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Original Article

Coadministration of glycogen-synthase kinase 3 inhibitor with morphine attenuates chronic morphine-induced analgesic tolerance and withdrawal syndrome

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Abstract

Background: Glycogen-synthase kinase 3 (GSK3) is involved in many signaling pathways and is associated with a host of high-profile pathophysiological states. However, its role in morphine tolerance, especially naloxone-precipitated withdrawal syndrome, has not been well investigated. The present study was undertaken to study the role of GSK3 in chronic morphine exposure.
Methods: Adult male Sprague–Dawley rats were subjected to intraperitoneal (i.p.) injections of morphine (10 mg/kg) twice daily for 6 consecutive days, and tail-flick tests were conducted to evaluate changes of morphine-induced antinociception. GSK3 inhibitor, SB216763 or SB415286, was i.p. injected prior to morphine to investigate the influences on morphine tolerance. There were four groups receiving morphine plus vehicle (2% dimethyl sulfoxide), morphine plus SB216763 (0.6 mg/kg) or SB415286 (1.0 mg/kg), GSK3 inhibitor alone, or dimethyl sulfoxide: as the control group. On Day 7, naloxone (i.p., 1 mg/kg) was administered and naloxone-precipitated withdrawal behaviors were individually compared between groups.
Results: Repeated morphine exposure in this study led to progressive shortening of tail-flick latencies and produced six of nine observed naloxone-precipitated withdrawal behaviors. Coadministration with SB216763 or SB415286 significantly prevented antinociceptive tolerance and alleviated parts of withdrawal syndrome. Both inhibitors could similarly reverse withdrawal behaviors including grooming, chewing, and ptosis, but did not affect withdrawal behaviors of penis licking and defecation.
Conclusion: The results demonstrate the importance of GSK3 in reducing chronic morphine-induced tolerance and withdrawal syndrome. Although GSK3 is involved in diverse physiological functions, aiming at GSK3-related pathway could still be a potential tool to improve therapeutic quality in clinical morphine treatment.

Keywords: glycogen synthase kinase; morphine tolerance; naloxone; withdrawal

1. Introduction

Morphine is widely used in relieving pain in medical care such as cancer pain, chronic pancreatitis, neuropathic pain, and somatic pain in aged patients who are contraindicated to

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invasive treatments. However, chronic morphine exposure is often accompanied with the development of analgesic tolerance and risk of withdrawal symptoms, which force patients to escalate the doses to achieve an adequate analgesic effect and be very cautious of abrupt cessation of morphine use. Also, chronic morphine users easily become dependent on drugs and are susceptible to addiction. All the above conditions ultimately diminish the clinical usefulness of morphine and lead to socioeconomical problems.[1−3]

A growing list of molecules, such as excitatory amino acid receptors,[4−7] adenyl cyclase/cAMP/PKA pathway,[8,9] MAPK/ERK,[10] protein kinase C translocation and activation, and nitric oxide release,[11,12] have been recognized as the targets altered by repeated opiate administrations, and are advocated to be involved in morphine tolerance. Meanwhile, new evidence has revealed that phosphatidylinositol 3-kinase/Akt cascade may also participate in regulating opiate-induced responses via phosphorylating the downstream effector, glycogen synthase kinase-3 β (GSK3β), particularly after chronic administration of morphine. Supporting data indicate that acute application of opioid agonists stimulated μ-opioid receptor and subsequently enhanced Akt activity *in vitro*[13,14] and *in vivo*[15]; however, repeated morphine treatments significantly depressed the Akt phosphorylation level.[15] In addition, coinjections of GSK3 inhibitors, such as lithium salt,[16,17] BIO, or SB216763,[18] with morphine were found to attenuate the chronic morphine-induced tail-flick tolerance in rodents in responses to thermal stimulation. Parkitna et al[18] further claimed that reversal efficacy was always associated with an increase in abundance of GSK3β phosphorylation.

GSK3 is a cellular Ser/Thr kinase originally found to be involved in glucose synthesis, and was soon recognized as an important intracellular signaling involved in regulating cell cycle, development, oncogenesis, and neuroprotection.[19] Lithium, a nonselective GSK3 inhibitor has long been used in bipolar disease, and recently, diverse types of GSK3 inhibitors have been proposed as potential therapeutic agents for diseases such as diabetes, Alzheimer's disease, and colon cancer[20] in preclinical studies. Although the significance of GSK3 in modulating morphine function has been stated,[17,18,21,22] the only tested effect on tolerance was given via intrathecal route, but no data regarding withdrawal syndrome are available. We designed this study using systemic administration of morphine with different GSK3 inhibitors to investigate the effects on nociceptive thresholds and naloxone-precipitated withdrawal behaviors following chronic morphine exposure. The study's purposes were to explore the role of GSK3 signaling pathway in ameliorating chronic morphine-induced complications and serve as a preclinical study for potential clinical application.

2. Methods

2.1. Animal preparations

The study was performed on male Sprague−Dawley rats (weighing 200−250 g). Rats were housed in groups of three in an environment of 23 ± 0.5°C with a 12-hour dark/light cycle. Food and water were available *ad libitum*. All experimental procedures were conducted in accordance with guidelines approved by the Guidelines for the Care and Use of Experimental Animals of Shin-Kong Memorial Hospital (Taipei, Taiwan), which was based on the Codes for Experimental Use of Animals from the Council of Agriculture, Taiwan.

2.2. Drug injections

There were two types of GSK3 inhibitors, SB216763 and SB415286 (Tocris Cookson Ltd., Bristol, Avon, UK), used in this study. They were initially dissolved in dimethyl sulfoxide (DMSO) and were then diluted to adequate concentrations for injection with a final DMSO concentration of 2%. Therefore, 2% DMSO solution was used as a vehicle in the control group. The selected doses of SB216763 and SB415286 had been reported in previous heart reperfusion studies for providing a cardioprotective effect.[22−25] Animals were randomly divided into four groups (at least 7 rats in each group) as follows: (1) morphine plus vehicle group (Mor-Veh): morphine was intraperitoneally (i.p.) injected at a dose of 10 mg/kg 30 minutes after vehicle administrations (2% DMSO at a volume of 1 mL/kg, i.p.) twice/day (at 09:00 and 17:00) for 6 successive days; (2) morphine plus GSK3 inhibitor (Mor-S2 or Mor-S4) group: SB216763 (i.p., 0.6 mg/kg) or SB415286 (i.p., 1 mg/kg) prior to morphine injections at the same time course in the Mor-Veh group; (3) DMSO (Veh) group: animals were subjected to i.p. DMSO as the naïve control; and (4) SB (S2 or S4) group: rats received SB216763 or SB415286 alone as the sham control. Drugs in Veh and SB control groups were given according to the abovementioned protocol for 6 days. All four groups received an injection of morphine (i.p., 10 mg/kg) on the morning of Day 7. To investigate morphine abstention-induced withdrawal syndrome, subcutaneous naloxone (1 mg/kg) was injected in the Mor-Veh, Mor-S2, and Mor-S4 groups 2 hours after the last morphine injections on the Day 7 of the experiment.

2.3. Nociceptive threshold tests

Tail-flick response to an external heat nociception was used to evaluate the analgesic effect of morphine. The test latency was defined as the duration of a withdrawal reaction to a radiant heat projection from an algesic machine (Tail Flick Analgesia Meter MK-303B, Muromachi Kikai, Tokyo, Japan). The heat intensity of the light bulb was set to result in basal latencies within 6−8 seconds, and the cutoff time was set at 15 seconds. The tail flick responses to different treatments were examined 30 minutes after drug injections on Day 1, Day 2, Day 3, Day 5, and Day 7. Baseline withdrawal threshold, i.e., "basal latency", was determined 2 days prior to the experiment. At each time-point, three tail flick tests separated by at least 2-minute intervals were measured and averaged as the "test latency". The percentage of maximal possible antinociceptive effect (MPE) representing the analgesic result is defined by the equation: MPE% = [(test latency − basal latency)/(cutoff time − basal latency)] × 100.

2.4. Withdrawal behavior observation

Animals in the Mor-Veh and Mor-S2 or Mor-S4 groups ($n = 6$ each) were subcutaneously injected with naloxone at a dose of 1 mg/kg 2 hours after morphine on the morning of Day 7. Data from a separate group of naïve rats receiving saline alone for 7 days was used as the control group (Veh) for comparison. Immediately after naloxone injection, the rat was placed in a transparent Plexiglas box for observation of two classes of withdrawal behaviors, a method modified from previous study.[26] One class was measured by occurrences, such as grooming, mouth chewing, teeth chattering, penis licking, wet-dog shake, and jumping by numbers of bouts, whereas the other class was evaluated by durations within 30 minutes, including ptosis and rearing (or exploring). Stools, either dry or watery, during this period were weighed. The withdrawal score was calculated as the sum of occurrence numbers or time for all signs.

2.5. Statistical analysis

In the morphine tolerance experiment, tail-flick latencies at different time points among the four groups were compared via two-way analysis of variance (ANOVA; SigmaStat, Systat Software, San Jose, CA, USA). In addition, repeated measurement of ANOVA was applied to assess the daily changes prior to and after the drug administrations in individual groups, and one-way ANOVA with *post hoc* Tukey's test was applied to compare between-group differences after drug treatments at each time point. One-way ANOVA with *post hoc* Tukey's test was used for the comparison of withdrawal behaviors among multiple groups. Differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of GSK3 inhibitor on morphine antinociception and tolerance

As expected, initial morphine injections elicited strong antinociception, evidenced by significant increase of withdrawal latencies in the tail-flick test (Fig. 1). Repeated administration of morphine in the Mor-Veh group led to tolerance phenomenon, demonstrated by the gradual decline of MPE% from a value of nearly 100% on Day 1, Day 2, and Day 3 to 59.06 ± 21.06% ($p = 0.02$ vs. Day 3) and 84.33 ± 10.60% ($p = 0.03$ vs. Day 3) on Day 5, and then to 22.38 ± 23.90% ($p = 0.034$ vs. Day 5) and 26.99 ± 26.38% ($p = 0.001$ vs. Day 5) on Day 7 in Fig. 1A and 1B, respectively. Coinjection of SB216763 with morphine (the Mor-S2 group) did not differ in MPE% from those in the Mor-Veh group on Day 1, Day 2, and Day 3; however, SB216763 preserved antinociceptive responses and showed significant difference on Day 5 (86.68 ± 18.46% in the Mor-S2 group vs. Mor-Veh, $p = 0.039$) and Day 7 (84.53% ± 14.15% in the Mor-S2 vs. Mor-Veh, $p < 0.001$; Fig. 1A). SB415286 had a similar effect on morphine tolerance but showed less evident

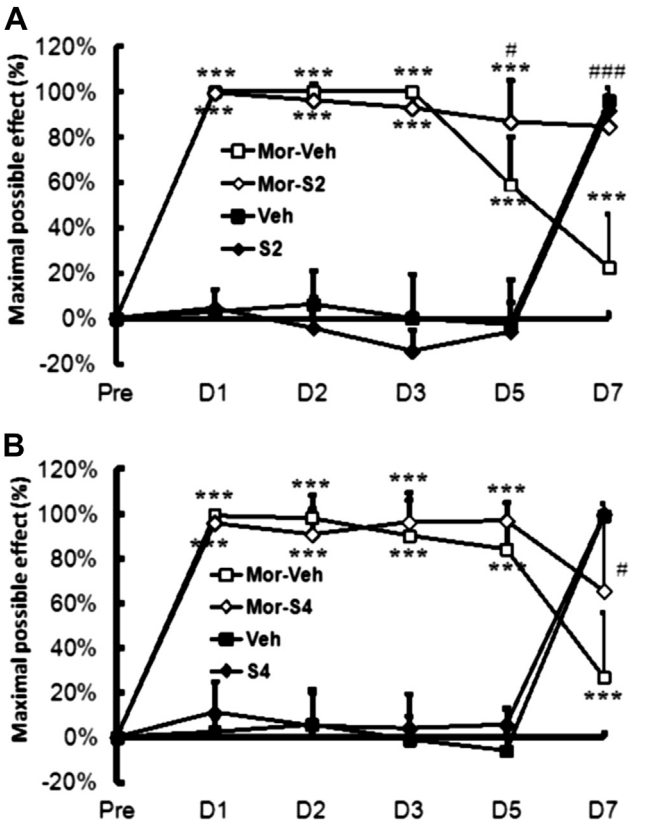

Fig. 1. Effect of two GSK3 inhibitors, SB216763 and SB415286, on the development of morphine tolerance. Morphine was intraperitoneally (i.p.) injected at a dose of 10 mg/kg twice daily for 6 consecutive days and once in the morning on Day 7. Tail-flick latencies were measured 30 minutes after morphine administrations. (A) SB216763 (i.p., 0.6 mg/kg) or vehicle (2% dimethyl sulfoxide: DMSO, 1 ml/kg) were injected 30 min prior to morphine in the Mor-S2 group or the Mor-Veh group, respectively. Two control groups (the Veh and S2 group) received i.p. injections of 2% DMSO or SB216763, respectively ($n = 8$ or $n = 9$ for each group). (B) SB415286 (i.p., 1.0 mg/kg) or vehicle (2% DMSO, 1 ml/kg) were injected following the same protocol. ($n = 7–9$ for each group) All data are presented as means ± standard deviation. *** $p < 0.001$; vs. the Veh group; # $p < 0.05$, ### $p < 0.001$; the Mor-S2 (A) or Mor-S4 (B) group vs. the Mor-Veh group.

(Fig. 1B). However, neither DMSO in 2% concentration (the Veh group) nor SB treatment (the S2 or S4 group) showed basal changes in MPE% following repeated injections. The tail flick responses to morphine injection on Day 7 in both control groups exhibited normal analgesic responses, indicating that GSK3 inhibitor *per se* did not alter physiological thermal threshold.

3.2. Effects of GSK3 inhibitor on morphine withdrawal syndrome

Both GSK3 inhibitors, SB216763 and SB415286, not only reversed chronic morphine-induced tolerance, but also showed prevention of morphine withdrawal syndrome in a similar pattern. Behavioral signs of naloxone-precipitated morphine withdrawal symptoms appeared immediately after naloxone injection and persisted during the 30-minute observation in the

Mor-Veh and Mor-S2/S4 groups in comparison with the control (Veh) group (Figs 2 and 3). Due to very low incidence of wet dog shakes, jumping, and rearing behaviors in all groups, they were excluded from our plot. Various levels of naloxone-precipitated withdrawal behaviors appeared after chronic morphine exposure and those with significant differences included grooming, chewing, ptosis, chattering, penile licking, and defecation (stool weight). Comparing between the Mor-Veh group and the Mor-S2/S4 group, both inhibitors could similarly reduce behaviors of grooming, chewing, and ptosis, but had no effect on diarrhea (stool weight). There were milder variations in drug effect between the two inhibitors. Teeth-chattering was inhibited only by SB216763 (Fig. 2) and penile licking only by SB415286 (Fig. 3). Most signs of the withdrawal syndrome disappeared 2 hours after naloxone injection.

4. Discussion

Morphine is an exogenous opioid mainly acting at the μ-opioid receptors as well as affecting a great diversity of receptors and intracellular signal factors to produce a wide-range of cellular functions. When given chronically, morphine leads to disorders such as tolerance and withdrawal phenomenon, which are commonly identified in chronic pain patients.[27] In this study, intraperitoneal injection of the GSK3 inhibitor prior to morphine administration significantly attenuated the development of analgesic tolerance. Furthermore, we proved that several withdrawal behaviors including grooming, chewing, ptosis, and chattering were effectively alleviated by coadministration of GSK3 inhibitor and morphine. These findings strongly support the involvement of GSK3 in modifying chronic morphine-induced complications.

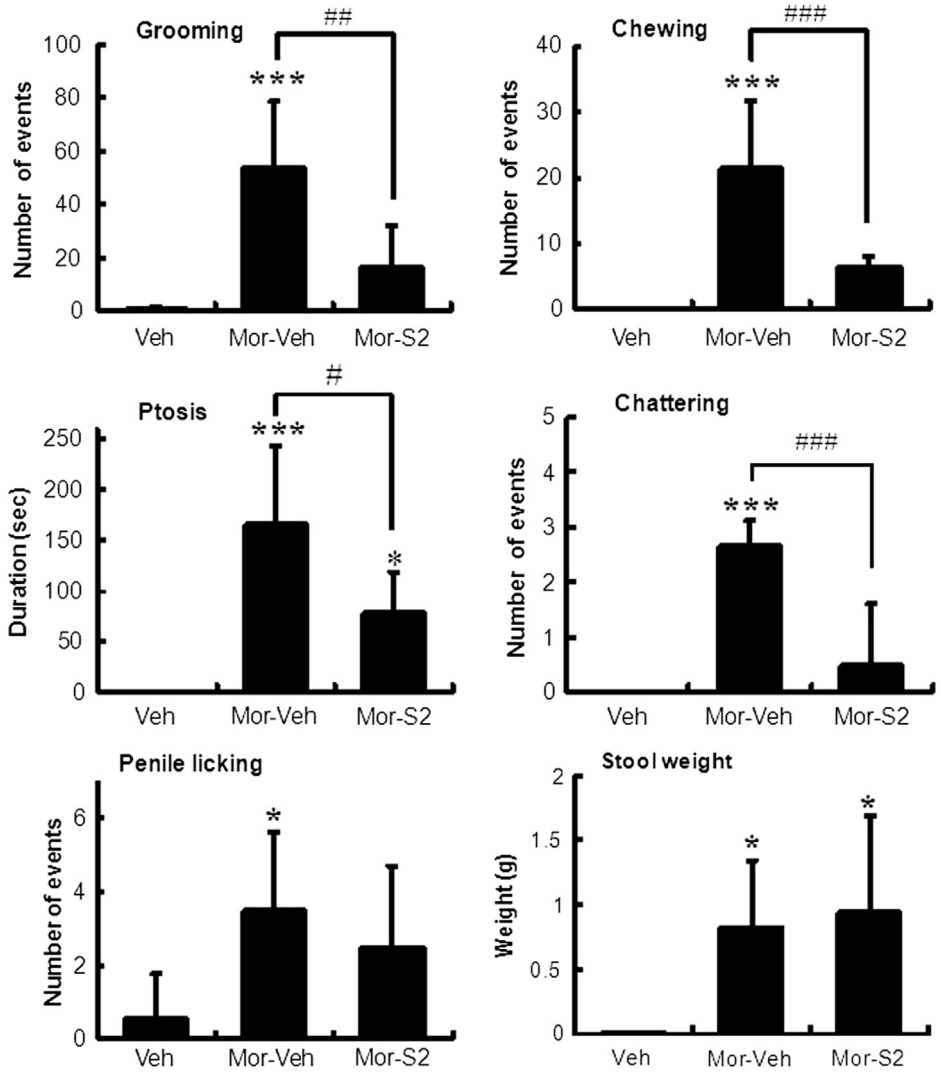

Fig. 2. Consistent coadministration of morphine with SB216763 alleviated naloxone-precipitated morphine withdrawal behaviors. Naloxone was subcutaneously injected at a dose of 1 mg/kg 2 hours after the last morphine/vehicle administration. Rats were subjected to 7-day morphine or vehicle (2% dimethyl sulfoxide: DMSO), and SB216763 (0.6 mg/kg) was injected 30 minutes prior to morphine. Veh: 2% DMSO; Mor-Veh: Morphine plus 2% DMSO; Mor-S2: morphine plus SB216763. Data are presented as means ± standard deviation. One-way ANOVA with *post hoc* Tukey's test, * $p < 0.05$, *** $p < 0.001$ vs. the Veh group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ between the Mor-Veh and the Mor-S2 ($n \geq 6$ for each group).

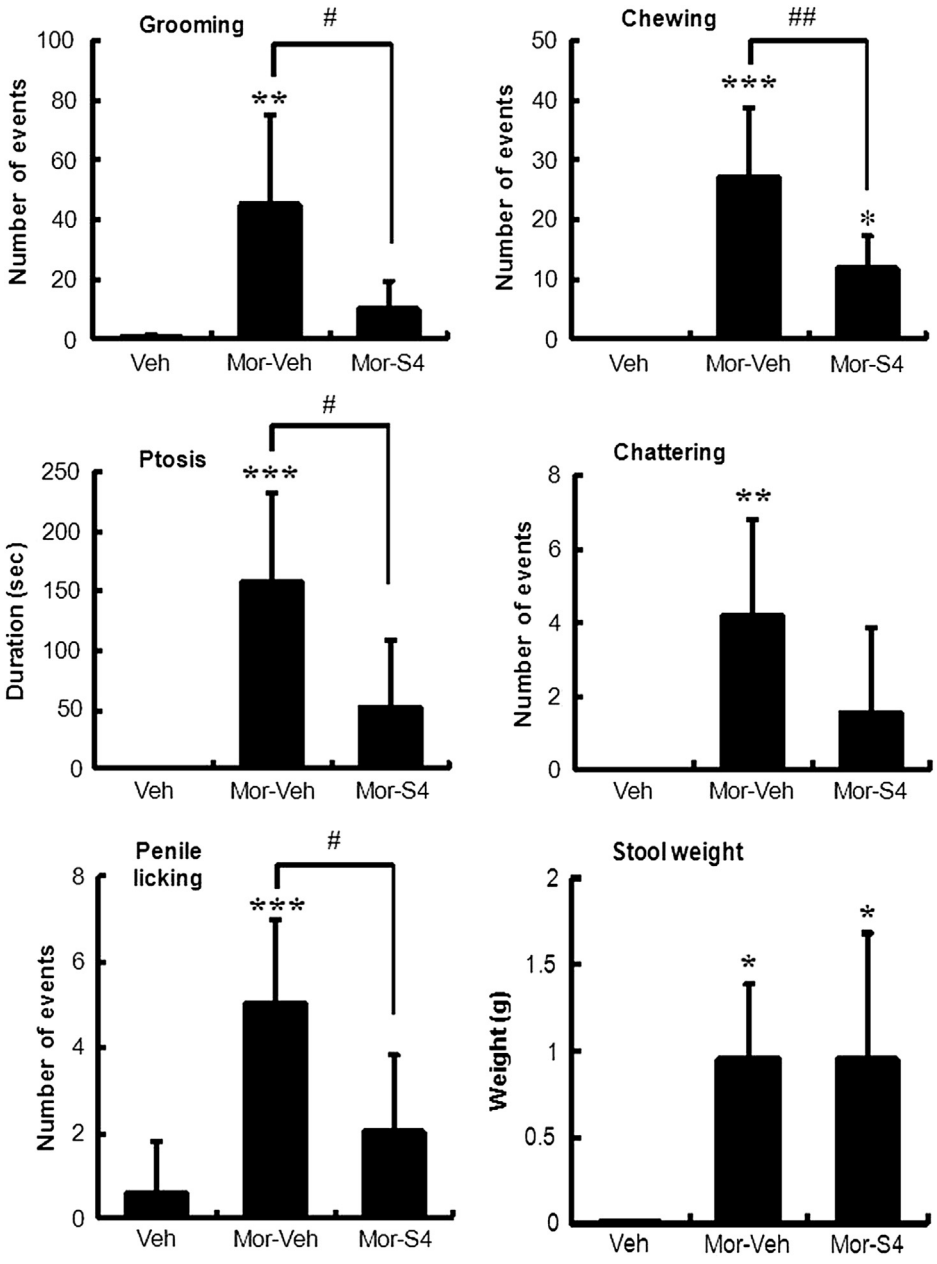

Fig. 3. Consistent coadministration of morphine with SB415286 alleviated naloxone-precipitated morphine behaviors. Naloxone was subcutaneously injected 2 hours after the last morphine/vehicle administration. Veh: 2% dimethyl sulfoxide; Mor-Veh: Morphine plus 2% dimethyl sulfoxide; Mor-S4: morphine plus SB415286 (1.0 mg/kg). Data are presented as means ± standard deviation. One-way ANOVA with *post hoc* Tukey's test, * $p < 0.05$, ** $p < 0.05$, *** $p < 0.001$ vs. the Veh group; # $p < 0.05$, ## $p < 0.01$ between the Mor-Veh and the Mor-S4 ($n \geq 6$ for each group).

4.1. Pharmacological considerations

The participation of GSK3β in opioid signaling was initially noted by alterations of analgesic effectiveness and dependence behavior when animals were coadministrated with morphine and lithium salt, the latter was recognized as a GSK3 inhibitor.[18,28] However, the precise therapeutic effect of lithium is ill-defined due to the nonselective effect of lithium on GSK3β. For example, administration of lithium alone was found to produce longer response latency in a hot-plate escape test,[29] whereas no antinociceptive effect was observed in a tail-flick test.[16] Dehpour et al[16] reported that lithium modulated morphine-induced analgesia depending on the lithium quantity, in that doses below 1 mg/kg depressed morphine antinociception but a higher dose of the drug at a dose of 10 mg/kg potentiated the effect. More recently, Parkitna et al[18] reported that with intrathecal injection of two specific GSK3 inhibitors, BIO (5 ng, 0.5 ng, and 0.05 ng) or SB216763 (50 ng, 5 ng, 0.5 ng, 0.05 ng), 30 minutes prior to i.p. morphine administration (10 mg/kg, twice/day) for 8 consecutive days, the tail flick latencies were maintained close to 90% of baseline level when SB216763 was above a dose of 5 ng. Because interthecal injection is not a feasible technique for clinical routine practice, we proved in this study that

systemic administration could equally produce the effect. Comparing the doses between the two studies, i.p. SB215763 at a dose of 0.6 mg/kg is larger than that given via the intrathecal route, by about 20,000 times. Undoubtedly, the extreme high potency in Parkitna and colleagues' study[18] can be attributed to the intrathecal agent acting directly on the spinal cells, whereas the i.p. injected agent has to be systemically absorbed and overcome the blood−brain barrier. Nevertheless, interthecal injection of the drug at the lumbar levels may have a property of localized distribution, which makes it unable to predict to what extent SB215763 could spread to the supraspinal level and whether SB215763 could affect brain reward/adaptive-related structure, which controls most substance abstinence-induced withdrawal syndromes.[30,31] It is rational to presume that systemic delivery of SB216763 provides better central nervous distribution and equilibrium of effect on both analgesic tolerance and withdrawal syndromes.

The two GSK3 inhibitors used in this study, SB216763 and SB415286, showed similar effects on morphine tolerance and withdrawal syndrome, however, their potencies may differ and can only be confirmed when dose-effect analyses are available. In addition to numerous cellular studies exploring GSKβ activities in various biological functions, there were few *in vivo* studies with direct animal evidence, and fewer to show the dose-response relationship. Among them, only two *in vivo* studies indicated that systemic SB215763 injection could penetrate the blood−brain barrier to produce central nervous system effect.[32,33] With so few preclinical studies, the present study can be a high-value reference and encourage more pharmacodynamic experiments in animals.

4.2. Possible mechanism underlying Akt/GSK3-mediated morphine effects

The present study suggests that chronic morphine exposure could alter the basal activity of GSK3 distributed in the nervous system, and that the active form of GSK3 may play a crucial role in the induction of tolerance and withdrawal symptoms. Growing evidence has yielded to one contention, that altered Akt/GSK3 signaling may be related to the development of tolerance and withdrawal behaviors. In a cell line transfected with the μ-opioid receptor cDNA, Akt has been validated to be activated in opioid signaling because the application of opioid agonists, morphine or D-Ala2, N-Me-Phe4, Gly5-ol enkephalin (DAMGO) enhanced the immunoreactive signal of the phosphorylated Akt[13,14] within 2 hour. Further, Muller and Unterwald[15] directly examined the influence of acute and chronic morphine exposure on Akt activity in rats, and found that in the nucleus accumbens, a region that dominates the dependence behaviors, phospho-Akt level was significantly upregulated upon acute morphine exposure, but phospho-Akt was inhibited by chronic morphine administrations for 6 days or 10 days. In particular, these changes were effectively abolished by pretreatment with naltrexone, indicating that the Akt activity is inversely regulated depending on whether there are acute or chronic morphine actions on opioid receptors. We speculated that prolonged morphine administration may upregulate GSK3 activity in specific brain and spinal cord regions via modulating phosphorylated-Akt levels to enhance morphine tolerance, withdrawal behaviors, and dependence. Finally, GSK3 is a multi-functional kinase constitutively acting in diverse physiological processes by interacting with Wnts, hedgehog, growth factors, and many G-protein-coupled ligands.[34] To improve the specificity of GSK3 on morphine-induced side effects, investigations on molecular mechanisms are necessary to differentiate the selectivity in functions of Akt/GSK3β modulating cascade.

In conclusion, based on the present findings, we conclude that inhibition of GSK3 activity improves the quality of prolonged morphine administration. It is therefore implied that modulating GSK3-mediated activity could be a useful therapeutic option to treat chronic morphine-induced complications.

Acknowledgments

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