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Research article

Morphine administration induces change in anxiety-related behavior via Wnt/β-catenin signaling

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HIGHLIGHTS

- Treatment with a low dose of morphine activates Wnt signaling.
- Morphine-exposed rats exhibited less cautious behavior, which was restored by treatment with DKK1.
- The decrease of dendritic spines after up-regulation of Wnt signaling caused by morphine treatment may have a role in cautious behavior change.

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ABSTRACT

Chronic morphine administration is known to decrease anxiety-related behavior, which may lead to morphine-seeking and other social problems. Recent studies have revealed that Wnt/β-catenin signaling plays an important role in anxiety-related behavior. We used HT22 cells, which were derived from primary mouse hippocampal neuronal cultures, to explore the relationship between Wnt signaling and morphine exposure. Many techniques, such as western blot analysis, immunofluorescence and luciferase assays, were utilized. We also examined anxiety-related behaviors and dendritic spines in Male Sprague-Dawley (SD) rats after chronic morphine injection and stereotaxic injection of Dkk1. The cell cultures indicated that morphine treatment induced β-catenin expression. The rats that received morphine injection entered open pathways more often in elevated plus maze, spent a greater proportion of time in the interior zone of open field test, and showed less dendritic spine than their vehicle-injected counterparts. However, the injection with Dkk1 significantly prevented this change. Our study demonstrated that Wnt signaling is activated by morphine exposure. The use of Dkk1 before morphine treatment induced a decrease of β-catenin indicated that frizzled receptor(FZD) and LDL receptor-related protein 5/6(LRP5/6) may be crucial to the activity of wnt signaling after morphine exposure. Additional investigation involving animals suggested that the less anxiety observed in the SD rats after morphine treatment could be caused by the loss of dendritic spines and that this may be related to Wnt/β-catenin signaling.

1. Introduction

1 Wnt proteins bind to a cell surface receptor named frizzled receptor(FZD) [9,10] and to LDL receptor-related protein 5/6(LRP5/6). The binding of Wnt proteins to receptors induces the accumulation of β-catenin that leads to the formation of the TCF/LEF-β-catenin complex and controls the expression of downstream target genes. Previous studies have indicated that the Wnt/β-catenin signaling pathway is activated after morphine administration in the rat spinal cord [23]. Moreover, long-term exposure to cocaine in Sprague-Dawley (SD) rats results in less anxiety and more gene expression of Wnt/β-catenin signaling compared with their vehicle-injected counterparts [24]. Thus, we hypothesized that activation of Wnt signaling might also be involved in the decrease in anxiety induced by morphine exposure.

The down-regulation of Wnt/β-catenin signaling appears to cause more anxiety behavior, as indicated by significantly less time being spent in the open arms of an elevated plus maze (EPM) [12,21]. Morphine administration clearly reduces anxiety behavior [2,3,7]. Moreover, it is reported that the CA1 area of hippocampus is associated with anxiety that are important for the escape from a dangerous environment [15,18], and the low level of anxiety induces drug seeking, severe violent crime and other social

Abbreviations: FZD, frizzled family of wnt receptor; BSA, bovine serum albumin; DMEM, dulbecco's modificationof eagle's medium; EPM, elevated plus maze test.

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problems[8]. Therefore, we hypothesized that morphine exposure may induce less anxiety-related behavior in SD rats via Wnt signaling and that the inhibition of Wnt signaling by Dkk1, a protein that combines with LRP6 of the receptor complex to inhibit Wnt signaling [1], may be a treatment for behavior disorders brought on by morphine exposure. Dendritic spines are considered postsynaptic elements with critical properties for the information processing. Moreover, spine density may act as a predictor of high and low anxiety levels [19]. A reduction in dendritic spines following the activation of Wnt signaling has been well-described [25].

However, few studies have investigated whether inhibiton of Wnt Signaling by Dkk1 is able to exert protective effects against morphine induced decrease in anxiety in SD rats. Therefore, the aim of this study was to investigated protective effect of DKK1 against morphine induced behavior disorder and the possible mechanisms underlying these effects.

2. Experimental procedures

2.1. Animals and treatment

SD rats (7 weeks old, weighting 200–250 g), purchased from animal center of the Fourth Military Medical University in Xi'an, China, were used in this search. The animal care and procedures were carried out according to the National Institutes of Health guidelines for care and use of laboratory animals and were approved by the Ethics Committee of the Fourth Military Medical University (Xi'an, PR china). The experiments involving rats are in accordance with the ARRIVE guidelines which had been reported [4,14].

2.2. Dendritic spine density analysis

An experimenter blind to the treatment quantified spine density. All protrusions (irrespective of morphological characteristics) were counted as dendritic spine on an 80 μm dendrite stretch using NeuroLucida (MicroBrightField Inc., Williston, VT, USA) attached to Nikon microscope.

2.3. Experimental protocol

The rats were divided into 4 groups. The control group was treated with saline injections from day 1 to day 7 and an intracerebral PBS injection on day 4. The morphine group was treated with morphine injections at a dose of 10 mg/kg from day 1 to day 7 and an intracerebral PBS injection on day 4 [26]. The Dkk1 group was treated with saline injections from day 1 to day 7 and an intracerebral Dkk1 injection on day 4. The morphine+ Dkk1 group was treated with morphine injections from day 1 to day 7 and intracerebral Dkk1 injection on day 4. After 2 h of rest following the morphine or saline injection, we conducted the behavioral tests(n = 9 per group), Golgi-staining(n = 5 per group), and molecular studies(n = 5 per group)on day 7(3 days after exposure to Dkk1 or PBS). Moreover, After 2 h of rest following the morphine injection, rats from the morphine group were sacrificed on days 1, 4, and 7 for molecular studies(n = 5 per group).

2.4. Statistical analysis

Values were reported as means ± SEMs. Comparisons among multiple groups were assessed by one-way analysis of variance(ANOVA) followed by Bonferroni test. For behavioral studies, the data were analyzed with an ANOVA followed by tukey's multiple comparison. The significance level was set at $p < 0.05$. Statistics were calculated using Prism 5 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Treatment with a low dose of morphine activates Wnt signaling

HT22 cells were cultured in DMEM and treated with morphine for 0 h, 1 h, 12 h, 24 h, or 36 h. The results (Fig. 1A and B) indicated that the level of β-catenin reached a maximum concentration after 24 h of morphine treatment compared with control group ($p = 0.0013$, $F_{4,15} = 7.764$; control[0.45 ± 0.08] vs 24h[1.05 ± 0.15], $P < 0.05$; control[0.45 ± 0.08] vs 36h[1.01 ± 0.11], $p < 0.05$). We also observed that the signal of the TOPFlash reporter assay was increased in 24 h morphine treatment group compared with control group ($p < 0.0001$, $F_{3,16} = 26.35$; 9.44 ± 1.66 vs 2.74 ± 0.83, $p < 0.01$). Significant suppression of the luciferase activity was observed in the cells that also pretreated with 100 ng/ml of Dkk1 for 2 h before 24 h morphine treatment (2.93 ± 1.02, $p < 0.05$)[5]. The change of luciferase activity were also observed when the cells received 12 h and 36 h morphine administration(Fig. 1C–F; 12 h: $p = 0.0001$, $F_{3,16} = 13.85$; control[2.66 ± 0.55] vs morphine[7.70 ± 0.73], $P < 0.05$; morphine[7.70 ± 0.73] vs Dkk1[2.50 ± 0.84], $P < 0.05$; morphine[7.70 ± 0.73] vs morphine + Dkk1[2.40 ± 0.64], $P < 0.05$; 36 h: $p < 0.0001$, $F_{3,16} = 64.98$; control[2.49 ± 0.27] vs morphine[9.24 ± 0.50], $P < 0.01$; morphine [9.24 ± 0.50] vs Dkk1[1.90 ± 0.46], $P < 0.01$; morphine[9.24 ± 0.50] vs morphine + Dkk1[2.12 ± 0.41], $P < 0.01$). Further study that take into consideration the concentration of morphine revealed that the β-catenin level in 10 μM morphine treatment group (3.31 ± 0.44) was approximately 2 times higher than the control group (Supplementary Fig. 1; $p = 0.0011$, $F_{4,20} = 6.99$; 1.60 ± 0.32, $p < 0.05$).

3.2. The β-catenin level increased in both the cytoplasmic and nuclear fractions

The immunofluorescence results indicated that β-catenin translocated to the nucleus when the cells were treated with 10 μM morphine for 24 h (Fig. 2A). Western blotting revealed that an increased expression of β-catenin occurred in nuclear fractions after morphine treatment compared to control group. However, the expression of β-catenin decreased to a normal level when the cells were pretreated with 100 ng/ml of Dkk1 for 2 h before adding morphine. Moreover, Dkk1 attenuated the morphine induced β-catenin accumulation both in cytoplasmic fraction and total cell (Fig. 2B–G; nuclear: $p = 0.0034$, $F_{3,12} = 7.98$; control[0.52 ± 0.13] vs morphine[1.59 ± 0.25], $P < 0.01$; morphine[1.59 ± 0.25] vs morphine + Dkk1[0.46 ± 0.17], $P < 0.01$; cytoplasmic: $p = 0.0026$, $F_{3,12} = 8.57$; control[0.58 ± 0.07] vs morphine[1.54 ± 0.23], $P < 0.01$; morphine[1.54 ± 0.23] vs Dkk1[0.68 ± 0.12], $P < 0.01$; morphine[1.54 ± 0.23] vs morphine + Dkk1[0.80 ± 0.12], $P < 0.05$; total: $p < 0.0001$, $F_{3,12} = 19.62$; control[0.38 ± 0.05] vs morphine[0.81 ± 0.05], $P < 0.01$; morphine[0.81 ± 0.05] vs Dkk1[0.16 ± 0.05], $P < 0.01$; morphine[0.81 ± 0.05] vs morphine + Dkk1[0.38 ± 0.09], $P < 0.05$).

3.3. Morphine-exposed rats exhibited less cautious behavior, which was restored by treatment with Dkk1

We examined the β-catenin protein level during the morphine exposure period (Fig. 3A). The β-catenin level increased on day 1 and maintained a high level of expression on days 4 and 7 in CA1 area of hippocampus (Fig. 3B and C; $P < 0.0001$, $F_{3,16} = 62.22$; control[0.47 ± 0.04] vs 1 day[1.22 ± 0.05], $P < 0.01$; control[0.47 ± 0.04] vs 7 day[1.59 ± 0.10], $P < 0.01$). Western blotting revealed that the infusion of Dkk1 on days 4 produced a significant decrease in β-catenin ($p = 0.0021$, $F_{3,16} = 9.052$; morphine[1.63 ± 0.19] vs mor-