



Valproate attenuates the development of morphine antinociceptive tolerance

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ABSTRACT

Morphine is a potent opioid analgesic. Repeated administration of morphine induces tolerance, thus reducing the effectiveness of analgesic treatment. Although some adjuvant analgesics can increase morphine analgesia, the precise molecular mechanism behind their effects remains unclear. Opioids bind to the mu opioid receptor (MOR). Morphine tolerance may be derived from alterations in the intracellular signal transduction after MOR activation. Chronic morphine treatment activates glycogen synthase kinase 3 β (GSK3 β), whose inhibition diminishes morphine tolerance. Valproate is widely prescribed as an anticonvulsant and a mood stabilizer for bipolar disorders because it increases the amount of γ -aminobutyric acid (GABA) in the central nervous system. Although the activation of GABAergic neurons may be responsible for the chief pharmacologic effect of valproate, recent studies have shown that valproate also suppresses GSK3 β activity. We examined the effect of valproate on the development of morphine antinociceptive tolerance in a mouse model of thermal injury. Mice were treated with morphine alone or with morphine and valproate twice daily for 5 days. The resulting antinociceptive effects were assessed using a hot plate test. While mice treated with morphine developed tolerance, co-administration of valproate attenuated the development of tolerance and impaired the activation of GSK3 β in mice brains. Valproate alone did not show analgesic effects; nevertheless, it functioned as an adjuvant analgesic to prevent the development of morphine tolerance. These results suggest that the modulation of GSK3 β activity by valproate may be useful and may play a role in the prevention of morphine tolerance.

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Morphine is a potent opioid analgesic that is widely used for acute and chronic pain control [24]. Repeated administration of morphine induces tolerance, which reduces its effectiveness as an analgesic agent. Opioids bind to the mu opioid receptor (MOR) to activate various signaling molecules through heterotrimeric guanine nucleotide-binding proteins (G proteins). Chronic morphine tolerance may arise from adaptations in the intracellular signal transduction after MOR activation, as morphine does not effectively induce MOR phosphorylation or internalization [9]. Persistent activation of MOR on the cell surface may cause altered signal transduction including changes in MOR-coupled G proteins from G α to G α [5], increased activity of protein kinase C [11], and upregulation of *N*-methyl-D-aspartate receptor signaling [26]. Chronic morphine treatment also activates the cyclin-dependent kinase 5-glycogen synthase kinase-3 β (GSK3 β) signaling pathway, while inhibition of this pathway diminishes morphine tolerance and restores the efficacy of analgesic in rat [20] and mouse models [7].

Valproate is widely prescribed as an anticonvulsant and a mood stabilizer for bipolar disorders. It increases the amount of γ -aminobutyric acid (GABA) in the central nervous system [18]. Although the activation of GABAergic neurons may be responsible for the central pharmacologic effect of valproate, recent studies have shown that valproate also suppresses the activity of GSK3 β [2,14]. Since valproate use is frequent in clinical discourse, we examined the effect of valproate as an inhibitor of GSK3 β on the development of morphine antinociceptive tolerance in a mouse model of thermal injury.

All animal experimental procedures were in accordance with a protocol approved by the Institutional Animal Care Committee of Chiba University, Chiba, Japan. C57BL/6 male mice (22–27 g; aged 10–15 weeks; 69 mice) were used. All mice were provided with food and water *ad libitum* before the experiment.

The following reagents were used: sodium valproate (Sigma Chemical Co., Irvine, UK), morphine hydrochloride (Takeda Pharmaceutical Co., Tokyo, Japan), and SB216763 (Biomol International, Plymouth Meeting, PA, USA). The following antibodies were used: rabbit polyclonal antibody against MOR-1 (Chemicon, Temecula, CA, USA), mouse monoclonal antibody (mAb) against phospho-GSK3 β (Tyr279/Tyr216) (Upstate Biotechnology, Chicago, IL, USA), Cy-3-conjugated donkey antibody against rabbit IgG, and Cy-2-conjugated donkey antibody against mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

Abbreviations: MOR, mu opioid receptor; G proteins, heterotrimeric guanine nucleotide-binding proteins; GSK3 β , glycogen synthase kinase 3 β ; GABA, γ -aminobutyric acid.

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A hot plate test was carried out to assess the effects of a pharmacologic agent on the thermal nociceptive threshold of mice. Mice were placed on a 54.5 °C hot plate (Socrel hot-plate model DS37, Ugo Basile, Italy). The response latency to either a hind paw lick or a jump was recorded. In the absence of a response, the animals were removed from the hot plate after 60 s to avoid tissue injuries, and a 60 s latency was assigned as the response. Before drug administration, the hot plate latency was measured 3 times, and the average of the second and third measurements was used as the pre-drug response latency at 0 min. The hot plate latency was also measured at 5, 15, 30, 45, and 60 min following intraperitoneal drug injection. Each reagent was dissolved in 100 μ l saline for intraperitoneal injection. To confirm analgesia with morphine, the hot plate latency was measured at 5, 15, 30, 45, and 60 min after a single intraperitoneal injection with morphine (20 mg/kg, $n = 25$). To obtain control data, mice were injected with the vehicle (saline, $n = 2$) or valproate (150 mg/kg; $n = 5$) twice a day for 5 days, and the hot plate tests were performed on day 5 after the pharmacological agents were administered intraperitoneally. To evaluate the effect of valproate on morphine analgesia, 20 mg/kg morphine alone ($n = 12$) or morphine with valproate (50, 150, 300 mg/kg; $n = 5, 14, 5$, respectively) were administered intraperitoneally twice a day for 5 days. On day 5, more than 6 h after drug administration, mice were administered 20 mg/kg of morphine and then subjected to the hot plate test.

To analyze the effects of these drugs on animal performance in the hot plate test, the %MPE was calculated, where $\%MPE = ([\text{post-drug maximum response latency} - \text{pre-drug response latency}] / [\text{cut-off time (60 s)} - \text{pre-drug response latency}]) \times 100$. The post-drug maximum response latency was defined as the single longest response latency during the entire time course of the hot plate test. A higher %MPE represented a better analgesic effect.

Mice were deeply anesthetized with pentobarbital (Dainippon Sumitomo Pharma, Osaka, Japan) and fixed by transcardial perfusion with 4% paraformaldehyde in phosphate-buffered saline (PBS). Their brains were further immersion-fixed for 12 h in 4% paraformaldehyde at 4 °C. After fixation, the brains were dehydrated by immersing in increasing concentrations of ethanol, and subsequently embedded in paraffin wax. For immunofluorescence, sections (8 μ m) were incubated with 10% normal goat or bovine serum in PBS for 30 min to block nonspecific antibody binding and then incubated with a primary antibody in PBS for 1 h at room temperature. The sections were rinsed with PBS and then incubated with a mixture of Cy-3-conjugated anti-rabbit IgG and Cy-2-conjugated anti-mouse IgG in PBS for 1 h at room temperature. Subsequently, the sections were rinsed with PBS and mounted on glass slides with Perma Fluor (Immunon, Pittsburgh, PA, USA). Immunolocalization was observed with a fluorescence microscope using FITC/rhodamine filters and Plan-Neofluar 20 \times and 40 \times NA 0.75 objectives (Axiovert 200 M; Carl Zeiss, Oberkochen, Germany). The brightness and contrast were optimized using AxioVision 4.4 software (Carl Zeiss), and immunofluorescence images were captured with a digital camera (AxioCam MRm, Carl Zeiss). The mean grey values of cells with the background subtraction were used for densitometry.

To compare the hot plate %MPE, latencies, and other values between groups, one-way or two-way ANOVA was used followed by the Bonferroni post hoc test (GraphPad Prism 4.0, GraphPad Software, San Diego, CA, USA).

We evaluated analgesia induced with administration of a single dose of morphine (20 mg/kg) administration in our mice model with the hot plate test (Fig. 1A). The response latencies reached the 60 s cut-off point 30 min after injection. The response latencies of the valproate injection group and the saline control group (NS) did not differ significantly (Fig. 1A). Thus, valproate alone did not affect the nociceptive threshold of mice.

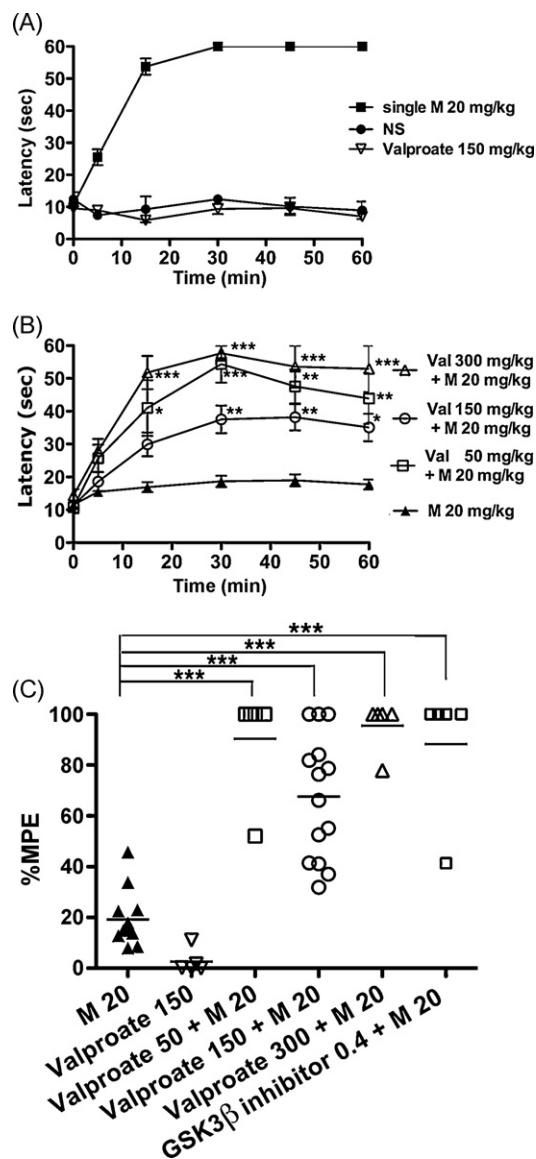


Fig. 1. Valproate attenuated the development of morphine tolerance. (A) The graph represents the latent responses (0–60 s) of mice administered morphine once (single M), saline twice a day for 5 consecutive days (NS), or 150 mg/kg of valproate twice a day for 5 consecutive days (mean \pm SEM). (B) The graph represents the latent responses (0–60 s) of mice administered morphine alone (M, $n = 12$), 50 mg/kg of valproate and morphine, 150 mg/kg valproate and morphine, and 300 mg/kg valproate and morphine twice a day for 5 consecutive days (mean \pm SEM; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, two-way ANOVA with the Bonferroni post hoc test). (C) The distribution of %MPE after the repetitive drug treatment for 5 days. The mean %MPEs of the mice that received valproate and morphine were significantly greater than those of mice treated with morphine alone. The mean %MPE of mice receiving GSK3 β inhibitor (0.4 mg/kg of SB216763) and morphine ($n = 5$) was also significantly greater than that of mice treated with morphine alone (*** $P < 0.001$, one-way ANOVA with the Bonferroni post hoc test).

Subsequently, to evaluate the effect of valproate on morphine tolerance, mice were administered morphine alone or a combination of morphine and valproate intraperitoneally twice a day for 5 consecutive days. On day 5, more than 6 h after the drug administration, mice were administered morphine alone (20 mg/kg) and then subjected to the hot plate test (Fig. 1B). We found that the response latencies were significantly reduced, indicating that morphine tolerance was induced by repeatedly injecting morphine (Fig. 1B, M20). On the other hand, the latent responses of mice co-administered valproate and morphine were significantly longer

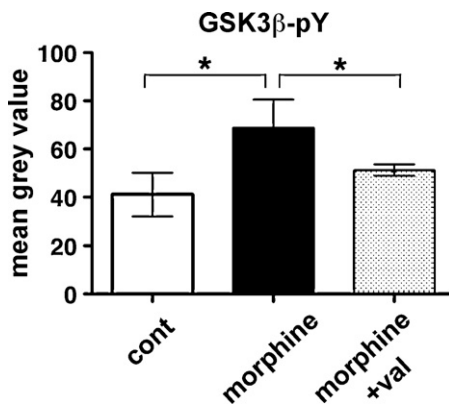


Fig. 2. GSK3 β activation is reduced in mice administered valproate and morphine. Wild-type mice were injected with 20 mg/kg morphine alone (morphine), 150 mg/kg valproate and morphine (morphine + val), or saline (cont) twice a day for 5 days. The brains were sectioned and double-immunostained with anti-phospho-GSK3 β (Tyr216) and anti-MOR. Based on densitometric analysis, MOR-immunopositive neurons in the PAG matter of mice injected with morphine alone showed a significantly greater enhancement of expression of tyrosine-phosphorylated GSK3 β after chronic morphine treatment than mice treated with morphine and valproate (arbitrary unit; $n=10$; mean \pm SD; $^*P<0.05$, one-way ANOVA with the Bonferroni post hoc test). A representative immunohistochemistry result is shown in [Supplementary Fig. 1](#).

than those of mice that received morphine alone at 15, 30, 45, and 60 min after drug administration ([Fig. 1B](#)).

The mean %MPEs of mice co-administered valproate and morphine were significantly greater than that of mice administered morphine alone ([Fig. 1C](#)). These results indicated that valproate alone does not provide analgesic effect, but it does inhibit the development of morphine antinociceptive tolerance.

GSK3 β inhibition by specific inhibitors such as SB216763 and (2'Z, 3'E)-6-bromoindirubin-3'-oxime diminishes the development of morphine tolerance in rats after chronic intrathecal morphine treatment [20]. In addition, we verified that GSK3 β inhibition by SB216763 suppressed the development of morphine antinociceptive tolerance in our mouse models [7]. Furthermore, we co-administered SB216763 (0.4 mg/kg) and morphine (20 mg/kg) twice a day for 5 days. The response latencies of mice following this co-administration were significantly longer than those of control mice that received morphine alone following the last morphine injection on day 5. The mean %MPE of mice that received both SB216763 (0.4 mg/kg) and morphine was significantly greater than that of mice treated with morphine alone on day 5 ([Fig. 1C](#)). SB216763 alone did not provide analgesic effect [7]. Thus, the inhibition of GSK3 β prevented the development of morphine tolerance.

Since morphine and valproate were injected intraperitoneally, both spinal and supraspinal neurons might have been affected. Neurons exhibiting MOR expression in the periaqueductal gray (PAG) matter contribute to morphine tolerance [1,19,27]. After chronic intraperitoneal injection of morphine alone or the combination of morphine with valproate for 5 days, mouse brains were sectioned and double-immunostained with antibodies against MOR and tyrosine-phosphorylated GSK3 β . We examined 3 mice in each group. Mice that were administered the combination of morphine and valproate remained sensitive to morphine analgesia with 100% MPE (mean). On the other hand, mice administered morphine alone revealed diminished analgesia with 15.3% MPE (mean). MOR-immunopositive neurons in the PAG region of mice that were injected morphine alone showed a significantly greater enhancement of tyrosine-phosphorylated GSK3 β expression than those of mice injected with morphine and valproate ([Fig. 2](#) and [Supplementary Fig. 1](#)).

These observations suggest that chronic MOR stimulation by repetitive morphine injections may activate GSK3 β , which might be related to the development of morphine tolerance. Mice injected with morphine and valproate remained morphine-sensitive, and the inhibition of GSK3 β by valproate may have thus contributed to the attenuation of the development of morphine tolerance.

This study shows that systemic administration of valproate suppressed the development of morphine antinociceptive tolerance in a mouse model. Although administration of valproate alone did not exhibit an analgesic effect, it functioned as an adjuvant analgesic to prevent the development of morphine tolerance.

Chronic morphine administration may alter the signal transduction through persistent MOR activation. We thus examined the role of GSK3 β as a ventral signaling molecule in the MOR signaling pathway. GSK3 β is a serine/threonine kinase that plays critical roles in diverse intracellular signaling systems [12]. Its kinase activity is inactivated by the phosphorylation of Ser9, while the same is enhanced by the dephosphorylation of Ser9 and the phosphorylation of Tyr216. The p90 ribosomal S6 kinase [25], Akt [6], protein kinase C [10], and protein kinase A [8] have been demonstrated to phosphorylate GSK3 β at Ser9. While acute activation of MOR inactivates GSK3 β by the phosphorylation of Ser9 through the PI3K/Akt pathway [12], the chronic activation of MOR may activate GSK3 β , which is associated with the development of morphine tolerance [20]. ZAK1 [15], Fyn tyrosine kinases [16], and transient increases in intracellular Ca^{2+} [13] have been reported to activate GSK3 β by inducing phosphorylation of Tyr216; however, the regulatory mechanism for the activation of GSK3 β remains uncertain in comparison to the mechanism responsible for its inactivation. Although the precise molecular mechanism of MOR-mediated GSK3 β activation remains uncertain, it is hypothesized that morphine administration induces an elevation in cytosolic Ca^{2+} levels by both an influx of extracellular Ca^{2+} and a release of Ca^{2+} from its intracellular stores [21]. Persistent activation of MOR on the cell surface may cause an upregulation of N-methyl-D-aspartate receptor signaling [26], leading to an influx of extracellular Ca^{2+} , which would possibly lead to the activation of GSK3 β .

Activation of MOR triggers signal transduction, leading to the induction and activation of transcriptional factors such as activator protein-1 (AP-1), which may cause alterations of gene expression related to the development of tolerance and dependence. Acute treatment of MOR with morphine stimulates AP-1 DNA-binding activity, while chronic treatment normalizes it [3]. FosB-/- mice, as compared to fosB+/+ mice, developed enhanced tolerance to morphine-induced analgesia [23]. Thus, AP-1 seems to be involved in the development of morphine tolerance. GSK3 β has been reported to be a negative regulator of growth factor-induced activation of the c-Jun N-terminal kinase [17], and the phosphorylation of recombinant human c-Jun proteins in vitro by GSK3 β also decreases its DNA-binding activity [4]. On the other hand, the inhibition of GSK3 β appears to be required for effective morphine analgesia because co-administration of morphine and GSK3 β inhibitors was found to prevent the development of morphine tolerance, as shown in previous studies [7,20]. Although the precise mechanism by which the activation of GSK3 β causes morphine tolerance is unclear, the activation of GSK3 β possibly leads to the decrease in the DNA-binding activity of AP-1 as well as the development of morphine tolerance.

In this study, we did not observe any dose-dependent effect for valproate when 50–300 mg/kg of valproate was administered twice a day for 5 days. Although valproate concentrations were not evaluated, we believe that concentrations less than 50 mg/kg might also be effective in preventing the development of morphine antinociceptive tolerance. In the clinical setting, up to 1200 mg/day of valproate is prescribed; therefore, approximately 10 mg/kg of valproate should be administered twice a day. Although the dosage

used in our mouse experiments did not drastically differ from those used in the human clinical setting, a smaller dose of valproate should be tested for future clinical applications.

This study has demonstrated that the co-administration of valproate with morphine suppressed the development of morphine tolerance. Since valproate has multiple biological effects [22], inactivation of GSK3 β as well as GABAergic action may contribute to the attenuation of morphine tolerance. Further investigation is required to elucidate the relationship between morphine tolerance and GSK3 β signaling. The modulation of GSK3 β activity by valproate may be a potentially viable clinical application for preventing morphine tolerance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.neulet.2010.08.084](https://doi.org/10.1016/j.neulet.2010.08.084).

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