**Article Type: Original Article**

**Title page**

# Propofol attenuates postoperative hyperalgesia via regulating spinal GluN2B-p38MAPK/EPAC1 pathway in an animal model of postoperative pain

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**Short title**: Propofol inhibits postoperative pain

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**Conflicting interest**: The authors declare no conflicts of interest.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/ejp.1349

**ABSTRACT**

**BACKGROUND** Total intravenous anaesthesia with propofol has been shown to reduce postoperative pain in some clinical studies, but knowledge of its underlying analgesic mechanism remains limited. In this study, we compared the analgesic effects of propofol versus isoflurane in an animal model of postoperative pain and evaluated its underlying molecular mechanisms.

**METHODS** Plantar incision was made in the hind paws of rats under general anaesthesia with 2.5% of inhalational isoflurane (isoflurane group) or intravenous infusion of propofol (1.5 mg $kg^{-1}$ $min^{-1}$, propofol group). Mechanical allodynia was assessed by paw withdrawal threshold before and after incision. Spinal dorsal horns (L3-L5) were harvested 1 h after incision to assess the level of phosphorylated GluN2B, p38MAPK, ERK, JNK and EPAC using Western blot and immunofluorescence.

**RESULTS** Mechanical allodynia induced by plantar incision peaked at 1 hour and lasted for 3 days after incision. It was significantly less in propofol group compared with isoflurane group in the first 2 hours following incision. The incision-induced increases in phosphorylated GluN2B, p38MAPK, and EPAC1 were significantly reduced in propofol group. The number of spinal dorsal neurons co-expressed with EPAC1 and c-Fos after the incision was significantly lower in the propofol group.

**CONCLUSION** Propofol reduced pain responses in an animal model of postoperative pain and suppressed the spinal GluN2B-p38MAPK/EPAC1 signalling pathway. Since the p38MAPK/EPAC pathway plays a critical role in the development of postoperative hyperalgesia, our results provide evidence-based behavioural, molecular and cellular mechanisms for the analgesic effects of propofol when used for general anaesthesia.

**SIGNIFICANCE** These findings may provide a new mechanism for the postsurgical analgesic effect of propofol, which is particularly interesting during the subacute period after surgery as it is the critical period for the development of persistent postsurgical pain.

**Key Words:** Propofol, analgesia, postoperative pain, GluN2B, p38 MAPK, EPAC

## 1. Introduction (628 words)

Management of postoperative pain can be a challenge. Evidence suggests that more than half of patients following surgical procedures report inadequate postoperative pain relief (Chou *et al.*, 2016) and 10-50% of these patients experience persistent postsurgical pain (Kehlet *et al.*, 2006). Propofol is a commonly used general anaesthetic for induction and maintenance of general anaesthesia. Total intravenous anaesthesia (TIVA) with propofol has been shown to reduce acute postoperative pain after surgery compared with inhalational anaesthesia in some clinical studies (Chan *et al.*, 2016; Qiu *et al.*, 2016). However, this is not a consistent finding, and other clinical studies have shown no difference in postoperative analgesia (Fassoulaki *et al.*, 2008; Pokkinen *et al.*, 2014). Use of propofol as an adjunctive analgesic remains controversial and the underlying mechanisms of propofol in postoperative pain relief are largely unknown.

After surgery, patients develop acute postoperative pain that is characterized by mechanical hypersensitivity (pain increased by ambulation, cough or light touch on the surgical wound). N-methyl-D-aspartate (NMDA) receptors expressed widely in the central nervous system play an important role in the generation and maintenance of central sensitization that triggers the development of hyperalgesia and allodynia (Kim *et al.*, 2012; Tong & MacDermott, 2014). NMDA receptors include GluN1 and GluN2 subunits, and the latter consists of four types of subunits: GluN2A, 2B, 2C, and 2D. GluN2B subunit, mainly expressed at synapses of lamina I in the spinal dorsal horn, plays a key role in nociceptive signal transmission (Kim *et al.*, 2012; Tong & MacDermott, 2014). Our recent study in an animal model of formalin-induced inflammatory pain demonstrated that pre-emptive administration of propofol produced preventive analgesic effects on inflammatory pain by regulating neuronal GluN2B-containing NMDA receptor and extracellular signal-regulated kinase1/2 (ERK1/2) pathway in the spinal dorsal horn (Qiu *et al.*, 2017). MAPK (mitogen-activated protein kinase) family, including ERK1/2, p38 MAPK, and c-Jun N-terminal kinase (JNK), transduces a wide range of extracellular stimuli into various intracellular responses by both transcriptional and non-transcriptional regulations (Ji *et al.*, 2009). In neurons, calcium influx through NMDA receptors leads to activation of MAPK pathways (Takeda & Ichijo, 2002) and activation of MAPKs in spinal neurons is involved in the induction and maintenance of pain hypersensitivity under different pain conditions (Ji *et al.*, 2009).

Interestingly, a recent study reported that phosphorylation of p38MAPK (p-p38MAPK) induced by plantar incision contributed to the development of nociceptive hypersensitivity by increasing the expression of exchange protein directly activated by cyclic adenosine monophosphate (EPAC) in dorsal root ganglions (DRGs) (Matsuda *et al.*, 2017). EPAC is a newly identified cAMP target proteins that, as required effectors, play a critical role in the development of inflammatory and postoperative hyperalgesia (Hucho *et al.*, 2005; Singhmar *et al.*, 2016; Matsuda *et al.*, 2017). Intraplantar injection of EPAC agonist led to long-lasting mechanical hyperalgesia (Hucho *et al.*, 2005). Inhibition of p-p38MAPK during surgery not only prevented the induction of neuronal EPAC expression and EPAC-induced nociceptor sensitization, but also blocked the development of pain hypersensitivity sustained during the subacute postsurgical period (Matsuda *et al.*, 2017). Increased neuronal EPAC expression driven by activation of p38MAPK was a key component leading to long-lasting nociceptive hypersensitivity after plantar incision (Matsuda *et al.*, 2017) or inflammation (Wang *et al.*, 2013). However the upstream molecules that activate the p38MAPK/EPAC cascade in postoperative hyperalgesia remain unknown. Investigating how propofol regulates the neuronal p38MAPK/EPAC pathway in the spinal dorsal horn after incision will enhance our understanding of propofol's analgesic mechanism for postoperative pain.

In this study, we aimed to compare the effects of propofol infusion versus inhalational isoflurane on GluN2B subunit-containing NMDA receptor and downstream MAPK/EPAC pathway in a plantar incision induced postoperative pain model in rats. We hypothesized that propofol would exert anti-nociceptive effects after operation by regulating the GluN2B /MAPK/EPAC cascade at the spinal dorsal horn level.

## 2. Materials and Methods (1185 words)

### 2.1 Animal Preparation

Male adult Sprague Dawley rats weighing approximately 250-300 g were housed in cages with *ad libitum* food and water on a standard 12:12 h light/dark cycle. Animal experiments were conducted under the guide from the Committee on the Use of Live Animals in Teaching and Research from the University of Hong Kong (CULATR No. 3383-14).

### 2.2 Experimental Design

Rats were randomly allocated into three groups using an online software (www.randomization.com), namely naïve group (without any treatment, 3 cohorts of n=5), isoflurane group (receiving plantar incision under general anaesthesia with 2.5% of isoflurane inhalation, Abbott Laboratories, Berkshire, UK, 3 cohorts of n=5), and propofol group (receiving plantar incision under general anaesthesia with intravenous infusion of propofol via an implanted catheter into the lateral tail vein with an infusion rate of 1.5 mg $kg^{-1}$ $min^{-1}$, B. Braun, Melsungen, Germany, 3 cohorts of n=5). The dose of propofol used in animals was equivalent to a human dose of 0.2 mg $kg^{-1}$ $min^{-1}$ for maintenance of general anaesthesia according to the guide for dose conversion between animals and human (Nair & Jacob, 2016). General anaesthesia was conducted with either 2.5% of isoflurane (Isoflurane group) or 1.5 mg $kg^{-1}$ $min^{-1}$ of propofol (Propofol group) for 30 min and plantar incision was performed under general anaesthesia.

Half an hour of recovery time was allowed after plantar incision, when all the rats meet the recovery criteria (i.e. recovery of the righting reflex) (Li *et al.*, 2012). Mechanical allodynia of both contralateral and ipsilateral paws was measured before and up to 72 h after planter incision using mechanical threshold by a research assistant who was not involved in providing general anaesthesia and performing plantar incision (i.e. blinded to the type of general anaesthesia). Mechanical allodynia was recorded up to 3 days after plantar incision because the reduction of paw withdrawal threshold (PWT) caused by plantar incision showed no significant difference beyond 3 days as reported by previous studies (Brennan *et al.*, 1996; Whiteside *et al.*, 2004). The detailed experimental design is illustrated in Figure 1.

### 2.3 Plantar Incision

Plantar incision was used as a postoperative pain model. The procedure was performed as previously described (Brennan *et al.*, 1996). Briefly, under general anaesthesia with either isoflurane or propofol, the plantar aspect of right hind paw was disinfected with 10% povidone-iodine solution followed by 75% of ethanol. A 1-cm longitudinal incision was made with a No. 11 blade, starting at 0.5 cm far from the end of the talus bone and extending to the toe, through skin and fascia of plantar aspect of the paw. The flexor digitorum brevis

muscle was elevated and incised twice. The muscle origin and insertion remained intact. The skin was closed with suture (5-0 Nylon). The procedure of plantar incision was completed within 30 min. After surgery, the rats were kept in temperature-supported cages for 30 min to recover from anaesthesia, which was ensured by the recovery of the righting reflex described previously (Li *et al.*, 2012).

### 2.4 Assessment of Mechanical Allodynia

Mechanical allodynia was assessed by measuring the mechanical threshold, which was a response of an animal to an innocuous mechanical stimulus. Mechanical threshold was performed by testing the right hind paw withdrawal response to von Frey filaments of the Electrovonfrey apparatus (IITC Life Science, Woodland Hills, CA, USA). Rats were held on a metal mesh floor with a transparent plastic dome, and the plantar surface of the paws could be touched through the metal mesh. Each rat was accommodated to the environment 30 min before the test. During the assessment, a von Frey filament was pressed perpendicularly against the center of hind paw's plantar surface with continuous force. The withdrawing of hind paw within 6~8 sec represented a positive response. Any hind paw movement caused by locomotion was not recorded as a positive response. If the rat failed to withdraw its paw, then a stiffer filament was used until a positive response was observed. In this study, the range of probe was from 0.4 g to 74 g. The force (in gram) was shown on the screen of the Electrovonfrey apparatus which was recorded in each positive response. The assessment was repeated 3 times for each rat at approximately 1-min intervals. The average values of the three repetitions were recorded as the final mechanical threshold.

### 2.5 Western Blot

Lumbar spinal dorsal horns from L3-L5 segments were collected 1 h after surgery. The tissue was homogenized in ice-cold Laemmli buffer (50 mM Tris-HCl, pH7.5, 0.5% SDS, 5% 2-mercaptoethanol) with 1% protease inhibitor cocktail (MilliporeSigma, St. Louis, MO, USA). The supernatant was collected after protein denaturation by boiling for 5 min. Proteins were separated, transferred, and detected with primary antibodies including anti-phospho- or pan-

GluN2B (Merck Millipore, Darmstadt, Germany), ERK1/2, p38 MAPK, JNK, Epac1/2 (Cell Signaling Technology, Danvers, MA, USA), and the housekeeping protein GAPDH (Merck Millipore, Darmstadt, Germany). Membranes were then incubated with secondary antibodies goat anti-rabbit or mouse IgG (Cell Signalling Technology, Danvers, MA, USA) for 1 h at room temperature. Protein bands were visualized on X-ray film after incubation with enhanced chemiluminescence (Bio-Rad Laboratories, Hercules, CA, USA). Densitometry of the bands was analyzed by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

### 2.6 Immunofluorescence Staining

One hour after the operation, rats were anaesthetized with sodium pentobarbital and then perfused with 4% paraformaldehyde solution through the cardiovascular system. Lumbar spinal segments L3-5 were collected and embedded in tissue freezing medium at -80°C after dehydration. Cryosections were cut transversely at 15 μm by a cryostat (Leica Microsystem, Wetzlar, Germany). The sections were then blocked with 10% normal goat serum in phosphate-buffered saline (PBS) at room temperature for 1 h and followed by incubation with primary antibody(s), including mouse anti-c-Fos (1:100, Abcam, Cambridge, UK), mouse anti-Epac1-mixed with rabbit anti-NeuN antibody and (1:500, Abcam), rabbit anti-Epac2 antibody (1:100, Cell Signaling Technology Inc) mixed with mouse anti-NeuN antibody (1:500, Abcam) at 4$^{o}$C for overnight. After washing with PBS, the sections were incubated with fluorescent-conjugated secondary antibodies (1:500, goat anti-mouse IgG conjugated with Alexa Fluor 488 or 568 and goat anti-rabbit IgG conjugated with Alexa Fluor 568 or 488, Thermo Fisher Scientific Corporation). The sections were mounted with mounting medium with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Vector Laboratories, Burlingame, CA, USA). The immunoreactive cells were identified under a confocal microscope (LSM 780, Carl Zeiss, Oberkochen, Germany). Cell counting for double-labelling immunohistochemistry against Epac1 or Epac2 with NeuN, or c-Fos with DPAI were performed using ImageJ software (Version 1.52c, NIH, USA), and expressed as percentage of double-labelled cells versus total cells under the same Regions of Interest (ROI).

### 2.7 Statistical Analysis

All data in this study are expressed as mean ± standard error of mean (SEM). The density of protein bands of Western blot was measured by ImageJ software normalized to corresponding loading control bands. Calculations were performed by GraphPad Prism software (GraphPad software Inc, CA, USA). Two-way ANOVA followed by Tukey's multiple comparisons was used to analyze the data of time course in behavioural test; and one-way ANOVA was used for the other analyses. In all statistical comparisons, *p* values less than or equal to 0.05 were considered as statistically significant.

## 3. Results (1157 words)

### 3.1 Propofol inhibited mechanical allodynia caused by plantar incision

In the isoflurane group, paw withdrawal threshold (PWT) of the ipsilateral hind paw was significantly decreased compared to that at baseline (55.2 ± 0.8 g), and was lowest at 1 h after plantar incision (20.8 ± 4.3 g, n=5, $p<0.01$). The PWT gradually recovered towards baseline from 2 h (30.1 ± 2.5 g, n=5, $p<0.01$), but the differences compared with baseline remained significant up to 72 h (47.4 ± 0.8 g, n=5, $p<0.01$) after incision (Fig. 2). In the propofol group, the ipsilateral PWT was also significantly decreased compared with baseline. The lowest PWT was also at 1 h after incision. However, the ipsilateral PWT was fully recovered at 72 h after the incision, compared with baseline (55.9 ± 1.0 g and 51.6 ± 2.6 g at baseline and 72 h after plantar incision, respectively, n=5, $p>0.05$, Fig. 2). The ipsilateral PWT values in both isoflurane and propofol group were significantly lower than those on the contralateral side after operation (Fig 2).

The ipsilateral PWT in the propofol group was significantly higher than isoflurane group at 1 h (33.3 ± 1.1 g and 20.8 ± 4.3 g for propofol group and isoflurane group, respectively, n=5, $p<0.05$) and 2 h (43.3 ± 1.3 g and 30.1 ± 2.5 g for propofol group and isoflurane group, respectively, n=5, $p<0.05$) after incision. We collected spinal tissue of L3-L5 from each treatment group at the 1 h time point following plantar incision for molecular and cellular signaling assessment because both degree of mechanical allodynia and the difference in

mechanical allodynia between the two groups was the greatest at this time point after the incision.

### 3.2 Propofol inhibited the expression of spinal phosphorylated GluN2B induced by plantar incision

The protein level of phosphorylated GluN2B (p-GluN2B) in ipsilateral dorsal horns was significantly increased 1 h after plantar incision in isoflurane group compared with naïve group (1.14 ± 0.07 and 0.39 ± 0.09 for isoflurane group and naïve group, respectively, n= 4, $p<0.01$). There was also a significant increase in p-GluN2B expression in the ipsilateral dorsal horn of propofol group compared to naïve group (0.67 ± 0.01 and 0.39 ± 0.09 for propofol group and naïve group, respectively, n=4, $p<0.05$). However, p-GluN2B expression in propofol group was significantly lower than isoflurane group (0.67 ± 0.01 and 1.14 ± 0.07 for propofol and isoflurane, respectively, n= 4, $p<0.05$, Fig. 3A). No difference was found in the contralateral lumbar dorsal horns among all other groups.

### 3.3 Propofol inhibited activation of spinal p38 MAPKs following plantar incision

One hour after the incision, the ratio of phosphorylated p38 MAPK (p-p38) to pan-p38 MAPK in the ipsilateral dorsal horns of L3-5 lumbar spinal cord was significantly elevated in isoflurane group compared with naive group (1.16 ± 0.02, n=4 and 0.70 ± 0.06, n=3 for isoflurane and naïve, respectively, Fig. 3B, $p<0.01$). The ratio of p-p38 to pan-p38 MAPK expression in the ipsilateral dorsal horn in the propofol group was significantly lower than the isoflurane group (0.88 ± 0.10 in the propofol treated group versus 1.16 ± 0.02 in the isoflurane treated group, $p<0.05$, Fig. 3B). The ratio of p-ERK to pan-ERK was significantly enhanced in both isoflurane group (1.31 ± 0.07, n=5, $p<0.01$) and propofol group (1.29 ± 0.04, n=5, $p<0.01$) compared with the naïve group (0.95 ± 0.04, n=5), and there were no difference between the propofol and isoflurane groups ($p>0.05$, Fig. 3C). There was no difference in the ratio of ipsilateral spinal p-JNK to pan-JNK between the three groups (Fig. 3D). There was

also no difference in the expression of p-p38, p-ERK, and p-JNK in the contralateral dorsal horn amongst the 3 groups.

### 3.4 Propofol inhibited the expression of EPAC1 but not EPAC2 following plantar incision

EPAC was recently reported to contribute to sustaining post-incisional pain driven by activation of neuronal p38MAPK in dorsal root ganglions (DRGs) (Matsuda *et al.*, 2017). As shown in Fig 4A, the ratio of EPAC1 to α-tubulin was increased in the ipsilateral dorsal horns of L3-5 lumbar spinal cord following plantar incision in the isoflurane group (1.57 ± 0.14, $p<0.05$, n=5) compared with naive group (1.13 ± 0.12, n=5). However, it was significantly lower in the propofol group (0.67 ± 0.14, n=5, $p<0.05$, two-way ANOVA followed by Tukey post hoc test) compared with isoflurane group. There was no statistical difference in EPAC1 expression in the contralateral dorsal horn among the different groups (n=5, P=0.1413, two way-ANOVA; mean±SD, 0.816±0.48, 1.384±0.51 and 0.821±0.48 for Naïve, isoflurane and propofol treated group, respectively). Spinal dorsal expression of EPAC2 was not altered among the different groups after plantar incision (Fig. 4B,n=5, p=0.1643, two way-ANOVA).

In accordance with the Western blot findings, immunofluorescence against EPAC1 or EPAC2 detected low levels of EPAC1 and EPAC2 immunoreactivities that were co-expressed with neuronal marker NeuN in the ipsilateral spinal dorsal horns in the naive group (Fig. 5A & 5D). The percentage of double labelled neurons of EPAC1 versus total neurons in the same regions of interest showed a significant increase 1 h after plantar incision in the isoflurane group (17.96% ± 2.22, $p<0.05$, n=4, Fig 5B) as compared to the naive group (9.47% ± 1.51, n=4). However, the percentage of spinal neurons co-expressing EPAC1 was significantly lower in the propofol group as compared to the isoflurane group (10.01% ± 1.86, n=4, $p<0.05$, two-way ANOVA followed by Tukey post hoc test, Fig 5C). Consistent with the Western findings, there were no significant differences in EPAC2 expression among the three groups (n=4, p=0.1689 as shown in Fig 5D-F).

### 3.5 Propofol prevented plantar incision induced spinal c-Fos expression

Compared to the naïve group (1.76 ± 0.67, n=3), the number of c-Fos-positive cells double labelled with DAPI in lamina I of spinal dorsal horn segments (L3-L5) was markedly increased 1 h after plantar incision in the isoflurane group (15.0 ± 0.5, n=4, $p<0.01$, Fig 6A). However, the number of c-Fos-positive cells double labeled with DAPI was significantly lower in the propofol group (Fig 6A) compared with the isoflurane group (5.3 ± 0.2, n=5 for propofol group and 15.0 ± 0.5, n=4 for isoflurane group, $p<0.01$ Fig. 6B, two-way ANOVA followed by Tukey post hoc test). In contrast, compared to the naïve group (1.0 ± 0.58, n=3), the number of c-Fos-positive cells in laminae III-V was significantly increased 1 h after the incision in both the isoflurane group (4.75 ± 0.63, n=4, $p<0.05$, two-way ANOVA with Tukey post hoc test) and propofol group (5.75 ± 0.63, n=4, $p<0.01$, two-way ANOVA with Tukey post hoc test). In lamina III-V, there was no significant difference in the numbers of c-Fos-positive cells between propofol and isoflurane groups (4.75 ± 0.63 and 5.75 ± 0.63 for for isoflurane group and propofol group, respectively, $p>0.05$, two-way ANOVA with Tukey post hoc test, Fig 6B). These results indicate that nociceptive signal transmission in the lumbar spinal dorsal horn after plantar incision was selectively inhibited by propofol treatment in the superficial spinal dorsal horn.

## Discussion (1345 words)

This study demonstrates for the first time that intravenous infusion of propofol provided superior postoperative analgesia compared with isoflurane in an animal model of postoperative pain. The analgesic property of propofol may be attributed to its inhibitory effect on the activity of GluN2B-containing NMDA receptor and the downstream molecules p38MAPK and EPAC1 in the spinal dorsal horn (Fig 7).

Our study showed that the densitometric ratio of ipsilateral phosphorylated-GluN2B to pan-GluN2B was increased within 1 h after incision. The suppression of propofol on phosphorylated GluN2B occurred 1 h postoperatively in the ipsilateral L3-5 dorsal horn, but no change was observed on the contralateral side. It has been reported that in cultured hippocampal neurons, GluN2B is one of the targets of propofol to inhibit the activation of NMDA receptors and modulate $Ca^{2+}$ influx through slow calcium ion channels (Orser *et al.*,

1995). Propofol was also found *in vivo* to inhibit lithium-pilocarpine-induced status epilepticus in rats via down regulation of GluN2B subunit expression (Wang *et al.*, 2012). However, the information about the effect of propofol on spinal neurons is rather limited. It was shown that intraperitoneal injection of propofol displayed an appreciable anti-nociceptive effect in acetic acid-induced writhing test in mice, and this effect was reversed by intrathecal injection of NMDA agonists but potentiated by NMDA receptor antagonist (Xu *et al.*, 2004). The present study not only confirms the above findings but also demonstrated that spinal NMDA receptor GluN2B subunit was an important target through which intravenous propofol reduced mechanical allodynia compared with inhalational isoflurane in an animal model of postoperative pain. However, whether propofol produces its inhibitory effect on GluN2B through a direct binding site on NMDA receptors or via an indirect detour remains unknown.

In neurons, calcium influx through NMDA receptors leads to activation of MAPK pathways (Takeda & Ichijo, 2002). The phosphorylation of spinal MAPKs, such as p38 and ERK1/2, is involved in the development and maintenance of mechanical hypersensitivity in acute postoperative pain model (Wen *et al.*, 2009). Previous studies demonstrated that phosphorylation of p38 MAPK was increased in both spinal dorsal neurons and microglia from 1 h to 3 d after plantar incision (Wen *et al.*, 2009; Hsu *et al.*, 2015). Intrathecal injection of p38 inhibitor 30 min before plantar incision attenuated incision-induced mechanical allodynia (Wen *et al.*, 2009). Similarly, ERK activation in spinal dorsal horns occurred minutes after plantar incision, whereas pre-treatment with MEK inhibitor U0126 attenuated pain responses (Mizukoshi *et al.*, 2013). Consistent with these previous reports, we found that the activation of spinal p38 MAPK and ERK was significantly increased 1 h after plantar incision. Interestingly, the expression of activated p38MAPK, but not ERK1/2, after plantar incision was significantly lower in rats given propofol compared to those given isoflurane. Unlike ERK1/2, p38 MAPK is mainly activated by inflammatory cytokines (Ji & Woolf, 2001). Our early study demonstrated that tissue injury without nerve damage after surgery initiated a cascade of inflammatory cytokines that were positively correlated to postsurgical pain intensity (Wang *et al.*, 2009). Perhaps, the current findings indicate that the analgesic property of propofol in the postoperative pain model is associated with its anti-inflammatory effect (Zheng *et al.*, 2017). Furthermore, studies have also shown that enhanced phosphorylated p38MAPK is important in the development of persistent postoperative pain in the rat spinal cord (Alkaitis *et al.*, 2010; Huang *et al.*, 2011). For instance, enhanced

phosphorylated p38 in spinal dorsal horn was evident in the rat model of acute postoperative pain, but it spontaneously returned to basal levels in association with resolution of acute allodynia (Wen *et al.*, 2009). The inappropriate persistence of the enhanced expression of p38MAPK phosphorylation is associated with the development and maintenance of chronic postsurgical pain (Jin *et al.*, 2003). The current findings provide supportive evidence that propofol infusion reduces acute postoperative pain when used during general anaesthesia, and that this may be due to its inhibitory effect on p38MAPK.

It is well known that cAMP signaling plays a key role in regulating pain sensitivity, and EPAC is a novel class of cAMP target proteins (Matsuda *et al.*, 2017) . Two isoforms of this protein, EPAC1 and EPAC2, have been identified in humans and rodents. Intraplantar injection of the EPAC agonists led to long-lasting mechanical hyperalgesia (Hucho *et al.*, 2005). Moreover, the expression of EPAC1 and EPAC2 markedly increased at the time when nociceptive hypersensitivity developed after plantar incision (Mizukoshi *et al.*, 2013; Huang & Gu, 2017). Particularly interesting, genetic deletion of EPAC1 protected against inflammation induced mechanical hyperalgesia whereas selective EPAC1 inhibition prevented or reversed existing inflammation or incision induced nociceptive hypersensitivity without affecting normal pain sensitivity (Hucho *et al.*, 2005; Singhmar *et al.*, 2016). It was found that in DRG neurons, phosphorylation of p38MAPK through regulation of neuronal EPAC induction contributed to the development of pain hypersensitivity after plantar incisions (Mizukoshi *et al.*, 2013; Matsuda *et al.*, 2017). Activation of p38MAPK after plantar incision increased EPAC expression and induced long-lasting pain hypersensitivity (Mizukoshi *et al.*, 2013; Matsuda *et al.*, 2017). In contrast, inhibition of p38MAPK during surgery prevented the development of long-lasting pain hypersensitivity by preventing the induction of EPAC and EPAC-induced nociceptor sensitization (Matsuda *et al.*, 2017). In this study, we demonstrated that EPAC1 but not EPAC2 was induced in spinal dorsal neurons after plantar incision. This is in line with the findings that EPAC1 levels were increased after plantar incision (Matsuda *et al.*, 2017) and carrageenan induced inflammation (Wang *et al.*, 2013). However, we did not observe changes in EPAC2 in the spinal dorsal horn after incision. The discrepant findings may be attributed to the different neuronal profile between neurons in DRG and spinal dorsal horn. But the more reasonable explanation behind this observation may be associated with the difference in check time points between our study carried out during an acute period and others in a subacute period after incision. EPAC1 seems more closely associated with the transition from acute to long-lasting hyperalgesia

after an incision or inflammatory stimulus (Wang *et al.*, 2013; Matsuda *et al.*, 2017). Nevertheless, acquired EPAC activity in neurons driven by p38MAPK activation is a key molecular event during the transition and development of chronic hyperalgesia after surgery. Thus, the p38MAPK/EPAC pathway has been proposed to be a promising target in acute postoperative pain management to prevent pain persisting beyond the immediate acute phase (Wang *et al.*, 2013). Our findings that both phosphorylated p38MAPK and EPAC1 were suppressed in the propofol group indicate that propofol may produce better postsurgical analgesia than isoflurane by inhibiting the neuronal GluN2B-p38MAPK/EPAC1 cascade in the spinal dorsal horn. This may prevent the transition from acute to chronic hyperalgesia after surgery.

In further support to the above findings, we observed that the expression of c-Fos protein in lamina I of the ipsilateral dorsal horn significantly increased 1 h after plantar incision compared to baseline in the isoflurane treatment group. Propofol reduced the quantity of c-Fos-positive neurons in the superficial dorsal horn to one third of isoflurane group, without interference in the deep layers of dorsal horn. C-Fos has been used as a neural marker to study nociception because it is rapidly expressed after plantar incision in neurons (Harris, 1998). A consistency has been observed in the distribution of c-Fos in the spinal cord after noxious stimulation, typically located in laminae I-II of the dorsal horn, corresponding to the distribution of noci-responsive neurons and terminal fields of primary nociceptive afferent fibers (Todd, 2010). The current findings that propofol suppressed the enhanced c-Fos expression after incision in the superficial dorsal horn without interference in the deep layers of dorsal horn indicate a selective analgesic effect of propofol that may be not associated with its general anaesthetic effect (Takechi *et al.*, 2013).

In conclusion, our data showed that intravenous infusion of propofol during plantar incision reduced mechanical allodynia compared with inhalational isoflurane in a rat model of postoperative pain. This analgesic property of propofol appears to be mediated by inhibiting neuronal GluN2B-containing NMDA receptor and downstream p38MAPK/EPAC1 signalling pathway at the spinal dorsal horn level. The selective inhibitory effects of propofol on p38 MAPK/EPAC1 and c-Fos expression in the superficial spinal dorsal horn provide further evidence-based molecular and cellular mechanisms for use of propofol as an adjunctive analgesic in general anaesthesia.

**Acknowledgements:** This study was supported by the research funding from the Department of Anaesthesiology, Li Ka Shing Faculty of Medicine, The University of Hong Kong.

**Conflicts of Interest:** All authors declare no conflicts of interest.

**Author Contributions**: QQ, LS, PG, XMW and SSW participated in study design, data collection and analyses, result interpretation, and drafting of the manuscript. XMW, SSW and CWC contributed to manuscript writing, editing and finalization. SSW, QL, and CWC contributed to manuscript critical reading and editing process. CWC was entirely responsible for the overall study design, overseeing data collection and analysis, as well as manuscript finalization. All authors read and approved the final manuscript.

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## Figure legends

**Figure 1** Schematic illustration of experimental design. Anaesthesia was induced by isoflurane and maintained either by 2.5% of isoflurane or 1.5 mg $kg^{-1}$ $min^{-1}$ of propofol for 30min during which plantar incision was performed. Von Frey test was carried out before incision and at1 h, 2 h, 5 h, 24 h, 48 h, and 72 h after incision. Spinal dorsal horns were harvested 1 h after incision (time of peak hypersensitivity) in two other cohorts of animals for protein assay via Western blot and immunofluorescence staining.

**Figure 2** Effect of propofol and isoflurane on paw withdrawal threshold (PWT) after plantar incision. Time course of both ipsilateral and contralateral mechanical thresholds in isoflurane and propofol groups after hind paw incision is illustrated. Significant differences in the ipsilateral paw withdrawal threshold between isoflurane and propofol groups were detected at 1 h and 2 h after plantar incision. ** $p < 0.01$ ipsilateral vs contralateral in propofol group; # $p < 0.05$, ## $p < 0.01$ ipsilateral vs contralateral in isoflurane group; $\phi\phi$ $p < 0.01$ propofol versus isoflurane group on ipsilateral side; two-way ANOVA with Tukey's multiple comparisons test, n=5.

**Figure 3** The expression of phosphorylated and pan-GluN2B subunit-containing NMDA receptors and MAPKs in the ipsilateral and contralateral spinal dorsal horns after plantar incision. (A) The protein level of phosphorylated GluN2B in the ipsilateral dorsal horns was significantly increased 1 h after plantar incision in both isoflurane and propofol groups compared to that in naïve animals. However, there was a significant reduction in propofol group compared to isoflurane group. (B) The ratio of p-p38 to pan-p38 in the ipsilateral dorsal horn was significantly increased 1 h after plantar incision in isoflurane group compared to naïve group. There was a significant reduction in propofol group compared to isoflurane group. (C) The ratio of p-ERK to pan-ERK was increased in both isoflurane and propofol groups compared to naïve group. (D) There was no change in p-JNK after plantar incision. No difference was detected in contralateral dorsal horns among all the groups. *$p$ < 0.05, ** $p$ < 0.01, n=5, one-way ANOVA with Tukey's multiple comparisons test.

**Figure 4** Western blotting showed that the expression of exchange protein directly activated by cyclic adenosine monophosphate (EPAC) in spinal dorsal horn following plantar incision in different treatment groups. (A) The protein level of EPAC1 in the ipsilateral dorsal horns was significantly increased after plantar incision in isoflurane treated groups compared to that in naïve animals. There was a significant reduction in EPAC1 expression in the propofol group compared to isoflurane group. (B) There was no change in EPAC2 after plantar incision. No significant difference was detected in the contralateral dorsal horns among all the groups. Data are presented as mean ± SE. *$p$ < 0.05, n=5, two-way ANOVA followed by Tukey's multiple comparisons test.

**Figure 5** Double-staining immunohistochemistry for exchange protein directly activated by cyclic adenosine monophosphate (EPAC) in spinal dorsal horn following plantar incision. (A-C) Spinal dorsal neuronal expression of EPAC1 in different treatment groups after incision. (D-F) Spinal dorsal neuronal expression of EPAC2 in different treatment groups after incision. EPAC1 and EPAC2 expression, indicated as green signals, colocalized with neuorns stained with NeuN, red signals. Scale bar = 20 μm. Insets show higher magnification of double labelled cells. Scale bar=5 μm. The percentage of EPAC1-double labeled neurons versus total neurons in the region of interest (ROI) in the ipsilateral spinal dorsal horn increased significantly in the isoflurane treated group compared to the naive group; whereas

it was significantly suppressed in the propofol treated group. There were no significant differences in EPAC2-double labelled spinal neurons among the treatment groups. Data are presented as mean ± SE. *$p < 0.05$ versus naive group or isoflurane group (n = 4 per group, one-way ANOVA followed by Tukey post hoc test).

**Figure 6** Distribution of c-Fos-positive cells in the spinal dorsal horn of naïve, isoflurane and propofol groups. (A) Confocal fluorescence images illustrated the c-Fos-positive cells (green) in lamina I-II and III-V. Dotted lines show the estimated edges of laminae I/II/III-V. Scale bar: 25 μm; (B) Analysis of confocal images showed less c-Fos-positive cells in lamina I in propofol group after incision, compared to isoflurane group. There was no difference in laminae III-V between these two groups. * $p < 0.05$, ** $p < 0.01$, n=5, two-way ANOVA with Tukey's posthoc test.

**Figure 7** Schematic diagram showing the proposed spinal GluN2B-contaning NMDA receptor - p38MAPK- EPAC1 signaling pathway underlying the analgesic effect of intravenous propofol on postoperative pain. Plantar incision results in the upregulation of phosphorylated GluN2B-contaning NMDA receptor, which subsequently induces an increase in calcium influx to activate p38MAPK and ERK1/2 signal transduction pathway, which drives upregulation of EPAC1 expression in spinal dorsal neurons. Intravenous propofol at an anaesthetic dose is proposed to inhibit incisional injury-induced activation of spinal GluN2B-p38MAPK-EPAC1 signaling pathway. This leads to suppression of central sensitization and may reduce sustained pain hypersensitivity. P: phosphorylation.

Fig. 1

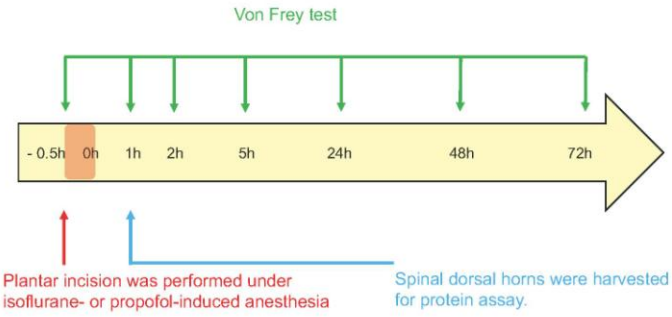



Fig. 2

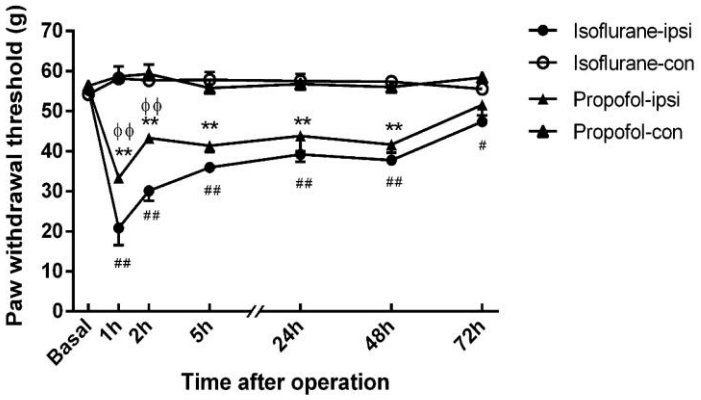
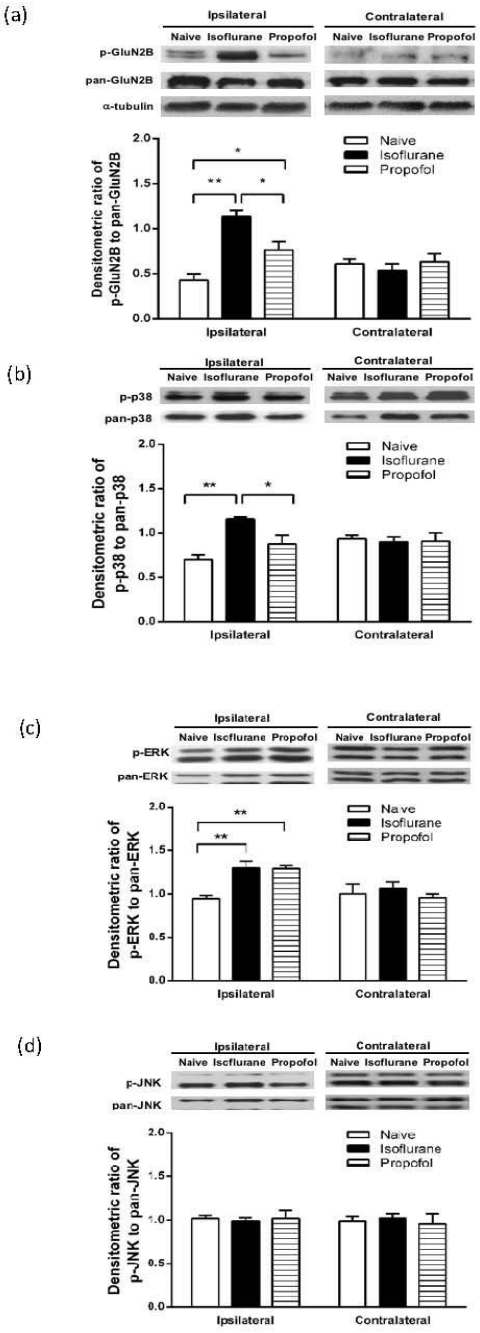
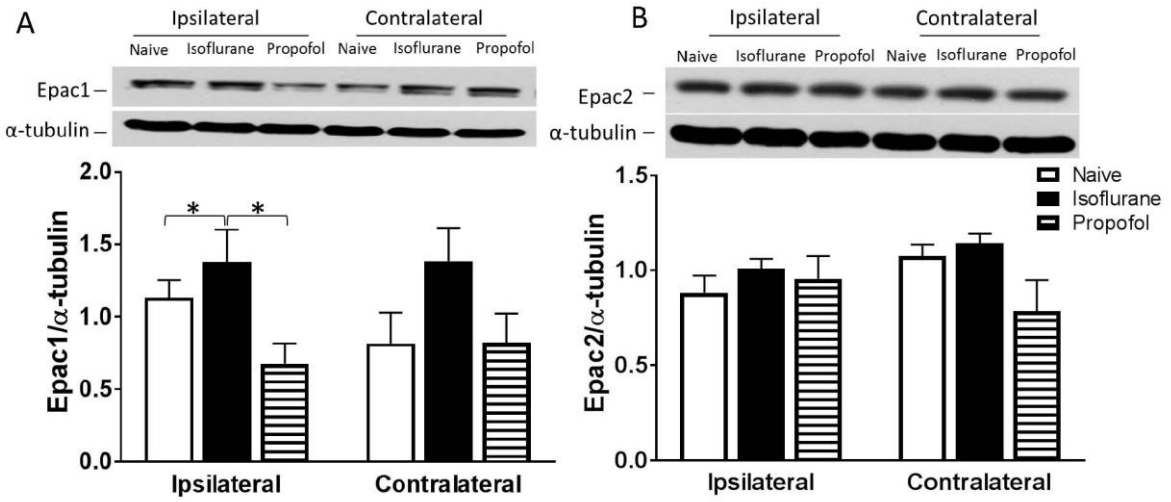
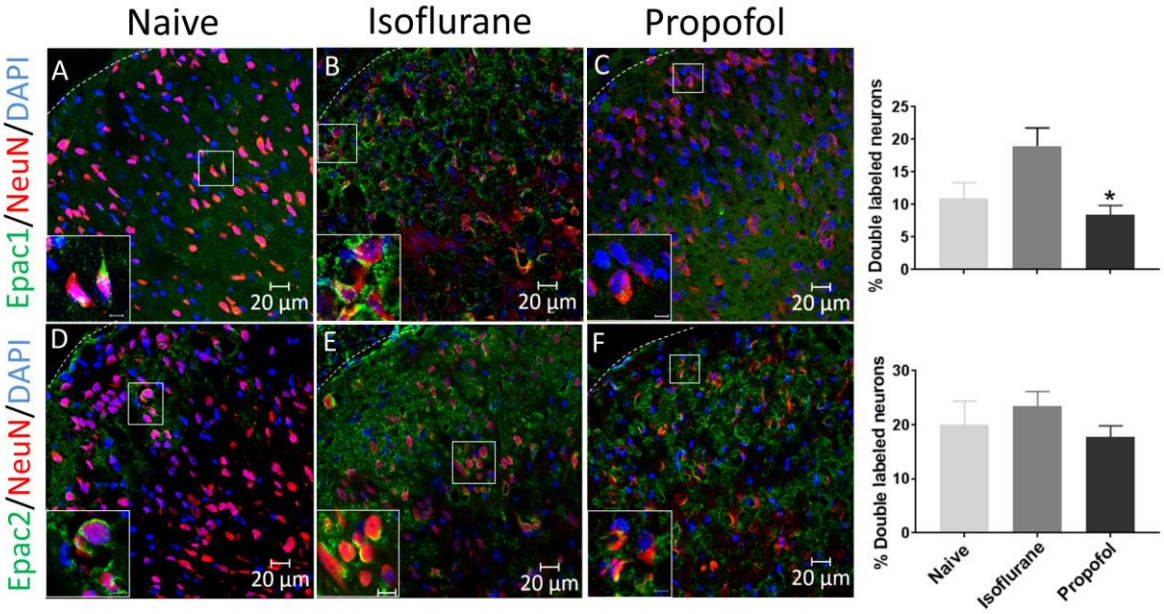
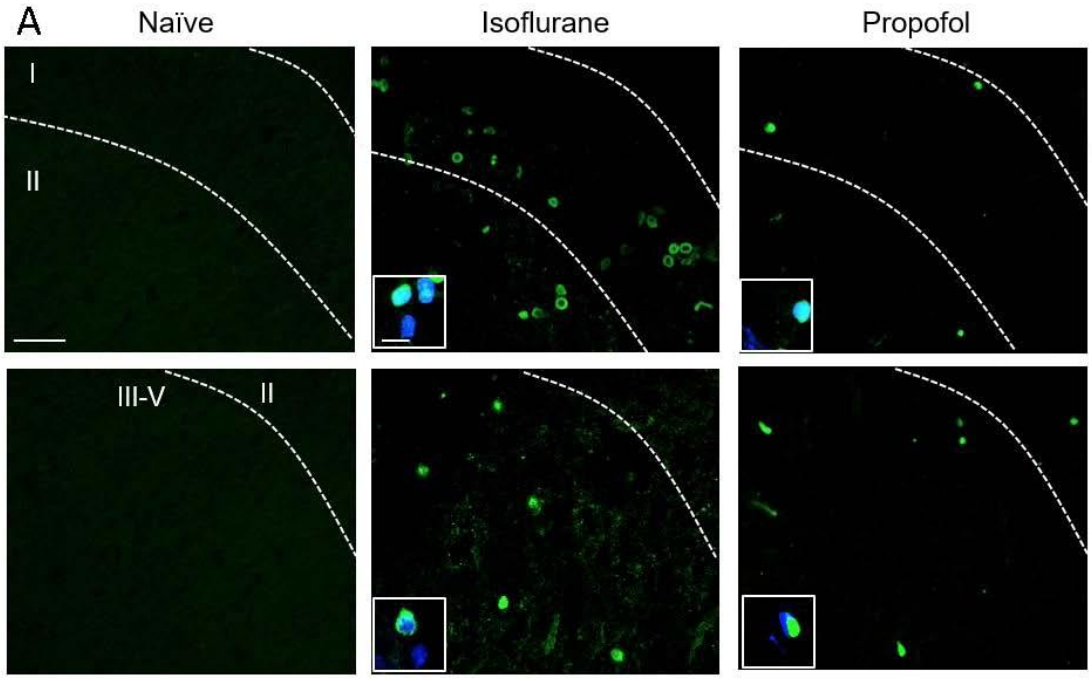


Fig. 3







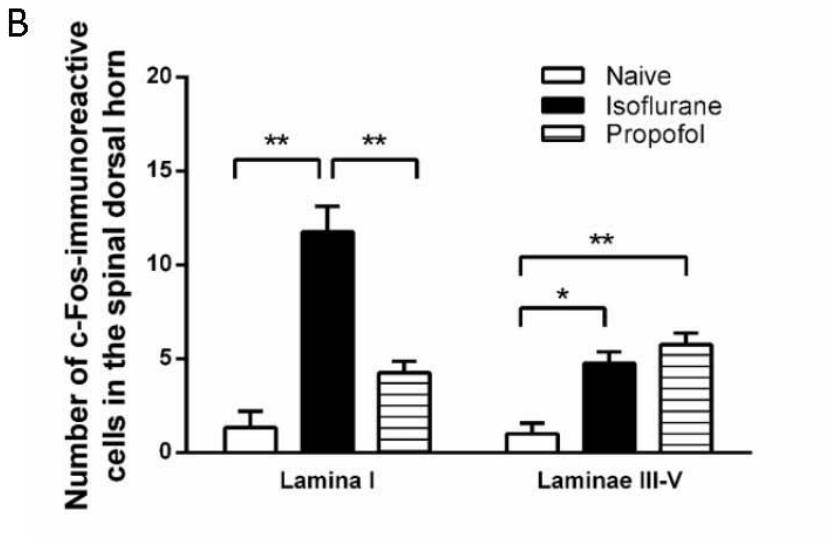



Fig. 7