

## **Galanin Receptor 1 Plays an Antinociceptive Effect via Inhibiting PKA Activation in the Nucleus Accumbens of Rats with Neuropathic Pain**

Ying ZHANG<sup>1#</sup>, Yi GAO<sup>2#</sup>, Chong-Yang LI<sup>3</sup>, Wei DONG<sup>4</sup>, Yan DONG<sup>1</sup>, Meng-Nan LI<sup>1</sup>, Ya-Nan LIU<sup>1</sup>, Shi-Lian XU<sup>1\*</sup>

<sup>1</sup>Department of Physiology, School of Basic Medicine, Kunming Medical University, Kunming, Yunnan, China

<sup>2</sup>Department of Colorectal Cancer Surgery, Third Affiliated Hospital, Kunming Medical University, Kunming, Yunnan, China

<sup>3</sup>Department of Tumor, Fourth Affiliated Hospital, Kunming Medical University, Kunming, Yunnan Province, China

<sup>4</sup>Department of Orthopedics, Third Affiliated Hospital, Yunnan Traditional Chinese Medical College, Kunming, Yunnan Province, China

### **\*Correspondence**

Shi-Lian XU, Department of Physiology, School of Basic Medicine, Kunming Medical University, Kunming, Yunnan Province 650500, China.

Tel.: 86-13099911387

E-mail address: [shilianxu@126.com](mailto:shilianxu@126.com) (S.L. XU).

<sup>#</sup>Ying ZHANG and Yi GAO contributed equally to this work.

**Short title:** The antinociception of GalR1 in neuralgia via inhibiting PKA.

## **Summary**

Galanin and galanin receptors (GalRs) have been reported to be involved in the transmission and modulation of nociceptive information in the central nervous system (CNS). However, the underlying mechanism of the antinociception of GalRs in neuropathic pain remains unclear. This study investigated the antinociception induced by galanin receptor 1 (GalR1) via protein kinase A (PKA) signalling pathway in the nucleus accumbens (NAc) of rats with neuropathic pain. A mononeuropathy model was replicated by ligation of the left sciatic nerve, following which the expression of phospho-PKA (p-PKA) in the NAc were markedly up-regulated at 14<sup>th</sup> and 28<sup>th</sup> day after ligation of sciatic nerve, and p-PKA expression was down-regulated by intra-NAc injection of GalR1 agonist M617, but the GalR1 antagonist M35 did not have an effect. We also found that M35 in the NAc blocked the M617-induced increase in the hindpaw withdrawal latencies (HWLs) of rats with mononeuropathy, but M35 alone had no effect on HWLs, and PKA inhibitor H-89 attenuated the M617-induced an increase in the HWLs. These results suggested that GalR1 induced an antinociception via inhibiting PKA activation, implying that GalR agonists may be potential and potent therapeutic options to treat chronic neuropathic pain.

## **Keywords**

Antinociception • galanin receptor 1 • protein kinase A • neuropathic pain • nucleus accumbens

## Introduction

Neuropathic pain caused by damage or disease is very common in the clinic, but its complex pathogenesis has hampered the effective treatment of neuropathic pain (Costigan *et al.* 2009). The development of current therapeutics that have been focusing on neuronal targets, including drugs such as opioid analgesics, NMDA receptor antagonists and sodium channel blockers (Dworkin *et al.* 2003). These drugs produce a brief pain relief in some patients, however, the side effects of these drugs also are notably, such as sedation, nausea and dizziness, as well as development of analgesic tolerance and addiction after opioid treatment, these side effects have greatly limited their universal use (Dworkin and Turk, 2011; Ho and Siau, 2009). Therefore, we need to find a safer and more effective analgesic.

Galanin was first separated from the porcine intestine. The function of galanin has been linked to the activation of specific galanin receptors (GalRs). Three galanin receptors (GalR1-3) have been identified (Branchek *et al.* 2000). The signalling pathways of three GalRs are essentially different (Lang *et al.* 2015). Our earlier research demonstrated that M617, a specific GalR1 agonist, has an antinociceptive effect in rats with neuropathic pain (Duan *et al.* 2015), indicating that GalR1 activation induced an antinociception effect. However, the signalling pathways mechanism of the antinociception of GalR1 in neuropathic pain is unclear.

Intracellular protein kinases play a significant role in the processing and development of chronic pain. Some studies have demonstrated that protein kinase A (PKA) activation plays an important functional role in the processing of nociceptive information (Hang *et al.* 2012; Hang *et al.* 2013; Zhu *et al.* 2014). This study investigated the antinociception induced by GalR1 via PKA signalling pathway in the rats with neuropathic pain.

The nucleus accumbens (NAc) is divided into two regions: the core and shell, which are

known to be the brain regions involved in pain modulation (Salgado and Kaplitt. 2015). Gear et al. reported that NAc acts an important effect in mediating the suppression of tonic or persistent pain (Gear and Levine. 2011). Infusion of N-acetylaspartylglutamate into the NAc significantly attenuated the pain induced by the activation of sensory nerves through optical stimulation (Watanabe *et al.* 2018). Previous studies in our laboratory have demonstrated that the expression of GalR1 was upregulated in the NAc of rats with inflammation or sciatic nerve ligation, intra-NAc injection of galanin or GalR1 agonist M617 induced dose-dependent increase on hindpaw withdrawal latencies (HWLs) to noxious thermal and mechanical stimulations in rats, suggesting galanin and GalR1 are involved in antinociceptive effects in the NAc of rats (Xu *et al.* 2012; Duan *et al.* 2015; Yang *et al.* 2015). However, the interaction mechanism is unclear between galanin and GalRs as well as downstream signal molecule in pain modulation in the NAc. In this study, we intended to investigate the involvement of PKA in the GalR1-induced antinociception in the NAc of rats with neuropathic pain.

## **Methods**

### *Animals*

Male Sprague-Dawley rats (weighing 180-250 g) were obtained from the experimental Animal Center of Kunming Medical University (Kunming, Yunnan, China). Rats were housed in cages with water and food available and libitum in a temperature-controlled ( $22 \pm 1$  °C) room with a normal day/night cycle. Experimental procedures were in strict accordance with the European Guidelines on Laboratory Animal Care.

### *Chemicals*

M617 (galanin(1–13)-Gln(14)-bradykinin(2–9)amide), M35 (galanin(1–13)-bradykinin(2–9)amide) and H-89 dihydrochloride ( $C_{20}H_{20}BrN_3O_2S \cdot 2HCl$ ) were purchased from Sigma-Aldrich

Inc. (St. Louis, MO, USA). M617 and M35 were dissolved in physiologic saline, H-89 was dissolved in 1% dimethyl sulfoxide (DMSO). The solutions were injected into the NAc at a total volume of one microliter.

#### *Neuropathic pain animal model*

The sciatic nerve ligation procedure for eliciting neuropathic pain was carried out as described previously (Bennett and Xie. 1988). Briefly, a rat was anaesthetized intraperitoneally with sodium pentobarbital (50 mg/kg), and the left sciatic nerve was exposed for approximately 1 centimetre at the level proximal to the sciatic trifurcation. Then, a total of 4 knots approximately 1 mm apart were loosely ligated with 4.0 chromic gut sutures.

In the sham control group, the same operation protocols were used without ligation of the sciatic nerve. In both groups, the contralateral limb remained un-operated.

#### *Behavioural test*

The hindpaw withdrawal latency (HWL) responses to noxious mechanical and thermal stimuli were tested as previously described (Xu *et al.* 2012; Yang *et al.* 2015; Zhang *et al.* 2017). A hot plate (Type YLS-6B, China) was used to test HWLs to noxious thermal stimulation. The temperature of the hot plate was kept within  $52\pm0.2^{\circ}\text{C}$ . The radiant heat source was focused on the planta of the hindpaw, and the HWL was recorded.

The Randall Selitto Test (Ugo Basile, Type 37215, Italy) was used to evaluate the HWL to noxious mechanical stimulation. A pusher was applied to the dorsal surface of the rat's hindpaw at a loading rate of 30 g/s, and the threshold required to initiate the struggle response was used as the HWL to mechanical stimulation.

Rats were individually acclimated to thermal and mechanical stimulation for 4-5 days before sciatic nerve ligation, and the HWLs were normally between 3-6 seconds. The HWL was

detected three times before intra-NAc injection to get an average value as the base HWL. The HWL was detected at 5<sup>th</sup>, 10<sup>th</sup>, 15<sup>th</sup>, 20<sup>th</sup>, 30<sup>th</sup>, 45<sup>th</sup> and 60<sup>th</sup> minute after intra-NAc injection, and each HWL was recorded as a percentage change from the basal HWL.

#### *Intra-NAc injection*

The rat was anesthetized using sodium pentobarbital (50 mg/kg, intraperitoneal injection) and placed in a stereotaxic instrument. A stainless steel guide cannula with outer diameter of 0.8 mm was penetrated to the NAc (anterior to Bregma: 1.7 mm; left or right to midline: 1.6 mm; ventral: 7.0 mm).

Rats recovered from the operation after 3 days. A Stainless steel needle with outer diameter of 0.4 mm was inserted into guide cannula and deepened 1.5 mm to the cannula. One microliter of solution was injected into the NAc.

#### *Western bolt assay*

Rats were anaesthetized with 5% isoflurane and euthanized. Then, the NAc tissue was promptly excised. The Western bolt assay procedure was performed as described previously (Duan *et al.* 2015; Yang *et al.* 2015). Primary antibodies against the following proteins were used: total-PKA (t-PKA, rabbit polyclonal anti-PKA alpha+beta, 1:200, Abcam), and phospho-PKA (p-PKA, rabbit polyclonal anti-PKA alpha+beta phospho, 1:300, Abcam). The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH, rabbit monoclonal, 1:5000, CellSignal) was also determined as an internal control. The bands were exposed to Fuji Medical X-Ray Film (Fuji Photo Film Co., Ltd, Karagawa, Japan) and the relative band density was detected by Image J software. The Western blot of the protein samples from the NAc of each experimental rat was repeated at least 3 times.

#### *Statistical analysis*

At the end of the experiments, the location of the needle tip was confirmed. As shown in

Fig. 1, only the results from rats whose needle tip was within the NAc were used for statistical analysis. Data were expressed as the mean  $\pm$  S.E.M. and analysed using GraphPad Prism 5 software. The statistical analyses were calculated using one-way analysis of variance test (ANOVA) followed by Tukey's multiple comparison tests or two-way repeated measure ANOVA followed by Bonferroni post-tests or Student's two tailed t-test. The p values  $< 0.05$  were considered to indicate a significant difference.

## Results

### *Up-regulated p-PKA expression in the NAc in rats with mononeuropathy*

At 7<sup>th</sup>, 14<sup>th</sup> and 28<sup>th</sup> day after sciatic nerve ligation, the NAc tissues were excised, and Western blot was performed to test the expression of p-PKA. The results showed that the expression of p-PKA was significantly up-regulated at 14<sup>th</sup> day (n=3; q=7.930; p<0.01) and 28<sup>th</sup> day (n=3; q=5.549; p<0.05) to differ from the data of sham group, but the difference in the expression of p-PKA was not significant at 7<sup>th</sup> day (n=3; q=3.701; p>0.05) after left sciatic nerve ligation, suggesting that the PKA signalling pathway may play a significant role in the transmission and modulation of neuropathic pain. The differences were determined by one-way ANOVA followed by Tukey's multiple comparison tests, as shown in Fig. 2.

### *M617 down-regulated p-PKA expression, and M35 had no effect on p-PKA expression in the NAc of neuropathic pain rats*

Our studies demonstrated that M617 had an antinociceptive effect for neuropathic pain in the NAc (Duan et al. 2015). This study investigated the involvement of the PKA signalling pathway in the GalR1 activation-induced analgesic effect in the NAc of neuropathic pain rats. At 14<sup>th</sup> day after sciatic nerve ligation, three groups of rats received an intra-NAc injection of 1 nmol M617 or 1 nmol M35 or 1  $\mu$ l saline, respectively. The NAc tissues were separated at 15 min after the injection,

and the expression of p-PKA was examined by Western blot. As shown in Fig. 3, the expression of p-PKA was significantly down-regulated after intra-NAc administration of M617 ( $n=3$ ,  $q=6.817$ ,  $p<0.01$ ), whereas intra-NAc administration of M35 did not have a significant effect ( $n=3$ ,  $q=0.9851$ ,  $p>0.05$ ). The differences were analysed by one-way ANOVA followed by Tukey's multiple comparison tests. The results showed that GalR1 plays an antinociceptive effect via inhibition of PKA activation in the NAc of rats with mononeuropathy, but blocking GalR1 had no effect on p-PKA expression, suggesting that there is other type of galanin receptor or signalling pathway related to antinociceptive effect via inhibiting PKA activation. M35 up-regulated p-PKA was counteracted by other type of galanin receptor activation or other signalling pathway down-regulated p-PKA.

*M35 in the NAc blocked the M617-induced increase in the HWLs of rats with mononeuropathy, but M35 alone had no effect on HWLs*

In order to study the effect of M35 further, seven rats were given an intra-NAc injection of 1  $\mu$ l of 0.9% saline, followed 5 min later by intra-NAc injection of 1 nmol of M35, as shown in Fig. 4, M35 alone had no effect on HWLs. Furthermore, two groups of rats ( $n=7$ ) received intra-NAc injection of 1 nmol M617, followed 5 min later by intra-NAc injection of 1 nmol of M35 or saline. Compared to the HWLs in the saline control group, the M617-induced increases in the HWLs to thermal (left hindpaw:  $F=40.59$ ,  $P<0.001$ ; right hindpaw:  $F=23.79$ ,  $P<0.001$ ) and mechanical (left hindpaw:  $F=69.91$ ,  $P<0.001$ ; right hindpaw:  $F=45.63$ ,  $P<0.001$ ) stimulation were blocked by M35, as shown in Fig. 4. The result showed that M35 in the NAc blocked the M617-induced increase in the HWLs of rats with mononeuropathy, but M35 alone had no effect on HWLs, which is consistent with M35 had no effect on p-PKA expression. The difference was analysed by two-way repeated measures ANOVA followed by Bonferroni post-tests.

*PKA inhibitor H-89 increased HWLs and attenuated the M617-induced an increase in the HWLs in*



In order to study the effect of intra-NAc injection of PKA inhibitor, seven rats were given an intra-NAc injection H-89 alone. As shown in Fig. 5, compared with the HWLs of the DMSO control group (n=7), the HWLs to thermal (left hindpaw:  $F=52.86$ ,  $P<0.001$ ; right hindpaw:  $F=31.24$ ,  $P<0.001$ ) and mechanical (left hindpaw:  $F=47.11$ ,  $P<0.001$ ; right hindpaw:  $F=61.44$ ,  $P<0.001$ ) stimulation were increased after intra-NAc injection 6  $\mu\text{g}$  of H-89, the differences were analysed by repeated measures two-way ANOVA followed by Bonferroni post-tests. This result suggested that the PKA inhibitor H-89 in the NAc eased pain-related behaviour in rats with neuropathic pain.

To further demonstrate that PKA is a downstream signalling molecule of GalR1, we injected the GalR1 agonist M617 to activate GalR1 to induce increases in HWLs first, then we injected PKA inhibitor H-89 to test whether the antinociceptive effect of M617 would be attenuated injection of H-89. Four groups of rats received intra-NAc injection of 1 nmol M617, followed 5 min later by intra-NAc injection of 1,3,6  $\mu\text{g}$  of the PKA inhibitor H-89 or DMSO as control group (n=6). The differences were analysed by repeated measures two-way ANOVA followed by Bonferroni post-tests. As shown in Fig. 5A and 5B, compared with the control group, the M617-induced increases in the HWLs to thermal stimulation were attenuated by intra-NAc injection of 3  $\mu\text{g}$  (left hindpaw:  $F=10.99$ ,  $P<0.01$ ; right hindpaw:  $F=3.2$ ,  $P=0.10$ ) and 6  $\mu\text{g}$  (left hindpaw:  $F=14.88$ ,  $P<0.01$ ; right hindpaw:  $F=8.83$ ,  $P<0.05$ ), but not 1  $\mu\text{g}$  H-89 (left hindpaw:  $F=2.94$ ,  $P=0.11$ ; right hindpaw:  $F=0.18$ ,  $P=0.68$ ). In addition, as shown in Fig. 5C and 5D, intra-NAc injection of 3  $\mu\text{g}$  (left hindpaw:  $F=9.96$ ,  $P<0.01$ ; right hindpaw:  $F=5.94$ ,  $P<0.05$ ) and 6  $\mu\text{g}$  (left hindpaw:  $F=13.14$ ,  $P<0.01$ ; right hindpaw:  $F=8.28$ ,  $P<0.05$ ) H-89 dose-dependently attenuated the antinociceptive effect induced by M617 with mechanical stimulation, but there was a non-significant effect after injection of 1  $\mu\text{g}$  H-89 (left hindpaw:  $F=1.79$ ,  $P=0.21$ ; right hindpaw:  $F=0.94$ ,  $P=0.35$ ), suggesting that the PKA inhibitor H-89

attenuated the M617-induced an increase in the HWLs in NAc of rats with mononeuropathy. These results also showed that GalR1 plays an antinociceptive effect via inhibition of PKA activation in the NAc of rats with mononeuropathy.

## **Discussion**

Previous studies have shown that galanin is involved in the processing and development of nociceptive information in the CNS (Xu *et al.* 2012; Yang *et al.* 2015; Zhang *et al.* 2017). Galanin plays its roles via three GalRs: GalR1, -R2 and -R3, all of them are G-protein coupled receptors (Webling *et al.* 2012; Lang *et al.* 2015). Studies have proven that intra-central nucleus of the amygdala (Li *et al.* 2012) and intra-cerebroventricular (Fu *et al.* 2011) injection of M617, a GalR1-specific agonist (Lundström *et al.* 2005), induced an analgesic effect in intact rats. Our previous study also demonstrated that GalR1 expression was up-regulated, and M617 in the NAc induced an increase in the HWLs of rats with neuropathic pain (Duan *et al.* 2015), but its underlying mechanism is unclear.

PKA is widely distributed in the CNS. Some studies have demonstrated that the activation of the PKA signalling pathway is associated with the processing and development of nociceptive information. Intrathecal injection of the PKA inhibitor H-89 relieved the pain behaviour in rats with bone cancer-induced pain (Huang *et al.* 2012; Zhu *et al.* 2014). Additionally, electrophysiological experiment has also proven that the PKA signalling pathway was involved in modulating neuronal excitability in response to acute or chronic noxious stimulation in single DRG or spinal cord neurons (Huang *et al.* 2012). In the present study, the results showed that p-PKA expression in the NAc was up-regulated significantly at 14<sup>th</sup> and 28<sup>th</sup> day after sciatic nerve ligation in rats, suggesting an involvement of PKA in the transmission and modulation of neuropathic pain in the NAc of rats. Therefore, we wanted to know whether the PKA pathway was involved in the

analgesic effect of GalR1 in the NAc of rats with neuropathic pain.

Interestingly, p-PKA expression was down-regulated by intra-NAc administration of GalR1 agonist M617, whereas there was no significant difference in p-PKA expression after intra-NAc administration of M35, a general antagonist of GalR1 and GalR2 with an 18-fold selectivity for GalR1 over GalR2 in vitro (Webling *et al.* 2012). These results implied that GalR1 activation might play important roles in antinociception via PKA inhibition in the NAc of rats with mononeuropathy. In order to study this effect further, rats received intra-NAc injection of M617, followed 5 min later by intra-NAc injection of PKA inhibitor H-89, the results showed that H-89 attenuated the M617-induced increase in the HWLs in NAc of rats with mononeuropathy, however intra-NAc injection of PKA inhibitor H-89 alone induced an increase in HWLs. Finally, we also found that M35 blocked the M617-induced increase in the HWLs of rats with mononeuropathy, but M35 alone had no effect on HWL, these results suggested GalR1 plays an antinociceptive effect via inhibition of PKA activation in the NAc of rats with mononeuropathy, but may suggest that there is other type of galanin receptor or other signal transduction pathway related to antinociceptive effect via inhibiting PKA activation.

In conclusion, this study showed that the involvement of PKA in neuropathic pain, GalR1 activation results in the inhibition of the PKA, leading to antinociception for neuropathic pain in the NAc of rats. This finding implied that GalR1 agonist could be potential and potent therapeutic option for neuropathic pain.

### **Conflict of Interests**

None declared.

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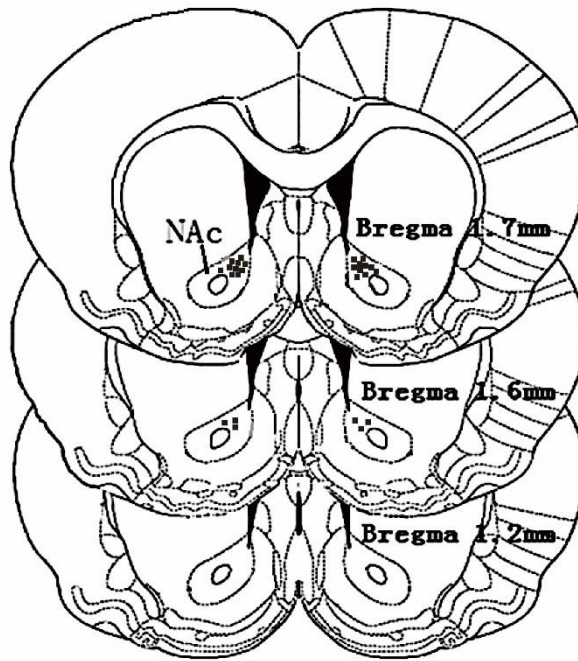
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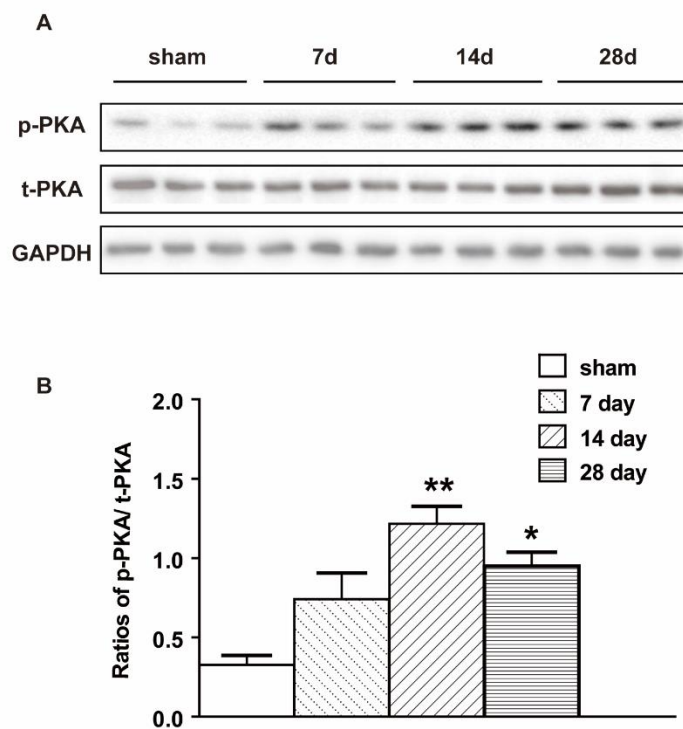
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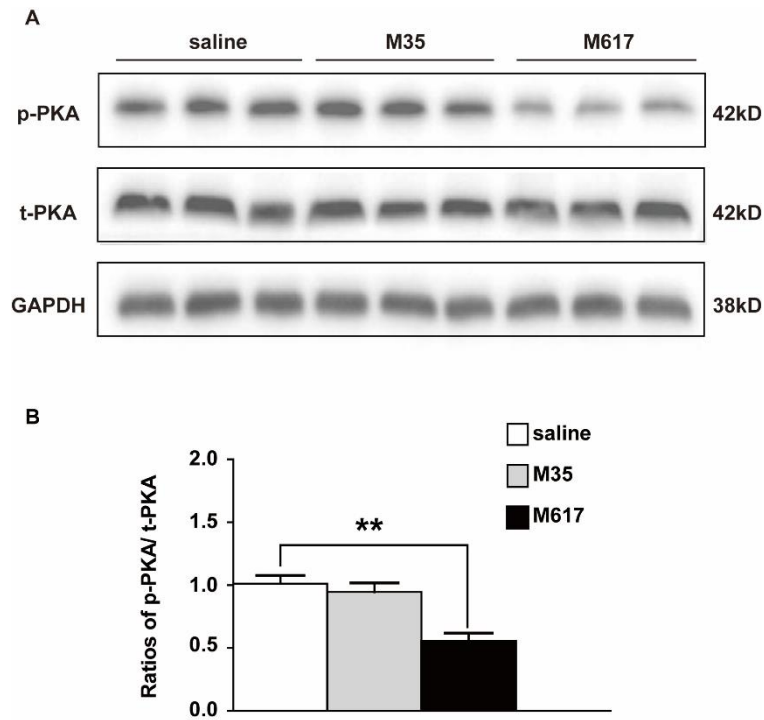


**Fig. 1** Illustration of the location of the injection needle tips



**Fig. 2** The expression of p-PKA in the NAc in neuropathic pain rats

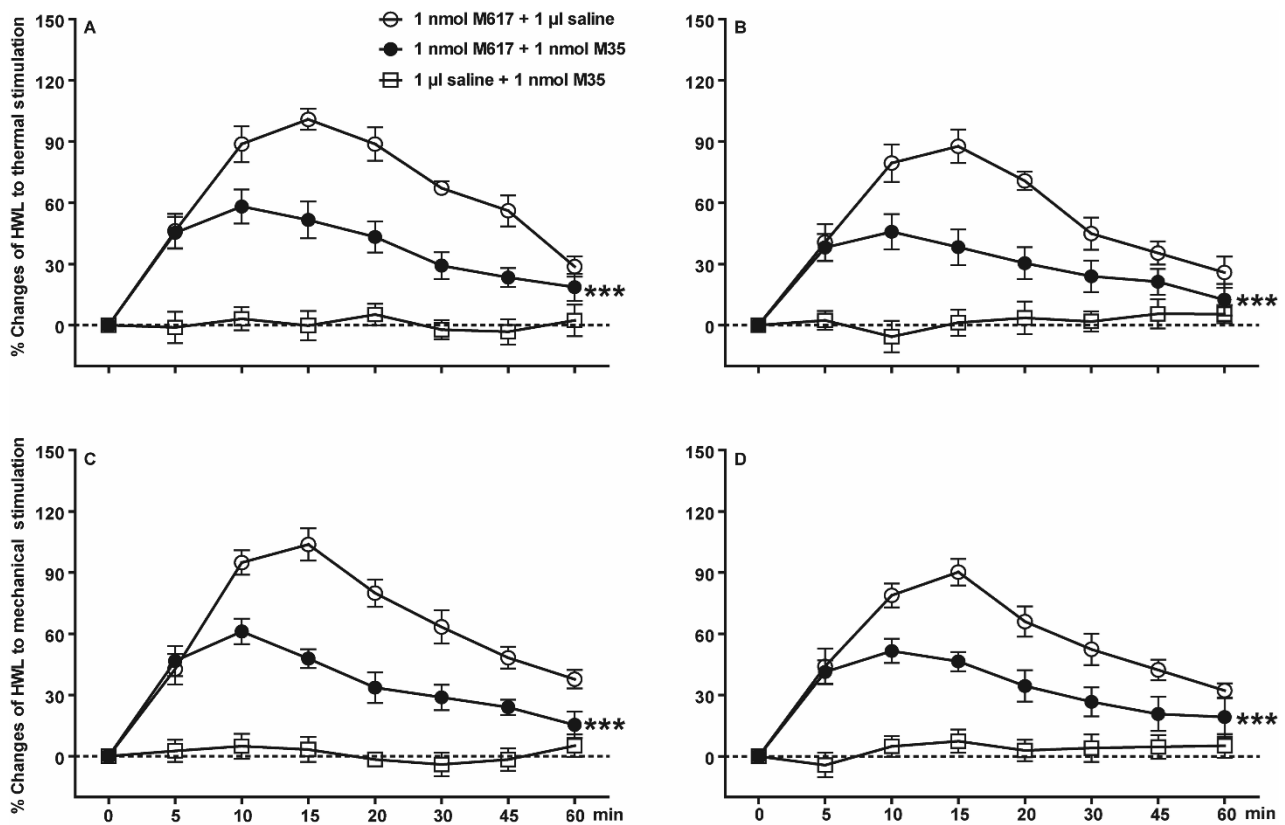
A: Representative Western blots of p-PKA, t-PKA and GAPDH in the sham control group and left sciatic nerve-ligated group. B: Histograms show the ratios of p-PKA/t-PKA. Data are presented as the mean  $\pm$  S.E.M. \* $P < 0.05$ , \*\* $P < 0.01$  compared with the control group.



**Fig. 3 Effects of M617 and M35 on p-PKA expression in the NAc of neuropathic pain rats**

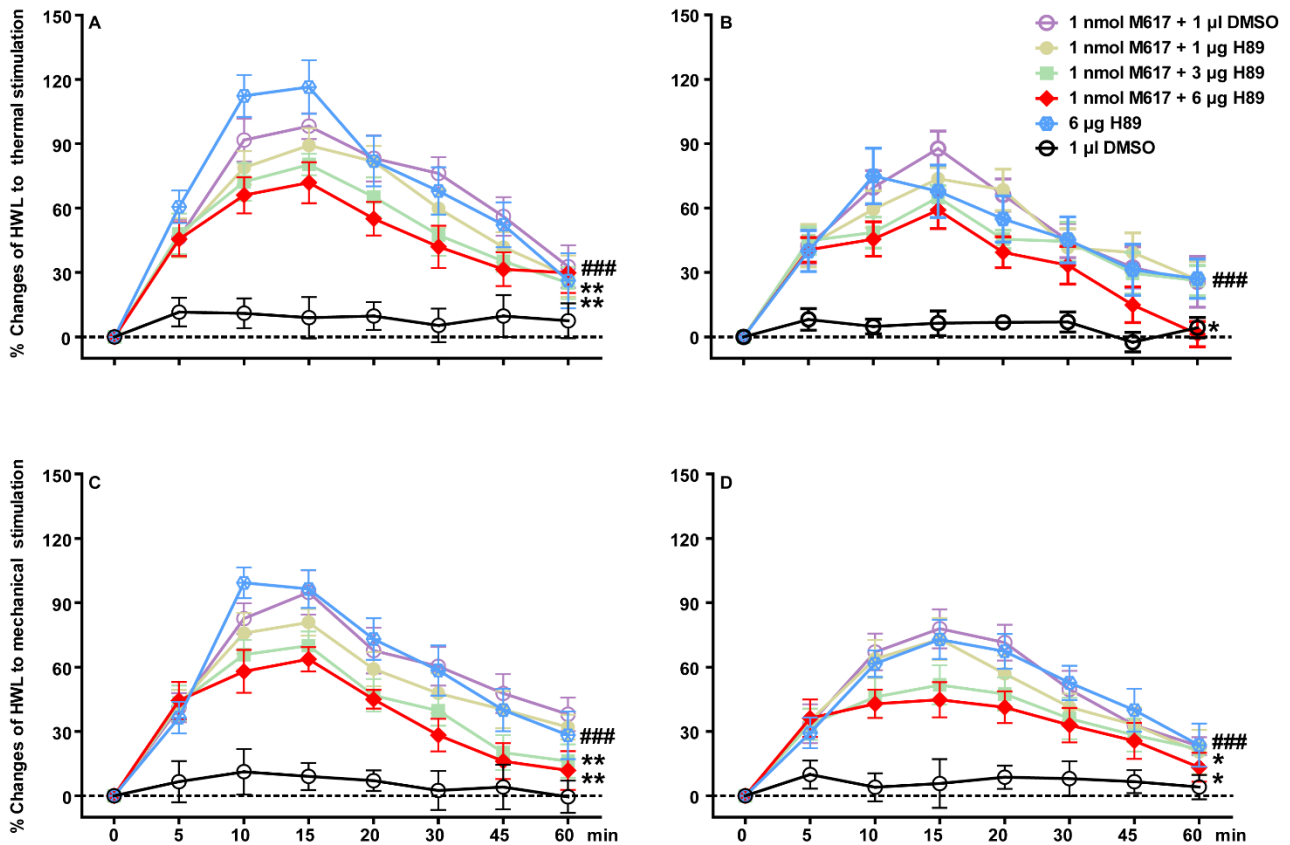
A: Representative Western blots of p-PKA, t-PKA and GAPDH in the saline control group and intra-NAc M617- and M35-injected groups. B: Histograms show the ratios of p-PKA/t-PKA. Data are presented as the mean  $\pm$  S.E.M. \*\* $p < 0.01$  compared with the sham group.





**Fig. 4 Effects of intra-NAc injection of M35 on the M617-induced antinociception in neuropathic pain rats**

A and B: % change in HWL to thermal stimulation; C and D: % change in HWL to mechanical stimulation; A and C: Left HWL; B and D: right HWL. Time=0 min: intra-NAc injection of 1 nmol M617; time=5 min: intra-NAc injection of 1 nmol M35 or 1 µl 0.9% saline as the control group. Data are presented as the mean  $\pm$  S.E.M. \*\*\*P<0.001, compared with the control group.



**Fig 5 Effects of intra-NAc injection of H-89 on M617-induced antinociception in rats with neuropathic pain**

NAc: nucleus accumbens; HWL: hindpaw withdrawal latency. A and B: %changes of HWL to thermal stimulation; C and D: %changes of HWL to mechanical stimulation; A and C: Left HWL; B and D: right HWL. Data are presented as mean  $\pm$  S.E.M. \* $P < 0.05$ , \*\* $P < 0.01$ , intra-NAc injection of 1 nmol M617, followed 5 min later by intra-NAc injection of 1,3,6  $\mu$ g of the PKA inhibitor H-89 or 1% DMSO as the control group, time=0 min: intra-NAc injection of 1 nmol of M617, time=5 min: intra-NAc injection of 1, 3, 6  $\mu$ g of H-89 or 1  $\mu$ l of DMSO. ### $P < 0.001$ , intra-NAc injection H-89 alone versus intra-NAc injection DMSO alone.