Effects of aquaporin 4 deficiency on the expression of spinal PKCα, PKCγ and c-Fos in naloxone-precipitated morphine withdrawal mice

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Abstract: The previous study indicated that aquaporin 4 (AQP4) deficiency attenuated opioid physical dependence. However, the underlying mechanism remains unknown. In the present study, the effects of AQP4 deficiency on the expression of three factors, protein kinase C (PKC) α, PKCγ and c-Fos in the spinal cord, which are known to be concerned with spinal neuronal sensitization and opiate dependence, were investigated in AQP4 knockout mice using Western blotting analysis. It was observed that AQP4 deficiency reduced the score of naloxone-precipitated abstinent jumping after repeated morphine administration compared with wild-type (P < 0.001). Meanwhile, the protein levels of PKCα and c-Fos in the spinal cord of AQP4 knockout mice were significantly higher than those in the wild-type mice; while the expression of PKCγ was decreased remarkably by AQP4 knockout during the withdrawal (P < 0.01). These data suggest that AQP4 deficiency-attenuated morphine withdrawal responses may be partially attributed to the changes in the spinal expression of PKCα, PKCγ or c-Fos.

Key words: aquaporin 4; c-Fos; mice; morphine withdrawal; spinal cord; protein kinase C

Research Paper

Opioids are widely used in management of many forms of acute and chronic pain. However, repeated use of opioids such as morphine can lead to opiate tolerance and dependence. Opioid dependence is a major health problem and delineation of the molecular mechanisms represents an important scientific challenge with sub-
stantial social impact. Several lines of evidence indicate that the spinal cord, the first central relay station for processing nociceptive information, is an important site in the development of opioid dependence and withdrawal, and the sensitization of spinal neurons is involved in this process \[1–7\]. The spinal sensitization is unmasked by the administration of naltrexone and naloxone, the opioid antagonists, and is manifested by c-Fos induction in the superficial laminae I/II of the spinal dorsal horn in withdrawing animals \[4, 5, 8\]. Moreover, it has been assumed that the translocation and activation of protein kinase C (PKC) in the spinal cord may be a critical step in the development of opiate tolerance and dependence \[9\]. Specially, the isoforms PKCα and PKCγ play different roles in morphine dependence and naloxone-precipitated withdrawal response \[1, 10, 11\].

Aquaporin 4 (AQP4) is a water-selective channel specifically expressed in astrocytes and ependymal cells in the central nervous system. Previously, revealed by AQP4 knockout mice, the main role of AQP4 is water selective transport between blood and brain cerebrospinal fluid compartments \[12\]. Recent reports have highlighted that AQP4 is concerned with neural signal transduction by modulating K⁺ and glutamate reuptake in astrocytes, neurotransmitter release in neurons, gap junctions and synapses formation between neural cells \[13–16\]. Given that glia contribute to morphine tolerance \[17, 18\], it is conceivable that astrocyte-expressed AQP4 may play an important role in the development of opioid dependence.

In our previous study, AQP4 deficiency did not affect chronic tolerance to morphine analgesia in tail flick tests \[19\]. However, it has been reported that AQP4 deficiency attenuated cocaine-induced locomotor activity \[20\] and inhibited the naloxone-precipitated abstinent jumping after repeated morphine administration \[21\]. The mechanism underlying these actions remains unknown. Considering the important site of spinal cord as well the key factors such as PKCα, PKCγ and c-Fos contributing to morphine dependence and abstinence, the present study was to investigate the effects of AQP4 deficiency on these proteins in mice spinal cord in naloxone-precipitated withdrawal from chronic morphine.

1 MATERIALS AND METHODS

1.1 Animals

Both AQP4 wild-type and knockout mice in the CD1 genetic background were provided by Prof. HU Gang in Nanjing Medical University. The absence of AQP4 protein in knockout mice was confirmed by Western blotting analysis. Two- to three-month-old mice were kept under environmentally controlled conditions (ambient temperature, 22 °C ± 1 °C; humidity, 50%–60%) on a 12/12 h light/dark cycle with food and water ad libitum. All animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals by the International Association for the Study of Pain (IASP) \[22\]. The experimental procedures were approved by Animal Care and Use Committee of Fudan University, and all efforts were made to minimize animal suffering and to reduce the number of animals used for the experiments.

1.2 Drug administration

In morphine-induced physical dependence and withdrawal procedure, wild-type and AQP4 knockout CD1 mice received a constant dose of morphine (70 mg/kg, i.p., morphine hydrochloride, Shengyang First Pharmaceutical Factory, China) or saline (10 mL/kg, i.p.) injection once daily at 2:00 p.m. for 4 consecutive days. Twenty-four hours after the last morphine or saline administration, the number of jumping was counted for 30 min just before or after naloxone injection (5 mg/kg, i.p., Sigma, St. Louis, MO, USA).

1.3 Western blotting analysis

Mice received repeated injection of morphine (70 mg/kg, i.p.) or saline (10 mL/kg, i.p.) once daily for 4 consecutive days. Twenty-four hours after the last administration or within 1 h after the injection of naloxone (5 mg/kg, i.p.), animals were sacrificed. The lumbar spinal cords (L4–6) were removed quickly. Collected tissues were homogenized in a lysis buffer (12.5 µL/mg tissue) containing a mixture of protease inhibitors (Roche) and phenylmethanesulphonyl fluoride (Sigma). Total protein samples were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5%–12% gels, Bio-Rad) and transferred onto polyvinylidene fluoride membranes (Millipore). The membranes were blocked with 5% non-fat milk dissolved in Tris-buffered saline containing 0.05% Tween-20 (TBST) at room temperature for 2 h and incubated overnight at 4 °C with a primary antibody (mouse anti-PKCα, 1:500, Santa Cruz; mouse anti-PKCγ, 1:1 000, Santa Cruz; rabbit anti-AQP4, 1:2 000, Chemicon; or rabbit anti-c-Fos, 1:1 000, Abcam). After being washed three times with TBST,
the membranes were incubated with the corresponding HRP-conjugated secondary antibody (1:1 000, Santa Cruz) for 2 h at room temperature. The blots were then visualized in ECL solution (Pierce) and exposed onto X-films. The developed X-films were scanned for data analysis. Each sample was repeated for three times under the same conditions. Negative control lanes lacking primary antibody were performed in parallel. GAPDH was used as a loading control.

1.4 Statistics
All data were presented as the mean ± SEM. Statistical analysis between the two groups was performed with Student t-tests. Statistical analysis for multiple comparisons was performed by a two-way ANOVA with genotype and treatment as the independent factors. The level of statistical significance was defined as $P < 0.05$.

2 RESULTS
The complete deficiency of AQP4 in the spinal cord of knockout mice was confirmed as previously [19]. Repeated administration of morphine in a constant dose manner (70 mg/kg, i.p., once daily for 4 consecutive days) resulted in physical dependence, in which naloxone precipitation induced withdrawal syndrome. Since locomotor activities before naloxone precipitation were similar in wild-type and AQP4 knockout mice [21], so abstinent jumping, the main withdrawal symptom in mice, was monitored. As shown in Fig. 1A, after 4 days’ administration of morphine, there was no significant difference in the jumping number before naloxone injection between the wild-type and AQP4 knockout mice on day 5 ($F_{(1,28)} = 0.75, P = 0.49$, two-way ANOVA). Moreover, naloxone (5 mg/kg, i.p.) failed to induce abstinent jumping in both wild-type and AQP4 knockout mice in saline-treated group (10 mL/kg, i.p., once daily for 4 days) on day 5 (Fig. 1B). However, after repeated treatment with morphine (70 mg/kg, i.p.) once daily for 4 consecutive days, 24 h post the last morphine injection on day 5, naloxone (5 mg/kg, i.p.) induced significant withdrawal syndrome with the jumping number of 108.1 ± 17.8 within 30 min in wild-type mice; while the naloxone-precipitated jumping number was only 1.4 ± 1.1 in AQP4 knockout mice treated with the same schedule (Fig. 1B). The jumping percentage in wild-type mice was 100% (7 of 7), whereas that in AQP4 knockout mice was 25% (2 of 8). This indicated that morphine-induced physical dependence in AQP4 knockout mice was weaker than that in wild-type mice ($F_{(1,28)} = 19.25, P < 0.001$, two-way ANOVA). It suggests that AQP4 deficiency inhibits the development of morphine physical dependence, consistent with the previous report [21].

After the repeated treatment of saline (10 mL/kg, i.p., once daily for 4 days) or morphine (70 mg/kg, i.p., once daily for 4 days), the lumbar spinal cords (L4–6) of mice were quickly removed 24 h after the last injection, or within 1 h after the injection of naloxone (5 mg/kg, i.p., 24 h after the last morphine administration on day 5) for Western blotting analysis. As shown in Fig 2A–D,

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**Fig. 1.** The attenuation of AQP4 deficiency to morphine physical dependence. **A:** The abstinent jumping number was counted just before naloxone injection on day 5 in the saline (10 mL/kg, i.p., once daily for 4 days) or morphine (70 mg/kg, i.p., once daily for 4 days) treated mice. **B:** Naloxone injection was performed to induce abstinent jumping on day 5, 24 h after the last saline/morphine administration. Data were presented as mean ± SEM. ***$P < 0.001$ vs saline + naloxone group, ****$P < 0.001$ vs wild-type mice, two-way ANOVA followed by $t$-test.
the spinal expressions of PKCα and PKCγ were increased in morphine dependence and naloxone-precipitated withdrawal in wild-type or AQP4 knockout mice, compared with the corresponding saline control group ($P < 0.05$ or $P < 0.01$, t test). However, the protein level of PKCα in spinal cord of AQP4 knockout mice was much higher than that in the wild-type mice under withdrawal (Fig 2A–B; $P < 0.01$, t test). In contrast, the expression of PKCγ in AQP4 deficient mice was significantly lower than that in the wild-type mice of the sa-

Fig. 2. Changes in the expression of PKCα, PKCγ and c-Fos in spinal cord in morphine dependence and naloxone-precipitated withdrawal mice. A: Western blot results of PKCα expression. B: Statistic comparison of PKCα expression. C: Western blot results of PKCγ expression. D: Statistic comparison of PKCγ expression. E: Western blot results of c-Fos expression. F: Statistic comparison of c-Fos expression. All the spinal cord tissues for analysis were collected on day 5. The control group: chronic saline treated for 4 days (10 mL/kg, i.p., once daily). The dependence group: chronic morphine treated for 4 days (70 mg/kg, i.p., once daily). The withdrawal group: naloxone administered on day 5 after chronic morphine treatment (70 mg/kg, i.p., once daily for 4 days). Data were presented as mean ± SEM, $n = 4$ per group. *$P < 0.05$ vs wild-type control group, **$P < 0.05$ and ***$P < 0.01$ vs knockout control group, $$$P < 0.01$ vs wild-type mice, t test.
line control, dependence or withdrawal group, respectively (Fig 2C–D; \( P < 0.01 \), \( t \) test). Meanwhile, the expression of c-Fos, a marker of neuronal activity, was unchanged in morphine dependence, but remarkably up-regulated in naloxone-precipitated withdrawal in both wild-type and knockout mice spinal cord (Fig 2E–F; \( P < 0.05 \) or \( P < 0.01 \), \( t \) test). Nevertheless, the level of c-Fos in AQP4 knockout mice was much higher than that in the wild-type mice under withdrawal (\( P < 0.01 \), \( t \) test).

3 DISCUSSION

The communication between neurons and astrocytes is essential for many neural functions, including opioid analgesia, tolerance and dependence. Astrocytes tightly enwrap the vast majority of synapses and actively modulate synaptic communication via so-called “tripartite synapse” [23]. AQP4 localizes in the astrocyte plasma membrane in the mammalian brain and spinal cord, which is associated with neural signal transduction [13–16]. It is conceivable that AQP4 could modulate neuronal activity contributing to morphine dependence or withdrawal.

The spinal cord is an important site in the development of opioid dependence and withdrawal [1–7]. Lines of evidence indicate that PKC in the spinal cord is crucial in the development of opiate tolerance and dependence [1, 9–11, 24]. It has been found that the expressions of PKC\( \alpha \) and PKC\( \gamma \) are upregulated in rat spinal cord in naloxone-precipitated morphine withdrawal, and that the pretreatment of intrathecal PKC antagonist chelerythrine chloride decreases the scores of withdrawal jumping as well as the protein expressions of spinal PKC\( \alpha \) significantly [1]. In addition, intrathecal administration of PKC\( \alpha \) antisense oligonucleotides can hamper the development of morphine tolerance [24]; while PKC\( \gamma \) deficiency is able to enhance the analgesic efficiency of morphine in the spinal cord and relieve both the desensitization of \( \mu \) opioid receptors and morphine tolerance [11]. Here, consist with the previous report in rat [1], the protein expressions of spinal PKC\( \alpha \) and PKC\( \gamma \) were increased in morphine tolerance and naloxone-precipitated withdrawal in wild-type or AQP4 knockout mice. However, AQP4 knockout further increased PKC\( \alpha \) in the spinal cord after withdrawal, compared with the wild-type mice. In addition, PKC\( \gamma \) expression in AQP4 knockout mice was significantly lower than that in wild-type mice of the corresponding control, dependence or withdrawal group. It is likely that the PKC isoforms \( \alpha \) and \( \gamma \) may play different roles in the expression of naloxone-precipitated withdrawal and that the reduced abstinent jumping score is partially attributed to the changes in the expression of PKC\( \alpha \) and PKC\( \gamma \) by AQP4 deficiency.

It has been reported that AQP4 knockout increased PKC activity examined by substrate protein phosphorylation method, which was not affected by cocaine administration or withdrawal [25]; whereas the protein level of PKC isoforms was significantly changed by AQP4 deficiency following naloxone-precipitated morphine withdrawal in our study. There might be several reasons. First, different drugs were administered: cocaine vs. morphine; second, different tissues were examined: hippocampus vs spinal cord; third, different analysis methods were used: substrate protein phosphorylation method vs Western blotting; Fourth, there is a family of PKC; we only tested the isoforms PKC\( \alpha \) and PKC\( \gamma \), which had been reported to be closely related to morphine dependence and withdrawal; Last, the mechanisms underlying cocaine withdrawal and naloxone-precipitated morphine withdrawal may not be identical, in which AQP4 or PKC plays different roles.

The protein expression of the immediate early gene \( c-fos \) can reflect the sensitization of neurons [4, 5, 8]. In morphine tolerance and dependence, the spinal neurons are under latent sensitization and the expression of c-Fos remains unchanged. However, naloxone triggers these neurons to be sensitized, which is one of the cellular mechanisms underlying the withdrawal behavioral responses. Simultaneously, the spinal expression of c-Fos increases significantly parallel to abstinence [1, 5, 6].

The pattern of c-Fos expression in rat lumbar spinal cord following systemic withdrawal is primarily a consequence of increased activity in opioid receptor-containing circuits intrinsic to the dorsal horn and that the magnitude of c-Fos expression is normally dampened by supraspinal and primary afferent-derived inhibitory inputs [7]. In this study, unexpectedly, the c-Fos protein level in the AQP4 knockout mice’s spinal cords was much higher than that in the wild-type group; whereas the withdrawal jumping score was reduced significantly by AQP4 deficiency. The mechanism underlying morphine withdrawal syndrome is very complicated and involves a lot of factors, including both supra-spinal and spinal sites [2–7, 26–30]. Besides, the expression of c-Fos is also affected by supraspinal and primary afferent-derived inhibitory inputs [6]. Thus, the exact con-
nection between the increased spinal expression of c-Fos and the decreased abstinence jumping in the AQP4 deficient mice needs further studies to elucidate.

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