PHOSPHORYLATION OF CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE II (CAMKII) AND EXTRACELLULAR REGULATED KINASE (ERK) IN STRIATUM MEDIATE NICOTINE DEPENDENCE IN BALB/C MICE

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ABSTRACT

Objective: Nicotine is an active compound in tobacco and has a rewarding effect in the central nervous system (CNS), which may lead to dependence. Although nicotine dependence is elucidated by brain mechanisms, synaptic molecular substrates underlying the dependence remain unclear. We hypothesized that reward signaling is mediated by dopamine and glutamate receptors, in where calcium/calmodulin-dependent kinase II (CaMKII) and extracellular signal-regulated kinase (ERK) may mediate the synaptic signaling of dependence. Methods: To investigate the roles of both CaMKII and ERK on nicotine dependence were assessed by conditioned place preference (CPP) methods followed by dissection. One day after conditioning, preference scores were measured to evaluate nicotine dependence. Mice were sacrificed and their striatum dissected for immunoblotting analyses of CaMKII and ERK phosphorylation. Results: Nicotine-induced conditioned place preference as a symptom of nicotine dependence. CaMKII and ERK phosphorylation in striatum significantly increased along with the development of nicotine dependence. Conclusion: We should next apply pharmacological strategies to manipulate CaMKII and ERK signaling to propose an attractive therapeutic approach to inhibit nicotine dependence. Keywords: Nicotine dependence, CaMKII, ERK, Conditioned place preference, Preference score

INTRODUCTION

Nicotine is an active compound and the main addictive material in tobacco products; nicotine dependence symptoms are characterized by compulsive use, craving, tolerance from continued use and withdrawal upon cessation [1]. Nicotine dependence is a chronic brain disorder and a worldwide primary public health issue [2]. Nicotine binds with the receptor nicotinic acetylcholine receptors (nAChRs), which are pentamers consisted of α2, α4 α7, α10, and β2, β4 subunits [3]. nAChRs are widely distributed in the central nervous system (CNS), including cortical and limbic regions. These receptors are critical for drug addiction through stimulation of synaptic activity in the hippocampus, amygdala, ventral tegmental area (VTA), and nucleus accumbens (Nac) and striatum region [4].

nAChRs are ligand-gated ion channels that were activated by the endogenous neurotransmitter acetylcholine (Ach) and the exogenous tertiary alkaloid nicotine [5]. Activation of nAChRs by nicotine stimulates calcium influx. Calcium through nAChRs, especially via the alpha-bungarotoxin-sensitive alpha7-containing nAChRs, which is a very effective subtype of nAChRs on enhancing cytoplasmic calcium level [6]. Calcium entry through voltage-gated Ca2+ channels is critical in developing the Calcium/Calmodulin Protein Dependent Kinase (CaMKII) level [7]. On the other hand, influx Ca2+ intracellular to result in activation and phosphorylation of PYK2, turn on the RAS through tyrosine kinase receptor and upstream the activity of extracellular regulated kinase 1/2 (ERK1/2) [8].

CaMKII is the most important Ca2+-sensors changing glutamatergic activation into synaptic plasticity during learning and memory formation, This cascade is pivotal for Long-Term Potentiation (LTP) as basis for morphological adaptations at the synapse during learning process [9]. ERK is a part of mitogen-activated protein kinases (MAPKs), affected in the modulation of many cellular processes, including cell proliferation, differentiation, growth, and death of cells. The previous study showed cocaine induces phosphorylation of ERK during dependence conditions [10]. Accumulating evidence supports ERK-dependency in molecular adaption, morphological plasticity, and behavioral performance such as nicotine like behaviour [11]. Here, we performed the experiment to investigate the roles of CaMKII and ERK on nicotine dependence conditions. These observations led us to the discovery of the new mechanism of nicotine-induced conditioned place preference through CaMKII and ERK. Moreover, this research will provide a new approach to prevent nicotine dependence by inhibiting the phosphorylation of CaMKII and ERK.

MATERIALS AND METHODS

Materials

Male BALB/c mice aged 8 w (20–30 g) were purchased from SLC (Hamamatsu, Japan). Mice were housed in a room with a 12/12-hour light/dark cycle (lights on at 09:00). Room conditions were temperature controlled at 22.0±2 °C with a relative humidity of 55%±15%. Mice had free access to food and water. All experimental animal procedures were approved by the Committee on Animal Experiments at Tohoku University, and studies were conducted following committee guidelines. Every effort was designated to minimize suffering and limit the number of animals used. Nicotine Hydrogen Tartrate was obtained from Sigma Aldrich. Phosphorylation of CaMKII antibody gift from Professor Kohji Fukunaga. Phosphorylation of ERK1/2 was purchased from cell signaling. Rabbit Secondary antibody was bought from Abcam company.

The testing apparatus for the conditioned place preference consisted of three compartments measuring 12.7 cmx6.5 cmx12.7 cm (width x length x height) in size. The middle compartment was grey, called the neutral compartment. Two conditioning compartments differed in color and floor texture. Compartment A was white with a quadrangular sieve (mesh). The other compartment (B) was black with stainless steel floors. Each compartment was separated by two doors (fig 1).

The immunoblotting analyses performed used Bio-rad apparatus, Protein separation by gel electrophoresis 1. Load equal amounts of...
protein (20 µg) into the wells of a mini (8.6 x 6.7 cm) or midi (13.3 x 8.7 cm) format SDS-PAGE gel, along with molecular weight markers. 2. Run the gel for 5 min at 50 V. 3. Increase the voltage to 100 - 150 V to finish the run in about 1 h.

Preference ratio observed by counting the time spent in nicotine compartment and total time in all compartment using stopwatch

Immunoblot analysis
Mice were sacrificed immediately after preference score conditioning calculation, Striatum were dissected out form the brain mice. The tissues were stored in liquid nitrogen for temporary and then stored at -80 °C until use. Western blot analysis was started as described. Striatum region samples were homogenized in 200 µl homogenizing buffer containing 4 µmol ethylenediaminetetraacetic acid (EDTA), 50 µmol Tris-HCl (pH 7.4), 1 µmol Na3VO4, 0.5% Triton X-100, 10 µmol EDTA, 40 µmol sodium pyrophosphate, 50 µmol NaF, leupeptin 25 µg ml−1, pepstatin A 50 µg ml−1, trypsin inhibitor and 1 mm dithiothreitol (DTT), 100 µmol calyculin A 50 µmol l−1. 10 minutes centrifugation at 15 000 r. p. m 4 °C was used to delete insoluble particle. After determining protein concentration in supernatants using Bradford’s solution using a spectrophotometer, samples were boiled in 100 °C incubator for 3 minutes in Laemmli buffer [13].

The samples containing equivalent amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) at 500 V and 40 mA. Proteins were transferred to an Immobilon polyvinylidene difluoride (PVDF) membrane (pore = 0.45 µm) (Millipore) for 2- h at 70 V. After blocking with TTBS solution (50 mm Tris-HCl, pH 7.5, 150 mm NaCl and 0.1% Tween 20) containing 5% of fat free milk powder for 1-h at room temperature, membranes were incubated overnight at 4 °C with Anti-phospho-CaMKII (1:5000) [14]. Anti-phospho-ERK (1:2000; Cell Signaling lot number 9101) [15].

Figures of the band were developed using an ECL immunoblotting detection system (Amersham Biosciences, Piscataway, NJ, USA) and were visualized on X-ray film (Fuji Film, Tokyo, Japan). Autoradiographic films were scanned for densitometry analysis (Lasergraphics, Irvine, CA, USA) and quantitative analyses were used Image Gauge version 3.41 (Fuji Film, Tokyo, Japan).

RESULTS
Nicotine administration generates nicotine-induced conditioned place preference as a symptom of nicotine dependence (fig 2) which is characterized by elevation of the value of preference ratio.
Nicotine administration induced nicotine dependence (fig. 2) which is characterized by increase of the value of preference ratio as a symptom nicotine dependence, preference ratio elevate 0.59 and 0.73 on 14 d and 28 d nicotine injection respectively compared to nicotine pre-administration condition on normal mice 0.44 (fig. 2A) [16]. According to the preference ratio, the time spent on nicotine compartment significantly enhanced on 28 d of administration of nicotine even though the enhancement of time in nicotine compartment even though pre-conditioning condition (fig. 2B). Moreover, our research shows if the change of time in nicotine compartment dramatically elevated more than 100 seconds in 28 d after nicotine administration (fig. 2C).

Since two important mediators in memory formation, particularly in long-term potentiation of neurons, were recognized and mediated by CaMKII and ERK 1/2, we analyzed the concentration of phosphorylation of both protein using western blot methods. The nicotine dependence was associated with elevation of activity of phosphorylation CaMKII and ERK1/2 auto-phosphorylation on striatum of mice brains (fig. 3. A and 3. B). The level of CaMKII phosphorylation markedly increased more than 2.5 fold compared to the level of CaMKII Phosphorylation on vehicle group 1 fold. In line with phosphorylation of CaMKII, Autophosphorylation of ERK 1/2 expression on striatum region also enhanced around 175%, comparable to vehicle treatment group 100%. ERK1/2 phosphorylation dramatically increases almost 75% if we compare to the vehicle group as an effect of nicotine administration for 28 consecutive days.

DISCUSSION
Nicotine is the neuroactive compound and addictive agent in tobacco, in the present study the administration of nicotine 0.5 mg/kg for 14 and 28 d is sufficient to induce dependence in mice. Nicotine successfully increases the preference ratio as a symptom of nicotine dependence on CPP methods, CPP is a familiar method to evaluate the rewarding effect of nicotine [17–20]. This protocol involves passive administration of the drug on one side of a conditioning apparatus, this method is substantially different from the drug self-administration method [21]. In the drug self-administration method, repeated self-infusions are required to establish substance addiction behaviors. It is likely that repeated exposure affects receptor transduction mechanisms associated with tolerance and sensitization [21]. Moreover, CPP is the preferred method for rapid screening and can be used with many mouse strains with high sensitivity [22].

The prolonged nicotine exposure for 28 d results in neural adaptation following receptor desensitization and upregulation of nAChR [23, 24], chronic nicotine exposure selectively up-regulates the density of α4β2 to stimulates nicotine addiction in rats induction by nicotine [25]. Not different with α4β2 the α7 as homo-oligomer nAChRs also involved in nicotine dependence, approved by the high expression of α7nAChR on striatum in a nicotine dependence rat model [26], on striatum nAChR bind with the nicotine and induce dependence. nAChRs is abundant in the family of ligand-gated ion channels that is expressed broadly throughout the central nervous system.
system and peripheral nervous system, and in non-neuronal cells [27]. Calcium intracellular influx through nAChRs, particularly via the α-bungarotoxin-sensitive α7-containing nAChRs, is a very effective way to raise cytoplasmic calcium concentration [6]. Calcium ions are one of the most important intracellular messengers known, and impacts almost every aspect of cellular life, including generating the proteins and their downstream effectors such as CaMII and ERK1/2 [28].

Nicotine increases activity of CaMII in the striatum region (fig. 3A) [29]. CaMII in striatum may correlate by long-term potentiation on memory formation by nicotine and strengthen the memory concerned with convenient feeling during nicotine administration [13]. In addition, lack of CaMII generates memory deterioration, the deficiency of CaMII mice proposed to abolish the memory formation on remembering the nicotine-paired compartment and failed to evoke CPP [30, 31]. Moreover, the ERK1/2 phosphorylation has been increased in striatum region nicotine dependence condition (fig. 3B) due to calcium influx that was enhanced by stimulation of nAChR [32]. Influx Ca2+intracellular to result in activation and phosphorylation of PKC, in turn the RAS is activated through the tyrosine kinase receptor and upstream the activity of ERK1/2 [8]. Besides that, ERK1/2 activation through β-adrenergic receptor plays a dual role in cell proliferation; it phosphorylates Stat3 at Ser727 and regulates cell proliferation [33]. Accumulation of ERK1/2 autoprophosphylation influences molecular adaptation, morphological plasticity, and behavioral performance such as nicotine like behaviour [11].

CONCLUSION
CaMII and ERK phosphorylation significantly increased along with the development of nicotine dependence. We should next apply pharmacological strategies to manipulate CaMII and ERK signaling. In particular, disruption of reconsolidation by disrupting CaMII and ERK signaling may propose an attractive therapeutic approach to inhibit nicotine dependence.

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AUTHORS CONTRIBUTIONS
All the authors contributed equally.

CONFLICT OF INTERESTS
Declared none

REFERENCES
1. Benowitz NL. Nicotine addiction. Prim Care. 1999;26(3):611-31. doi: 10.1016/s0002-4554(05)70120-2 PMID 10436290.
2. Henningfeld JE, Miyamoto K, Jasinski DR. Abuse liability and pharmacodynamic characteristics of intravenous and inhaled nicotine. J Pharmacol Exp Ther. 1985;234(1):1-12. PMID 40095494.
3. Changeux JP. Nicotine addiction and nicotinic receptors: lessons from genetically modified mice. Nat Rev Neurosci. 2010;11(6):389-401. doi: 10.1038/nrneuro.2009.98 PMID 20485364.
4. Leslie FM, Mojica CY, Reynaga DD. Nicotinic receptors in addiction pathways. Mol Pharmacol. 2013;83(4):753-8. doi: 10.1124/mol.112.003659 PMID 23247824.
5. Albuquerque ES, Pereira ER, Alkondon M, Rogers SW. Mammalian nicotinic acetylcholine receptors: from structure to function. Physiol Rev. 2009;89(1):317-120. doi: 10.1152/physrev.00015.2008 PMID 19126755.
6. Shen JX, Yakei JL. Nicotinic acetylcholine receptor-mediated calcium signaling in the nervous system. Acta Pharmacol Sin. 2009;30(6):673-80. doi: 10.1038/aps.2009.64 PMID 19448647.
7. Xiao RP, Cheng H, Lederer WJ, Suzuki T, Lakatua EG. Dual regulation of Ca2+-calmodulin-dependent kinase II activity by membrane voltage and by calcium influx. Proc Natl Acad Sci USA. 1994;91(20):9659-63. doi: 10.1073/pnas.91.20.9659 PMID 7937825.
8. Tahara S, Fukuda K, Kodama H, Kato T, Miyoshi S, Ogawa S. Potassium channel blocker activates extracellular signal-regulated kinases through Pyk2 and epidermal growth factor receptor in rat cardiomyocytes. J Am Coll Cardiol. 2001;38(5):1554-63. doi: 10.1016/s0735-1097(01)01558-3 PMID 11691539.
9. Sheng M, Kim MJ. Post synaptic neural transmission and plasticity mechanisms. Science. 2002;298(5594):776-80. doi: 10.1126/science.1075333 PMID 12399578.
10. Valjent E, Corvol JC, Pages C, Besson MJ, Maldonado R, Caboche L. Involvement of the extracellular signal-regulated kinase cascade for cocaine-rewarding properties. J Neurosci. 2000;20(23):8701-9. doi: 10.1523/JNEUROSCI.20-23-08701.2000 PMID 11102476.
11. Chwang WB, O’Riordan KJ, Levinson JM, Sweatt JD. ERK/MAPK regulates hippocampal histone phosphorylation following contextual fear conditioning. Learn Mem. 2006;13(3):322-8. doi: 10.1101/lm.152906 PMID 16741283.
12. Benowitz NL, Porchet H, Sheiner L, Jacob P. Nicotine absorption and cardiovascular effects with smokeless tobacco use: comparison with cigarettes and nicotine gum. Clin Pharmacol Ther. 1989;46(4):23-8. doi: 10.1016/0009-9236(89)90411-0 PMID 2639001.
13. Wilar G, Shinoda Y, Sasaoka T, Fukunaka K. Crucial role of dopamine D2 receptor signaling in nicotine-induced conditioned place preference. Mol Neurobiol. 2019;56(12):7911-28. doi: 10.1007/s12035-019-1635-x PMID 31129809.
14. Fukunaka K, Goto S, Miyamoto E. Immunohistochemical localization of CaMII-α/calmodulin-dependent protein kinase II in rat brain and various tissues. J Neurochem. 1985;41(4):1070-8. doi: 10.1111/j.1471-4159.1985.tb03070.x PMID 30473161.
15. Roux PP, Blenis J. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev. 2004;68(2):320-44. doi: 10.1128/MMBR.68.2.320-344.2004 PMID 15187187.
16. Risinger FO, Oakes RA. Nicotine-induced conditioned place preference and conditioned place aversion in mice. Pharmacol Biochem Behav. 1999;51(2-3):457-61. doi: 10.1016/0091-3057(95)00007-7 PMID 9767638.
17. Benowitz NL. Nicotine place preference: a large range of doses in rats. Psychopharmacol Bull. 2004;38(4):234-4. doi: 10.1111/j.1471-8849.2004.00424.x PMID 15104212.
18. Benowitz NL, Goldberg SR. Nicotine induces conditioned place preference over a large range of doses in rats. Psychopharmacology (Berl). 2005;178(4):481-92. doi: 10.1007/s00213-004-2021-5 PMID 15765262.
19. Brielmaier JM, McDonal GS, Smith RF. Nicotine place preference in a biased conditioned place preference design. Pharmacol Biochem Behav. 2008;89(1):94-100. doi: 10.1016/j.pbb.2007.11.005 PMID 18086490.
20. Carkagetti DJ, Schechter MD. Nicotine place preference using the biased method of conditioning. Prog Neuropsychopharmacol Biol Psychiatry. 1994;18(5):925-33. doi: 10.1016/0091-3057(94)90108-2 PMID 7972862.
21. Baro MT, Bevins RA. Conditioned place preference: what does it add to our preclinical understanding of drug reward? Psychopharmacology (Berl). 2000;153(1):31-43. doi: 10.1007/s002130050856 PMID 11255927.
22. Cunningham CL, Dickson SD, Grahame NJ, Okorn DM, Mc Mullin CS. Genetic differences in cocaine-induced conditioned place preference in mice depend on conditioning trial duration. Psychopharmacology (Berl). 1999;146(1):73-80. doi: 10.1007/s002130050190 PMID 10445967.
23. Govind AP, Veizina P, Green WN. Nicotine-induced upregulation of nicotinic receptors: underlying mechanisms and relevance to nicotine addiction. Biochem Pharmacol. 2009;78(7):755-65. doi: 10.1016/j.bcp.2009.06.011 PMID 19540212.
24. Rogers SW, Gahringer LC. Upregulation of nicotinic acetylcholine receptor alpha4+beta2 through a ligand-independent PI3Kbeta mechanism that is enhanced by TnAlpha and the la2/p38MAPK pathways. PLoS ONE. 2015;10(11):e0143319. doi: 10.1371/journal.pone.0143319 PMID 26619354.
25. Nguyen HN, Rasmussen BA, Perry DC. Subtype-selective upregulation by chronic nicotine of high-affinity nicotinic receptors in rat brain demonstrated by receptor...
autoradiography. J Pharmacol Exp Ther. 2003;307(3):1090-7. doi: 10.1124/jpet.103.056408. PMID 14560040.

26. Nomikos GG, Schilström B, Hildebrand BE, Panagis G, Grenhoff J, Svensson TH. Role of alpha7 nicotinic receptors in nicotine dependence and implications for psychiatric illness. Behav Brain Res. 2000;113(1-2):97-105. doi: 10.1016/s0166-4328(00)00204-7. PMID 10942036.

27. Hurst R, Rollemo H, Bertrand D. Nicotinic acetylcholine receptors: from basic science to therapeutics. Pharmacol Ther. 2013;137(1):22-54. doi: 10.1016/j.pharmthera.2012.08.012, PMID 22925690.

28. Gubbins EJ, Gopalakrishnan M, Li J. Alpha7 nAChR-mediated activation of MAP kinase pathways in PC12 cells. Brain Res. 2010;1328:1-11. doi: 10.1016/j.brainres.2010.02.083, PMID 20211606.

29. Jackson KJ, Muldoon PP, Walters C, Damaj MI. Neuronal calcium/calmodulin-dependent protein kinase II mediates nicotine reward in the conditioned place preference test in mice. Behav Pharmacol. 2016;27(1):50-6. doi: 10.1097/FBP.0000000000000189, PMID 26292186.

30. Picconi B, Gardoni F, Centonze D, Mauceri D, Cenci MA, Bernardi G, Calabresi P, Di Luca M. Abnormal Ga2-calmodulin-dependent protein kinase II function mediates synaptic and motor deficits in experimental parkinsonism. J Neurosci. 2004;24(23):5283-91. doi: 10.1523/JNEUROSCI.1224-04.2004, PMID 15190099.

31. Silva AJ, Paylor R, Wehner JM, Tongeawa S. Impaired spatial learning in alpha钙-calcium-calmodulin kinase II mutant mice. Science. 1992;257(5067):206-11. doi: 10.1126/science.1321493, PMID 1321493.

32. Nakayama H, Numakawa T, Ikeuchi T, Hatanaka H. Nicotine-induced phosphorylation of extracellular signal-regulated protein kinase and CREB in PC12 cells. J Neurochem. 2001;79(3):489-98. doi: 10.1046/j.1471-4159.2001.00602.x, PMID 11701752.

33. Chen RJ, Ho YS, Guo HR, Wang YJ. Rapid activation of Stat3 and ERK1/2 by nicotine modulates cell proliferation in human bladder cancer cells. Toxicol Sci. 2008;104(2):283-93. doi: 10.1093/toxsci/kfn086, PMID 18448488.