様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

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研究課題名（和文）Does the combination of nicotine and ethanol facilitate or block dopaminergic neuron damage in Parkinson's disease models?

研究課題名（英文）Does the combination of nicotine and ethanol facilitate or block dopaminergic neuron damage in Parkinson's disease models?

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研究成果の概要（和文）：DA、DOPAC、HVA レベル、TH 発現、Ser31 の増加によって証明されるように、エタノール (EtOH、2.0 および 3.0 g/kg) またはニコチン (NIC、1.0 および 2.0 mg/kg) 単独では、MPTP 治療の効果が逆転しました。 リン酸化。MPTP モデルにおける線条体および海馬の DA ニューロンに対する修復効果を示します。 EtOH (2.0 g/kg) と NIC (1.0 mg/kg) を同時投与すると、DA および HVA 組織含有量、TH 発現、および Ser31 リン酸化がさらに増加し、相加効果が示されました。

研究成果の学術的意義や社会的意義
Our results show that EtOH and NIC induce similar increases in brain DA and TH via TH phosphorylation activation in MPTP model mice. EtOH and NIC showed an additive effect in combination, suggesting that their co-application could be a potent therapeutic strategy for treating PD.

研究成果の概要（英文）：EtOH (2.0 and 3.0 g/kg) alone reversed the effects of MPTP treatment in both studied brain regions, as evidenced by an increase in DA, DOPAC, and HVA levels, TH expression, and Ser31 phosphorylation compared to the control, indicating restorative effects on striatal and hippocampal DA neurons in the MPTP model. Likewise, NIC (1.0 and 2.0 mg/kg) alone reversed MPTP treatment effects, with treated mice showing increased DA, DOPAC, and HVA contents, TH expression, and Ser31 phosphorylation compared to control mice. Co-administration of EtOH (2.0 g/kg) and NIC (1.0 mg/kg) further increased DA and HVA tissue contents, TH expression, and Ser31 phosphorylation, indicating an additive effect.

研究分野：Neuroscience

キーワード：Ethanol　nicotine　dopamine

様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９（共通）

１．研究開始当初の背景

Nicotine and ethanol (EtOH) are the two most commonly-abused substances and are often consume together. Unfortunately, EtOH has been found to be the largest single factor leading to fatal injuries. Research has shown a confounding link between nicotine and reduced risk of PD (Quik et al., 2012). By contrast, EtOH and acetaldehyde (ACE) induce a reduction in the dopamine (DA) levels in the brain, even if contradictory data are present in the literatures. The EtOH- or ACE-induced decrease in DA may lead to enhance toxin-induced PD (Corsini et al., 1987; Zuddas et al., 1989). These opposing findings generate considerable interest in how these drugs act in combination on PD to contribute to the degenerative changes observed in PD brain, for instance, mitochondrial dysfunction, oxidative stress, as well as alterations in other molecular and cellular functions. Here, we will examine the question whether the combination of the two (nicotine and EtOH) would facilitate or block nicotine own protection in mouse models of PD. To approach this, we measured DA and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT) and homovanillic acid (HVA), tyrosine hydroxylase (TH), Ser31 phosphorylation in the striatum and hippocampus of WT mice.

２．研究の目的

We used toxin-induced mouse models of PD for the first time to test both the individual and combined effects of nicotine, EtOH and/or ACE on protection against dopaminergic neuron injuries. The rationale for considering nicotine and EtOH is based on previous findings that nicotine and EtOH appear to be mutually reinforcing (Larsson and Engel, 2004), although these two drugs are rather dissimilar in their mechanisms of action and their effects. In spite of the dissimilarity and the high prevalence of combined use of the two, there are no reports to examine the effects of nicotine and EtOH combination in animal models of PD. ACE is highly toxic and its combination with nicotine appears to be more addictive than nicotine alone (Belluzzi et al., 2005). To assess the role of ACE, Aldh2 knockout (Aldh2-KO) mouse line is generated that lacks expression of human aldehyde dehydrogenase 2, resulting in ACE accumulation.

３．研究の方法

Animals

C57BL/6N (WT) mice were purchased from CLEA Japan (Tokyo, Japan). All experiments were conducted with male mice. Each was 10–12 weeks old and weighed 24–28 g. All animals were housed in controlled conditions: 23 ± 3˚C, 50%–70% humidity, and 12-h–12-h light–dark cycle. All animal experiments were approved by the Kagawa University Animal Investigation Committee.

MPTP injection

Mice received intraperitoneal (I.P.) injection of MPTP hydrochloride (20 mg/kg; Sigma-Aldrich) in saline twice daily for two successive days at 1 h interval, and the total dose per mouse is 80 mg/kg. Mice were euthanized 7 days after the final injection. Mice receiving saline (vehicle)-only injections served as controls. All procedures involving MPTP were conducted in strict accordance with published safety and handling guidelines (Jackson-Lewis and Przedborski, 2007).

Open field activity

Open field activity were determined before treatment and at day 3, 5 and 7 after the first MPTP injection. The open field task took place in a square, plexiglas box (dimension: 31 cm length x 29 cm width x 30 cm height) surrounded by a white paper. Mouse was placed in the center of the testing chamber and allowed to freely move for 10 min adaptation, followed by a 10 min recording. The movements were recorded by an overhead video tracking system and stored on a computer. After the experiment is completed, computer tracking programs analyzed the total distance traveled and velocity over time. The apparatus was cleaned with cotton pad wetted with 10% ethanol each time before next session and dried between each test to remove odor trails.

Experimental groups

The MPTP-treated mice were divided into 8 groups (n=8/group): a. control, b. nicotine 0.5 mg/kg, c. nicotine 1.0 mg/kg, d. nicotine 2.0 mg/kg, e. EtOH 1.0 g/kg (20% w/v), f. EtOH 2.0 g/kg, g. EtOH 3.0 g/kg, h. nicotine 1.0 mg/kg + EtOH 2.0 g/kg. All injections were administered i.p. The nicotine and EtOH treatment was adjusted based on the results of previous work (Ryan et al., 2001; Jamal et al., 2010).

Brain tissue preparation

Mice were sacrificed by cervical dislocation. Brains were removed and rinsed with ice-cold isotonic saline. One half of the striatum and hippocampus was placed in a 2-ml tube for DA and its metabolites analysis ex vivo with HPL-ECD, and the other half was placed in a 2-ml tube for protein analysis. The tubes were stored at −80°C until use.

Ex vivo HPLC-ECD

The tissue samples were homogenized using a Polytron® homogenizer (Kinematica AG, Lucerne, Switzerland) in 0.2 M perchloric acid (10 µl/mg of tissue) including 100 µM EDTA-2Na and 1 ng/µl (10 ul) isoproterenol (Tokyo Company Industry ltd, Japan) as an internal standard (IS). Sample kept on ice for 30 min and then centrifuged at 15,000 x g at 4 °C for 15 min. The supernatants were then filtered through 0.45 µm Minisart sterile filters (Sartorius Stedim Biotech GmbH, Germany) and maintained pH 3.0 by adding 1 M Na-acetate. 10 µL of the resulting solution was injected into the HPLC-ECD for determining the levels of DA and its metabolites.

In order to determine the *ex vivo* concentrations of monoamines and their metabolites in the brain, we used an HPLC system equipped with an ECD-300 (Eicom, Japan). The main operative conditions for HPLC were as follows: column (EicompaK SC-5ODS; 3.0 mm × 150 mm), oven temperature of 25°C, detector, oxidation potential (+ 750 mV versus Ag/AgCl reference analytical electrode), mobile phase: 83% citrate-acetate buffer (pH 3.5) containing 17% methanol, 190 mg/l sodium octane sulfonate and 5 mg/l EDTA-2Na at a flow rate of 0.23 ml/min. The samples were analyzed for 30 min. The chromatograms were recorded with a PowerChrom (AD Instruments, Sydney, Australia). Stock standard solutions of 1 ng/µl NE, DA, and 5-HT and their metabolites were purchased from Eicom (Japan) and stored at 4 °C until use.

Western blotting

The striatum and hippocampus was homogenized using a Polytron® homogenizer in 0.4 ml of RIPA lysis buffer (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Added 4µL PMSF solution, 4µL sodium orthovanadate solution and 4µL protease inhibitor cocktail solution to 0.4 ml of RIPA buffer. After centrifugation at 10,000 × g at 4 °C for 10 min, the supernatant was used for WB analysis. The protein

content of the supernatant was determined using the Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA), with bovine serum albumin (Sigma-Aldrich Corp., St. Louis, MO, USA) as the standard. Samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis with molecular weight markers (Bio-Rad Laboratories, Inc.), and then transferred to polyvinylidene difluoride membranes. Membranes were incubated with primary antibodies against TH (1:4000; Cell Signaling Technology, Massachusetts, USA), Ser31 (1:1000; Cell Signaling Technology), and β-actin (1:2000; Wako Pure Chemical Industries, Ltd., Osaka, *Japan*), and then with corresponding horseradish peroxidase-linked secondary antibodies. Band intensities were evaluated using an ImageQuant LAS 4000 (GE Healthcare Japan). The relative protein expressions were normalized to those of β-actin in each sample.

４．研究成果

EtOH (2.0 and 3.0 g/kg) alone reversed the effects of MPTP treatment in both studied brain regions, as evidenced by an increase in DA, DOPAC, and HVA levels, TH expression, and Ser31 phosphorylation compared to the control, indicating restorative effects on striatal and hippocampal DA neurons in the MPTP model. Likewise, nicotine (1.0 and 2.0 mg/kg) alone reversed MPTP treatment effects, with treated mice showing increased DA, DOPAC, and HVA contents, TH expression, and Ser31 phosphorylation compared to control mice. Neither EtOH nor nicotine dose altered 3-MT content in either studied brain region. Co-administration of EtOH (2.0 g/kg) and nicotine (1.0 mg/kg) further increased DA and HVA tissue contents, TH expression, and Ser31 phosphorylation, indicating an additive effect. These results show that EtOH and nicotine induce similar increases in brain DA and TH via TH phosphorylation activation in MPTP model mice. EtOH and nicotine showed an additive effect in combination, suggesting that their co-application could be a potent therapeutic strategy for treating PD.

５．主な発表論文等

〔雑誌論文〕　計0件

〔学会発表〕　計0件

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

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７．科研費を使用して開催した国際研究集会

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８．本研究に関連して実施した国際共同研究の実施状況

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