主論文の要旨

Muscarinic signaling regulates voltage-gated potassium channel KCNQ2 phosphorylation in the nucleus accumbens via protein kinase C for aversive learning

（忌避学習においてムスカリンシグナル伝達は蛋白質キナーゼCを介して側坐核の電位依存性カリウムチャネルKCNQ2のリン酸化を制御する）

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【Introduction】

The nucleus accumbens (NAc) plays critical roles in emotional behaviors, including aversive learning. In the NAc, 95% of neurons are medium spiny neurons (MSNs), which are subdivided into dopamine D1 receptor (D1R)-expressing MSNs and dopamine D2 receptor (D2R)-expressing MSNs. Additionally, 1–2 % of NAc neurons are cholinergic interneurons that release acetylcholine (Ach). The Ach is abundant in the striatum and NAc area of the brain. Aversive stimuli (e.g., Foot-shock, tail pinch) increase the release of Ach in the NAc. Ach modulates synaptic plasticity of MSNs, which express muscarinic M1 receptor, by activating its M1 receptor, regulating cellular excitability. However, it remains to fully elucidate the mechanism underlying neuronal excitability triggered by Ach.

The phosphorylation of KCNQ2 at threonine 217 (T217) was predicted by mass spectrometry, but the responsible kinase remains unknown and needs to be determined both *in vitro* and *in vivo*. In this study, we found that PKC phosphorylated KCNQ2 at T217 *in vitro* and *in vivo* downstream of muscarinic signaling and that aversive stimulus, such as electric foot-shock (eFS), increased the phosphorylation level of KCNQ2 at T217. We also found that conditional deletion of KCNQ2 in the NAc enhanced eFS-induced aversive learning.

【Materials and Methods】

Striatal and accumbal slice culture

For the striatal and accumbal slice preparation in biochemical assays, mice were sacrificed humanely and the brain was immediately removed after decapitation. Striatal/accumbal slices (350 μm) were prepared using a VT1200S vibratome in cold oxygenated Krebs-HCO_3^- buffer. The dissected slices were stimulated with PKC activator PEP005 (1 μM) for 90 min after pretreatment with the PKC inhibitor GF109203X (10 μM) for 90 min. The treated slices were kept in liquid nitrogen and stored in a freezer at −80°C until assayed.

Electric foot shock

An electric foot shock experiment was carried out in two sessions: habituation (day 1) followed by electric foot shock induction (day 2) (0.4 mA, 60 Hz for 2 s). VU0255035 (35 mg/kg) was injected intraperitoneally for 30 min before electric foot shock. Ten minutes after foot shock, the mice were decapitated, and the NAc was punched out using a 2 mm diameter biopsy punch. The tissues were solubilized in 300 μL of 1X SDS-urea buffer followed by overnight rotation at 4°C, subsequently performing the western blot analyses with the indicated antibodies. For immunohistochemistry, after 10 min of electric shock, mice were perfused with 4% paraformaldehyde (PFA). Coronal brain slices (20 μm

thickness) were post-fixed with 4% PFA for 10 min, followed by incubation with the indicated antibodies. After incubation of primary antibody overnight, the slices were washed three times with PBS and later incubated with secondary antibodies (goat anti-rat Alexa Fluor 488, 1:1000; goat anti-rabbit Alexa Fluor 555, 1:1000, DAPI 1:2000 in Blocking one P) for 1 h at RT. A confocal microscope (LSM780, Carl Zeiss, Jena, Germany) was used for image observation and image acquisition. For image acquisition, we used a 63X objective with identical pinhole, dwell time, gain and laser settings.

【Results and Discussion】

PKC activator, PEP005 stimulated the phosphorylation of KCNQ2 at T217 in striatal/accumbal slices (Figure 1A-C). The increased phosphorylation of KCNQ2 was significantly blocked by pretreatment with PKC inhibitor GF109203X (Figure 1A-C). PEP005 also confirmed the increased phosphorylation of well-known PKC substrates, the NMDA receptor NR1 subunit at S890 and myristoylated alanine-rich C-kinase substrate (MARCKS) at S152/S156 in striatal/accumbal slices (Figure 1B, D, E), whereas this phosphorylation was prevented by pretreatment with GF109203X. These results demonstrated that PKC phosphorylated KCNQ2 at T217 in the mouse striatum/NAc.

Application of electric foot shock induced the phosphorylation of KCNQ2 at T217, NR1 at S890 and MARCKS at S152/156, whereas preinjection of M1R antagonist VU0255035 suppressed the phosphorylation of these proteins (Figure 2A-E). The immunofluorescence intensity of P-KCNQ2 and the number of P-KCNQ2 T217-positive cells were increased in the NAc by electric foot shock (Figure 2G-H). Taken together, these results indicated that aversive stimulus enhanced the phosphorylation of KCNQ2 at T217 in MSNs of the NAc.

To investigate the role of KCNQ2 in aversive learning, KCNQ2-cKO mice were employed in a motivated passive avoidance test with electric foot shock. In the passive avoidance test, step-through latency was recorded in the test session 24 h after the training session, where electric foot shock was applied. Compared with the control mCherry injection, knockout of *Kcnq2* in the NAc significantly increased the step-through latency (Figure 3A-B). These results indicated that conditional deletion of *Kcnq2* facilitated aversive learning and suggested that KCNQ2 negatively regulated aversive behavior.

【Conclusions】

In this study, we found that PKC directly phosphorylates KCNQ2 at T217 in response to muscarinic stimulation (Figure 1 and 2) and conditional deletion of *KCNQ2* in the NAc enhanced electric foot shock-induced aversive learning (Figure 3). PKC participates in muscarinic receptor-mediated inhibition of KCNQ channel activity. Thus, muscarinic signaling likely inhibits the M-current and increases the neuronal excitability of MSNs in the NAc through the phosphorylation of KCNQ2 at T217 by PKC for aversive learning.

T217 is located at the large intracellular domain named the S4–S5 linker region of KCNQ2, which plays a critical role in the regulation of voltage-gated channel activity. Phosphatidylinositol-4,5 bisphosphate (PIP_2) interacts with the S4–S5 linker region and increases the current amplitude and voltage sensitivity of the KCNQ2 channel. Thus, it is conceivable that adding a phosphate moiety to the S4-S5 linker region by phosphorylation at T217 prevents the interaction with PIP_2. KCNQ2 Phosphorylation may reduce the M current by modulating the PIP_2 affinity, thereby increasing neuronal firing.