Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

Phosphatidylinositol 3-kinase mediates pain behaviors induced by activation of peripheral ephrinBs/EphBs signaling in mice

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ARTICLE INFO

ABSTRACT

EphBs receptors and their ephrinBs ligands are present in the adult brain and peripheral tissue and play a critical role in modulating multiple aspects of physiology and pathophysiology. Our recent evidence has shown that ephrinBs acted as a sensitizer to participate in peripheral sensitization and hyperalgesia induced by activation of peripheral ephrinBs/EphBs signaling. In the present study, we explored the role of phosphatidylinositol 3-kinase (PI3K) in ephrinB1-Fc-induced pain behaviors. Intraplantar injection of ephrinB1-Fc produced a time- and dose-dependent increase of PI3K-p110γ expression and of phosphorylation of AKT in skin of injection site. Pre-treatment with PI3K inhibitor wortmannin or LY294002 prevented activation of peripheral AKT by ephrinB1-Fc. The activated AKT expressed in peripheral nerve terminals and DRG peptide-containing and small non-peptide-containing neurons. Inhibition of peripheral PI3K signaling dose-dependently prevented and reversed pain behaviors and spinal Fos protein expression induced by intraplantar injection of ephrinB1-Fc. Furthermore, pre-treatment with PI3K inhibitor wortmannin or LY294002 prevented ephrinB1-Fc-induced ERK activation in a dose-dependent manner. These data demonstrated that PI3K and PI3K crosstalk to ERK signaling mediated pain behaviors induced by activation of peripheral ephrinBs/EphBs signaling in mice.

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1. Introduction

Inflammatory or injury-induced sensitization of the peripheral nociceptors (also termed peripheral sensitization) manifests as an exaggerated pain response to a noxious stimulus (hyperalgesia) and/or the perception of a non-noxious stimulus as noxious (allodynia). Peripheral sensitization increases the firing of small-diameter sensory neurons, which communicate noxious information to the dorsal horn of spinal cord, and augments synaptic function in spinal cord, and then induces central sensitization, which may be a major cellular mechanism for converting acute nociceptive injury into chronic pain status. A large body of evidence supports the notion that activation of peripheral receptor tyrosine kinase (RTK) system (e.g. NGF/TrkA, BDNF/TrkB and GDNF/Ret) and the related downstream signaling pathways contributes to the development of peripheral sensitization (Pezet and McMahon, 2006; Nicol and Vasko, 2007; Bogen et al., 2008). Ephrin/Eph signaling, the largest family of RTK system, is involved in diverse aspects of development, such as tissue patterning, angiogenesis, axon guidance,

and synapse formation. (Tessier-Lavigne, 1995; Klein, 2004, 2009). Recent advances indicate that Eph receptors and ephrin ligands are present in the adult brain and peripheral tissue and play a critical role in modulating multiple aspects of physiology and pathophysiology (for example, activity-dependent synaptic plasticity, regulation of pain threshold, epileptogenesis, inflammation response, excitotoxic neuronal death etc.) (Gerlai, 2001; Yamaguchi and Pasquale, 2004; Goldshmit et al., 2006; Pasquale, 2008; Song et al., 2008a, b). Interestingly, several Eph receptors and ephrin ligands are also expressed in the adult rat spinal cord and the dorsal root ganglion (DRG) (Willson et al., 2002; Bundesen et al., 2003; Willson et al., 2003). Bundesen et al. reported that EphB2 receptor was present in the laminas I–III of the dorsal horn and on small- and medium-sized DRG neurons but not upon large-diameter neurons (Bundesen et al., 2003), which are two important sites for modulation of nociceptive information. Our recent evidence showed that ephrinBs acted as a sensitizer to participate in peripheral sensitization and hyperalgesia induced by activation of peripheral ephrinBs/EphBs signaling via a MAPKs dependent mechanism (Cao et al., 2008). In many both physiological and pathological conditions, activation of MAPK signaling pathway is usually companied with activation of phosphoinositide 3-kinase (PI3K) signaling and there is a crosstalk between the two signaling pathways (Menges and McCance, 2008; Cakir and Grossman, 2009; Russo et al., 2009). Thus, we want to know whether PI3K is involved in peripheral sensitization and

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hyperalgesia induced by activation of peripheral ephrinBs/EphBs signaling.

PI3K, which phosphorylates the D3 position of the inositol ring of phosphoinositides and thereby generates intracellular signaling molecules, has been demonstrated to be essential for a plethora of physiological and pathological processes (Castellino and Durden, 2007; Endersby and Baker, 2008; Kim et al., 2008). It is well established that activation of PI3K signaling is involved in the modulation of nociceptive information and peripheral and central sensitization produced by intense noxious stimuli (Zhuang et al., 2004; Malik-Hall et al., 2005; Stein et al., 2006; Sun et al., 2006, 2007; Xu et al., 2007; Zhu and Oxford, 2007; Zhu et al., 2007; Bogen et al., 2008; Pezet et al., 2008). Importantly, PI3K mediated peripheral sensitization and hyperalgesia induced by activation of peripheral RTK system NGF/TrkA and GDNF/Ret signaling (Zhuang et al., 2004; Malik-Hall et al., 2005; Stein et al., 2006; Zhu and Oxford, 2007; Zhu et al.,2007). Moreover, several lines of evidence have shown that regulation of PI3K pathway is associated with EphBs receptors activation (Steinle et al., 2002, 2003). These studies supported that PI3K may be as a downstream effector to modulate of peripheral sensitization and pain behaviors related to ephrinBs/EphBs signaling.

2. Methods

2.1. Animals

Adult, male Kunming mice (20–25 g) were employed in the present studies. Mice were housed under a 12-h/12-h light–dark cycle regime, with free access to food and water. The animals were provided by Experimental Animal Center of Xuzhou Medical College. All experimental protocols were approved by the Animal Care and Use Committee of the college and were in accordance with the Declaration of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 80-23, revised 1996).

2.2. Drug application

Wortmannin, an irreversible PI3K inhibitor, and a reversible competitive PI3K inhibitor LY294002 were purchased from Sigma (St. Louis, MO). Both PI3K inhibitors were dissolved in 1% DMSO. EphrinB1-Fc was purchased from R&D Systems Inc. and dissolved in saline. The dosages of drugs are based on the results of preliminary experiments. The usage and dose for each drug were presented in the parts of results and figure legends.

Drugs or vehicles were administered under isoflurane anesthesia in a volume of 10 μl into the plantar surface of the right hind paw using a 25-μl Hamilton syringe with a 28-gauge needle. The needle was inserted into the plantar skin proximal to the midpoint of the hind paw and advanced about 10 mm so that it reached the midpoint of the hind paw, where the solution was injected forming a bleb that disappeared within 10 min.

2.3. Measurement of thermal hyperalgesia

Thermal hyperalgesia was measured using the paw withdrawal latency according to the method described by Hargreaves et al. (1988). In brief, mice were placed in clear plastic chambers (7×9×11 cm) and allowed to acclimatize to their environment for 1 h before testing. The radiant heat was directed to the plantar surface of each hindpaw that was flush against the glass or site of injection of solution through the glass plate. The nociceptive endpoints in the radiant heat test were the characteristic lifting or licking of the hindpaw. The time to the endpoint was considered the paw withdrawal latency (PWL). The radiant heat intensity was adjusted to obtain basal PWL of 12–15 s. An automatic 20-s cutoff was used to prevent tissue damage. Thermal stimuli were delivered 3 times to each hind paw at 5-min intervals.

2.4. Measurement of mechanical allodynia

Mechanical allodynia was assessed by using von Frey filaments (North Coast Medical Inc., San Jose, CA), starting with 0.31 g and ending with 4.0 g filament as cutoff value. Animals were placed in individual plastic boxes (20×25×15 cm) on a metal mesh floor and allowed to acclimate for 1 h. The filaments were presented, in ascending order of strength, perpendicular to the plantar surface with sufficient force to cause slight bending against the paw and held for 6–8 s. Brisk withdrawal or paw flinching were considered as positive responses. The paw withdrawal threshold (PWT) was determined by sequentially increasing and decreasing the stimulus strength (the "up- and-down" method), and the data were analyzed using the nonparametric method of Dixon, as described by Chaplan et al. (1994).

2.5. Immunohistochemistry

Mice were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and undergone sternotomy, intracardially perfuse with 20 ml saline followed by 100 ml 4% ice-cold paraformaldehyde in 0.1 mol/l phosphate buffer (PB). The spinal cord of L_{4-5} were removed, post-fixed in 4% paraformaldehyde for 3 h, and subsequently allowed to equilibrate in 30% sucrose in PB overnight at 4 °C. Thirty-μm transverse series sections were cut on a cryostat and every fifth section was collected in PB. After washing in phosphate buffer saline (PBS), the tissue sections were incubated in PBS containing 5% normal goat serum and 0.3% TritonX-100 at room temperature for 30 min, followed by primary polyclonal rabbit-anti-Fos antibody (1:1000) (Santa Cruz Biotechnology, CA, USA) for the spinal cord slices at 4 °C for 48 h. The sections were then incubated in biotinylated goat anti-rabbit (1:200) at 37 °C for 1 h and in avidin–biotin-peroxidase complex (1:100) (Vector Labs, CA, USA) at 37 °C for 2 h. Then, they are washed 3 times (10 min) with PBS. Finally, the sections were treated with 0.05% diaminobenzidine (DAB) for 5–10 min. Sections were rinsed in PBS to stop the reaction, mounted on gelatin-coated slides, air-dried, dehydrated with 70%–100% alcohol, cleared with xylene, and cover-slipped for microscopic examination.

For analysis the change of Fos protein expression, we examined 5 spinal cord sections per animal, selecting the sections with the greatest number of positive neurons. For each animal, we recorded the total number of positive neurons (I–V laminas) in the ipsilateral spinal cord. All positive neurons were counted without considering the intensity of the staining.

For immunofluorescence staining, the sections of skin were incubated overnight at 4 °C with rabbit anti-phospho-AKT antibody (Cell Signaling Technology, 1:200) followed by Rhodamine Red™-X goat anti-rabbit IgG (Invitrogen, 1:100) and DRG sections with a mixture of rabbit anti-phospho-AKT antibody (Cell Signaling Technology, 1:200) and mouse anti-substance P(SP) (Santa Cruz, 1:250) or anti-IB4 antibody (Sigma, 1:200) followed by a mixture of Rhodamine Red™-X goat anti-rabbit IgG and FITC-monoclonal rat anti-mouse IgG1(Invitrogen, 1:100) for 2 h at room temperature. Nonspecific staining was determined by excluding the primary antibodies. Sections were rinsed, mounted, and cover-slipped with glycerol containing 2.5% of anti-fading agent DABCO (Sigma) and stored at −20 °C in the dark. Images were captured using a laser scanning confocal microscopy (LSCM, LeiCa TCS SP2, Germany).

The number of immunoreactive neuronal profiles was counted in a blinded fashion. Four sections of the DRG were randomly selected from each mouse. Four mice were included in each group. At least a total of 500 neurons from each mouse were measured. Neuronal cell bodies were identified by the presence of a nucleus. The density threshold for positive staining was determined by averaging three cell bodies in each section that were judged to be minimally positive. All neurons for which the mean density exceeded the threshold were counted as positive, and the positive cells were expressed as a

percentage of total counted neurons. The area of the pAKT neuronal profiles was measured and displayed as size-frequency distributions. Percentages of size frequency were calculated as follows: (number positive neurons within a size range/total number of positive neurons) × 100. To distinguish cell-size-specific changes, we divided the DRG neurons into small ($<600\,\mu m^2$), medium (600–$1200\,\mu m^2$), and large ($>1200\,\mu m^2$) neurons according to their cross-sectional area.

2.6. Western blot analysis

The skins of injection site of mice were quickly extracted and stored in liquid nitrogen. Tissue samples were homogenized in lysis buffer containing (in mM): Tris 20.0, sucrose 250.0, Na_3VO_4 0.03, $MgCl_2$ 2.0, EDTA 2.0, EGTA 2.0, phenylmethylsulfonyl fluoride (PMSF) 2.0, dithiothreitol (DTT) 1.0, protease inhibitor cocktail 0.02% (v/v), pH 7.4. The homogenates were centrifuged at 5000 g for 30 min at 4 °C. The supernatant was collected and protein concentration was performed according to Bradford (1976) using BSA as standard. The protein samples were stored at −80 °C.

Protein samples were dissolved in 4 × sample buffer (in mmol/L, pH 6.8): Tris–HCl 250.0, Sucrose 200.0, DTT 300.0, 0.01% Coomassie brilliant blue-G, and 8% sodium dodecyl sulfate (SDS), and denatured at 95 °C for 5 min, then the equivalent amounts of proteins (30 μg) were separated by using 10% SDS-polyacrylamide gel (PAGE) electrophoresis and transferred onto nitrocellulose membrane. In addition, the gels stained with Coomassie blue were used to confirm the equal amounts of protein loaded on each lane. The membranes were incubated overnight at 4 °C with the following primary antibodies: primary polyclonal rabbit anti-PI3K/p110γ (Santa Cruz, 1:250) or anti-β-actin antibody (Santa Cruz,1:200) or primary polyclonal rabbit anti-pAKT (1:400) or t-AKT(1:400) or primary polyclonal rabbit anti-pERK or t-ERK antibody (Santa Cruz, 1:400). The membranes were extensively washed with Tris-Buffered Saline Tween-20 (TBST) and incubated for 1 h with the secondary antibody conjugated with alkaline phosphatase (1:1000) at room temperature. The immune complexes were detected by using a NBT/BCIP assay kit (Sigma). Western blot densitometry analysis of signal intensity was performed using Adobe Photoshop software (Adobe, CA, USA) and phosphorylation levels of AKT or ERK from densitometry were normalized to total AKT or ERK. Expression of PI3K was normalized to β-actin. The blot density from control groups was set as 100%.

2.7. Statistical analysis

Data are expressed as mean ± S.E.M. Statistical analysis between two samples was performed using Student's *t*-test. Statistical comparison of more than two groups was performed using one-way analysis of variance (ANOVA) followed by a Tukey's post hoc test. The significance of any differences in thermal latency and mechanical threshold in behavior test was assessed using two-way ANOVA. 'Time' was treated as 'within subjects' factor and 'treatment' was treated as a 'between' subjects factor. Statistical analyses of data were generated using GraphPad Prism 5 (Graph Pad Software Inc., San Diego, CA, USA). $P<0.05$ was considered as statistically significant.

3. Results

3.1. Intraplantar injection of ephrinB1-Fc induced a time-dependent and dose-dependent activation of peripheral PI3K and AKT

Previous studies have shown that injection of p110γ, the catalytic subunit PI3K, in the dorsum of the rat hind paw induced hyperalgesia (Malik-Hall et al., 2005). Knockout PI3K-p110γ or treatment with a PI3K-p110γ inhibitor suppresses the progression of joint inflammation and damage in two distinct mouse models of rheumatoid arthritis (Camps et al., 2005). These results suggested that peripheral PI3K-p110γ involved the development of inflammation pain. Therefore, regulation of PI3K-p110γ may be involved in EphrinB1-Fc-induced pain behaviors in peripheral. To determine whether intraplantar (i.pl.) injection of ephrinB1-Fc activates PI3K/AKT pathway, we measured the time course and dose-dependent relationship of expression of PI3K-p110γ and phosphorylation of downstream kinase AKT (S473) as an indicator of activation of PI3K pathway in the skin of injection site. As shown in Fig. 1, i.pl. injection of ephrinB1-Fc (0.5 μg) increased the expression of skin PI3K-p110γ, reaching maximal level on 30 min and returning to baseline level on 8 h after injection (Fig. 1a). The same injection caused an increase of phosphorylation of AKT on already detectable 10 min after injection, which reached its peak by 30 min to 1 h and lasted at least up to 8 h after injection (Fig. 1b). To further determine ephrinB1-induced activation of PI3K/AKT pathway, we picked up 30-min time point to perform dose–response experiments. I.pl. injection of ephrinB1-Fc (0.02, 0.1, and 0.5 μg) produced a dose-dependent increase of skin PI3K-p110γ expression and of phosphorylation of AKT (Fig. 1c, d). To explore the role of PI3K in AKT activation by ephrinB1-Fc, we intraplantarly injected two PI3K inhibitors wortmannin or LY294002 at 30 min before ephrinB1-Fc injection, then test the expression of skin pAKT at 30 min after ephrinB1-Fc injection. We found that both pre-treatments prevented the increase of pAKT expression (Fig. 2). It suggested that PI3K contributed to activation of peripheral AKT by ephrinB1-Fc. In our previous experiments, to exclude a nonspecial effect of the Fc portion, human Fc was used as a control for ephrinB1-Fc. We found that no significant hyperalgesia was detected after injection of human Fc. Therefore, vehicle for ephrinB1-Fc was used as its control in the present study.

3.2. AKT in peripheral sensory neurons and terminals was activated by i.pl. injection of ephrinB1-Fc

Ephrin ligands and Eph receptors are expressed on epidermal. Ephrin/Eph signaling pathway was involved in the development of rat cutaneous sensory innervations both during normal development and following skin injury. Furthermore, Eph receptors including EphBs and EphAs are expressed on DRG neurons, suggesting that these sensory neurons have the capacity to respond to the presence of ephrins in the epidermis (Moss et al., 2005). These studies provided a reasonable speculation that PI3K signaling in peripheral sensory system may be activated by i.pl. injection of ephrinB1-Fc. To confirm this point, we performed the pAKT immunofluorescence staining in the skin of injection site and DRG at 30-min after i.pl. injection of ephrinB1-Fc. We found that pAKT positive nerve fibers were barely detected in saline-injected hindpaw skin (Fig. 3a), but many fibers were labeled in the epidermis of the ephrinB1-Fc-injected hindpaw (Fig. 3a). Next, we want to know whether the bodies of sensory nerve fibers (DRG neurons) were activated by i.pl. injection of ephrinB1-Fc. Our results showed that there was a substantial increase in the number of pAKT positive neurons in DRG at 30 min after i.pl. injection of ephrinB1-Fc in the hind paw. The percentage of labeled cells in the saline group was 13.4 ± 2.15; however, that in the ephrinB1-Fc group was 29.2 ± 5.67 (Fig. 3b, c, e). Most of these pAKT positive neurons were small-to-medium-diameter sensory neurons (Fig. 3f). The pAKT positive neurons were colocalized with substance P (SP), a maker for peptide-containing neurons, and a small non-peptide-containing neurons maker IB4 positive neurons (Fig. 3c, d). These results indicated that i.pl. injection of ephrinB1-Fc could induced AKT activation in peripheral sensory nerve terminals and its neurons. In other words, activation of PI3K pathway in peripheral sensory system at least partially mediated hyperalgesia induced by i.pl. injection of ephrinB1-Fc. However, our study couldn't rule out involvement of peripheral non-neuronal cell such as the Langerhans cells, T cells, endothelial cells etc. in this process.
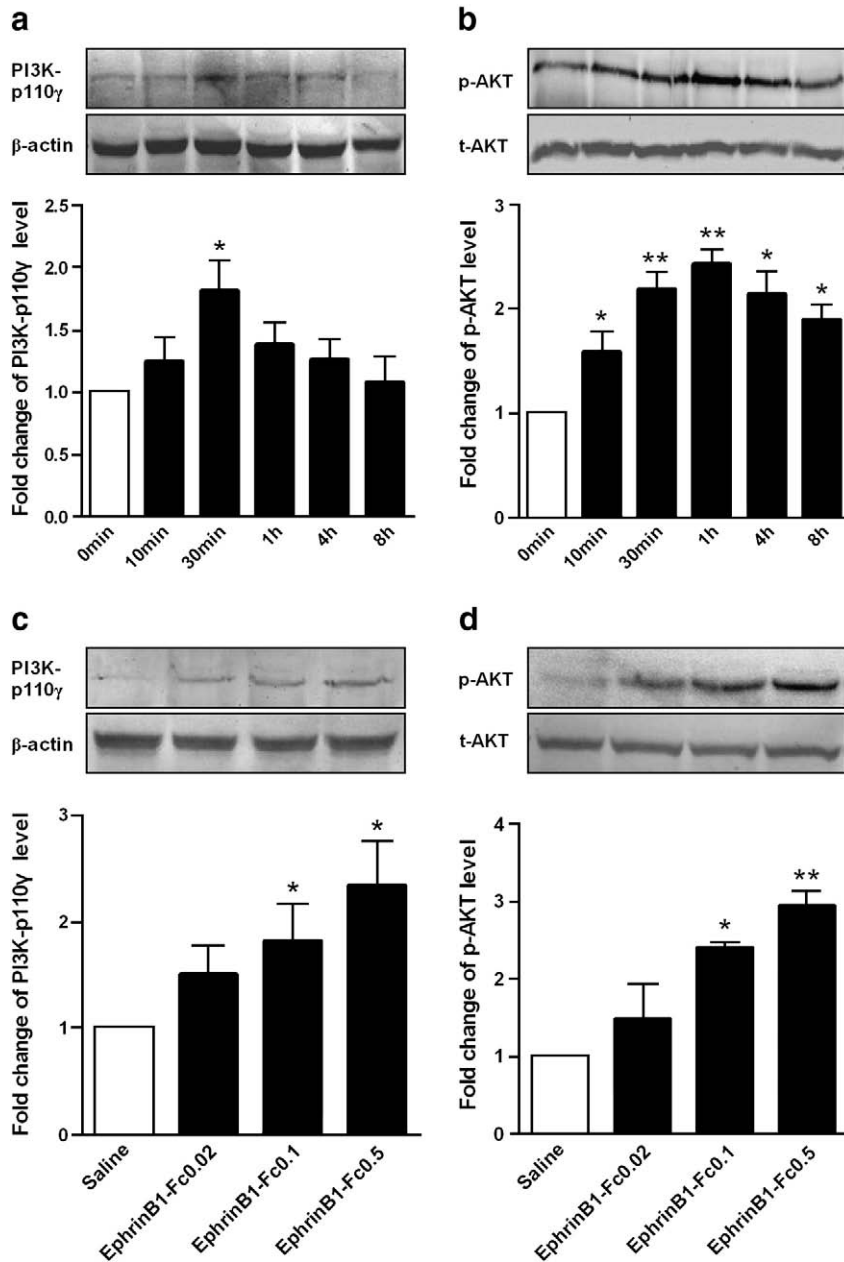
Fig. 1. Intraplantar injection of ephrinB1-Fc induced a dose- and time-dependent increase of peripheral PI3K-p110γ and pAKT expression. The expression of skin PI3K-p110γ and pAKT was assayed at 0, 10-min, 30-min, 1-h, 4-h, and 8-h time-points in the time-dependent experiments and at 30-min time-point in the dose-dependent experiments after ephrinB1-Fc injection. (a, b) The representative bands (top) for the expression of PI3K-p110γ or pAKT in skin at different time points after injection of ephrinB1-Fc (0.5 μg) and the quantitative data (bottom) for the expression of PI3K-p110γ or pAKT. The fold change for the density of PI3K-p110γ or pAKT normalized to β-actin or total AKT for each sample, respectively. The fold change of PI3K or pAKT level in 0 time point groups was set at 1 for quantifications. $^*P<0.05$, $^{**}P<0.01$, compared with 0 time point group, $n = 4$–6 mice in each group. (c, d) The representative bands (top) for the expression of PI3K-p110γ or pAKT in skin after injection of various dose of ephrinB1-Fc (0.02, 0.1, and 0.5 μg). The quantitative data (bottom) for the expression of PI3K-p110γ or pAKT normalized to β-actin or total AKT for each sample, respectively. The fold change of PI3K or pAKT level in 0 time point groups was set at 1 for quantifications. $^*P<0.05$, $^{**}P<0.01$, compared with 0 time point group, $n = 6$ mice in each group.

3.3. Inhibition of peripheral PI3K prevented and reversed pain behaviors induced by i.pl. injection of ephrinB1-Fc

I.pl. injection of ephrinB1-Fc induced thermal hyperalgesia and mechanical allodynia, which was associated with activated PI3K pathway. Therefore, inhibition of PI3K should alleviate ephrinB1-Fc-induced pain behaviors in theory. As we expected, pre-treatment with wortmannin (0.016, 0.08, and 0.4 μg in 1% DMSO), an irreversible PI3K inhibitor, or a reversible competitive PI3K inhibitor LY294002 (0.2, 1, and 5 μg in 1% DMSO), not DMSO, at 30 min before i.pl. injection of ephrinB1-Fc, prevented thermal hyperalgesia and mechanical allodynia

induced by i.pl. injection of ephrinB1-Fc (0.5 μg) in a dose-dependent manner. Compared to DMSO-ephrinB1-Fc group, PWL and PWT were significantly elevated in W0.4-ephrinB1-Fc and LY0.5-ephrinB1-Fc group at 0.5–4 h after i.pl. injection of ephrinB1-Fc ($p<0.05$ or $p<0.01$) (Fig. 4a). The calculated area under curve (AUC) (-2–24 h) was significantly increased in W0.4-, W0.08-, or LY5-ephrinB1-Fc group in PWL test, and in W0.4-, W0.08-, LY5- or LY1-ephrinB1-Fc group in PWT test ($p<0.05$ or $p<0.01$) (Fig. 4a). These results suggested that activation of PI3K pathway was involved in the initiation of pain behaviors induced by ip.l. injection of ephrinB1-Fc. Then we asked if PI3K pathway also participated in its maintenance process. To address
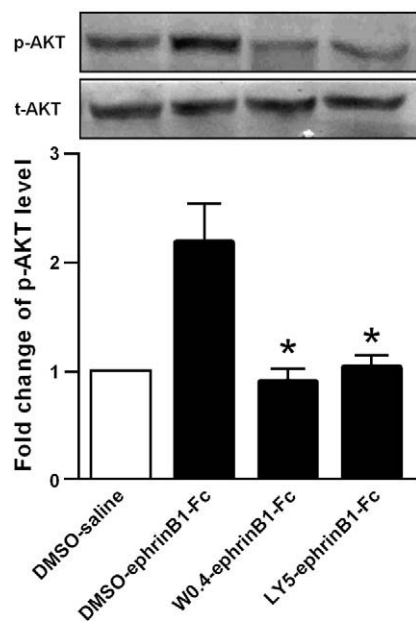
Fig. 2. PI3K mediated peripheral AKT activation by i.pl. injection of ephrinB1-Fc. PI3K inhibitor wortmannin (0.4 μg) or LY294002 (5-μg) or DMSO was intraplantarly injected at 30 min before i.pl. ephrinB1-Fc (0.5 μg). The expression of pAKT in the skin of injective site was assayed at 30 min after ephrinB1-Fc injection. The representative bands (top) and the quantitative data (bottom) for the expression of peripheral pAKT. The fold change for the density of pAKT normalized to total AKT for each sample. The fold change of pAKT level in DMSO-saline group was set at 1 for quantifications. $^*P<0.05$, compared with DMSO-saline group, $n=6$ mice in each group.

this question, wortmannin (0.016, 0.08, and 0.4 μg) or LY294002 (0.2, 1, and 5 μg) was administrated at 1 h after i.pl. injection of ephrinB1-Fc (0.5 μg). We found that post-treatment with both PI3K inhibitors dose-dependently reversed the established thermal hyperalgesia and mechanical allodynia by i.pl. injection of ephrinB1-Fc (Fig. 4b). Compared with ephrinB1-Fc-DMSO group, the calculated area under curve (AUC) (−2–8 h) (inside bar figure) was significantly increased in ephrinB1-Fc-W0.4-, -LY1 and -LY5 group in both PWL and PWT test, suggesting that PI3K pathway is required for the maintenance of pain behaviors induced by i.pl. injection of ephrinB1-Fc. To exclude systemic roles of PI3K inhibitors, we injected with the PI3K inhibitors in the contralateral paw. This injection didn't prevent and reverse the hyperalgesia induced by i.pl. injection of EphrinB1-Fc (data no shown), suggesting that the effect of i.pl. injection of the inhibitors in inhibiting pain behavior is mediated by a local peripheral effect and not by a systemic effect.

3.4. Inhibition of peripheral PI3K prevented and reversed spinal Fos protein expression induced by i.pl. injection of ephrinB1-Fc

The expression of Fos protein also may be a useful tool to examine the effectiveness of different analgesic regimens (Munglani et al., 1996; Coggeshall, 2005). To further clarify the analgesic effect of inhibition of PI3K on pain induced by i.pl. injection of ephrinB1-Fc, we investigated the effect of pre-treatment or post-treatment with PI3K inhibitors on spinal Fos protein expression induced by i.pl. injection of ephrinB1-Fc (0.5 μg). DMSO, wortmannin (0.4 μg) or LY294002 (5 μg) was intra-plantarly administrated at 30 min before or after i.pl. injection of ephrinB1-Fc (0.5 μg) and spinal Fos protein expression was assayed at 1 h after ephrinB1-Fc injection in pre-treatment experiments and at 2 h in post-treatment experiments. The present results had shown that both pre-treatment and post-treatment with both PI3K inhibitors partially inhibited the spinal Fos expression induced by i.pl. injection of ephrinB1-Fc (Fig. 5a, b, c, and d).

3.5. PI3K activity is required for ERK activation induced by i.pl. injection of ephrinB1-Fc

Our recent study has shown that peripheral ERK activation was involved in hyperalgesia induced by i.pl. injection of ephrinB1-Fc (Cao et al., 2008). Pre- or post-treatment with MEK inhibitor prevented or reversed hyperalgesia induced by i.pl. injection of ephrinB1-Fc. Moreover, ERK is a potential downstream target of PI3K. Thus, we want to know whether peripheral PI3K pathway contributes to ERK activation induced by ephrinB1-Fc. To address this question, various doses of PI3K inhibitor wortmannin (0.016, 0.08, and 0.4 μg) or LY294002 (0.2, 1, and 5 μg) were intraplantarly injected into the right hindpaw at 30 min before ephrinB1-Fc injection. We measured activation of peripheral ERK by western blot using a phosphopeptide-specific antibody that recognizes phosphorylated ERK(pERK) at 30 min after ephrinB1-Fc injection. We found that both wortmannin and LY294002 prevented ephrinB1-Fc-induced ERK activation in a dose-dependent manner (Fig. 6a, b).

4. Discussion

The present study demonstrated that (1) ephrinB1-Fc-induced hyperalgesia was companied with the increase of peripheral PI3K-p110γ expression and activation of AKT, a downstream target of PI3K; The activated AKT was expressed in peripheral nerve terminals and DRG peptide-containing and small non-peptide-containing neurons. (2) Inhibition of peripheral PI3K prevented and reversed pain behaviors and spinal Fos protein expression induced by i.pl. injection of ephrinB1-Fc. (3) PI3K activity is required for ERK activation induced by i.pl. injection of ephrinB1-Fc. These data provided direct evidence that PI3K mediated pain behaviors induced by activation of peripheral ephrinBs/EphBs signaling in mice.

PI3K are widely expressed in the skin, particularly in the epidermis (including keratinocytes), where peripheral nociceptors are distributed in this level, and soma of peripheral nociceptors DRG (Zhuang et al., 2004; Fecker et al., 2007; Sun et al., 2007; Xu et al., 2007; Müller et al., 2008). Ample and growing evidence showed that PI3K was involved in peripheral sensitization induced by various stimuli (Malik-Hall et al., 2005; Sun et al., 2007; Zhu and Oxford, 2007; Zhu et al., 2007; Bogen et al., 2008). For example, injection of P110γ, a catalytic subunit of PI3K, in the rats hind paw induced PI3K-mediated robust hyperalgesia (Malik-Hall et al., 2005). Intradermal injection of capsaicin induced the increased phosphorylation of pAKT in DRG and nerve fibers in the epidermis of the stimulated hindpaw (Zhuang et al., 2004). Pre- or post-treatment with PI3K inhibitors wortmannin or LY294002 dose-dependently suppressed or reversed the capsaicin induced-heat hyperalgesia (Zhuang et al., 2004). Intradermal injection of PI3K inhibitor wortamannin or PKB/AKT inhibitor IV with capsaicin inhibited the changes of exploratory behavior produced by capsaicin injection in a dose-dependent manner (Sun et al., 2007). A large body of evidence supports the notion that activation of peripheral receptor tyrosine kinase (RTK) system and the related downstream signaling pathway contribute to the development of peripheral sensitization (Pezet and McMahon, 2006; Nicol and Vasko, 2007). For example, activation of TrkA or TrkB receptor tyrosine kinases by peripheral administration of nerve growth factor (NGF) or brain-derived neurotrophic factor (BDNF) produces hypersensitivity and pain in humans and reduces nociceptive threshold in animal models of pain. The enhanced level of neurotrophin release in inflamed human skin contributes to peripheral sensitization. Blockage of NGF/TrkA or BDNF/TrkB system also attenuates excitability of sensory neurons and prevents mechanical and thermal hyperalgesia secondary to local injection of Complete Freund's adjuvant (CFA) or carrageenan (Sammons et al., 2000; Amaya et al., 2004; Zhao et al., 2006). Furthermore, PI3K activation mediates NFG, BDNF or GDNF-induced peripheral sensitization and hyperalgesia (Malik-Hall et al.,
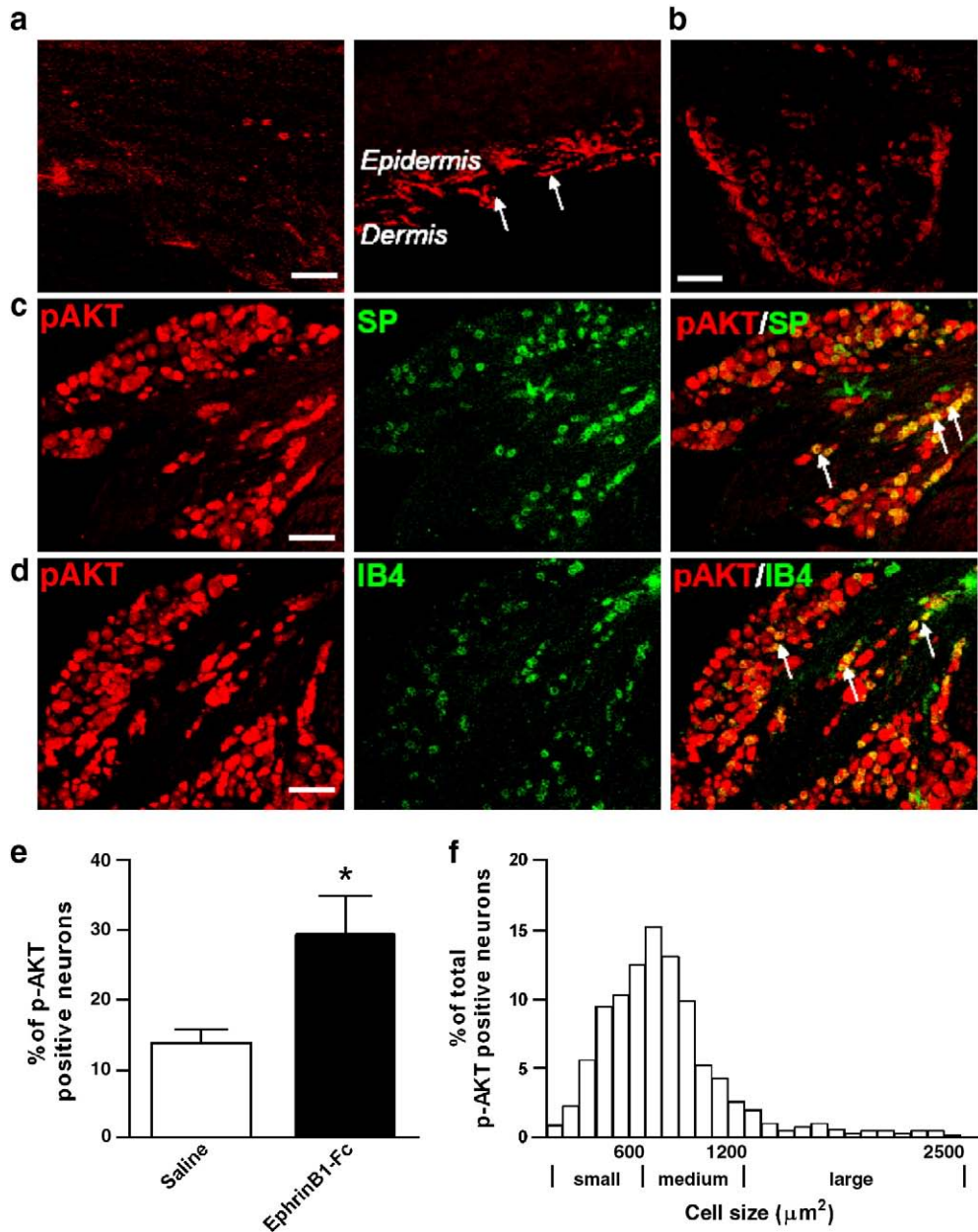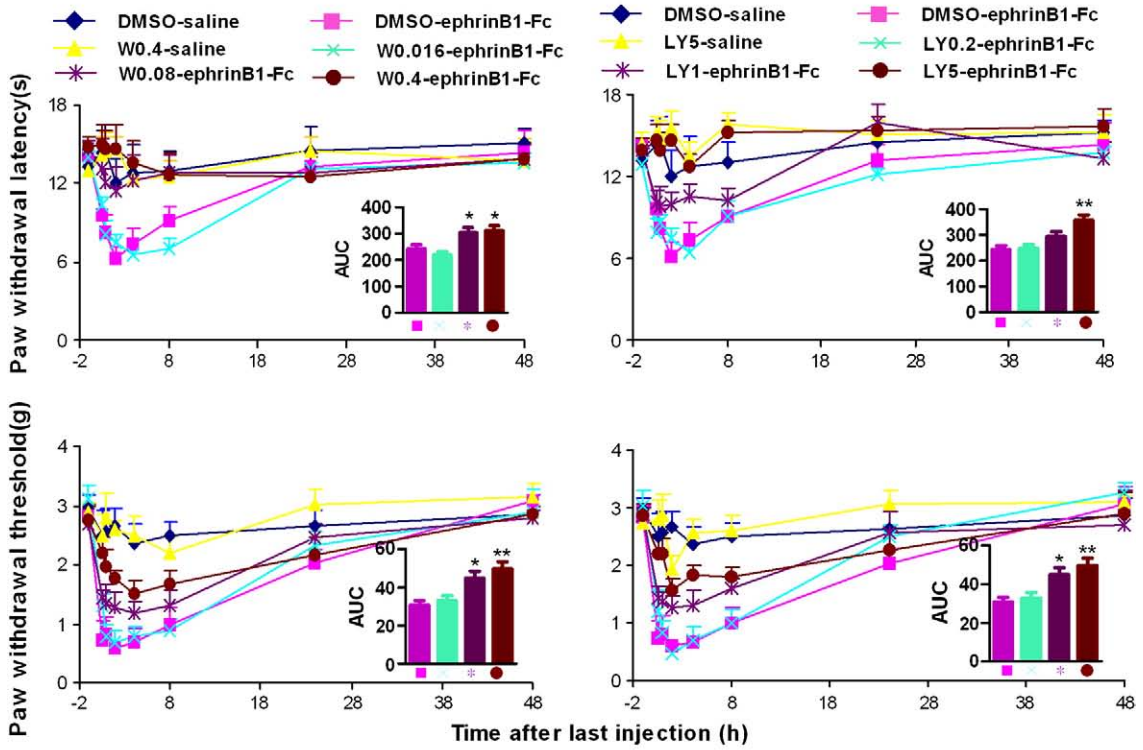
Fig. 3. AKT in peripheral sensory neurons and terminals was activated by i.pl. injection of ephrinB1-Fc. The pAKT immunofluorescence for the skin of injective site or pAKT/SP and pAKT/IB4 double immunofluorescence for DRG was performed at 30 min after i.pl. injection of ephrinB1-Fc. (a) The expression of pAKT was detected in the sensory nerve fibers and significantly increased in i.pl. ephrinB1-Fc group (right) than in i.pl. saline group (left). (b) The DRG pAKT expression in i.pl. saline group. (c) The expression for pAKT, SP, or pAKT/SP double-label in DRG in i.pl. ephrinB1-Fc group. (d) The expression for pAKT, IB4, or pAKT/IB4 double-label in DRG in i.pl. ephrinB1-Fc group. Bar = 100 μm. (e) Quantification of the percentage of pAKT positive neurons in DRG at 30 min after ephrinB1-Fc injection. $^*P<0.05$, compared with saline group, $n = 4$ mice in each group. (f) Size-frequency distribution histogram of pAKT neuron profiles in the DRG at 30 min after ephrinB1-Fc injection.

2005; Bogen et al., 2008). Our recent study indicated that ephrinBs acted as a sensitizer, which coupled with second-messenger systems through their EphBs receptors, to participate in peripheral sensitization relevant to hyperalgesia (Cao et al., 2008). In the present study, we found 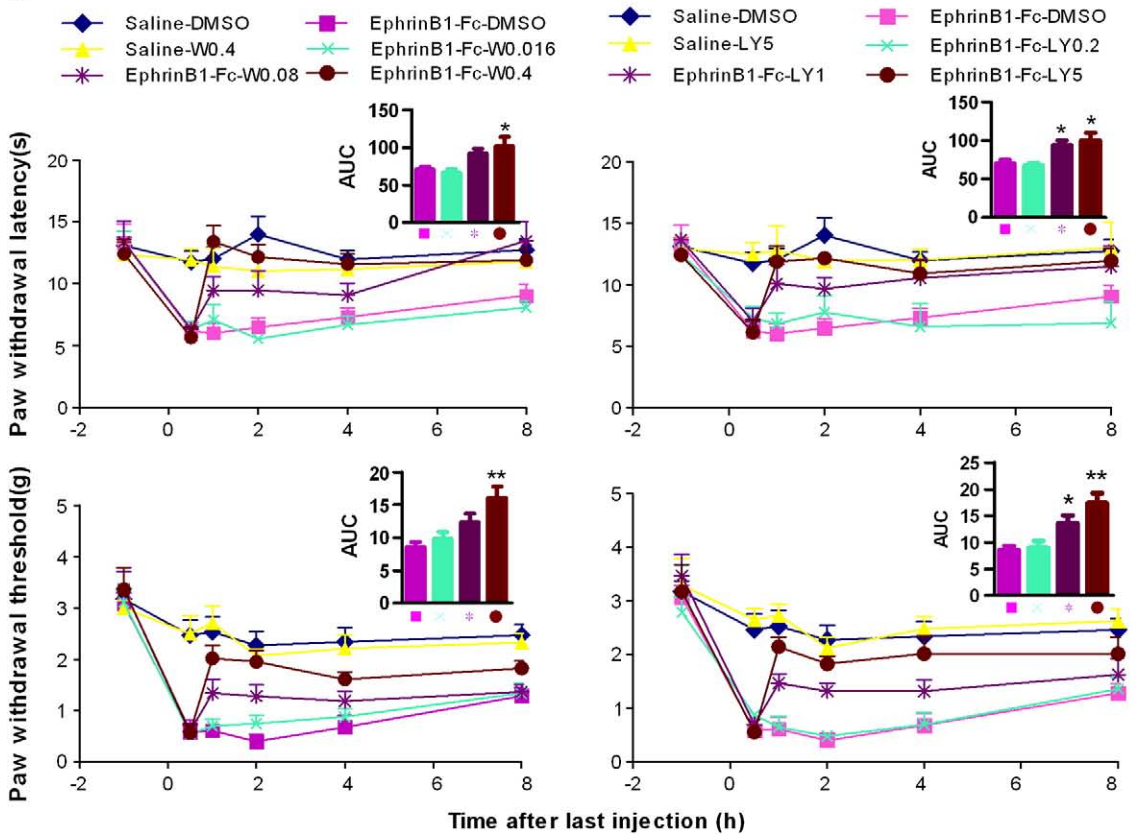that ephrinB1-Fc-induced hyperalgesia was companied with the time- and dose-dependently increase of peripheral PI3K-p110γ expression and activation of AKT. Increased phosphorylation of pAKT was prevented by pre-treatment with PI3K inhibitors wortmannin and LY294002. Inhibition of peripheral PI3K prevented and reversed pain behaviors and spinal Fos protein expression induced by i.pl. injection

Fig. 4. Inhibition of PI3K prevented and reversed pain behavior induced by i.pl. ephrinB1-Fc. PI3K inhibitors were injected at 30 min before or 1 h after ephrinB1-Fc injection. Paw withdrawal latency to the radiant heat and paw withdrawal threshold to mechanical stimuli were recorded at 0.5 h, 1 h, 2 h, 4 h, 8 h, 24 h and 48 h after ephrinB1-Fc (0.5 μg) injection in the pre-treatment experiment and at 0.5 h, 1 h, 2 h, 4 h, and 8 h after PI3K inhibitors in post-treatment experiment. (a) Pre-treatment with various dose of wortmannin (0.016, 0.08, and 0.4 μg) or LY294002 (0.2, 1, and 5 μg) prevented ephrinB1-Fc-induced thermal hyperalgesia (Top) and mechanical alloynia (bottom) in a dose-dependent manner. The calculated area under curve (AUC) (−2–24 h) (inside bar figure) was significantly increased in W0.4-, W0.08-, or LY5-ephrinB1-Fc group in PWL test, and in W0.4-, W0.08-, LY5- or LY1-ephrinB1-Fc group in PWT test, $^*P<0.05$, $^{**}P<0.01$ compared with DMSO-ephrinB1-Fc group, $n = 8$–10 mice in each group. (b) Post-treatment with wortmannin (0.016, 0.08, and 0.4 μg) or LY294002 (0.2, 1, and 5 μg) dose-dependently reversed the established thermal hyperalgesia (top) and mechanical alloynia (bottom) by ephrinB1-Fc (0.5 μg). Compared with ephrinB1-Fc-DMSO group, the calculated area under curve (AUC) (−2–8 h) (inside bar figure) was significantly increased in ephrinB1-Fc-W0.4-, -LY1 and -LY5 group in both PWL and PWT test, $^*P<0.05$, $^{**}P<0.01$ compared with ephrinB1-Fc-DMSO group, $n = 8$–10 mice in each group.
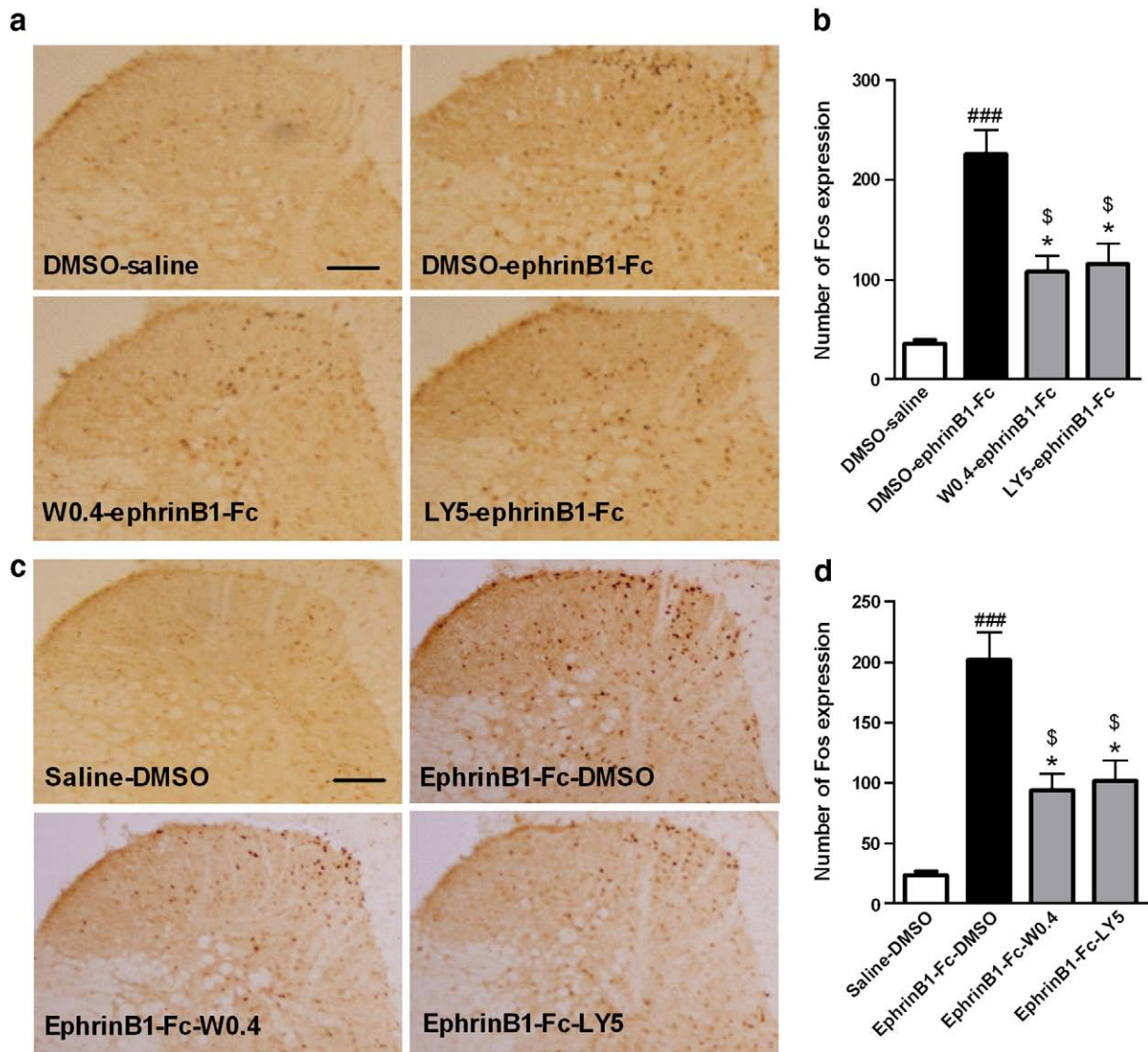
Fig. 5. Inhibition of PI3K partially inhibited or reversed the increased expression of spinal Fos protein by i.pl. ephrinB1-Fc. PI3K inhibitor wortmannin (0.4 μg) or LY294002 (5 μg) or DMSO was intraplantarly injected at 30 min before or 1 h after i.pl. ephrinB1-Fc (0.5 μg). Spinal Fos protein expression was assayed at 1 h in pre-treatment experiments (a, b) or 2 h in post-treatment experiments (c, d) after ephrinB1-Fc injection. (a, c) Representative Fos immunohistochemical staining and the quantitative data (b, d) of Fos expression in the spinal cord of mice in pre-treatment experiments or post-treatment experiments. $^{###}P<0.001$, compared with saline-DMSO or DMSO-saline group, $^{*}P<0.05$, compared with DMSO-ephrinB1-Fc or ephrinB1-Fc-DMSO group, $^{$}P<0.05$, compared with saline-DMSO or DMSO-saline group, $n=6$ mice in each group, Scale bar = 100 μm.

of ephrinB1-Fc. These results indicated that, like other RTK systems, pain behaviors induced by activation of peripheral ephrinBs/EphBs signaling were mediated by activation of peripheral PI3K. In the present study, we found that the time course of expression of PI3K-p110γ by i.pl. injection of ephrinB1-Fc was not consistent with that of pAKT expression. Expression of PI3K-p110γ had a significant increase only at 30-min timepoint after i.pl. injection of ephrinB1-Fc. However, expression of pAKT had a significant increase at all detected timepoint. We think that the following reasons may contribute to this point. (1) The expression of PI3K-p110γ is not regulated by EphBs receptors. (2) Minor change of PI3K activity could induce significant pAKT activation. (3) The change of pAKT expression was easier to be detected than that of PI3K. (4) Other signaling pathways were involved in activation of pAKT induced by ephrinB1-Fc.

Some studies have indicated that PI3K signaling was involved in the roles of ephrinBs/EphBs system in some physiological and pathological conditions. For example, EphB4 receptor and its ligand ephrin B2 were involved in human retinal endothelial cell and microvascular endothelial cell proliferation and migration through

activation of the PI3K pathway (Steinle et al., 2002, 2003). PI3K was involved in ephrinA5- mediated growth cone collapse. Signaling through ephrin-A ligand leads to activation of Src-family kinases, AKT phosphorylation, and inhibition of antigen receptor-induced apoptosis (Holen et al., 2008). The present study further supported and extended this view.

Both nerve terminals (nociceptors) and non-neuronal cell may be involved in peripheral sensitization and pain behaviors induced by activation of peripheral ephrinBs/EphBs system. PI3K are widely expressed not only in the nociceptors, but also in peripheral non-neuronal cells (for example: keratinocytes in the epidermis). The present results showed that i.pl. injection of ephrinB1-Fc induced PI3K pathway activation in both sensory terminals and neurons, indicating that peripheral sensory system at least in part mediated pain behaviors induced by i.pl. injection of ephrinB1-Fc. Keratinocytes, the major cell mass of the epidermis, act as a 'signal transducer', is capable of converting exogenous stimuli into the production of cytokines, adhesion molecules and chemotactic factors. It has been shown that keratinocytes play an important role in the pathogenesis
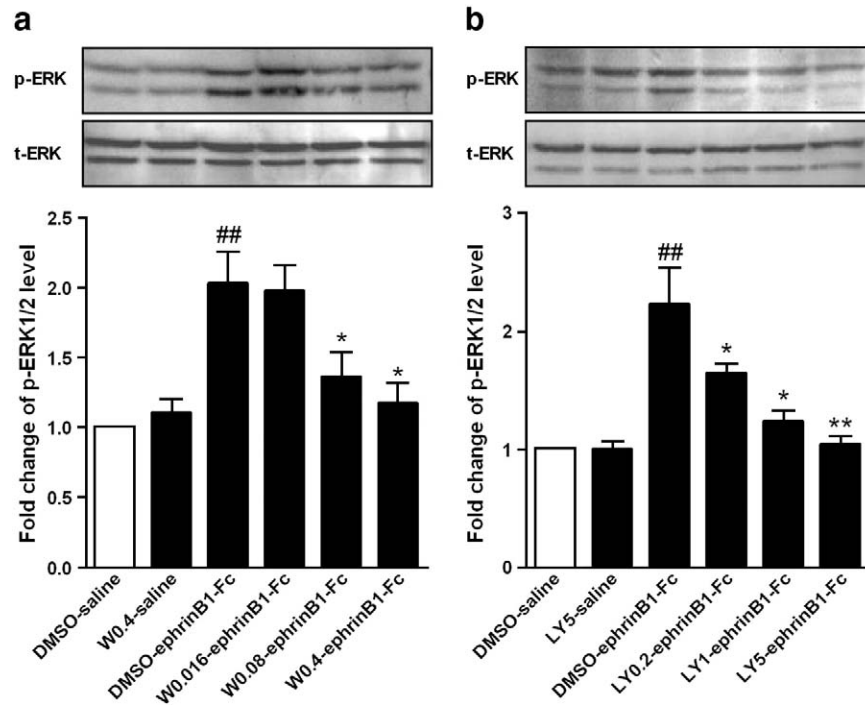
Fig. 6. PI3K mediated peripheral ERK activation induced by i.pl. ephrinB1-Fc. PI3K inhibitor wortmannin (0.016, 0.08, and 0.4 µg) or LY294002 (0.2, 1, and 5 µg) or DMSO was intraplantarly injected at 30 min before i.pl. ephrinB1-Fc (0.5 µg). The expression of peripheral pERK was assayed at 30 min after i.pl. ephrinB1-Fc injection. Pre-treatment with both wortmannin (a) and LY294002 (b) dose-dependently inhibited peripheral ERK activation induced by i.pl. ephrinB1-Fc. The representative bands (top) and the quantitative data (bottom) for the expression of peripheral pERK after i.pl. ephrinB1-Fc (0.5 µg). The fold change for the density of each pERK normalized to total ERK for each sample. The fold change of pERK level in DMSO-saline group was set at 1 for quantifications. $^{##}P<0.01$, compared with DMSO-saline group, $^{*}P<0.05$, $^{**}P<0.01$ compared with DMSO-ephrinB1-Fc group, $n=6$ mice in each group.

of cutaneous inflammatory disease by producing proinflammatory cytokines such as interleukin 1a (IL-1a), IL-1b, IL-6, IL-8, and tumor necrosis factor a (TNF-a) (Albanesi et al., 2005; Tüzün et al., 2007). Keratinocytes are also implicated in sensory transduction and can influence nociception (Southall et al., 2003; Lee and Caterina, 2005; Zhao et al., 2008). PI3K signaling plays an important role in keratinocytes motility, proliferation, differentiation, apoptosis, survival, inflammation response, wounding repair etc. (Sayama et al., 2002; Umeda et al., 2003; Pankow et al., 2006; Fitsialos et al., 2007). Therefore, it is possible that PI3K in non-neuronal cells also mediates pain behavior induced by activation of peripheral ephrinBs/EphBs system.

Our recent study suggested that peripheral ERK activation was involved in pain behaviors induced by activation of peripheral ephrinBs/EphBs system. EphrinB1-Fc-induced hyperalgesia was companied with the time- and dose-dependently increase of peripheral pERK expression. Inhibition of peripheral ERK prevented and reversed pain behaviors and spinal Fos protein expression induced by i.pl. injection of ephrinB1-Fc. Pre-treatment with EphB1-Fc, which inhibited EphBs receptors, suppressed the pain behaviors and the increase of skin pERK expression induced by formalin. Increasing evidence had showed that crosstalk between PI3K and ERK signaling played a prominent role in many physiological and pathological conditions. Both PI3K and ERK are major downstream targets of RTK. Crosstalk between PI3K and Ras has been shown in previous studies carried out in DRG neurons (Jones et al., 2003). Crosstalk from the PI3K to the ERK/MEK signaling pathway contributed to NGF-induced mechanical hyperalgesia (Zhuang et al., 2004; Malik-Hall et al., 2005). The involvement of interaction between PI3K and ERK pathways has been shown in a pain model of thermal hyperalgesia (Zhuang et al., 2004). In the present study, PI3K inhibitors dose-dependently blocked ERK activation induced by i.pl. injection of ephrinb1-Fc. Our results support this link in

peripheral level in ephrinB1-Fc-induced pain model. Our recent study has showed that peripheral NMDA receptor contributed to activation of ERK by ephrinB1-Fc (Cao et al., 2008). Previous results indicated that NMDA receptor-mediated $[Ca^{2+}]i$ increase can activate ERK pathways in a PI3K dependent manner (Chandler et al., 2001; Perkinton et al., 2002). Therefore, we thought that exogenous ephrinBs-Fc or increased expression of ephrinBs by tissue or inflammation directly activates EphBs receptors in nociceptors or non-neuronal cells. Activated EphBs receptors can modulate the NMDA receptor function via a Src-dependent mechanism and then enhance NMDAR-mediated Ca^{2+}. This $[Ca^{2+}]i$ elevation in turn directly facilitates ERK and AKT signaling via PI3K pathways. The activated ERK and AKT signaling further phosphorylated NMDA receptor (Sánchez-Pérez et al., 2006), which increases membrane excitability and induces nociceptor sensitization. On the other hand, the activated ERK and AKT signaling could modulate the expression of transcriptional factors, such as CREB and Fos, and promotes the transcription of downstream genes. The increased expression of pain-related genes may contribute to the maintenance of ephrinBs/EphBs signaling-induced hyperalgesia.

In conclusion, PI3K signaling pathway mediates pain behaviors induced by activation of peripheral ephrinBs/EphBs system in mice and there is a crosstalk between PI3K and ERK signaling in this process. These findings may have important implications for exploring the roles and mechanisms of ephrinBs/EphBs system underlying physiological and pathological pain and other CNS diseases.

Acknowledgements

References

Albanesi C, Scarponi C, Giustizieri ML, Girolomoni G. Keratinocytes in inflammatory skin diseases. Curr Drug Targets Inflamm Allergy 2005;4:329–34.

Amaya F, Shimosato G, Nagano M, Ueda M, Hashimoto S, Tanaka Y, et al. NGF and GDNF differentially regulate TRPV1 expression that contributes to development of inflammatory thermal hyperalgesia. Eur J NeuroSci 2004;20:2303–10.

Bogen O, Joseph EK, Chen X, Levine JD. GDNF hyperalgesia is mediated by PLCgamma, MAPK/ERK, PI3K, CDK5 and Src family kinase signaling and dependent on the IB4-binding protein versican. Eur J NeuroSci 2008;28:12–9.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.

Bundesen LQ, Scheel TA, Bregman BS, Kromer LF. Ephrin-B2 and EphB2 regulation of astrocyte–meningeal fibroblast interactions in response to spinal cord lesions in adult rats. J Neurosci 2003;23:7789–800.

Cakir M, Grossman AB. Targeting MAPK (Ras/ERK) and PI3K/Akt pathways in pituitary tumorigenesis. Expert Opin Ther Targets 2009;13:1121–34.

Camps M, Rückle T, Ji H, Ardissone V, Rintelen F, Shaw J, et al. Blockade of PI3Kgamma suppresses joint inflammation and damage in mouse models of rheumatoid arthritis. Nat Med 2005;11:936–43.

Cao JL, Ruan JP, Ling DY, Guan XH, Bao Q, Yuan Y, et al. Activation of peripheral ephrinBs/EphBs signaling induces hyperalgesia through a MAPKs-mediated mechanism in mice. Pain 2008;139:617–31.

Castellino RC, Durden DL. Mechanisms of disease: the PI3K-Akt-PTEN signaling node-an intercept point for the control of angiogenesis in brain tumors. Nat Clin Pract Neurol 2007;3:682–93.

Chandler LJ, Sutton G, Dorairaj NR, Norwood D. N-methyl-D-aspartate receptor-mediated bidirectional control of extracellular signalregulated kinase activity in cortical neuronal cultures. J Biol Chem 2001;276:2627–36.

Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TJ. Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 1994;53:55–63.

Coggeshall RE. Fos, nociception and the dorsal horn. Prog Neurobiol 2005;77:299–352.

Endersby R, Baker SJ. PTEN signaling in brain: neuropathology and tumorigenesis. Oncogene 2008;27:5416–30.

Fecker LF, Stockfleth E, Nindl I, Ulrich C, Forschner T, Eberle J. The role of apoptosis in therapy and prophylaxis of epithelial tumours by nonsteroidal anti-inflammatory drugs (NSAIDs). Br J Dermatol 2007;156:25–33.

Fitsialos G, Chassot AA, Turchi L, Dayem MA, LeBrigand K, Moreilhon C, et al. Transcriptional signature of epidermal keratinocytes subjected to in vitro scratch wounding reveals selective roles for ERK1/2, p38, and phosphatidylinositol 3-kinase signaling pathways. J Biol Chem 2007;282:15090–102.

Gerlai R. Eph receptors and neural plasticity. Nat Rev Neurosci 2001;2:205–9.

Goldshmit Y, McLenachan S, Turnley A. Roles of Eph receptors and ephrins in the normal and damaged adult CNS. Brain Res Rev 2006;52:327–45.

Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 1988;32:77–88.

Holen HL, Shadidi M, Narvhus K, Kjøsnes O, Tierens A, Aasheim HC. Signaling through ephrin-A ligand leads to activation of Src-family kinases, Akt phosphorylation, and inhibition of antigen receptor-induced apoptosis. J Leukoc Biol 2008;84:1183–91.

Jones DM, Tucker BA, Rahimtula M, Mearow KM. The synergistic effects of NGF and IGF-1 on neurite growth in adult sensory neurons: convergence on the PI 3-kinase signaling pathway. J Neurochem 2003;86:1116–28.

Kim MS, Rådinger M, Gilfillan AM. The multiple roles of phosphoinositide 3-kinase in mast cell biology. Trends Immunol 2008;29:493–501.

Klein R. Eph/ephrin signaling in morphogenesis, neural development and plasticity. Curr Opin Cell Biol 2004;16:580–9.

Klein R. Bidirectional modulation of synaptic functions by Eph/ephrin signaling. Nat Neurosci 2009;12:15–20.

Lee H, Caterina MJ. TRPV channels as thermosensory receptors in epithelial cells. Pflugers Arch 2005;451:160–7.

Malik-Hall M, Dina OA, Levine JD. Primary afferent nociceptor mechanisms mediating NGF-induced mechanical hyperalgesia. Eur J NeuroSci 2005;21:3387–94.

Menges CW, McCance DJ. Constitutive activation of the Raf–MAPK pathway causes negative feedback inhibition of Ras–PI3K–AKT and cellular arrest through the EphA2 receptor. Oncogene 2008;27:2934–40.

Moss A, Alvares D, Meredith-Middleton J, Robinson M, Slater R, Hunt SP, et al. Ephrin-A4 inhibits sensory neurite outgrowth and is regulated by neonatal skin wounding. Eur J NeuroSci 2005;22:2413–21.

Müller EJ, Williamson L, Kolly C, Suter MM. Outside-in signaling through integrins and cadherins: a central mechanism to control epidermal growth and differentiation? J Invest Dermatol 2008;128:501–16.

Munglani R, Fleming BG, Hunt SP. Remembrance of times past: the significance of c-fos in pain. Br J Anaesth 1996;76:1–4.

Nicol GD, Vasko MR. Unraveling the story of NGF-mediated sensitization of nociceptive sensory neurons: ON or OFF the Trks? Mol Interv 2007;7:26–41.

Pankow S, Bamberger C, Klippel A, Werner S. Regulation of epidermal homeostasis and repair by phosphoinositide 3-kinase. J Cell Sci 2006;119:4033–46.

Pasquale EB. Eph-ephrin bidirectional signaling in physiology and disease. Cell 2008;133:38–52.

Perkinton MS, Ip JK, Wood GL, Crossthwaite AJ, Williams RJ. Phosphatidylinositol 3-kinase is a central mediator of NMDA receptor signaling to MAP kinase (Erk1/2), Akt/PKB and CREB in striatal neurones. J Neurochem 2002;80:239–54.

Pezet S, McMahon SB. Neurotrophins: mediators and modulators of pain. Annu Rev Neurosci 2006;29:507–38.

Pezet S, Marchand F, D'Mello R, Grist J, Clark AK, Malcangio M, et al. Phosphatidyli-nositol 3-kinase is a key mediator of central sensitization in painful inflammatory conditions. J Neurosci 2008;28:4261–70.

Russo SJ, Mazei-Robison MS, Ables JL, Nestler EJ. Neurotrophic factors and structural plasticity in addiction. Neuropharmacology 2009;56:73–82.

Sammons MJ, Raval P, Davey PT, Rogers D, Parsons AA, Bingham S. Carrageenan-induced thermal hyperalgesia in the mouse: role of nerve growth factor and the mitogen-activated protein kinase pathway. Brain Res 2000;876:48–54.

Sánchez-Pérez AM, Llansola M, Felipo V. Modulation of NMDA receptors by AKT kinase. Neurochem Int 2006;49:351–8.

Sayama K, Yamasaki K, Hanakawa Y, Shirakata Y, Tokumaru S, Ijuin T, et al. Phosphatidylinositol 3-kinase is a key regulator of early phase differentiation in keratinocytes. J Biol Chem 2002;277:40390–6.

Song XJ, Cao JL, Li HC, Zheng JH, Song XS, Xiong LZ. Upregulation and redistribution of ephrinB and EphB receptor in dorsal root ganglion and spinal dorsal horn neurons after peripheral nerve injury and dorsal rhizotomy. Eur J Pain 2008a;12:1031–9.

Song XJ, Zheng JH, Cao JL, Liu WT, Song XS, Huang ZJ. EphrinB-EphB receptor signaling contributes to neuropathic pain by regulating neural excitability and spinal synaptic plasticity in rats. Pain 2008b;139:168–80.

Southall MD, Li T, Gharibova LS, Pei Y, Nicol GD, Travers JB. Activation of epidermal vanilloid receptor-1 induces release of proinflammatory mediators in human keratinocytes. J Pharmacol Exp Ther 2003;304:217–22.

Stein AT, Ufret-Vincenty CA, Hua L, Santana LF, Gordon SE. Phosphoinositide 3-kinase binds to TRPV1 and mediates NGF-stimulated TRPV1 trafficking to the plasma membrane. J Gen Physiol 2006;128:509–22.

Steinle JJ, Meininger CJ, Forough R, Wu G, Wu MH, Granger HJ. Eph B4 receptor signaling mediates endothelial cell migration and proliferation via the phosphatidylinositol 3-kinase pathway. J Biol Chem 2002;277:43830–5.

Steinle JJ, Meininger CJ, Chowdhury U, Wu G, Wu MH, Granger HJ. Role of ephrin B2 in human retinal endothelial cell proliferation and migration. Cell Signal 2003;15:1011–7.

Sun RQ, Tu YJ, Yan JY, Willis WD. Activation of protein kinase B/Akt signaling pathway contributes to mechanical hypersensitivity induced by capsaicin. Pain 2006;120:86–96.

Sun R, Yan J, Willis WD. Activation of protein kinase B/Akt in the periphery contributes to pain behavior induced by capsaicin in rats. Neuroscience 2007;144:286–94.

Tessier-Lavigne M. Eph receptor tyrosine kinases, axon repulsion, and the development of topographic maps. Cell 1995;82:345–8.

Tüzün Y, Antonov M, Dolar N, Wolf R. Keratinocyte cytokine and chemokine receptors. Dermatol Clin 2007;25:467–76.

Umeda J, Sano S, Kogawa K, Motoyama N, Yoshikawa K, Itami S, et al. In vivo cooperation between Bcl-xL and the phosphoinositide 3-kinase-Akt signaling pathway for the protection of epidermal keratinocytes from apoptosis. FASEB J 2003;17:610–20.

Willson CA, Irizarry-Ramirez M, Gaskins HE, Cruz-Orengo L, Figueroa JD, Whittemore SR, Miranda JD. Upregulation of EphA receptor expression in the injured adult rat spinal cord. Cell Transplant 2002;11:229–39.

Willson CA, Miranda JD, Foster RD, Onifer SM, Whittemore SR. Transection of the adult rat spinal cord upregulates EphB3 receptor and ligand expression. Cell Transplant 2003;12:279–90.

Xu JT, Tu HY, Xin WJ, Liu XG, Zhang GH, Zhai CH. Activation of phosphatidylinositol 3-kinase and protein kinase B/Akt in dorsal root ganglia and spinal cord contributes to the neuropathic pain induced by spinal nerve ligation in rats. Exp Neurol 2007;206:269–79.

Yamaguchi Y, Pasquale EB. Eph receptors in the adult brain. Curr Opin Neurobiol 2004;14:288–96.

Zhao J, Seereeram A, Nassar MA, Levato A, Pezet S, Hathaway G, Morenilla-Palao C, Stirling C, Fitzgerald M, McMahon SB, Rios M, Wood JN, London Pain Consortium. Nociceptor-derived brain-derived neurotrophic factor regulates acute and inflammatory but not neuropathic pain. Mol Cell Neurosci 2006;31:539–48.

Zhao P, Barr TP, Hou Q, Dib-Hajj SD, Black JA, Albrecht PJ, et al. Voltage-gated sodium channel expression in rat and human epidermal keratinocytes: evidence for a role in pain. Pain 2008;139:90-105.

Zhu W, Oxford GS. Phosphoinositide-3-kinase and mitogen activated protein kinase signaling pathways mediate acute NGF sensitization of TRPV1. Mol Cell Neurosci 2007;34:689–700.

Zhu W, Xu P, Cuascut FX, Hall AK, Oxford GS. Activin acutely sensitizes dorsal root ganglion neurons and induces hyperalgesia via PKC-mediated potentiation of transient receptor potential vanilloid I. J Neurosci 2007;27:13770–80.

Zhuang ZY, Xu H, Clapham DE, Ji RR. Phosphatidylinositol 3-kinase activates ERK in primary sensory neurons and mediates inflammatory heat hyperalgesia through TRPV1 sensitization. J Neurosci 2004;24:8300–9.