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Original Article

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 were dissected out 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. In particular, disruption of reconsolidation by disrupting CaMKII and ERK signaling may propose an attractive therapeutic approach to inhibit nicotine dependence.

Keywords: Nicotine dependence, CaMKII, ERK, Conditioned place preference, Preference score

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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 by main 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 Ca^{2+}channels is critical in develop the Calcium/Calmodulin Protein Dependent Kinase (CaMKII) level [7]. On the other hand, Influx Ca^{2+}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 Ca^{2+}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 adaptation, 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%±5%. 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 buyed from Abcam company.

The testing apparatus for the conditioned place preference consisted of three compartments measuring 12.7 cmx46.5 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.

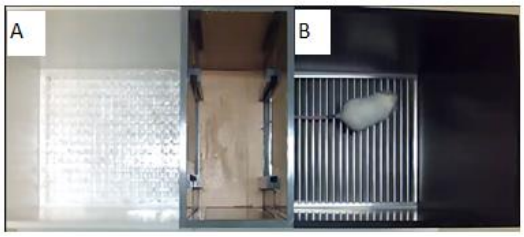

Fig. 1: Conditioned place preference box

Nicotine-induced conditioned place preference

Mice were divided into two groups, nicotine treatment group n=6 and vehicle treatment group n=6. Mice were first habituated to the CPP apparatus for five days; Acclimatization was performed for five days prior to pre-conditioning. It is designed to remove any environmental stress, including the weighing and testing rooms, CPP apparatus, and drug administration. Followed by a pre-conditioning test to determine the nicotine-paired compartment. Mice entered conditioning training for one month in which 0.5 mg/kg nicotine was administered intraperitoneally, followed by confinement in the designated compartment of CPP apparatus for 30 minute [12]. Four hours later, the same procedure was repeated, only this time saline was given instead of nicotine and the mouse was confined in the opposite of nicotine compartment. One day after conditioning, preference scores were measured three times to evaluate the nicotine dependence on preconditioning, 2 w conditioning and 4 w conditioning. Preference scores were calculated using this formula:

$$\text{Preference Score} = \frac{\text{Sojourn time in nicotine compartment (s)}}{\text{Total time spent in all compartments (s)}}$$

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 µm ethylene glycol tetraacetic acid (EGTA), 50 µm Tris–HCl (pH 7.4), 1 µm Na_3VO_4, 0.5% Triton X-100,, 10 µm EDTA,, 40 µm sodium pyrophosphate, 50 µm NaF, leupeptin 25 µg ml^{-1}, pepstatin A 50 µg ml^{-1}, trypsin inhibitor and 1 mm dithiothreitol (DTT), 100 µm calyculin A 50 µgml^{-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].

Pictures 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.

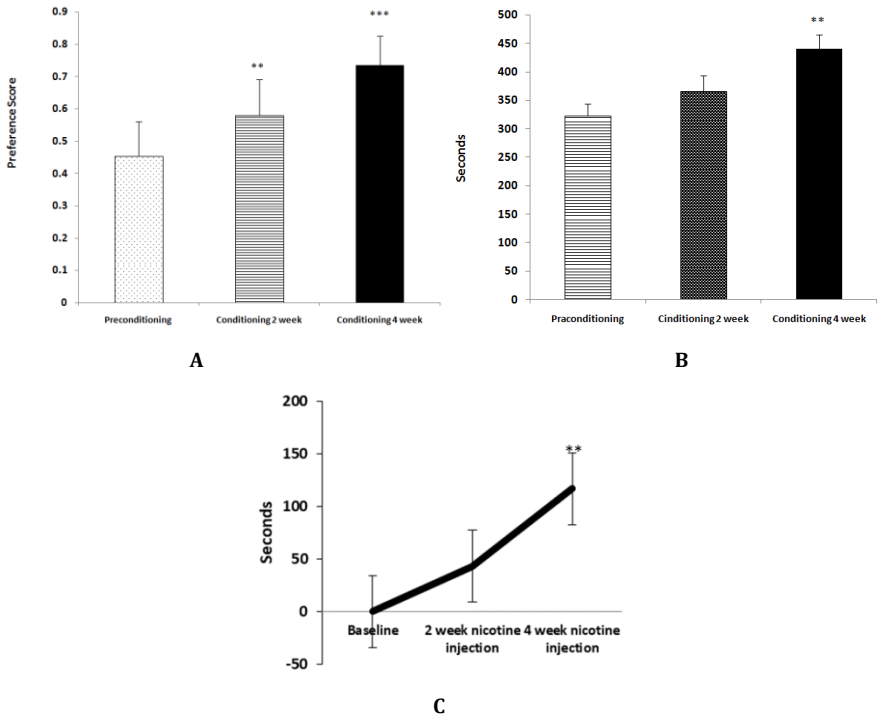

Fig. 2: Nicotine-induced conditioned place preference. A. Preference score of Nicotine dependence in 4 w nicotine administration (n=6). B. Time in Nicotine paired compartment after nicotine administration in 28 consecutive days (n=6). C. Δ Time in Nicotine paired compartment (n=6). Data were presented as average±SEM and analyzed using one-way ANOVA followed by multiple comparisons between baseline condition and the results obtained 2 w and 4 w after nicotine administration post hoc Tukey tests. Significantly different was considered *p<0.05 were compared by preconditioning or baseline condition

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 after 14 administration was not differently significant compared by preconditioning 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).

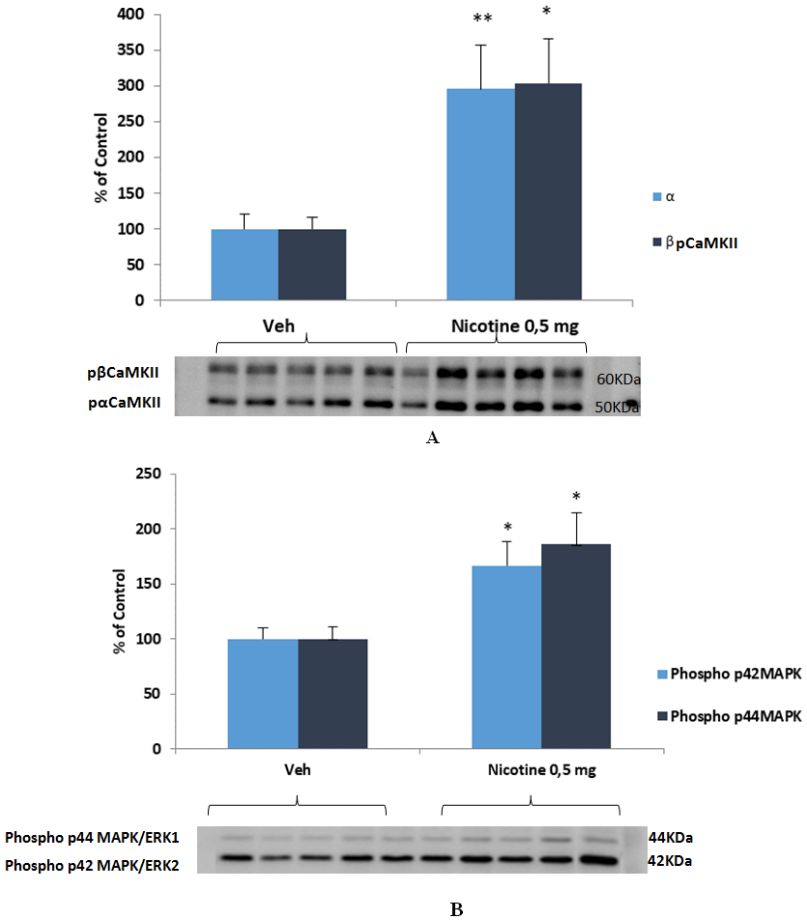

Fig. 3: The expression of phosphorylation CaMKII and ERK nicotine dependence on striatum brain region. A. Nicotine Induces Enhancement of CaMKII autophosphorylation in striatum (n=5). B. Enhancement of ERK1/2 phosphorylation in striatum stimulated by nicotine administration (n=5). Data were presented as average±SEM and analyzed using one-way ANOVA followed by multiple comparisons between vehicle condition and the results 4 w after nicotine administration post hoc Tukey tests. Significantly different was considered *p<0.05 were compared by vehicle group

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 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 CaMKII and ERK1/2 [28].

Nicotine increases activity of CaMKII in the striatum region (fig. 3A) [29]. CaMKII 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 CaMKII generates memory deterioration, the deficiency of CaMKII 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 Ca^{2+}intracellular to result in activation and phosphorylation of PYK2, 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-adrenoceptors plays a dual role in cell proliferation; it phosphorylates Stat 3 at Ser727 and regulates cell proliferation [33]. Accumulation of ERK1/2 autophosphorylation influences molecular adaptation, morphological plasticity, and behavioral performance such as nicotine like behaviour [11].

CONCLUSION

CaMKII and ERK phosphorylation significantly increased along with the development of nicotine dependence. We should next apply pharmacological strategies to manipulate CaMKII and ERK signaling. In particular, disruption of reconsolidation by disrupting CaMKII 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

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