

# Wnt3a Inhibitor Attenuates Remifentanil-Induced Hyperalgesia via Downregulating Spinal NMDA Receptor in Rats

This article was published in the following Dove Press journal:  
*Journal of Pain Research*

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**Purpose:** The upregulation of spinal NMDA receptor is a crucial mechanism in remifentanil-induced hyperalgesia (RIH). Wnt3a/ $\beta$ -catenin pathway plays an important role in neuropathic pain. We hypothesized that wnt3a inhibitor (iwp-2) could downregulate the expression of NR<sub>2</sub>B subunit in NMDA receptor, in order to relieve RIH.

**Materials and Methods:** The study has 2 phases. The phase 1 study is designed by different doses of iwp-2 groups to create an appropriate iwp-2 dose used in RIH alleviation. The phase 2 study is designed to prove that the wnt3a inhibitor could downregulate the activation of the NR<sub>2</sub>B to inhibit RIH in rats. Thermal hyperalgesia (PWTL) and mechanical allodynia (PWMT) were evaluated after RIH. The area under the PWTL and PWMT curves (AUC) were calculated. The amount of activated NR<sub>2</sub>B subunit, c-fos, NF- $\kappa$ B,  $\beta$ -catenin, wnt3a and p-GSK-3 $\beta$  (Ser9) were detected in the lumbar spinal cord.

**Results:** Remifentanil infusion could induce overexpression of  $\beta$ -catenin and wnt3a in rats. Iwp-2 (60 $\mu$ M, 120 $\mu$ M, 180 $\mu$ M) could dose-dependently inhibit thermal hyperalgesia and mechanical allodynia in rats. In phase 2 study, both NR<sub>2</sub>B subunit antagonist Ro25-6981 and iwp-2 decreased the amount of activated NR<sub>2</sub>B, enhanced p-GSK-3 $\beta$  (Ser9), reduced  $\beta$ -catenin, c-fos and NF- $\kappa$ B in the lumbar spinal cord ( $p < 0.001$ ). In comparison with the group iwp-2, the group of Ro25-6981 had more benefit in reversing hyperalgesia, including higher AUC value of PWTL ( $p = 0.022$ ) and PWMT ( $p = 0.035$ ).

**Conclusion:** Remifentanil exposure could induce overexpression of wnt3a and enhance the production of  $\beta$ -catenin in the spinal dorsal horn. Inhibition of wnt3a response was capable of attenuating RIH in alleviating hyperalgesia-related behavioral parameters, as well as reducing overexpression of c-fos, NF- $\kappa$ B, NR<sub>2</sub>B in spinal dorsal horn.

**Keywords:** remifentanil, hyperalgesia, iwp-2, NR<sub>2</sub>B, wnt3a/ $\beta$ -catenin

## Introduction

Remifentanil, a kind of potential analgesic, is commonly used in the induction and maintenance in general anesthesia. Due to its ultra-short acting character, remifentanil could be used in high doses during the operations. However, nowadays remifentanil was found to have paradoxical nociceptive effect, characterized as hypersensitivity to allodynia and thermal stimulation, the effect of which could counteract its own antinociception.<sup>1</sup> Consumption of postoperative analgesics would increase after the exposure of remifentanil.<sup>2-5</sup> In animal research, intravenous remifentanil anesthesia could lead to remifentanil-induced hyperalgesia (RIH) and could dramatically exacerbate pre-existing pain after surgery. In most of the

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animal researches, RIH was noticed to begin at 2 h and peak at 48 h after remifentanyl exposure.<sup>1,4</sup>

Recent reports have demonstrated that NMDA receptor-mediated synaptic plasticity was essential to chronic pain.<sup>6,7</sup> RIH has confirmed to be attributed to the phosphorylation of NR<sub>2</sub>B subunit in NMDA receptor. NMDA antagonists could dose-dependently relieve RIH<sup>8</sup> and reverse the phosphorylation of NR<sub>2</sub>B subunit.<sup>9</sup> While the downstream molecular mechanism is still poorly understood.

The wnt3a/ $\beta$ -catenin pathway is a set of evolutionarily conserved signals which takes part in the development like dendrite morphogenesis and synapse formation. Recently, the effect of this pathway involved in chronic neuropathic pain<sup>10</sup> has been elucidated clearly. Wnt3a/ $\beta$ -catenin pathway was found to be a key target to mediate sensory neuron excitability, especially in spinal dorsal neuron.<sup>10–14</sup> The activation of NMDA receptor caused the upregulation of  $\beta$ -catenin proteins.<sup>15</sup> Wnt3a/ $\beta$ -catenin antagonist could abolish the activation of NMDA receptor.<sup>15</sup> Moreover, wnt antagonist can prevent neuronal apoptosis induced by NMDA receptor-mediated excitotoxicity in vivo and in vitro models.<sup>10</sup>

In this study, we speculated that the spinal inhibition of wnt3a/ $\beta$ -catenin signaling pathway could reverse the over-expression of NR<sub>2</sub>B subunit in NMDA receptor, hence provide theoretical basis of the treatment on RIH.

## Materials and Methods

### Animals

The experimental procedures were approved by the Animal Care Committee, Wenzhou Medical University. The animal procedures were performed in accordance with the Declaration of the National Institutes of Health Guide for Care and Use of Laboratory Animals, USA. Adult male SD rats (220  $\pm$  20g) had an acclimation period for at least 10 days. All animals were maintained at controlled temperature (23  $\pm$  0.5°C) and relative humidity (55  $\pm$  10%) with 12 h light: 12 h dark cycle. They were housed in individually ventilated cages with food and water freely available.

### Test Drugs

Remifentanyl hydrochloride (Ultiva) (batch number: 90A05161, Ren Fu Co., Yichang, China)

GluN<sub>2</sub>B antagonist Ro25-6981 (R7150, Sigma-Aldrich Co., St. Louis, MO, USA)

Iwp-2 (681671, EMD Chemicals, Darmstadt, Germany).

Remifentanyl was infused through caudal vein. Both Ro25-6981 and iwp-2 were injected intrathecally through PE-10 tube which had been implanted at spinal L<sub>3-4</sub> intervertebral space in advance. All of the test drugs were given under sevoflurane anesthesia (induction, 3.0%, maintenance, 1.5%. Maruishi Pharmaceutical Co., Ltd., Japan) by a nose mask.

## Experiment Related Procedures

Intrathecal catheters (PE-10 tube, ID0.28, OD0.61, Smiths Medical, UK) were implanted between L<sub>3-4</sub> intervertebral space under sevoflurane anesthesia. 20 $\mu$ L of 2% lidocaine was administrated through this catheter 3 days later. Only the rats showing lower limb paralysis after lidocaine test were included for further experiments. All following experiments including RIH model establishment were carried out 7 days later after intrathecal catheter implantation.

Under sevoflurane anesthesia, remifentanyl was infused intravenously at a rate of 1.2 $\mu$ g $\cdot$ kg<sup>-1</sup> $\cdot$ min<sup>-1</sup> for 60 min. During infusion, a longitudinal incision was made in the plantar surface of the right hind paw to simulate surgical stress.<sup>9</sup> After hemostasis, the incision was ligated and covered with antiseptic gauze. Ro25-6981 and iwp-2 were dissolved in 1% DMSO and injected intrathecally 30 min before remifentanyl infusion. A schematic diagram of the experimental protocol is shown in [Figure 1](#).

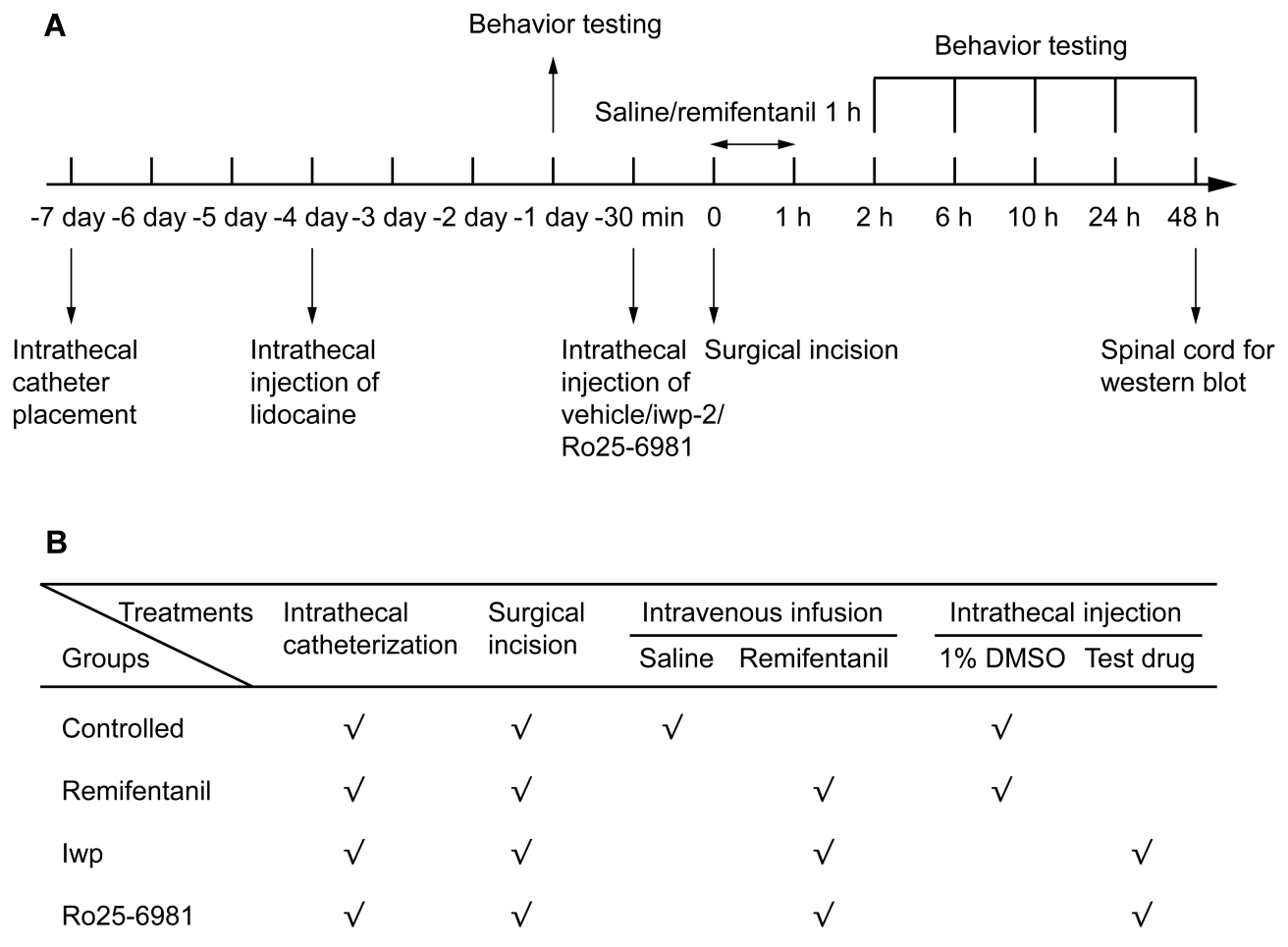
Both the intrathecal catheters implantation and surgical incision were performed by Gao independently.

## Experiment Protocol

### Phase I: Iwp-2 Dose Determination in RIH

Thirty rats were randomly divided into five groups (n = 6 in each group): group C (controlled group); group R (rats with RIH model); group iwp<sub>low</sub> (intrathecal (i.t.) 60 $\mu$ M iwp-2 in rats with RIH model), group iwp<sub>medium</sub> (i.t. 120 $\mu$ M iwp-2 in rats with RIH model) and group iwp<sub>high</sub> (i.t. 180 $\mu$ M iwp-2 in rats with RIH model). 10 $\mu$ L different doses of iwp-2 were injected using a microinjection syringe 30 min before RIH model establishment. An additional 20 $\mu$ L normal saline was administered to flush the catheter.

PWMT and PWTL tests were performed at -24 h, 2 h, 6 h, 10h, 24 h and 48 h after remifentanyl exposure. After behavior tests, the L<sub>4-6</sub> segments of the spinal cord were quickly removed for Western blot analysis. Expression of NR<sub>2</sub>B, wnt3a,  $\beta$ -catenin, and production of c-fos, NF- $\kappa$ B were measured (n = 3 in each group).



**Figure 1 (A, B)** Schematic diagram of the experimental protocol. **(A)** The timeline of experiment. **(B)** Experimental intervention in each group.

## Phase 2: The Effect of Test Drugs in RIH

Twenty-four rats were randomly divided into four groups ( $n = 6$  in each group): group C; group R (rats with RIH model); group iwp (i.t.  $180\mu\text{M}$  iwp-2 in rats with RIH model); group Ro (i.t.  $1.5\mu\text{g}$  Ro25-6981 in rats with RIH model).  $10\mu\text{L}$  both iwp-2 and Ro25-6981 were injected separately using a microinjection syringe 30 min before RIH model establishment.

Behavioral tests were performed at  $-24$  h, 2 h, 6 h, 10h, 24 h, and 48 h after remifentanil exposure. After the behavioral test, the  $L_{4-6}$  segments of the spinal cord were removed for Western blot analysis. Expression of  $\text{NR}_2\text{B}$ , wnt3a,  $\beta$ -catenin, c-fos, NF- $\kappa\text{B}$ , GSK- $3\beta$ , p-GSK- $3\beta$  (Ser9) was measured ( $n = 3$  in each group). The amount of nuclear  $\beta$ -catenin was also detected ( $n = 3$  in each group).

## Behavior Test

### Paw Withdrawal Mechanical Threshold (PWMT)

PWMT was assessed by electronic von Frey anesthesiometer (IITC INC, Life Science instrument, CA, USA)

and recorded by transducer (ALMEMO 2450, Ahlborn, Germany). Before testing, each animal was acclimatized for 10 min. Through a mesh bottom ( $1\times 1$  cm), a 0.8-mm diameter straight filament was applied vertically to right plantar surface adjacent to the wound (approximately 1 mm). Paw withdrawal or licking was defined as a positive response. The test was repeated 3 times with an interval of 5 min. The mean PWMT was defined as the average value from the 3 tests. A maximal cut-off value of 25 g was applied to prevent tissue damage.

### Paw Withdrawal Thermal Latency (PWTL)

PWTL was evaluated by testing equipment (Model 336, Series 8, IITC INC, Life Science instrument, CA, USA). Rats were fixed in a clear plastic chamber with a glass floor and allowed to habituate for 10 min before testing. An infrared heat source under the glass was focused on the right plantar surface adjacent to the wound until withdrawal was observed. A cut-off time of 30 s was established to prevent tissue damage. The test was repeated 3 times

with an interval of 5 min. The mean PWTL was defined as the average value from the 3 tests.

## Western Blotting

After the last behavioral test at 48 h, the L<sub>4-6</sub> segments of rats were quickly removed and frozen at  $-80^{\circ}\text{C}$ . Tissue samples were homogenized in lysis buffer containing a cocktail of protease inhibitors (Sigma-Aldrich Co.). The lysate was centrifuged at 13,000 rpm for 20 min at  $4^{\circ}\text{C}$  and supernatant was collected as the total proteins. To extract nuclear protein, an NE-PER Nuclear Cytoplasmic Extraction Reagent kit (Pierce, Rockford, IL, USA) was used. Protein samples were separated by SDS-PAGE (8% or 12%) gel and transferred onto PVDF membrane (Millipore, MA). The following primary antibodies were used: anti-c-Fos (1:2000, ab190289, abcam, UK), anti-NF- $\kappa\text{B}$  p65 (1:1000, ab7970, abcam, UK), anti-Wnt3a (1:1000, ab219412, abcam, UK), anti- $\beta$ -catenin (1:5000, ab32572, abcam, UK), anti-phospho-GSK-3 $\beta$  (Ser9) (1:1000, 5558, CST, USA), anti-GSK-3 $\beta$  (1:1000, 12456, CST, USA), anti-NMDAR2B (1:1000, 4212, CST, USA),  $\beta$ -actin (1:1000, 4970, CST, USA) or Histone H3 (1:2000, 4499, CST, USA) were used as endogenous control. The membrane was washed with TBST buffer and further incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:10,000, BL003A, Biosharp Co., China) and visualized in ECL system. The intensity of each band was assayed in Chemi-Doc XRS imaging system (Bio-Rad, CA) and analyzed by ImageJ software (NIH, Bethesda, MD). The results were expressed as the percentage of  $\beta$ -actin or Histone H3 immunoreactivity.

## Statistical Analysis

Data were presented as the mean  $\pm$  SD or mean  $\pm$  SEM. We calculated the time-effect curves (AUC) data, depicting the PWMT and PWTL values over time. The AUC was measured during 48 h after RIH according to the trapezoidal rule by multiplying the time interval with PWMT or PWTL values. The statistical analyses of Western blot, AUC and behavioral tests were analyzed using one-way ANOVA with Bonferroni Correction.  $P < 0.05$  was considered statistically significant. Statistical analysis was done using SPSS 17.0 software.

## Results

### Iwp-2 Doses in RIH Model

There were no significant differences among all groups in the baselines of PWMT and PWTL ( $p > 0.05$ ).

In the different doses of iwp-2, it was revealed that iwp-2 could dose-dependently inhibit RIH (Figure 2). Compared with the other two doses of iwp-2 (120 $\mu\text{M}$  and 60 $\mu\text{M}$ ), the high dose group (180 $\mu\text{M}$ ) could markedly decrease remifentanyl evoked high levels in PWMT and PWTL from 2 h to 48 h after surgery ( $p < 0.001$ ). Compared with group R, the medium dose of group iwp could effectively inhibit PWMT and PWTL from 2 h to 10 h. However, it failed to reverse the reduction of PWTL at 48h ( $p = 0.076$ ) and PWMT at 24 h or 48 h ( $p = 1.0$ ).

As shown in Figure 3, compared with group C, RIH model group markedly induced overexpression in c-fos, NR<sub>2</sub>B and NF- $\kappa\text{B}$ , as well as evoked an elevation in wnt3a,  $\beta$ -catenin production ( $p < 0.001$ ). Iwp-2 dose-dependently inhibited remifentanyl-induced activation of wnt3a/ $\beta$ -catenin signaling pathway and subsequently reverse overexpression of c-fos, NR<sub>2</sub>B and NF- $\kappa\text{B}$  ( $p < 0.001$ ).

### The Role of Iwp-2 to Reverse NR2B Activation in Order to Alleviate RIH Behavioral Tests

Test drugs were administrated 30 min before remifentanyl infusion and plantar incision. Compared with group R, pretreatment with iwp-2 could increase the thresholds of PMWT and PMWL in group iwp ( $p < 0.001$ ). Compared with group iwp, pretreatment with i.t. Ro25-6981 was more effective in ameliorating remifentanyl-induced thermal hyperalgesia at 24 h ( $p = 0.018$ ) and 48 h ( $p = 0.013$ ), as well as less mechanical allodynia at 24 h ( $p = 0.023$ ) (Figure 4). Ro25-6981 had more excellence performance in higher AUC of the PMWT ( $p = 0.022$ ) and PMWL ( $p = 0.035$ ) curves in comparison with group iwp (Table 1).

### Expression of Proteins in Spinal Dorsal Horn

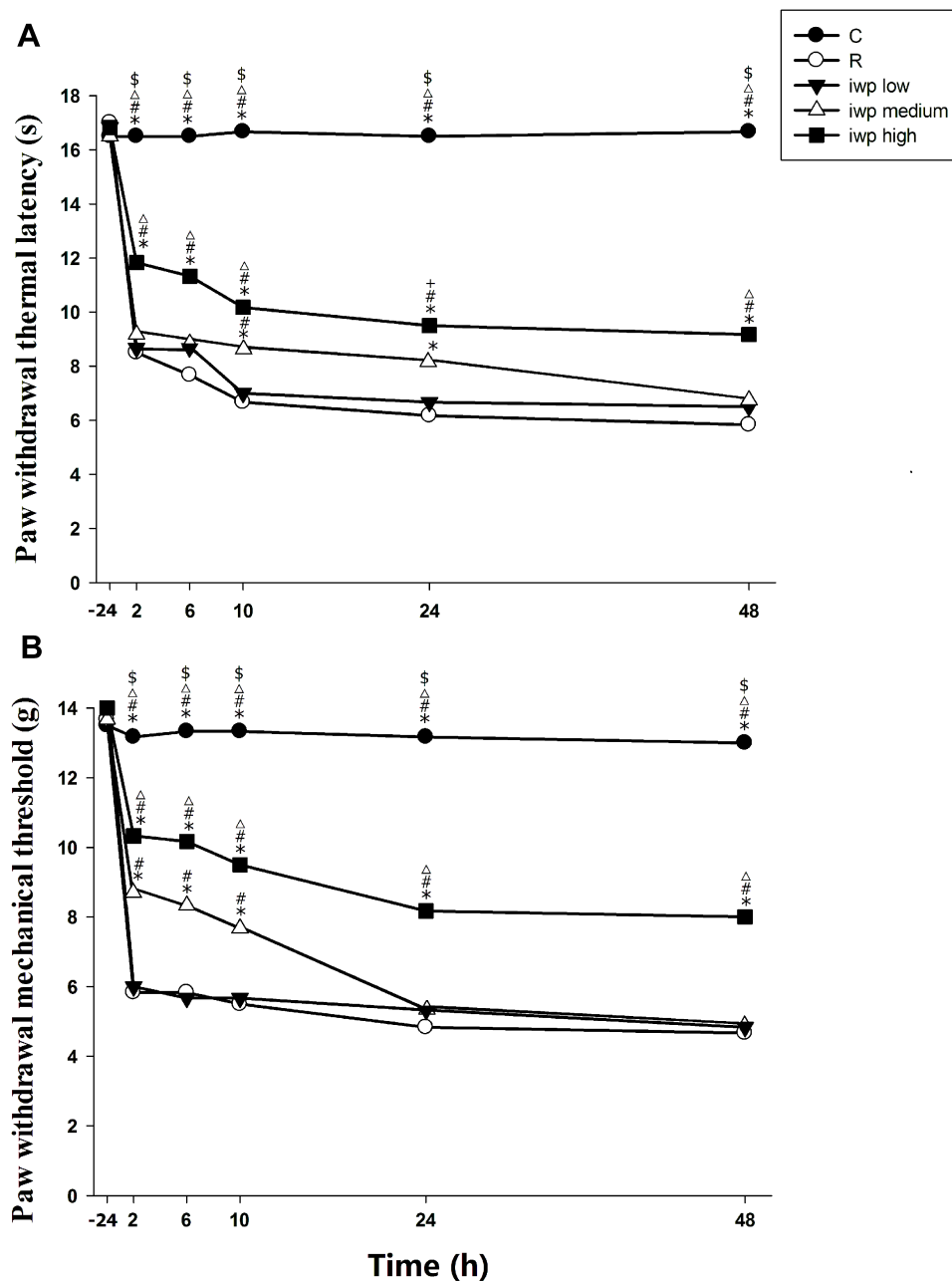
Both iwp-2 and NMDA antagonist Ro25-6981 enhanced the RIH induced phosphorylation of GSK-3 $\beta$ , inhibited

**Table 1** Characteristics of AUC Values (0–48 h)

	C (n = 6)	R (n = 6)	Iwp-2 (n = 6)	Ro (n = 6)
AUC-PWTL	757.00 $\pm 20.23$	330.33 $\pm 27.62$	531.00 $\pm 18.67^{\#\Delta+}$	491.67 $\pm 13.68^{\#\Delta}$
AUC-PWMT	476.50 $\pm 23.3$	208.67 $\pm 11.62$	355.67 $\pm 10.84^{\#\Delta+}$	326.33 $\pm 16.99^{\#\Delta}$

**Notes:** Groups' allocation: C: Group Control; R: Group remifentanyl; iwp-2: Group iwp-2 plus remifentanyl; Ro: Group Ro25-6981 plus remifentanyl. Data were expressed as mean  $\pm$  SD.  $\#P < 0.001$  compared with Group C,  $\Delta P < 0.001$  compared with Group R,  $*P < 0.05$  compared with Group Ro.

**Abbreviations:** AUC, area under the curve; PWTL: paw withdrawal thermal latency; PWMT, paw withdrawal mechanical threshold.



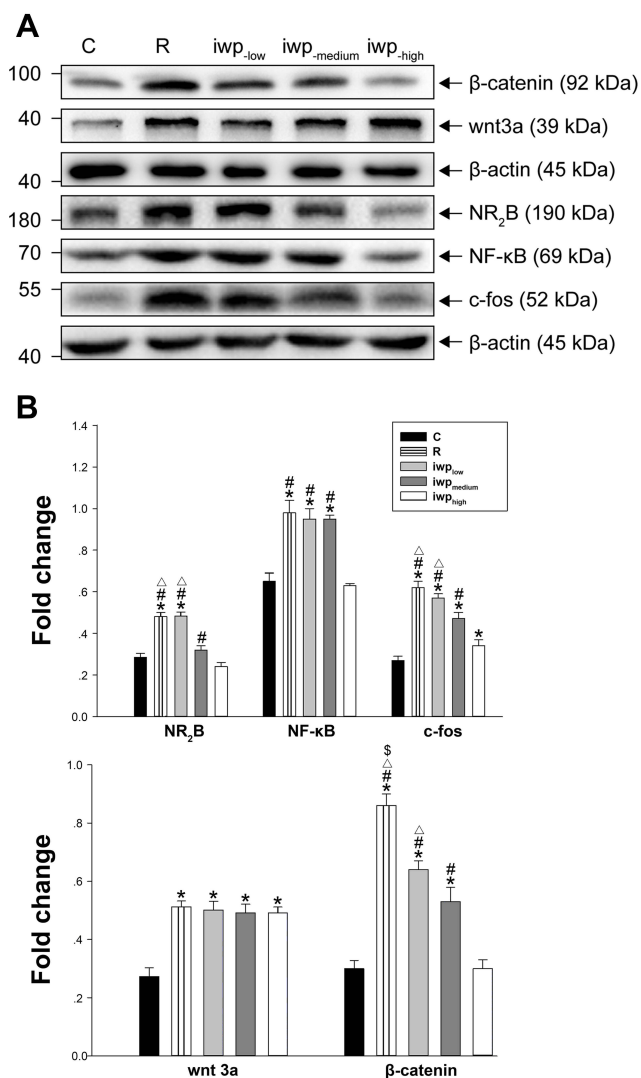
**Figure 2 (A, B)** Effect of different doses of iwip-2 in remifentanyl-induced hyperalgesia. Paw withdrawal thermal latency (A) and paw withdrawal mechanical threshold (B) were evaluated at -24 h before incision and at 2, 6, 10, 24 and 48 h after remifentanyl infusion. Groups allocation: (C) Group Control; (R) Group remifentanyl; iwip<sub>low</sub>: Group remifentanyl plus 60µM iwip-2; iwip<sub>medium</sub>: Group remifentanyl plus 120µM iwip-2; iwip<sub>high</sub>: Group remifentanyl plus 180µM iwip-2. Data were expressed as means ± SEM (n = 6). \*P < 0.001 compared with Group R, #P < 0.001 compared with Group iwip<sub>low</sub>, ΔP < 0.001 compared with Group iwip<sub>medium</sub>, \$P < 0.001 compared with iwip<sub>high</sub>, +P < 0.05 compared with Group iwip<sub>medium</sub>.

production and nuclear transport of  $\beta$ -catenin, hence inhibit the activity of wnt3a/ $\beta$ -catenin pathway ( $p < 0.001$ ). Whereas neither iwip-2 ( $p = 0.566$ ) nor Ro25-6981 ( $p = 1.000$ ) was failed in the inhibition of wnt3a production in comparison with group C. Compared with the pretreatment of group Ro, iwip-2 had less effect on inhibiting the upregulation of NR<sub>2</sub>B subunit in NMDA receptor ( $p < 0.001$ ) (Figure 5).

## Discussion

Opioid used for analgesics during operation may potentially induce hyperalgesia, characterized as hypersensitive to pain and more consumption of postoperative analgesics. This study demonstrated that RIH decreased PWMT and PWTL to mechanical and thermal stimulation. The progression of RIH was associated with rapid-onset and long-lasting expression of





**Figure 3 (A, B)** Bands of Western blot for levels of test proteins after different doses of i.t. iwp-2 administration in remifentanyl-induced hyperalgesia. Values for relative density of the proteins among groups. Protein is expressed relative to  $\beta$ -actin ( $n = 3$ ). Groups allocation: C: Group Control; R: Group remifentanyl; iwp<sub>low</sub>: Group remifentanyl plus 60  $\mu$ M iwp-2; iwp<sub>medium</sub>: Group remifentanyl plus 120  $\mu$ M iwp-2; iwp<sub>high</sub>: Group remifentanyl plus 180  $\mu$ M iwp-2. \* $P < 0.001$  compared with Group C,  $^{\$}P < 0.001$  compared with iwp<sub>low</sub>,  $^{\Delta}P < 0.001$  compared with Group iwp<sub>medium</sub>,  $^{\#}P < 0.001$  compared with Group iwp<sub>high</sub>.

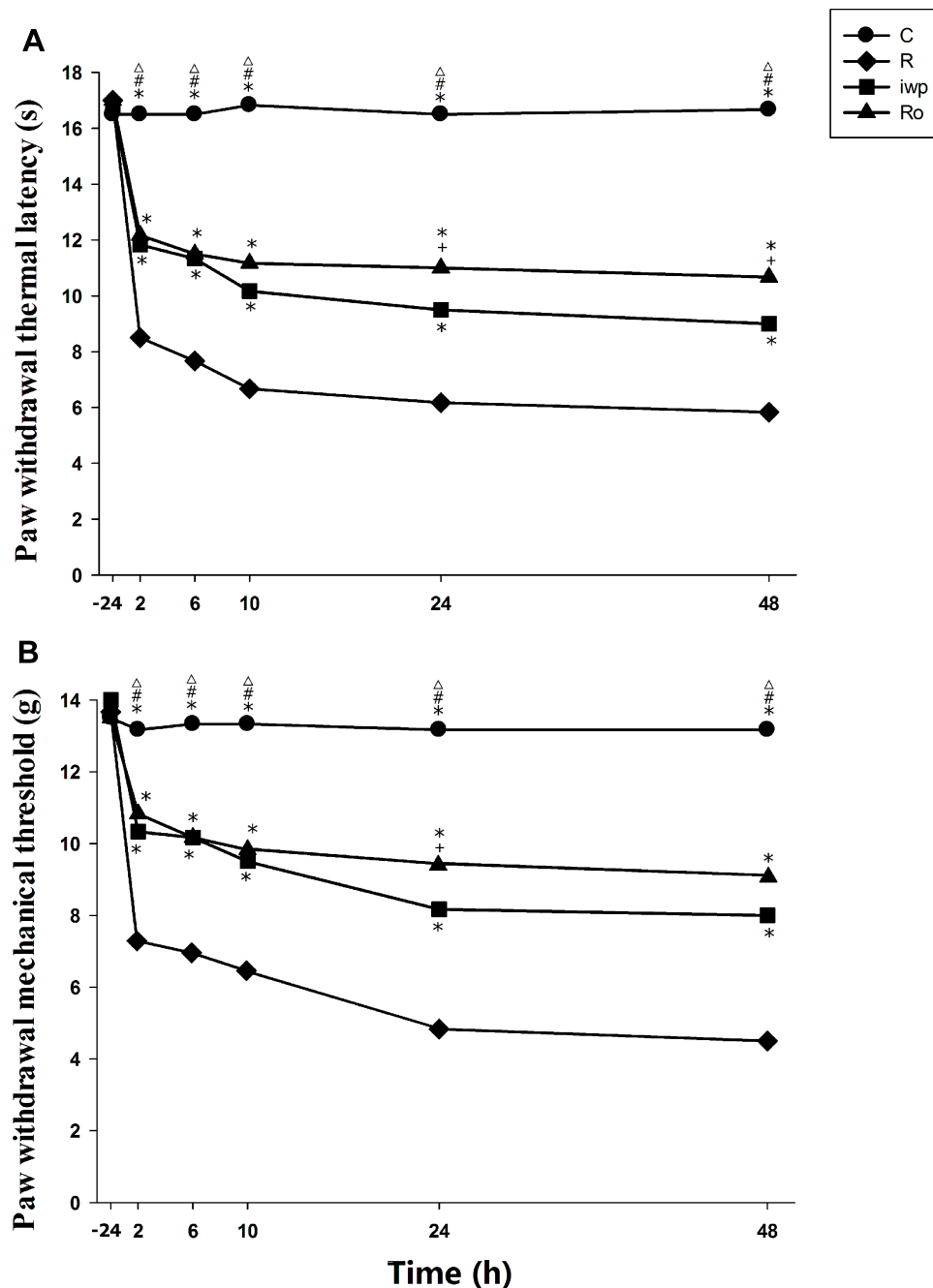
wnt3a and c-fos in dorsal horn neurons. Via inhibiting the activation of NMDA receptor subtype NR<sub>2</sub>B, spinal wnt antagonist iwp-2 could decrease the amount of the c-fos and ameliorate the postoperative hyperalgesia. Until now, it is the first study to delineate the modulation of Wnt3a/ $\beta$ -catenin pathway to reverse RIH through inhibiting activation of NR<sub>2</sub>B subunit.

C-fos, which is an immediate early gene and the third message in the message transmission, is generally taken as a marker for activation status of neurons. Pain, resulting from nociceptive neuron proliferation and activation, can cause overexpression of c-fos.<sup>16</sup>

NF- $\kappa$ B is a nuclear transcription factor which could reflect the degree of inflammation and pain. The release of glutamate in the dorsal horn could initiate NMDA activation and subsequently enhance the expression of NF- $\kappa$ B. NMDA antagonist could provide antinociceptive effect by inhibiting NF- $\kappa$ B nuclear translocation and activation.<sup>17</sup> The upregulation of NMDA and NF- $\kappa$ B was detected after remifentanyl infusion.<sup>18</sup> Wnt antagonist could prevent the RIH to reverse hypersensitivity and NF- $\kappa$ B activation after operations.

The mechanism of RIH is still unclear. RIH is usually attributed to the activation of NMDA receptor. The activation of NMDA receptor in spinal dorsal horn has been investigated extensively for the pain processing pathways in different models of pain.<sup>19</sup> Remifentanyl was previously proved to excite NMDA receptor subunits directly in vivo study.<sup>20</sup> Overexpression and phosphorylation of NR<sub>2</sub>B subunit in spinal dorsal horn are closely correlated with central nociceptive hypersensitivity. Author's earlier studies have demonstrated that various antagonists of NR<sub>2</sub>B subunit could dose-dependently alleviate the thermal and mechanical hyperalgesia and reverse the process of RIH.<sup>8</sup> Through enhancing NR<sub>2</sub>B subtype activity, the increased amount of glutamate could result in calcium influx and trigger more propagation of the stimulus to the central nervous system.<sup>21</sup>

Wnt3a/ $\beta$ -catenin signaling pathway is crucial in the mechanism of neuropathic pain.<sup>10-13</sup> Increasing evidence also suggests that wnt-signaling pathway is critically involved in chronic constriction injury, diabetic peripheral neuropathy, chemotherapy-induced neuropathic pain and other nerve injury models.<sup>10-12,22-24</sup> Extensively studies have found that wnt pathway was indispensable to maintain synaptic plasticity in neurons.<sup>25</sup> Via wnt-signaling pathway, NMDAergic neuron was stimulated through negatively modulate central inhibitory synaptic transmission.<sup>26,27</sup>  $\beta$ -catenin, the dominant downstream activator, could reflect the activity of the wnt3a/ $\beta$ -catenin pathway. Once the pathway initiated,  $\beta$ -catenin would be promoted to accumulate and translocate into nucleus. Upregulated  $\beta$ -catenin in the spinal cord dorsal horn was observed in various neuropathic pain rodents.<sup>28,29</sup> Besides,  $\beta$ -catenin is found to modulate synaptic plasticity and neuronal remodeling in vivo research.<sup>12</sup> Stimulation of wnt-signaling pathway could promote the release of excitatory NMDA substance.<sup>30</sup> So, we looked into the effect of wnt inhibitor on alterations of NMDA receptors expression when RIH occurs.



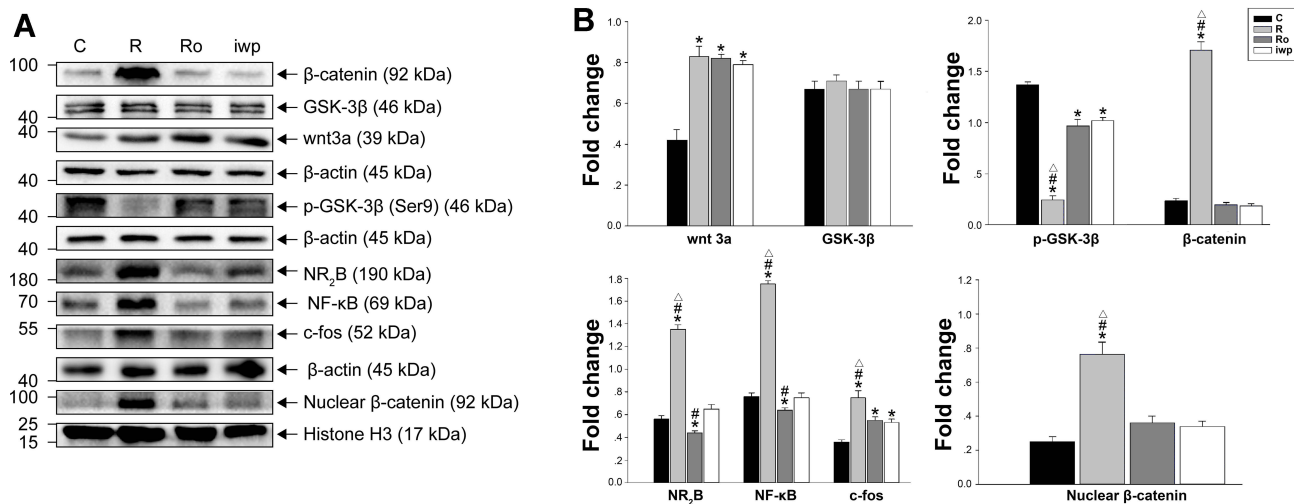
**Figure 4 (A, B)** Effect of NMDA antagonist and iwp-2 in remifentanyl-induced hyperalgesia. Ro25-6981 and iwp-2 were administered intrathecally 30 min before remifentanyl infusion and surgical incision. Paw withdrawal thermal latency (**A**) and paw withdrawal mechanical threshold (**B**) were evaluated at -24 h before incision and at 2, 6, 10, 24 and 48 h after remifentanyl infusion. Groups allocation: R: Group remifentanyl; C: Group Control; Ro: Group Ro25-6981 plus remifentanyl; iwp: Group iwp-2 plus remifentanyl. Data were expressed as mean  $\pm$  SEM (n = 6). \*P < 0.001 compared with Group R, #P < 0.001 compared with Group Ro, <sup>^</sup>P < 0.001 compared with Group iwp, +P < 0.05 compared with Group iwp.

In the trial, the overexpression of wnt3a and  $\beta$ -catenin in spinal dorsal horn after remifentanyl exposure reveals that the pathway participated in the initiation of RIH.

The nuclear protein of  $\beta$ -catenin was involved in the development of the neuropathic pain.<sup>10</sup> When the wnt canonical pathway is activated, the increased amount of  $\beta$ -catenin would migrate into the nucleus and initiate the

transcription of the wnt target genes. To distinguish the nuclear  $\beta$ -catenin level from the overall protein, we detected the level of nuclear  $\beta$ -catenin in the spinal sample. The amount of nuclear  $\beta$ -catenin, like the overall  $\beta$ -catenin presented, was also elevated in the study.

NMDA receptor-dependent synaptic transmission is modulated by activity of GSK-3 $\beta$ .<sup>31</sup> Interestingly, NMDA



**Figure 5 (A, B)** Bands of Western blot for levels of test proteins after Ro25-6981 or iwip-2 administration in remifentanyl-induced hyperalgesia. Values for relative density of the proteins among groups. Protein is expressed relative to  $\beta$ -actin ( $n = 3$ ) or Histone H3 ( $n = 3$ ). Groups allocation: R: Group remifentanyl; C: Group Control; Ro: Group Ro25-6981 plus remifentanyl; iwip: Group iwip-2 plus remifentanyl. \* $P < 0.001$  compared with Group C, <sup>#</sup> $P < 0.001$  compared with Group iwip,  $\Delta$  $P < 0.001$  compared with Group Ro.

antagonist could also present antinociceptive and anti-inflammatory effects through inhibition of GSK-3 $\beta$  activity.<sup>32</sup> However, the amount of the GSK-3 $\beta$  was not changed after remifentanyl exposure in this study. GSK-3 $\beta$  (Ser9) phosphorylation activation was reported to decrease the NR<sub>2</sub>B activity and alleviate RIH after remifentanyl infusion in animals.<sup>33</sup> In this research, both wnt antagonist iwip-2 and NMDA antagonist were found to enhance the phosphorylation of GSK-3 $\beta$  (Ser9) and subsequently lessen the expression of c-fos, NR<sub>2</sub>B and NF- $\kappa$ B after remifentanyl exposure.

Iwip-2 in this study was failed in reducing the over-expression of wnt3a which was otherwise slightly upregulated when exposed to remifentanyl, the result was consistent with the previous research.<sup>34</sup> It means that the overall synthesis of wnt3a would not be altered by the inhibition of wnt3a response.

Compared with group iwip-2, NR<sub>2</sub>B antagonist had better performance in the improvement of the behavioral tests, including higher threshold of thermal hyperalgesia and mechanical allodynia. It meant that except wnt3a/ $\beta$ -catenin pathway, other unknown pathways and confounding factors were also involved in RIH and NR<sub>2</sub>B activation. Further research about wnt3a involvement in the RIH should be performed in the future.

The doses of i.t. iwip-2 in this study was based on Zhang's trial.<sup>10</sup> Unlike the repetitive i.t. administration of 60 $\mu$ M iwip-2 (20 $\mu$ M multiple 3 times) to ameliorate chronic neuropathic pain in that trial, i.t. iwip-2 in this study was given in a single dose. In clinic, remifentanyl was only infused in operations. So it was unrealistic to

repetitive i.t. iwip-2 before or after operations only to prevent RIH. 180 $\mu$ M of iwip-2, rather than 60 $\mu$ M, was proved to be effective in the study.

## Limitation

Although wnt3a antagonist could inhibit the activation of NR<sub>2</sub>B subunit in NMDA receptor, iwip-2 could not have the same excellent antinociceptive effect as the NR<sub>2</sub>B antagonist Ro25-6981 did in the trial. Maybe more doses of iwip-2 could provide equal analgesic effect with the NMDA antagonist. Better understanding of the wnt3a/ $\beta$ -catenin pathway can result in much needed improved treatment strategies for RIH.

## Conclusion

This research highlighted the involvement of wnt3a/ $\beta$ -catenin pathway in RIH. It was announced that spinal wnt3a level was elevated when exposure to remifentanyl. Spinal blocking of wnt3a response has the potential to alleviate pain-associated behavioral parameters, inhibit the upregulation of  $\beta$ -catenin, c-fos, NF- $\kappa$ B and NR<sub>2</sub>B in spinal dorsal horn. The mechanism of the wnt3a inhibitor to reverse RIH might be attributed to downregulation of  $\beta$ -catenin and increase of GSK-3 $\beta$  phosphorylation. Effective blockage of wnt3a signaling pathway provided a basis for exploring the therapeutic potential in RIH.

## Abbreviations

RIH, remifentanyl-induced hyperalgesia; AUC, area under the curve; NMDA, N-Methyl-D-aspartic acid; PWMT,



paw withdrawal mechanical threshold; PWTL, paw withdrawal thermal latency.

## Data Sharing Statement

The raw data required to reproduce these findings can be shared: DOI:10.6084/m9.figshare.11876454 at <http://figshare.com/account/home>.

## Acknowledgments

We would like to thank Fangfang Liu PhD from Peking University for statistical preparation. Prince Henry Asamoah and Husain Alsari from school of international studies of Wenzhou medical university provided substantive suggestions in writing and manuscript editing.

## Author Contributions

Yuan Gao: Conceptualization, Methodology, Formal analysis, Writing and Reviewing. Songyi Zhou: Methodology, Formal analysis, Data curation, Writing. Yizhao Pan: Formal analysis, Data curation. Lijun Gu: Formal analysis, Investigation. Yuting He: Methodology, Supervision. Jiehao sun: Conceptualization, Methodology, Software, Writing, Reviewing, Editing, Funding acquisition. All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

## Funding

This work was supported by the Chinese National Natural Science Funding [81701094]; Zhejiang Provincial Natural Science Funding [LY20H090008] and Wenzhou Science and Technology Bureau [Y20180121].

## Disclosure

All of the authors have seen the original study data, reviewed the analysis of the data, and approved the final manuscript. The authors report no conflicts of interest in this work.

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