Role of Dopamine D2 Receptor/Fatty Acid-Binding Protein 3 Signaling in Nicotine-Induced Addiction

Role of Dopamine D2 Receptor/Fatty Acid-Binding Protein 3 Signaling in Nicotine-Induced Addiction（ニコチン依存症におけるドパミン D2 受容体と脂肪酸結合タンパク質 3 シグナルの役割）

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[Background]

Smoking was reported as the second leading reason for disability and early death in 2015. Smoking causes blood vessels to become thicker and narrower, which could increase cardiac beat and blood pressure. Moreover, smoking leads to 90% of all lung cancer deaths, and causes approximately 80% of all deaths from chronic obstructive pulmonary disease (COPD). Nicotine is a predominant addictive compound present in tobacco and exerts physical and psychological dependence through activating nicotinic acetylcholine receptors in the central nervous system (CNS). Nicotine exerts its physiological function by interacting with nicotinic acetylcholine receptors (nAChRs) in the ventral tegmental area (VTA), through binding with pentameric ligand-gated ion channels. Numerous investigations have reported that dopamine D1 receptors (D1Rs) are fully engaged in nicotine-induced addiction, and participate in signaling pathways related with addiction. Recently, Emerging evidence demonstrated that not only D1Rs, dopamine D2 receptors (D2Rs) are also critical for the acquisition of nicotine-induced behaviors. Our previous data suggested that D2R knockout ($D2R^{-/-}$) mice failed to exhibit conditioned place preference (CPP) scores following 28 days of consecutive nicotine administration.

Long-chain polyunsaturated fatty acids (LCPUFAs) are fatty acids with 18-20 carbons or more, and are abundantly expressed in the brain and retina. It is necessary for fatty acid-binding proteins (FABPs) to function as cellular shuttles to transport LCPUFAs to proper intracellular compartments since LCPUFAs are not soluble in water. Among FABPs, FABP3 is the particular isoform expressed in neurons in the mature brain. FABP3 is associated with D2R, and especially correlated with D2LR and binds with D2LR at the insert region of 29 amino acid sequences (G242-V270) in the third cytoplasmic loop. Moreover, previous investigations defined that fatty acid-binding protein 3 (FABP3) is critical for dopamine D2 receptor (D2R) function in the mouse striatum, suggesting that D2R and FABP3 signaling mediates the extrapyramidal behaviors in mice. In this context, we hypothesize that FABP3 mediates the nicotine-induced CPP in mice. To define how FABP3 elicits the D2R-mediated psychomotor behaviors, nicotine-induced CPP behaviors were investigated in FABP3 null ($FABP3^{-/-}$) mice.

[Methods]

Nicotine-induced CPP behaviors were evaluated with preference scores using CPP apparatus. The acclimatization phase persisted for 4 days. In this period, each mouse was placed in the neutral compartment with the two guillotine doors open and was allowed to freely explore all three compartments for a total of 10 min. On day 5, the preconditioning test was performed. Each mouse was placed in the neutral compartment for 5 min with two guillotine doors closed. Afterwards, two guillotine doors were removed, and each mouse was allowed to access all three compartments for 15 min. The formula for documenting CPP preference score was as follows: Preference score = Retention time in related conditioning compartment/Total time in all conditioning compartments × 100%. Subsequently, in the conditioning phase, mice were administered nicotine or saline and were confined in the related compartments with guillotine doors closed for 30 min daily for 14 consecutive days. CPP preference scores were calculated using the same method as described above. In the withdrawal phase, mice received saline administration prior to

placement in the related compartments for 5 days. In the nicotine relapse phase, mice were administered saline or nicotine once. Thirty minutes later, each mouse was placed in the neutral compartment in turn, and allowed to freely explore for the whole three compartments for 15 min.

Afterwards, the responsiveness of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) autophosphorylation and extracellular signal-regulated kinase (ERK) phosphorylation levels were examined by immunoblotting assay using nucleus accumbens (NAc) and hippocampal CA1 region extracts. The neuronal activities of NAc neurons with D1Rs and D2Rs were evaluated by calculating the number of immunoreactive double-positive cells with c-Fos and autophosphorylated CaMKII immunoreactivities.

[Results]

In CPP behavioral tests, we observed significantly increased CPP scores in 14 consecutive days of nicotine-treated WT mice in the comparison of base level of precondition in the CPP conditioning phase. However, the failure of CPP induction in FABP3$^{-/-}$ mice following 14 consecutive days of nicotine exposure was observed in the nicotine conditioning phase. Identical to the results in the conditioning phase, the inhibition of nicotine-induced CPP scores were also observed in the withdrawal and nicotine relapse phases **(shown in Figure 1)**.

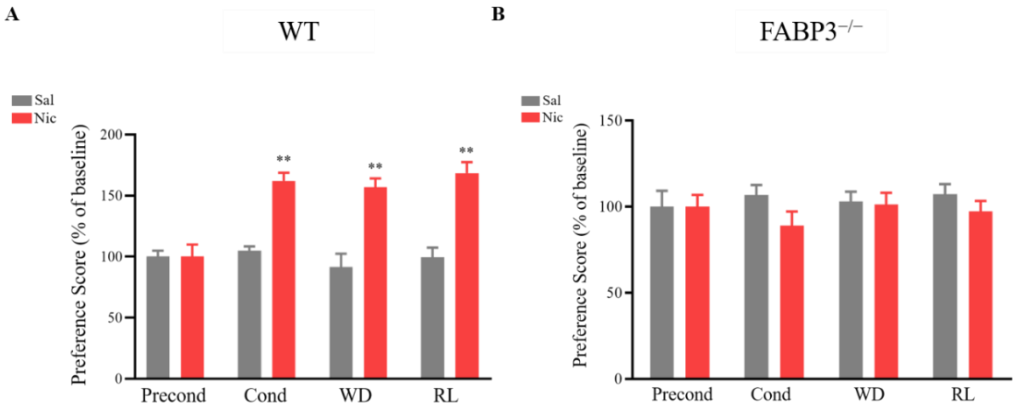

Figure 1. Significantly increased CPP was observed in WT mice, whereas failed CPP induction was observed in FABP3$^{-/-}$ mice. **A** Nicotine administration of 14 consecutive days successfully elevates CPP scores in the nicotine conditioning phase. In the withdrawal and nicotine relapse phases, elevations of CPP scores were also observed in WT mice. ** $p < 0.01$ vs. preconditioning nicotine group. **B** Nicotine-induced CPP was totally abolished in the conditioning phase in FABP3$^{-/-}$ mice. Furthermore, nicotine-induced CPP was not observed in withdrawal and nicotine relapse phases in FABP3$^{-/-}$ mice. *Error bars* represent SEMs. *Precond*, preconditioning; *Cond*, conditioning; *WD*, withdrawal; *RL*, relapse; *Sal*, saline; *Nic*, nicotine; *WT*, wild type; *FABP3$^{-/-}$*, FABP3 knockout.

Consistent with failed induction of CPP behaviors, FABP3$^{-/-}$ mice showed lack of responsiveness of CaMKII autophosphorylation and ERK phosphorylation levels in the context of nicotine exposure in the NAc and the hippocampal CA1 region. Interestingly, we observed a significantly increased basal level of CaMKII autophosphorylation in saline-treated FABP3$^{-/-}$ mice NAc relative to that in WT mice. Although we did not observe significantly increased basal levels of ERK phosphorylation in FABP3$^{-/-}$ NAc, the trend toward enhancement of ERK phosphorylation levels was observed **(shown in Figure 2).**

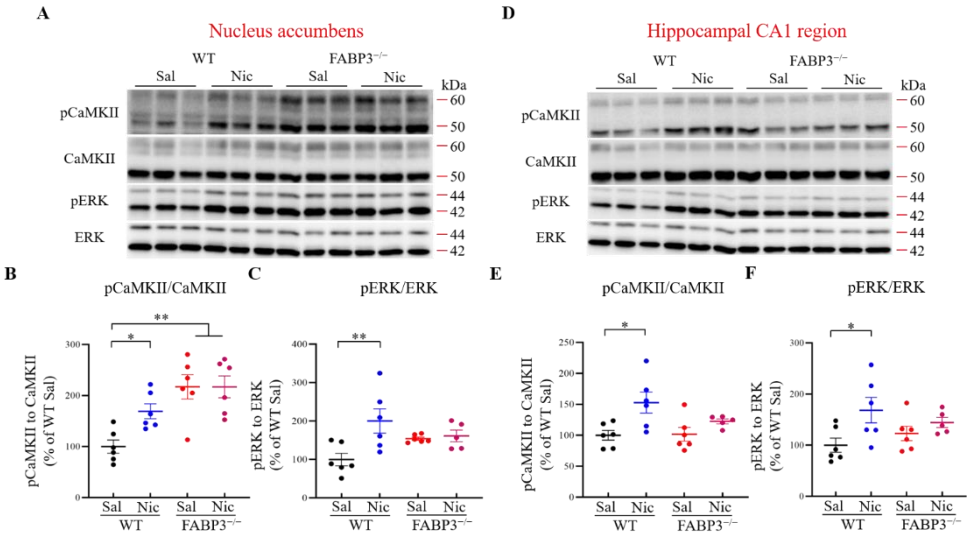

Figure 2. Levels of CaMKII and ERK in mice NAc and hippocampal CA1 region following CPP behavioral tests. A Representative images of autophosphorylated CaMKII, CaMKII, phosphorylated ERK, and ERK bands in brain tissue of the NAc. **B** CaMKII autophosphorylation levels significantly enhanced following chronic nicotine administration in WT mice NAc, whereas an increased baseline of CaMKII autophosphorylation levels and a lack of response of CaMKII autophosphorylation to nicotine were observed in FABP3$^{-/-}$ mice NAc. Autophosphorylated CaMKII: * $p < 0.05$, ** $p < 0.01$ vs. WT saline-treated group. **C** A lack of response of ERK phosphorylation to nicotine was observed in FABP3$^{-/-}$ mice NAc. Phosphorylated ERK: * $p < 0.05$. ** $p < 0.01$ vs. WT saline-treated group. **D** Representative images of autophosphorylated CaMKII, CaMKII, phosphorylated ERK, and ERK bands in the hippocampal CA1 region. **E** CaMKII autophosphorylation levels significantly enhanced following chronic nicotine administration in WT mice hippocampal CA1 region. However, a lack of responsiveness in terms of CaMKII autophosphorylation to nicotine was observed in FABP3$^{-/-}$ mice hippocampal CA1 region. Autophosphorylated CaMKII: * $p < 0.05$ vs. WT saline-treated group. **F** A lack of response of ERK phosphorylation to nicotine was observed in FABP3$^{-/-}$ mice hippocampal CA1 region. Phosphorylated ERK: * $p < 0.05$ vs. WT saline-treated group. *Sal*, saline; *Nic*, nicotine; *WT*, wild type; *FABP3*$^{-/-}$, FABP3 knockout. *Error bars* represent SEMs.

Subsequently, we observed that c-Fos levels were closely correlated with CaMKII autophosphorylation levels in D2R-positive cells in the NAc. We found a significantly increased number of D2R-positive cells and lack of responsiveness of nicotine-induced elevation in the number of D2R-positive cells in FABP3$^{-/-}$ mice. We then analyzed c-Fos activities in D2R-positive cells. As a result, a significantly increased number of D2R/c-Fos double-positive cells and lack of responsiveness of c-Fos activities to chronic nicotine treatment were observed in FABP3$^{-/-}$ mice NAc **(shown in Figure 3)**.

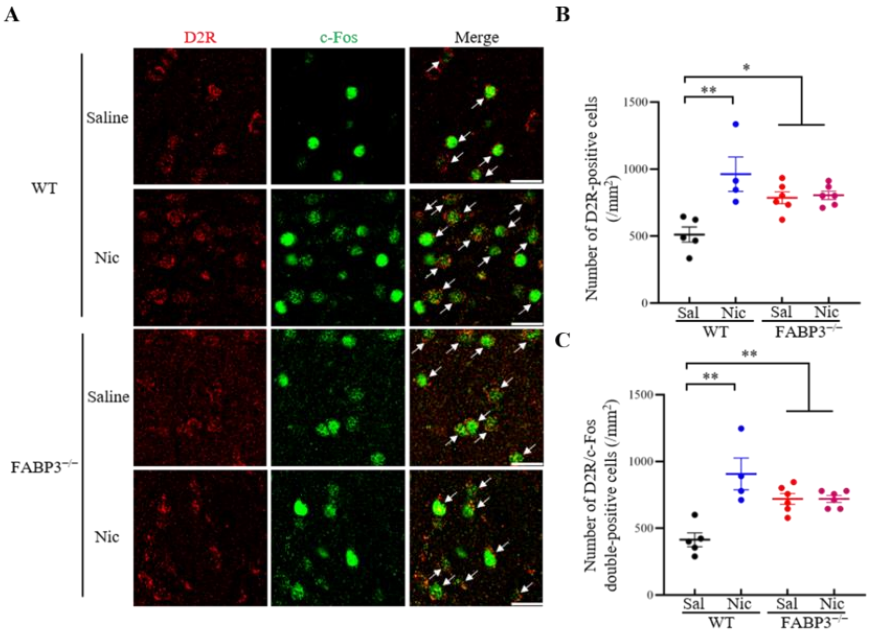

Figure 3. The number of D2R-positive cells and c-Fos levels in D2R-positive cells in mice NAc. A Representative images of D2R (red)/c-Fos (green) double-positive cells in the NAc in WT and FABP3$^{-/-}$ mice. **B** Quantification of the number of D2R-positive cells in the NAc in WT and FABP3$^{-/-}$ mice following saline or chronic nicotine administration, respectively. A significantly elevated number of D2R-positive cells and lack of responsiveness of nicotine-induced elevation in the number of D2R-positive cells were observed in FABP3$^{-/-}$ mice NAc. * $p < 0.05$, ** $p < 0.01$ vs. WT saline-treated group. **C** Quantification of the number of D2R/c-Fos double-positive cells in the NAc in WT and FABP3$^{-/-}$ mice following saline or chronic nicotine administration, respectively. A significantly elevated number of D2R/c-Fos double- positive cells and lack of responsiveness of c-Fos activities to chronic nicotine were observed in FABP3$^{-/-}$ mice NAc. ** $p < 0.01$ vs. WT saline-treated group. *Sal*, saline; *Nic*, nicotine. *Scale bars*, 25 μm. *Error bars* represent SEMs. *Arrows* indicate double-positive cells.

[Conclusion]

In summary, we demonstrate for the first time that FABP3$^{-/-}$ mice failed to establish nicotine-induced CPP behaviors in conditioning, withdrawal, as well as nicotine relapse phases. These impaired acquisition of CPP behaviors were closely correlated with lack of responsiveness of CaMKII autophosphorylation and ERK phosphorylation levels in the NAc and hippocampal CA1 region. Whereas, a significantly elevated CaMKII autophosphorylation level was observed in FABP3$^{-/-}$ mice NAc. Furthermore, an obviously decreased CaMKII autophosphorylation level in D1R-positive cells and a significantly increased CaMKII autophosphorylation level in D2R-positive cells were found in FABP3$^{-/-}$ mice NAc. Consistent with CaMKII autophosphorylation level, we observed that c-Fos level was identically robustly reduced in D1R-positive cells, while rapidly elevated in D2R-positive cells in FABP3$^{-/-}$ mice NAc. We provided interpretation of underlying mechanism that cAMP/Ca^{2+} signaling which was originally regulated by negative D2R/FABP3 signaling in WT mice NAc, was derepressed in FABP3$^{-/-}$ mice due to the aberrant D2R signaling through FABP3 deficiency, thereby causing significantly elevated basal levels of CaMKII autophosphorylation and CREB/c-Fos. These constitutive elevations failed to cause nicotine-induced CPP behaviors in FABP3$^{-/-}$ mice. Collectively, our results indicate that FABP3 could be a novel therapeutic target intended to treat nicotine-induced addiction, and other psychostimulant-induced addictive behaviors which affect DAergic systems in the CNS.

論 文 審 査 結 果 の 要 旨

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論 文 題 目：Role of Dopamine D2 Receptor/Fatty Acid-Binding Protein 3 Signaling in Nicotine-Induced Addiction（ニコチン依存症におけるドパミン D2 受容体と脂肪酸結合タンパク質３シグナルの役割）

賈文彬氏は、ニコチン誘発性の依存症における脂肪酸結合タンパク質(FABP)の関与を調べた。煙草には主な依存性物質としてニコチンが含まれており、喫煙は肺がんや慢性閉塞性肺疾患を誘発する主要なリスクファクターである。これまでの先行研究においては、ニコチン誘発性の症状が発現するためには、脳の線条体のドパミン２型受容体が必要であることが知られており、さらにドパミン２型受容体は、脳の様々な領域において、脂肪酸結合タンパク質の１つである FABP3 と共局在することが知られていた。賈文彬氏の研究では，FABP3 欠損マウスを用いて、ニコチン依存症の行動発現がどのように変化するか調べた。その結果、FABP3 欠損マウスでは、ニコチン誘発性の場所の嗜好性、ニコチン投与を停止した際に生じる不安症状が低下していることを明らかにした。この生化学的メカニズムを調べるために、脳の側坐核におけるドパミン D2 受容体と FABP3 の共局在に着目し、免疫組織化学染色法およびウエスタンブロット法を用いて、FABP3 が欠損するとドパミン２型受容体シグナルが正常に作動しないことを見出した。さらにウエスタンブロット法を用いて、これらのシグナル異常によって、カルモジュリン依存性プロテインキナーゼ II 自己リン酸化および CREB 結合タンパク質の基底発現レベルが恒常的に上昇することを見出した。同様の傾向は、側坐核のみならず脳の他の領域である海馬 CA1 野においても観察された。さらに詳細な細胞種の特定をするために、免疫組織科学的染色法およびウエスタンブロット法を用いて検討を進めたところ、FABP3 欠損マウスの側坐核ではドパミン１型受容体の陽性細胞において、カルモジュリン依存性プロテインキナーゼ II 自己リン酸化レベルが有意に減少することを見出した。併行して、ドパミン２型受容体の陽性細胞においては、カルモジュリン依存性プロテインキナーゼ II 自己リン酸化レベルが有意に増加することを見出した。こうしたカルモジュリン依存性プロテインキナーゼ II 自己リン酸化レベルの変化と一致して、FABP3 欠損マウスの側坐核においては、ドパミン１型受容体の陽性細胞では c-Fos タンパク質の発現量が減少した。このことは同細胞種では神経活動が低下していることを示唆している。一方で、ドパミン２型受容体の陽性細胞では c-Fos の発現量が増加した。このことは同細胞種では神経活動が増加していることを示唆している。以上のような生化学的反応の解析を通じて、賈文彬氏は、FABP3 欠損マウスにおいてニコチン依存症が生じないための脳の主要なメカニズムを発見し

た。これらの研究は、脳の FABP3 がドパミン作動システムに影響し、ニコチン依存症に対する新たな治療標的になる可能性を示唆する重要な成果である。

よって、本論文は博士（薬学）の学位論文として合格と認める。