. Cocaine (COC), a dopaminergic psychostimulant, has also been reported to be one of the "stressor-like" convulsion-inducing drugs, because physiological stressors such as a foot shock induced a dopamine (DA) transporter blockade similar to COC [4,5]. Furthermore, COC can be differentiated from other convulsants and stressors, because it is a drug of abuse, and the euphoric enhancement of locomotor activities can be observed even at toxic doses [6]. However, it is also possible that GABA receptor antagonists such as bicuculline (BIC), benzodiazepine receptor inverse agonists such as methyl 6,7-dimethoxy-4-ethyl-β-carboline-carboxylate (DMCM), and glutamate receptor agonists such as NMDA also function as stressors, because of their convulsion-inducing effects and the close correlation between their target receptors and the stress response [1–3]. Although these drugs, including COC, have different target receptors, previous studies on the convulsive profiles of each drug have reported no peculiar differences with respect to the induction of stress reactions [7].

It can be predicted that various anticonvulsant drugs function as therapeutic antagonists against convulsants, and attenuate both the toxic and the above stressor-like effects produced by convulsion-inducing drugs, including COC. However, among the convulsion-relieving drugs, ethanol (EtOH) is characterized by its own acute and chronic depressive toxicity [8]. Furthermore, EtOH can cause both stressor-like effects and convulsions as withdrawal symptoms by itself, and can enhance some of the effects of convulsant drugs [9–13]. In previous studies, however, low doses of EtOH attenuated the effects of stressors [14]. In particular, EtOH enhanced COC-induced euphoric effects and attenuated subjective pain, and is actually the most frequently coabused drug with COC [15,16]. Furthermore, there are reports that suggest that the anticonvulsant effects of EtOH against convulsant drugs may be related to both GABA (including benzodiazepine) and NMDA receptors [17–19]. Thus, it is possible that some of the stressor-like effects caused by these convulsant drugs could also be attenuated by EtOH.

In the present study, as one of the behavioral methods reported for evaluating the strength of stressor effects, the forced swimming test [20–22] was performed in groups of rats treated with a toxic, convulsant dose of COC and other convulsion-inducing drugs, after when no toxic behavioral symptoms including convulsions were observed. The swimming behaviors in these drug-treatment groups were then compared with groups of rats treated with a physiological stressor in order to detect any modifications of the swimming behaviors peculiar to the groups treated with the convulsion-inducing drugs, and which could not be induced by a physiological stressor (IM). Furthermore, based on the above-mentioned anticonvulsant-like effects of EtOH, modifications in the swimming behaviors caused by a non-toxic dose of EtOH were also examined, with special attention to the possible stress-relieving effects of EtOH against both physiological stress and convulsion-inducing drugs.

Results

Lethality and convulsive seizures caused by convulsion-inducing drugs before forced swimming (Table 1)

In the drug-treatment groups, the mortality rate, the severity of the convulsive seizures [percentage, score of the most frequent type of seizure (score of the most frequent seizure), score of the most severe type of seizure (score of the most severe seizure), and time to recovery from visible seizures in the surviving rats (time to recovery)], and the total number of rats examined are shown in Table 1. A total of five surviving rats from each group were subjected to an examination by the forced swimming test. Even with ethanol and other treatments, the values for the body weights were not significantly altered. Furthermore, no difference was observed in the weight between the surviving and dead animals. In the EtOH-cotreatment groups, the values for the parameters of the convulsive seizures were significantly ($p < 0.01$; two sample t test with Welch's correction) attenuated as compared to the corresponding non-EtOH groups [$t = 4.75$ with df (degree of freedom) = 13 (score of the most frequent seizures), $t = 6.38$ with df = 15 (score of the most severe seizures) and $t = 3.45$ with df = 8 (time to recovery) in the COC-EtOH vs. COC groups, $t = 3.64$ with df = 12 (score of the most frequent seizures), $t = 4.35$ with df = 13 (score of the most severe seizures) and $t = 4.06$ with df = 7 (time to recovery) in the BIC-EtOH vs. BIC groups, $t = 3.11$ with df = 12 (score of the most frequent seizures), $t = 3.71$ with df = 13 (score of the severe seizures) and $t = 4.93$ with df = 8 (time to recovery) in the DMCM-EtOH vs. DMCM groups, and $t = 4.16$ with df = 13 (score of the most frequent seizures), $t = 5.13$ with df = 13 (score of the most severe seizures) and $t = 4.72$ with df = 7 (time to recovery) in the NMDA-EtOH vs. NMDA groups]. However, within the non-EtOH groups (between the COCA, BIC, DMCM and NMDA groups) and within the EtOH-cotreatment groups (between the COCA-EtOH, BIC-EtOH, DMCM-EtOH and NMDA-EtOH groups), no significant difference [$p > 0.05$; one-way analysis of varience (ANOVA)] was observed for either the seizure scores or the time to recovery [$F(3, 36) = 0.13$ (score of the most frequent seizures), $F(3, 36) = 0.18$ (score of the most severe seizures) and $F(3, 16) = 0.23$ (time to recovery) within the non-EtOH groups, and $F(3, 36) = 0.13$ (score of the most frequent seizures), $F(3, 36) = 0.14$ (score of the most severe seizures) and $F(3, 16) = 0.52$ (time to recovery) within the EtOH-cotreatment groups].

Table 1: Mortality rate (%), and the percentage and severity of the convulsive seizures in the CV groups.

Treatment	Mortality Rate (%)	Presence of Convulsive Seizures (%)	Score of the most frequent seizure	Score of the most severe seizure	Time to recovery (min)
COCA 60 mg/kg (n = 10)	50.0	90.0	2.3 ± 0.9	2.4 ± 0.7	192 ± 30
BIC 5 mg/kg (n = 10)	50.0	80.0	2.1 ± 1.1	2.3 ± 1.0	205 ± 35
DMCM 10 mg/kg (n = 10)	50.0	80.0	2.0 ± 1.2	2.1 ± 1.0	198 ± 28
NMDA 200 mg/kg (n = 10)	50.0	80.0	2.2 ± 1.0	2.3 ± 0.8	209 ± 36
COCA 60 mg/kg + EtOH 1.5 g/kg (n = 11)	54.5	63.6	0.7 ± 0.4 a	0.7 ± 0.4 a	124 ± 26 a
BIC 5 mg/kg + EtOH 1.5 g/kg (n = 10)	50.0	60.0	0.6 ± 0.5 a	0.7 ± 0.5 a	118 ± 24 a
DMCM 10 mg/kg + EtOH 1.5 g/kg (n = 9)	44.4	66.7	0.7 ± 0.5 a	0.7 ± 0.5 a	106 ± 24 a
NMDA 200 mg/kg + EtOH 1.5 g/kg (n = 10)	50.0	70.0	0.7 ± 0.5 a	0.8 ± 0.5 a	106 ± 25 a

Both the most severe convulsive seizure and the most frequent type of seizure observed in each rat were scored, based on previously-reported evaluation methods using the common features of convulsive seizures [47,48]: score 0=no convulsive seizures, score 1=short-lasting (< 5 min) mild episodes of clonic convulsions, score 2=short-lasting episodes of clonic-tonic convulsions that caused a loss of the righting reflex, and score 3= episodic convulsive seizures continuous and violent enough to cause fatal respiratory disorders. Furthermore, the mean time to recovery from visible seizures in the surviving rats, after when no seizures were observed, is also shown, although all of the convulsive seizures and other toxic symptoms (e.g. abnormal locomotor activities) had disappeared before the 5 h time point. The data for the seizure scores represent means ± SD. a: significant ($p < 0.05$; two sample t test with Welch's correction) attenuation as compared to the non-EtOH groups. In the groups without EtOH cotreatment (between the COCA, BIC, DMCM and NMDA groups), and within the groups with EtOH cotreatment (between the COCA-EtOH, BIC-EtOH, DMCM-EtOH and NMDA-EtOH groups), no significant differences were observed ($p > 0.05$; one-way ANOVA) for the scores of the most severe convulsive seizure, the scores of the most frequent type of seizure, and the time to recovery in the surviving rats. Furthermore, each mean value for the most frequent type of seizure was not significantly different from the mean value for the most severe type of seizures ($p > 0.05$; two sample t test with Welch's correction). In all rats, there was no significant difference between the most severe score and the most frequent score ($p > 0.05$; two sample t test with Welch's correction), which suggests a similar severity profile within the non-EtOH groups, and within the EtOH-cotreatment groups.

Forced swimming test (Fig 1)

At 5 h after the treatment, both indices of swimming behaviors [time to immobility (time) and activity counts (counts), as explained in the Methods section] were significantly ($p < 0.05$; two sample t test with Welch's correction) attenuated in the BIC, DMCM, and IM groups as compared to the control group [$t = 4.19$ with $df = 6$ (time) and $t = 2.35$ with $df = 8$ (counts) in the BIC group, $t = 4.19$ with $df = 6$ (time) and $t = 2.51$ with $df = 8$ (counts) in the DMCM group, $t = 3.25$ with $df = 7$ (time) and $t = 3.05$ with $df = 7$ (counts) in the IM group]. However, in the COC group at this time point, both the time to immobility ($t = 1.63$ with $df = 8$, $P = 0.14$) and the activity counts ($t = 2.66$ with $df = 8$, $p < 0.05$) tended to be increased above control, suggesting enhanced swimming behaviors. At 12 h, the attenuated swimming behaviors in the BIC, DMCM, and IM groups returned to normal levels, which were not significantly different from the control ($p > 0.05$; two sample t test with Welch's correction). On the other hand, at the 12 h time point, a significant attenuation of the swimming behaviors as compared to the control group ($p < 0.05$; two sample t test with Welch's correction) was also observed in the COC and NMDA groups [$t = 3.35$ with $df = 8$ (time) and $t = 2.33$ with $df = 8$ (counts) in the COC group, $t = 4.42$ with $df = 7$ (time) and $t = 2.87$ with $df = 7$ (counts) in the NMDA group]. Nevertheless, none of the values differed significantly ($p > 0.05$; one-way ANOVA) from the control values at the 24 h time point [$F(5, 24) = 0.08$ (time), $F(5, 24) = 0.05$ (counts)].

In all groups, EtOH (1.5 g/kg) did not alter the suppression of the swimming behaviors as compared to the controls. Furthermore, in the EtOH-only group (control group with EtOH treatment), no significant modifications in the swimming behaviors were observed as compared to the control group without EtOH treatment, at any time point. At 5 h, the swimming behaviors were significantly attenuated ($p < 0.05$ for both time to immobility and activity counts; two sample t test with Welch's correction) in the BIC-EtOH, DMCM-EtOH, and IM-EtOH groups as compared to the control group with or without EtOH treatment [vs. control group without EtOH treatment: $t = 3.59$ with $df = 7$ (time) and $t = 2.20$ with $df = 8$ (counts) in the BIC-EtOH group, $t = 3.59$ with $df = 7$ (time) and $t = 2.18$ with $df = 8$ (counts) in the DMCM-EtOH group, and $t = 3.06$ with $df = 8$ (time) and $t = 2.45$ with $df = 8$ (counts) in the IM-EtOH group; vs. control group with EtOH treatment, $t = 3.59$ with $df = 7$ (time) and $t = 2.08$ with $df = 8$ (counts) in the BIC-EtOH group, $t = 3.59$ with $df = 7$ (time) and $t = 2.04$ with $df = 8$ (counts) in the DMCM-EtOH group, $t = 3.06$ with $df = 8$ (time) and $t = 2.31$ with $df = 8$ (counts) in the IM-EtOH group]. Furthermore, the values for the swimming behaviors were not significantly altered as compared to the corresponding non-EtOH

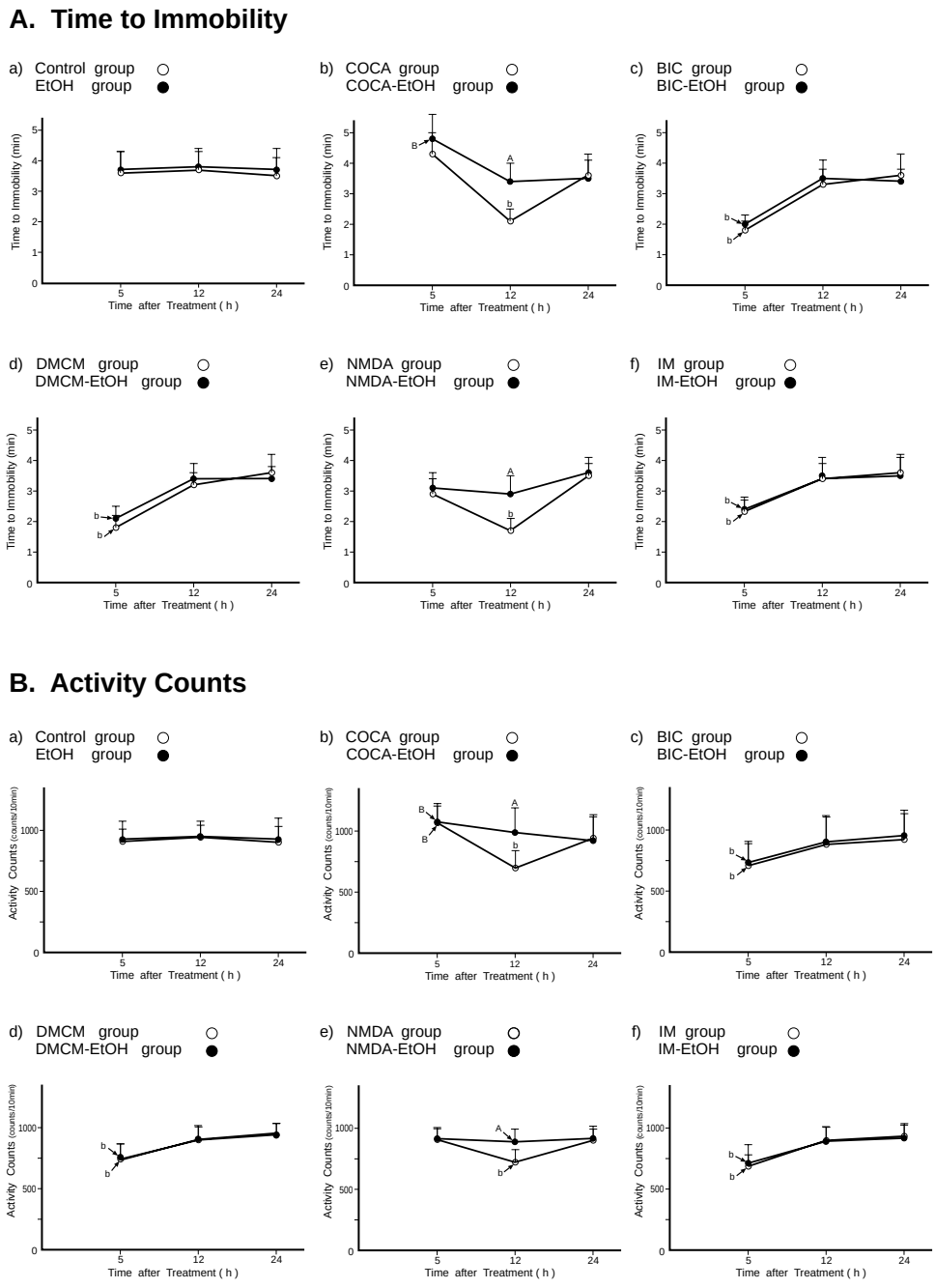

Figure 1

Time course of the forced swimming behaviors (time to immobility and activity counts), which were measured at the 5, 12 and 24 h time points after treatment. The data represent means ± SD (n = 5 for each group). A: significant ($p < 0.05$; two sample t test with Welch's correction) increase as compared to the non-EtOH group; B, b: significant ($p < 0.05$; two sample t test with Welch's correction) increase (B) or attenuation (b) as compared to the control group. In the EtOH-cotreated groups, "B" and "b" denote significant alterations as compared to the control groups with and without EtOH treatment.

groups, and had returned to the levels for the control group (with and without EtOH coadministration) at 12 h ($p > 0.05$ for both time to immobility and activity counts; two sample t test with Welch's correction). In the COC-EtOH group, the swimming behaviors were significantly increased above control levels at 5 h [vs. control group without EtOH treatment: $t = 2.53$ with $df = 8$ (time) and $t = 2.72$ with $df = 8$ (counts); vs. control group with EtOH treatment, $t = 2.53$ with $df = 8$ (time) and $t = 2.56$ with $df = 8$ (counts)], but returned to the levels for the control group (with and without EtOH coadministration) at 12 h ($p > 0.05$ for both time to immobility and activity counts; two sample t test with Welch's correction). At 12 h, the swimming behaviors were significantly increased ($p < 0.01$ for both time to immobility and activity counts; two sample t test with Welch's correction) as compared to the COC-only group [$t = 3.81$ with $df = 8$ (time) and $t = 3.04$ with $df = 7$ (counts)]. In the NMDA-EtOH group, none of the indices of swimming behaviors were significantly altered as compared to the control group (with and without EtOH coadministration) at any time point ($p > 0.05$ for both time to immobility and activity counts; two sample t test with Welch's correction), and the swimming behaviors at 12 h were significantly increased ($p < 0.01$ for both time to immobility and activity counts; two sample t test with Welch's correction) as compared to the NMDA-only group [$t = 3.15$ with $df = 7$ (time) and $t = 2.96$ with $df = 8$ (counts)]. Therefore, none of the values differed significantly ($p > 0.05$; one-way ANOVA) from the control values at the 12 h time point [$F(6, 28) = 0.54$ (time), $F(6, 28) = 0.52$ (counts)].

Throughout the experiments, a linear correlation was observed between the time to immobility (X) and the activity counts (Y), using simple regression analysis. All of the values could be approximated to the following equation: $Y = 715.66X + 43.53$ with $F(1, 178) = 12.09$ and $p < 0.01$. Therefore, in all treatment groups in which the swimming behaviors were suppressed, the time to immobility was also attenuated in proportion to the activity counts. In our observations, neither brief but violent swimming bursts nor prolonged slow swimming were observed.

Discussion

The present results revealed that the depressive effects reported for a physiological stressor (IM) [21,22] in the forced swimming test were also mimicked by toxic doses of convulsion-inducing drugs. However, the time course of the indices of swimming behaviors (time until immobility and activity counts) was different between each drug treatment group, although the doses used caused convulsive seizures of similar severity and duration (Table 1). The time course of the indices of swimming behaviors in the IM group was similar to the BIC and DMCM groups (Fig 1), and this is consistent with previous studies demonstrating the contribution of brain GABA and benzodiazepine receptors to stress-related behavioral alterations [1,3]. However, although very close relationships between stress and NMDA receptors [2] and DA transporters [5] have been reported, the depression of the swimming behaviors occurred at a later time point (12 h) in the COC and NMDA groups. The detailed mechanisms responsible for these delayed depressive effects have not been elucidated, but there are reports demonstrating delayed aftereffects of COC and NMDA at toxic doses which seemed to occur independently from the pharmacokinetics of the drugs, even after when the drugs were eliminated [23,24]. Although the values of the half-lives of NMDA, BIC and DMCM have not been elucidated, a previous study on the drug distribution suggests an earlier elimination of COC with a short half-life (about 30 min in rats) by hydrolysis to the inactive metabolites [25], in spite of the convulsive seizures at a similar severity to the other drug-treatment groups (Table 1) and the characteristic delayed stress-like responses in the forced swimming test (Fig 1). The delayed responses of brain DA transporters and NMDA receptors against stressors, including COC and NMDA, have been reported in the previous studies, and have been suggested to be correlated with prolonged excitation at the neuronal level, which has been characteristically observed for DA transporters and NMDA receptors after treatment with strong DA- or glutamate-neuron-selective drugs or stressors [23,24]. Furthermore, it is also possible that these responses were induced by mediators other than the drugs themselves. Since neuroendocrinal responses have been reported to be correlated with various stress responses [26–28], hormones such as those in the hypothalamic-pituitary axis [26,27] could be regarded as some of the proposed mediators. Although the coincidence with the delayed behavioral alterations peculiar to the COC- or NMDA-treated groups was not observed for representative hormones such as ACTH and corticosterone (the peak levels were observed earlier) in our previous study [29], it is still possible that some of the related neuroendocrinal systems contributed to the prolonged neuronal excitation and thus the delayed stress reaction on the swimming behaviors that was not induced by a physiological stressor such as IM. Furthermore, the stress responses related to some of the neuroendocrinal mediators could induce unfavorable effects on the body [30,31].

In the COC group, an early (5 h) enhancement of the swimming behaviors was characteristically observed, although the convulsive seizures, hyperactivity and other toxic alterations in locomotor activity known for high doses of COC [32] had disappeared by that time point. Unlike the other convulsion-inducing drugs, COC is known to be the drug of abuse, and has been reported to cause prolonged potentiation in DA neurons with a single treatment, even after the drug had been eliminated from

the body, and to enhance locomotor activities by rewarded stimulations or other motivations [33–35]. Therefore, it is possible in our experiment that the forced swimming behavior, which may save the life of the rat after putting it into the water, could function as such a driving motivation and thus enhance the swimming behaviors. Furthermore, our results showed that the psychostimulated conditions, as demonstrated by the alterations in the forced swimming behaviors, lasted for less than 12 hours, because no enhancement in the swimming behaviors was observed after the 12 h time point.

In spite of its equivalent convulsion-relieving effects (Table 1), the effects of EtOH on the depressed swimming behaviors were different. Although the present non-toxic dose of EtOH (1.5 g/kg IP) did not cause any further depressive modifications on the forced swimming behaviors (Fig 1), the BIC-, DMCM- and IM-attenuated swimming behaviors at the earlier (5 h) time point were not significantly modified by 1.5 g/kg EtOH. In contrast, the COC- and NMDA-attenuated swimming behaviors at the later (12 h) time point were significantly ameliorated as compared to the COC- or NMDA-only groups (Fig 1). These limited effects of EtOH in the COC- and NMDA-treated groups have not been sufficiently investigated. However, there are reports that suggest the presence of peculiar antagonistic effects of EtOH on DA and glutamate neurons [36,37], and thus there is a possibility that the delayed stressor-like effects caused by the agonistic ligands COC and NMDA were attenuated by EtOH. Furthermore, the appearance of euphoric effects, which could possibly be effective in attenuating the stressor effects, has been reported for EtOH treatments combined with some DA and NMDA receptor ligands [6,16,38]. In particular, with respect to combined COC-EtOH treatments, the euphoric effects of COC [34] have been reported to be enhanced by EtOH, possibly due to DA receptor-mediated endocrinal changes, the synthesis of the euphoric metabolite cocaethylene, etc [34,39]. On the other hand, NMDA alone has not been reported to cause any euphoric effects. However, a close correlation between EtOH-induced euphoric effects and NMDA receptors has been demonstrated by the effects of some NMDA receptor ligands, which modified the self-administration of EtOH [38]. The recovery from the NMDA-attenuated swimming behaviors in the present experiment might be correlated with these modified EtOH effects. However, the "favorable effects" of EtOH seem to be limited, considering that other studies have reported more severe toxic effects of cocaethylene as compared to COC [40], and enhanced toxicity of NMDA caused by chronic EtOH cotreatment [41].

Conclusions

The present results suggest that toxic doses of convulsion-inducing drugs caused depressive effects (attenuation of the time to immobility and activity counts) similar to a physiological stressor on the forced swimming behaviors in rats. However, depending on the target receptors, the effects of the drugs were different. The DA receptor-related drug COC and the glutamate receptor-related drug NMDA also attenuated the time until immobility and activity counts in the swimming behaviors, but the appearance of these stressor effects was delayed as compared to the other receptor related convulsive drugs and IM, which was consistent with a delayed response of the target receptors. Furthermore, the psychostimulant COC characteristically increased the swimming behaviors at an earlier time point, even when no spontaneous behavioral changes were observed in the cage. Following cotreatment with a non-toxic, convulsion-relieving dose of EtOH, the depressed swimming behaviors recovered only in the COC and NMDA groups. This may be due to the characteristic interference of EtOH at DA transporters and NMDA receptors, or the appearance of euphoric effects to relieve the stressor effects in the limited conditions for COC-EtOH and NMDA-EtOH combinations.

Methods

Animals and drug (immobilization stress) treatments

Adult male Wistar rats (280 ± 30 g) were purchased from the Shizuoka Laboratory Animal Center (Hamamatsu, Japan). They were housed under conditions similar to a previous report [42], with a 12 h/12 h light/dark cycle. The rats were kept in groups of 4 animals per cage (35 × 32 × 16 cm) with woodchip bedding, and were allowed water and lab chow *ad libitum*. The mean body weights in each group did not differ significantly (ANOVA). 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 Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1985). Following these guidelines, if any severe symptoms of pain induced by high doses of the drugs were observed, the experiment was stopped.

The doses of COC and the other convulsion-inducing drugs were selected based on previous studies [43–46]. The final doses used caused convulsive seizures of a similar severity (based on the criteria by De Sarro [47] and Braida [48]) and death in a similar percentage of rats, under the present conditions, in a preliminary trial: 60 mg/kg for COC hydrochloride (Takeda Chemical Industries, Ltd., Osaka Japan), 5 mg/kg for BIC (Tocris Cookson Inc., Ballwin, MO, USA), 10 mg/kg for DMCM (Sigma-Aldrich, Inc., Saint Louis, MO, USA), and 200 mg/kg for NMDA (Nacalai Tesque, Inc., Kyoto, Japan). All of the convulsion-inducing drugs were administered by an intraperitoneal (IP) injection of a mixed dimethylsulfoxide (DMSO)-distilled water (1:2) solution in a volume of 5

ml/kg body weight [7]. The group of rats treated with a physiological stressor underwent 10 min of immobilization stress [26], which was induced by placing the rat in a narrow space (diameter 18 cm) made in a vinyl bag with some breathing holes (IM group). In the EtOH-cotreated groups of rats, 1.5 g/kg EtOH solution (IP), which has been reported to cause euphoric effects but no fatal toxicity [8], was injected 15 min before the convulsion-inducing drugs or immobilization in a volume of 5 ml/kg. In the EtOH-only group, the EtOH solution was injected 15 min before a vehicle control solution, a mixture of DMSO and distilled water (1:2). In the control group, the same vehicle solution was administered instead of the drugs.

In the drug-treated groups, the mortality rate and the severity of the convulsive seizures [47,48] before each examination were also monitored and checked, using a video camera (SONY CCD-TRV90).

Forced swimming test

For the purposes of evaluating the strength of the stressor-like effects of each treatment, the forced swimming test was performed at 5, 12, and 24 h after the treatments, when all obvious drug-induced toxic behavioral symptoms had disappeared, in accordance with previous reports [20–22]. The phenomenon of immobility in water, which has been observed even under non-treatment conditions and has been reported to be accelerated by the loading of any stressor [21,22], was then compared with the non-treated, control group of rats (n = 5). The apparatus consisted of a plastic cylinder 40 cm in height and 35 cm in diameter containing 25 cm of water at 21–23°C.

Quantitative alterations in the swimming behaviors were then evaluated by: 1) the time to immobility, and 2) the activity counts. The time until immobility was defined to be the time after when only modest swimming movements necessary to avoid drowning were observed. The activity counts were recorded for 10 min, based on our preliminary observations that the time to immobility did not exceed 10 min in any group. The activity was counted using the counting instrument Supermex (Muromachi Kikai Co. Ltd., Tokyo, Japan) connected to the behavior analyzing system CompACT AMS (Muromachi Kikai Co. Ltd.) [49,50]. The sensor of the Supermex instrument was positioned directly over the cylinder at a distance of 20 cm from the water. This instrument can monitor swimming movements in all three planes of motion (sagittal, coronal and horizontal) as an infrared signal value using an infrared sensor with multiple Fresnel lenses, and then can evaluate the vigor of the swimming behaviors in addition to the swimming distance evenly as sets of activity counts. Therefore, by the combined evaluation of the activity counts using the present method plus the time to immobility, the overall magnitude of the swimming behaviors could be analyzed.

The time interval between the drug administration and each examination was consistent with the recovery time from the severe toxic symptoms which interfered with the swimming behaviors in our preliminary experiments (data not shown). During the earlier period (within 5 h after the drug administration), toxic symptoms such as convulsive seizures and unusual activity, which seemed to modify the swimming behaviors and exposed the rats to the danger of drowning, were observed. The interval between the repeated tests (5, 12 and 24 h time points) in the survivors was determined based on data from the non-treatment, control group which showed an absence of any significant alterations in the two parameters of the swimming behaviors (Fig 1).

Statistical analysis

For each treated and non-treated control group, the two above-defined indices for the forced swimming test, and the scores of the convulsive seizures were compared in order to evaluate the effects of each stressor with or without EtOH. A two sample t test with Welch's correction was performed after the data were subjected to a one-way analysis of variance (ANOVA) repeatedly at each time point [51]. For the comparison between the percentage of lethality and the convulsive seizures, Fisher's exact test was used [51]. For the examination of the relationship between the parameters for the swimming behaviors, a simple regression analysis was used. All of the comparisons were performed using statistical software packages and their manuals (OMS Publication, Saitama, Japan). Unless otherwise noted, p-values less than 0.05 were concluded to be statistically significant.

Authors' contributions

Author 1 (TH) designed the experiments and performed the behavioral experiments. Authors 2 and 3 (YY and KY) advised and improved the methods based on their previous or preliminary experiments, and also participated in the time course study.

Abbreviations

COC, cocaine; BIC, bicuculline; DMCM, methyl 6,7-dimethoxy-4-ethyl-β-carboline-carboxylate; NMDA, N-methyl-D-aspartate; EtOH, ethanol; GABA, γ-aminobutyric acid; DA, dopamine; DMSO, dimethylsulfoxide; IP, intraperitoneal; IM, immobilization stress; ANOVA, analysis of variance; df, degree of freedom

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