EJP
European Journal of Pain

ORIGINAL ARTICLE

Activation of ephrinB–EphB receptor signalling in rat spinal cord contributes to maintenance of diabetic neuropathic pain

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Funding sources
This work was partly supported by the National Natural Science Foundation of China (NSFC-81271241 and NSCF-81320108012), Peking University (BMU20120310) and a seed grant for PhD program from Ministry of Education of China (20130001110013).

Conflicts of interest
None declared.

This work should be attributed equally to the institutions.

Accepted for publication
7 June 2016

doi:10.1002/ejp.922

Abstract

Background: Diabetic neuropathic pain (DNP) is severe and intractable in clinic. The specific cellular and molecular mechanisms underlying DNP remain elusive and its treatment are limited. We investigated roles of EphB1 receptor in the development of DNP.

Methods: Diabetic neuropathic pain was produced in male, adult, Sprague-Dawley rats by a single i.p. streptozotocin (STZ) or alloxan. Western blot analysis and immunohistochemistry were used to analyse expression of EphB1 receptor as well as the activation of the glial cells and the pro-inflammatory cytokines in the spinal cord. DNP manifested as mechanical allodynia, which was determined by measuring incidence of foot withdrawal in response to mechanical indentation of the hind paw by an electro von Frey filament.

Results: Diabetic neuropathic pain and high blood glucose were exhibited simultaneously in around 70% of animals that received i.p. STZ or alloxan. Phosphorylation of EphB1, activation of the astrocytes and microglial cells, and level of tumour necrosis factor (TNF)-α and interleukin (IL)-1β in the spinal cord were significantly increased in rats with DNP. Spinal blocking EphB1 receptor activation in the late phase after STZ injection significantly suppressed the established mechanical allodynia as well as activation of the astrocytes and microglial cells and activity of TNF-α and IL-1β. However, spinal treatment of EphB1-Fc in the early phase after STZ injection did not prevent the induction of DNP.

Conclusions: EphB1 receptor activation in the spinal cord is critical to the maintenance, but not induction of diabetic pain. EphB1 receptor may be a potential target for relieving the established diabetic pain.

Significance: Activation of EphB1 receptor in the spinal cord is critical to maintaining the established diabetic neuropathic pain, but not to diabetic pain induction. Spinal blocking EphB1 receptor activation suppresses ongoing diabetic neuropathic pain.

1. Introduction

Diabetic neuropathic pain (DNP) is one of the most common complications of diabetes mellitus. DNP is severe and intractable and affects about 10–26% of millions of patients with diabetes (Davies et al., 2006; Quilici et al., 2009; Tomic et al., 2010; Alge-Priglinger et al., 2011; daCosta DiBonaventura et al., 2011; de Salas-Cansado et al., 2012; Sandercock et al., 2012; Otis et al., 2013). Despite decades of studies and numerous processes being implicated,

the specific cellular and molecular mechanisms that underlie the pathogenesis of DNP remain elusive and the clinical approaches for its treatment are thus limited. The multiple possible aetiologies which may underlie the pathogenesis of DNP may include the follows: (1) Hyperglycaemia-induced damages to nerve cells and decreases in neurovascular flow (Edwards et al., 2008); (2) Production of the pro-inflammatory cytokines including tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 (Vincent and Feldman, 2004; Brownlee, 2005; Edwards et al., 2008; Obrosova, 2009); (3) Perturbations in growth factors such as insulin-like growth factor (IGF), nerve growth factor (NGF) and neurotrophin 3 (NT-3) (Edwards et al., 2008; Obrosova, 2009); (4) Immune dysfunction (Obrosova et al., 2004); (5) Diabetes-related oxidant stresses such as mitochondrial oxidant stress, and other cellular stresses including endoplasmic reticulum (ER) stress (Edwards et al., 2008; Obrosova, 2009); and (6) Multifocal loss of myelinated and unmyelinated fibres (Kawashima et al., 2007; Arnold et al., 2013).

Eph receptors constitute the largest subfamily of receptor tyrosine kinases (RTKs) in mammals, with 14 members (Klein, 2009; Sheffler-Collins and Dalva, 2012), which play vital roles in transmitting external signals to the inside of many types of cells (Sloniowski and Ethell, 2012). Recently, we have demonstrated that EphB1 receptor signalling contributes greatly to the development and maintenance of chronic pain following nerve injury and bone cancer, as well as the long exposure to opiates (Han et al., 2008; Song et al., 2008a,b; Liu et al., 2009, 2011). DNP seen in the later phase of diabetes is a typical neuropathic pain, which is severe, intractable and with high prevalence. We hypothesized that ephrinB–EphB receptor signalling might play an important, but unknown role in DNP. Here, we show that activation of EphB1 receptor in the spinal cord is critical to the established DNP. Spinal blocking EphB1 receptor activation can greatly relieve DNP in rats with diabetes induced by intraperitoneal injection of streptozotocin (STZ).

2. Methods

2.1 Animals, drugs and drug administration

We purchased adult, male, Sprague-Dawley rats (200–220 g-wt) from the Department of Laboratory Animal Science of Peking University Health Science Center. We purchased EphB1-Fc (Sigma, St Louis, MO, USA) and immunoglobulin (Ig) G-Fc (Jackson Laboratories, West Grove, PA, USA). EphB1-Fc (5 μg) or IgG-Fc (5 μg) was dissolved in phosphorylate buffer solution (PBS) and then injected intrathecally (i.t., 20 μL) by means of lumbar puncture at the intervertebral space of L_4–L_6 for consecutive multiple injections.

2.2 Animal models of diabetes and peripheral nerve injury

Diabetes was induced in rats, after an overnight fast, by a single intraperitoneal injection (i.p.) of STZ (Sigma), 70 mg/kg, freshly dissolved in 0.1 mol/L citric acid buffer (CAB, pH 4.5) or alloxan (Sigma), 150 mg/kg, freshly dissolved in saline. Animals in the control group received an injection of an equivalent volume of citrate buffer or saline alone. A single or multiple measurements of blood glucose were made after injection of STZ or alloxan in each of the animals. The blood samples were collected from the tail vein blood vessel. Onset of diabetic conditions was defined as glucose levels >16.6 mmol/L. The rat model of chronic constriction injury of the sciatic nerve injury (CCI; Bennett and Xie, 1988) was used as a positive control in the experiments testing expression of EphB1 in the spinal cord. In brief, to produce the CCI model, the left common sciatic nerve was exposed at the mid-thigh level. Four ligatures (4–0 surgical catgut) were tied loosely around the sciatic nerve with about 1 mm between ligatures.

2.3 Assessment of mechanical allodynia

Mechanical allodynia in DNP rats was determined by measuring the incidence of foot withdrawal in response to mechanical indentation of the plantar surface of each hind paw using a sharp, cylindrical probe with a uniform tip diameter of approximately 0.2 mm (ALMEMO 2390-5 Electronic von Frey Anesthesiometer; IITC Life Science, San Diego, CA, USA). The probe was applied to six designated loci distributed over the plantar surface of each foot. The minimal force (in grams) that induced paw withdrawal was read off in the display. Two feet were measured in each rat and showed similar mechanical sensitivity in the conditions of control and DNP. The threshold of mechanical withdrawal in each animal was calculated by averaging the 12 (6 × 2) readings, and the force was converted into milli-newtons (mN). The protocols used for determining the pain-related behaviours are similar to those we have previously described (Song et al., 1999, 2008b; Zhang et al., 2013).

2.4 Western blotting

Protein precipitation procedures in conjunction with Western blots were employed to identify temporal expression of EphB1 protein and its phosphorylation (pEphB1) on tyrosine residues. Western blots containing precipitated EphB1 were first immunostained with a monoclonal antibody specific for phosphorylated tyrosine (4G10; Millipore, Bedford, MA, USA) to detect activated EphB1 receptors. (Bundesen et al., 2003; Richards et al., 2007; Liu et al., 2011). Protocols were similar to that we described previously (Liu et al., 2009, 2010, 2011). The entire spinal cord segments at L_4–L_5 were quickly removed from deeply anaesthetized rats. The tissues were homogenized in Radio-immunoprecipitation assay (RIPA) lysis buffer (Bio-Rad Laboratories, Hercules, CA, USA) containing a cocktail of protease inhibitor and phosphatise inhibitors (Sigma). Protein concentrations of the lysates were estimated using the bicinchoninic acid (BCA) method (with reagents from Pierce Biotechnology, Waltham, Massachusetts, USA). EphB1 was immunoprecipitated from 2 mg total protein/mL tissue lysate using an anti-EphB1 antibody 2 μg (Santa Cruz Technology, Paso Robles, CA, USA) complex with protein G-agarose (Invitrogen, Waltham, MA, USA). EphB1 and its phosphorylation were detected by anti-EphB1 antibody (Q20, Santa Cruz Technology) and phosphotyrosine antibody 4G10 (Millipore), respectively (Bundesen et al., 2003; Compagni et al., 2003; Richards et al., 2007; Liu et al., 2011). Whole-cell protein extracts lysates were used to identify temporal expression of protein levels of GFAP, IBA1, IL-1β, TNF-α and β-actin. The primary antibodies were used including GFAP (Millipore, 1:2000), IBA1 (1:1000; Abcam, Cambridge, UK), IL-1β (SCT, 1:1000), TNF-α (1:1000; Cell signaling, Danvers, MA, USA) and β-actin (1:2000; Bioworld, St. Louis Park, MN, USA). The filters were then developed by enhanced chemiluminescence reagents (Perkinelmer, Waltham, MA, USA) with secondary antibodies (Chemicon, Billerica, MA, USA). Data were analysed with a Molecular Imager (Gel DocTM XR, 170-8170) and the associated software Quantity One-4.6.5 (Bio-Rad Laboratories).

2.5 Immunohistochemistry

Sections of the spinal cord at segments L_4–L_5 were incubated with mouse monoclonal anti-GFAP (1:400) and anti-IBA1 (1:100). Morphological details were examined with a confocal microscope (FluoView FV1000, Olympus, Shinjuku, Tokyo, Japan). The protocols were similar to those described previously (Liu et al., 2009, 2010, 2011; Zhang et al., 2013). To quantitatively measure immunofluorescence of GFAP and IBA1 in the spinal dorsal horn, 12 sections from each rat spinal cord segment (three rats in one group) were photographed at the same exposure time to generate the raw data. The fluorescence images were converted to 8-bit grey and then inverted to black (GFAP+, IBA1+) and white images. After subtracting the background, the mean optical density (uncalibrated OD) of the interesting areas (laminae I–V of the DH of spinal cord) was measured using NIH ImageJ program (Bethesda, MD, USA).

2.6 Statistical analysis

SPSS Rel 15 (SPSS Inc., Chicago, IL, USA) was used to conduct all the statistical analyses. Alteration of expression of the proteins detected and the behavioural responses to mechanical stimuli over time among groups were tested with 1-way and 2-way ANOVA with repeated measures followed by Bonferroni post hoc tests, respectively. Results are expressed as means ± SEM. Statistical results are considered significant if $p < 0.05$.

3. Results

3.1 High blood glucose and mechanical allodynia induced by i.p. STZ and alloxan, respectively

We first established animal models of DNP by i.p. injection of STZ and alloxan, respectively. Following administration of STZ (70 mg/kg) and alloxan (150 mg/kg), respectively, 87% (STZ, $n = 226$) and 88% (alloxan, $n = 50$) of animals developed high blood glucose (>16.6 mmol/L). The average level of blood glucose increased to approximately 15–30 mmol/L during 9–42 days after injection of STZ or approximately 8–20 days after alloxan from approximately 6 mmol/L prior to injection of STZ or alloxan, respectively. In STZ group, the significantly increased level of blood glucose started within 7 days and kept gradually and aggressively increasing in a linear manner the following 14–42 days tested (Fig. 1A). In alloxan group, the increased level of blood glucose started within 5 days and kept gradually increasing in a linear manner until 20 days, the last examination (Fig. 1B).

Meanwhile, most of the animals that received injection of STZ or alloxan with increased level of

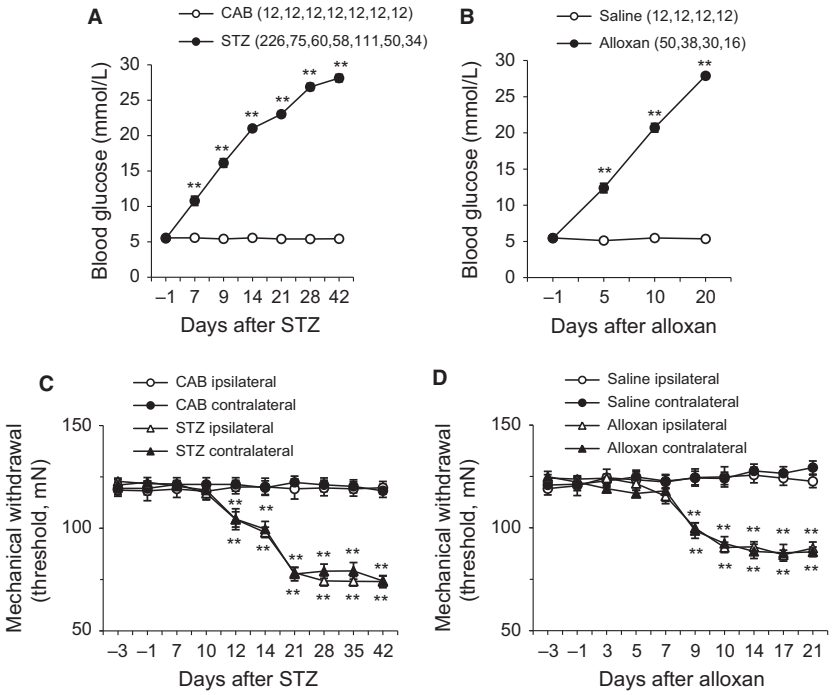

Figure 1 High blood glucose and mechanical allodynia following intraperitoneal injection of streptozotocin (STZ) or alloxan. (A, B) High blood glucose induced by STZ (A) and alloxan (B). Numbers of animals corresponding to each testing day are indicated in the parentheses. (C, D) Mechanical hypersensitivity (allodynia) manifested as significant decrease of the mechanical withdrawal thresholds induced by STZ (C) and alloxan (D), respectively. Twelve rats were included in each of the groups. There were approximately 20% animals that did not show mechanical allodynia were not included in the datasheet (see description in the first section of Results). All the rats included in C and D exhibited mechanical allodynia also had high blood glucose. Two-way ANOVA, $^{**}p < 0.01$ versus CAB (A), saline (B), CAB ipsilateral and CAB contralateral (C), or saline ipsilateral and saline contralateral (D).

glucose exhibited significant painful syndrome manifested as mechanical allodynia. In STZ group, among the 197 rats with high blood glucose, 141 (71.57%) developed painful syndrome, mechanical allodynia. The rest 56 (28.43%) animals with high level of glucose did not exhibit mechanical allodynia. In alloxan group, among 44 rats with high blood glucose, 77.27% (34/44) developed painful syndrome, mechanical allodynia. The rest 10 (22.73%) animals with high level of glucose did not exhibit mechanical allodynia. The STZ-induced mechanical allodynia started from 12 days, peaked at 21 days, and maintained at the peak level during 21–42 days (Fig. 1C). The alloxan-induced mechanical allodynia started within 9 days and lasted to 21 days, the last examination (Fig. 1D). Animals that received injection of alloxan without high level of blood glucose (12%, 6/50) did not exhibit mechanical allodynia. STZ and alloxan both induced high level of blood glucose and mechanical allodynia in similar patterns in most rats examined. The control vehicle treatment with CAB and saline, respectively, did not change the blood glucose and mechanical sensitivity (Fig. 1A–D).

Our data also showed that neither STZ nor alloxan treatment could induce significant changes in the thermal sensitivity, that is, no thermal hyperalgesia was induced in these rats treated with STZ or alloxan (data not shown). This observation is consistent with previous reports that STZ-diabetic rats did not develop thermal hyperalgesia (Chen and Pan, 2002; Wang et al., 2013), while diabetic mice did (Wang et al., 2013).

Among the animals that received i.p. STZ or alloxan, only those exhibited both high blood glucose and mechanical allodynia were included in the following studies and we defined these conditions as DNP.

3.2 Activity of EphB1 in the spinal cord after i.p. STZ or alloxan

To test the hypothesis that EphB receptor would be involved in DNP, we examined expression of EphB1 receptor protein and its phosphorylation (pEphB1) in the spinal cord in DNP rats. The results showed that i.p. STZ or alloxan significantly increased

expression of pEphB1, but not EphB1 (Fig. 2A and B). Our previous studies have shown that both EphB1 and pEphB1 can be greatly increased after nerve injury.(Song et al., 2008a,b) To confirm such a different expression of EphB1 and pEphB1 between DNP and peripheral nerve injury, we

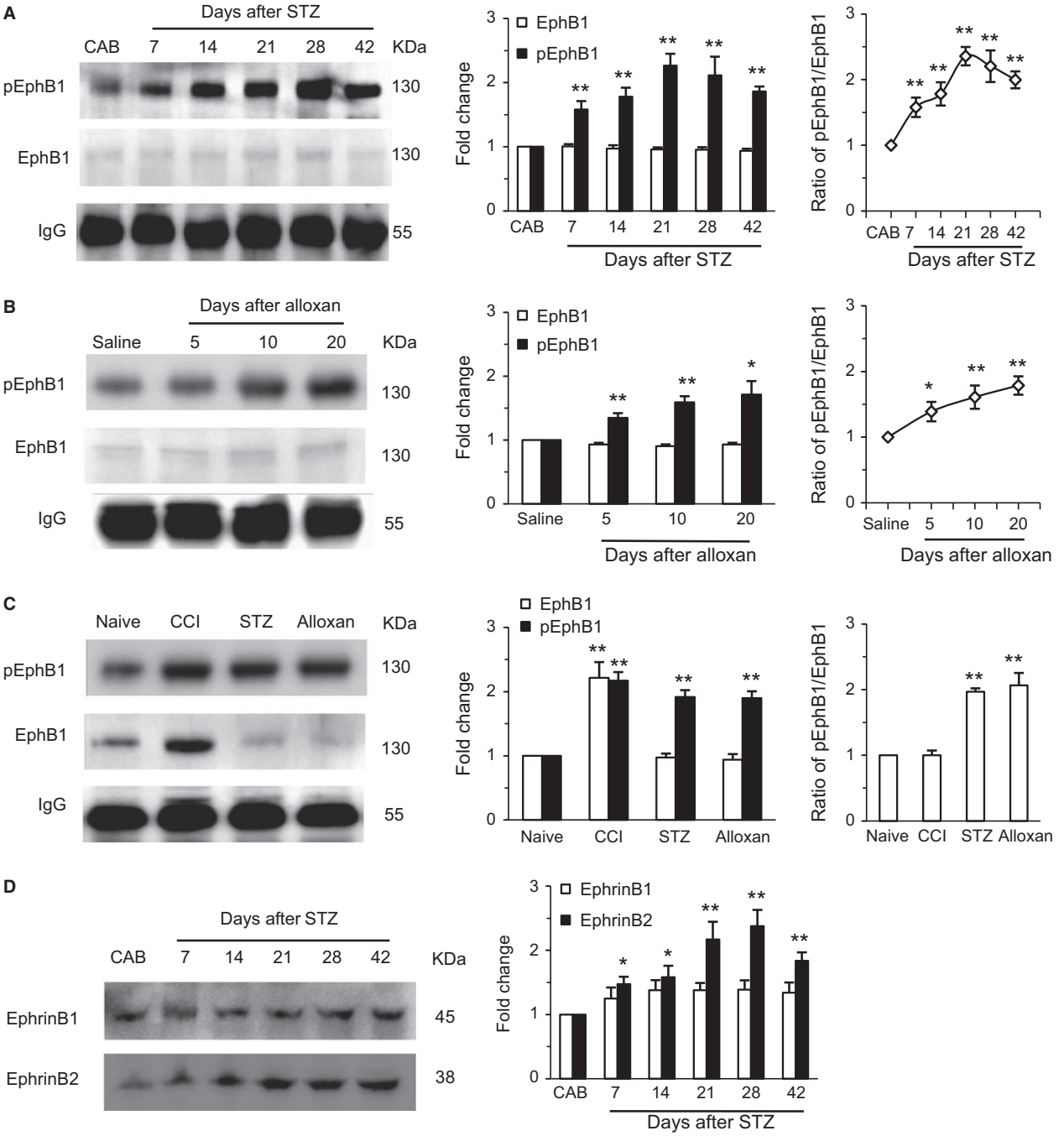

Figure 2 Expression of EphB1, ephrinB1, ephrinB2 and phosphorylation of EphB1 (pEphB1) in the spinal cord in DNP and CCI rats. (A, B) Western blot analysis showing time course of expression of EphB1 and pEphB1 after i.p. STZ (A) and alloxan (B), respectively. (C) Western blot analysis showing expressions of EphB1 and pEphB1 14 days after treatment of CCI, STZ, or alloxan. (D) Western blot analysis showing time course of expression of ephrinB1 and ephrinB2 after i.p. STZ. (A–D) Left: representative western blot bands; middle: data summary; right: ratio of pEphB1 and EphB1. Four samples were included in each of the groups. One-way ANOVA, $*p < 0.05$; $**p < 0.01$ versus vehicle (A, D), saline (B), or control (C).

examined and compared the expression of EphB1 and pEphB1 following treatment of STZ, alloxan, as well as the sciatic nerve injury (CCI treatment). The results confirmed that CCI increased expression of both EphB1 and pEphB1, while STZ and alloxan increased only pEphB1, but not EphB1 (Fig. 2C).

3.3 Spinal blocking EphB1 receptor signalling attenuates persistence, but not induction of STZ-induced mechanical allodynia

Given that phosphorylation (activity) of EphB1 in the spinal cord can be greatly increased in DNP rats, we examined whether spinal blocking EphB1 activity could suppress DNP, which manifested as mechanical allodynia in this study. The results showed that spinal administration of a single dose of EphB1-Fc (5 μg, i.t.) on 26 days, the late phase, after STZ injection, significantly inhibited the established mechanical allodynia. The inhibition started within 0.5 h and lasted for about 4 h (Fig. 3A) following EphB1-Fc treatment. Repetitive administration of EphB1-Fc (5 μg, i.t., and daily for 3 days on 26, 27 and 28 days after STZ injection) produced a significant, prolonged inhibition of mechanical allodynia (Fig. 3B).

To further test whether EphB1-Fc would affect the induction of DNP, the animals were treated with EphB1-Fc in the early phase after STZ injection when DNP had not been well developed. The results showed that repetitive i.t. EphB1-Fc, 5 μg, i.t., daily for three consecutive days on 9, 10 and 11 days after STZ injection, failed to prevent the development of DNP (Fig. 3C). Concerning that this 'early-injection' might not be early enough, we continued to perform additional behavioural tests to examine analgesic effects of spinal administration of EphB1-Fc given on the 7, 8, 9, 10 and 11 days after STZ treatment (earlier and covering longer period of time; the blood sugar started to increase greatly on the 7th day after STZ injection, see Fig. 1A). The results showed that such earlier-and-longer-treatment of EphB1-Fc again

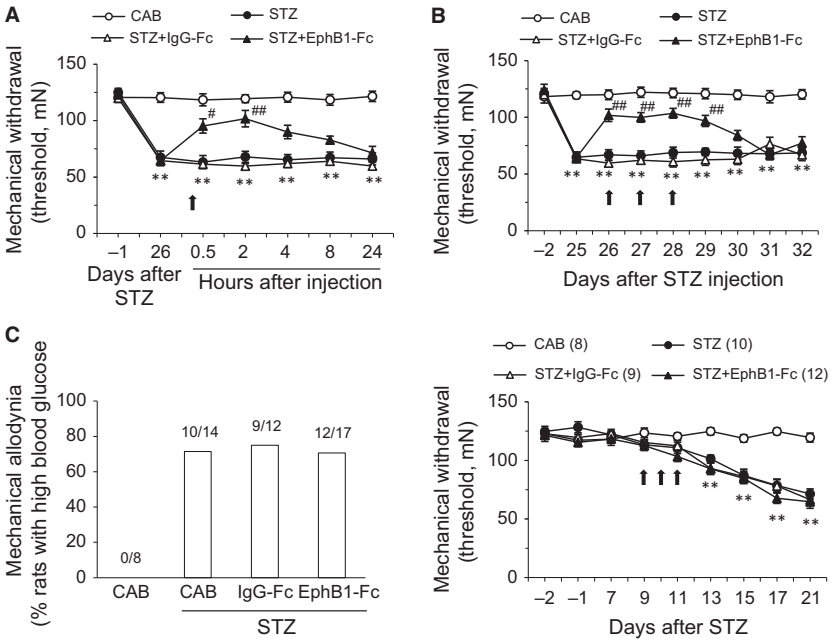

Figure 3 Intrathecal administration of EphB1 receptor-blocking reagent EphB1-Fc suppresses STZ-induced mechanical allodynia in rats. (A) A single dose of EphB1-Fc (5 μg) administrated in the late phase of DNP transiently reduced the persistence of mechanical allodynia. (B) Repetitive administration of EphB1-Fc (5 μg, daily for three consecutive days) in the late phase of DNP produced prolonged inhibition of the persistence of mechanical allodynia. Number of rats in A and B: eight rats were included in each of the groups and all rats with STZ treatment were first identified with high blood glucose and mechanical allodynia. (C) Repetitive administration of EphB1-Fc (5 μg, daily for three consecutive days) in the early phase after STZ injection did not prevent the induction of mechanical allodynia. Left: percentage of rats with high blood glucose exhibiting mechanical allodynia. The numbers shown on the top of each of the columns: number of rats with mechanical allodynia/total rats tested. The treatment did not change the percentage of rats showing mechanical allodynia. Right: effects of i.t. EphB1-Fc on mechanical allodynia. EphB1-Fc treatment did not prevent the induction of mechanical allodynia. Number of rats in each of the groups are indicated in the parenthesis. Those rats did not show mechanical allodynia shown in the left figure were not included in the right figure. Drug administration was indicated by the arrow(s) in the corresponding day point. Two-way ANOVA, $^{**}p < 0.01$ versus CAB, the vehicle control. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ STZ and STZ+IgG-Fc.

failed to prevent the development of STZ-induced mechanical allodynia (data not shown). These results indicated that EphB1 receptor signalling in the spinal cord may contribute to maintaining the established DNP, mechanical allodynia, but not its induction or production.

3.4 Spinal blocking EphB1 receptor activation suppresses STZ-induced activation of the astrocytes and IL-1β and TNF-α in the spinal cord

The astrocytes and microglial cells as well as the pro-inflammatory cytokines play important roles in DNP as well as other forms of neuropathic pain (Gao et al., 2010; Fernandes et al., 2011; Liao et al., 2011; Dauch et al., 2012; Lo et al., 2014; Ortmann and Chattopadhyay, 2014). Our Western blotting and immunohistochemical analysis showed that the astrocytes (GFAP) and microglial cells (IBA1) in the spinal cord were significantly activated in STZ-induced DNP. Repetitive spinal administration of EphB1 receptor blocker EphB1-Fc, 5 μg, daily for three consecutive days on 26, 27 and 28 days (the late phase after STZ injection), significantly inhibited the increased expression of GFAP, but not IBA1 (Fig. 4A and B). However, the same treatment of EphB1-Fc administrated on 9, 10 and 11 days (the early phase after STZ injection), failed to reduce the expression of GFAP and IBA1 (Fig. 4C). Similarly, repetitive spinal administration of EphB1 receptor blocker EphB1-Fc, 5 μg, daily for three consecutive days in the late phase on 26, 27, and 28 days after STZ injection significantly inhibited the increased activity of TNF-α and IL-1β (Fig. 5A). Meanwhile, such an EphB1-Fc treatment did not affect STZ-induced high blood glucose (Fig. 5B).

4. Discussion and conclusion

This study reveals that EphB1 receptor signalling in the spinal cord is critical to the maintenance of DNP and provides a new understanding of the pathogenesis of DNP. DNP induced by STZ or alloxan causes significant activation (phosphorylation) of the EphB1 receptor in addition to activation of the astrocytes and microglial cells as well as the pro-inflammatory cytokines IL-1β and TNF-α. Spinal administration of an EphB1 receptor-blocking reagent EphB1-Fc inhibits the established mechanical allodynia as well as activation of the astrocytes, IL-1β and TNF-α in the spinal cord. This study suggests that EphB1 receptor may be a potential target for relieving DNP.

Diabetic neuropathic pain is a severe, intractable chronic pain accompanying patients who suffer diabetes particularly in the later phase of the diabetes. The specific cellular and molecular mechanisms that underlie the pathogenesis of DNP are complex and remain elusive (Vincent and Feldman, 2004; Brownlee, 2005; Kawashima et al., 2007; Edwards et al., 2008; Obrosova, 2009; Arnold et al., 2013). Recently, we have demonstrated and identified that the ephrinB–EphB receptor signalling is one of the key mechanisms underlying neuropathic pain induced by peripheral nerve injury (Song et al., 2008a,b) and bone cancer (Liu et al., 2011, 2013). These studies have provided strong evidence that EphB1 receptor may be an effective therapeutic target for treatment of these painful conditions. In the present study, our results indicate that EphB1 receptor is involved in the maintenance of DNP and blocking EphB1 receptor activation can greatly reduce the established mechanical allodynia in DNP rats. This suggests that EphB1 receptor signalling may be a new mechanism underlying the pathogenesis of DNP and supports that EphB1 receptor may also be an effective pharmaceutical target for DNP treatment.

Pain caused by diabetic neuropathy is considered as a typical neuropathic pain and thus it is not surprised that DNP shares certain common neural mechanisms that underlie other forms of neuropathic pain such as that caused by peripheral nerve injury or bone cancer. However, our findings indicate that the roles of EphB1 receptor signalling in the pathogenesis of DNP are different from that in nerve injury- and bone cancer-induced pain. Our recent studies have shown that EphB receptor signalling in the spinal cord is critical to both induction and maintenance of neuropathic pain after nerve injury and bone cancer (Song et al., 2008a; Liu et al., 2013). However, in this study, EphB1 receptor activation in the spinal cord is found involved in only the maintenance, but not the induction of DNP, indicating the different mechanisms underlying induction of neuropathic pain after nerve injury and diabetes, respectively. Moreover, phosphorylation of EphB1 receptor in the spinal cord was activated in DNP status, while its protein expression of EphB1 receptors was not significantly altered (see Fig. 2A and B). EphB1 receptor activated by its ligand ephrins become extensively phosphorylated on the tyrosine residues. Upon phosphorylation of the tyrosine residues, the solvent-exposed juxtamembrane segment becomes available for interactions with downstream signalling proteins, such as, the specific

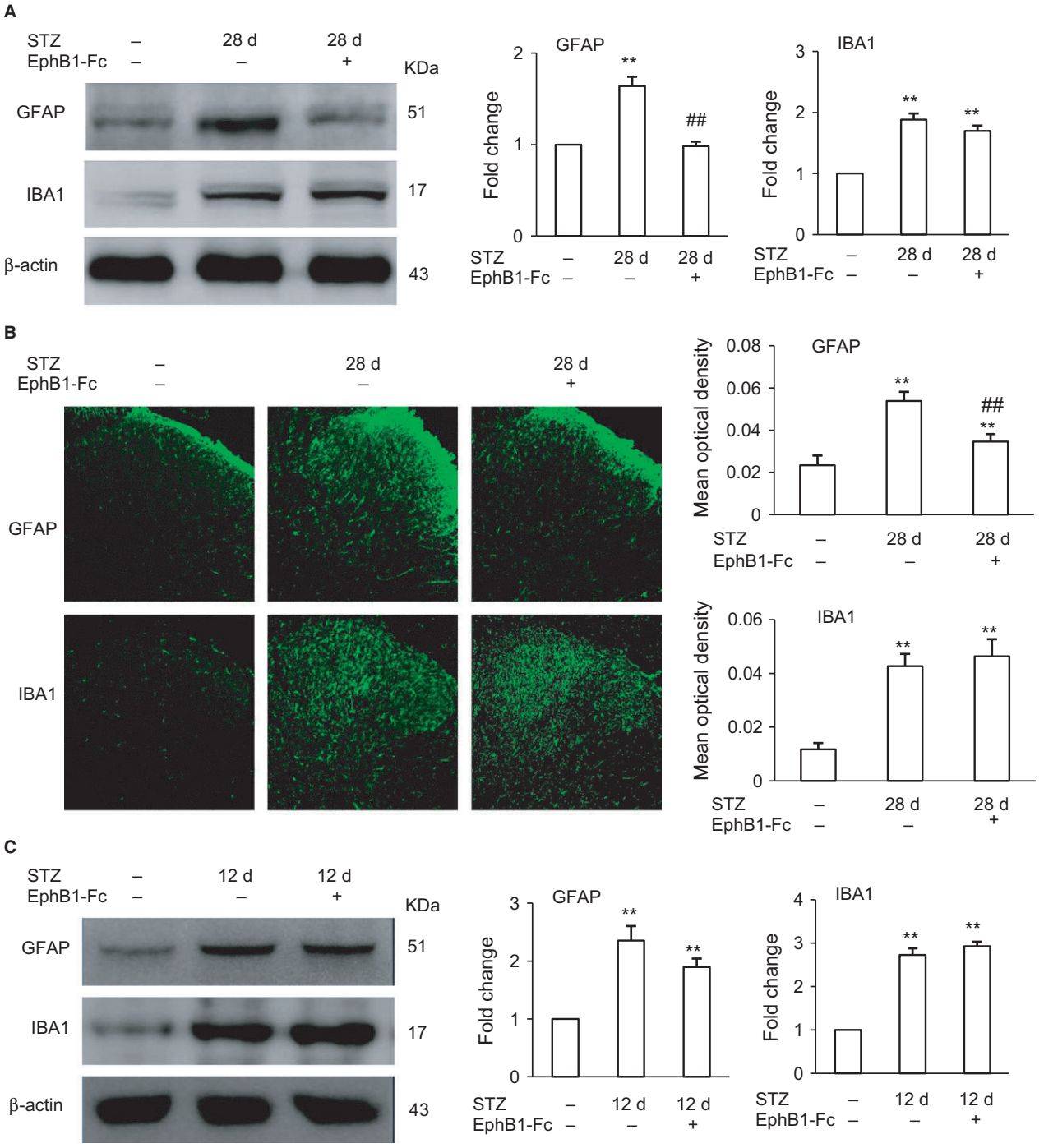

Figure 4 Effects of blocking EphB receptor activation on activation of the astrocytes (GFAP) and microglial cells (IBA1) in the spinal cord after STZ treatment. (A) Western blot showing effects of repetitive i.t. EphB1-Fc (5 μg, daily for three consecutive days on 26–28 days after STZ injection) on expression of GFAP and IBA1. Tissues were taken on the 28th day, 4 h after the last injection. Four spinal cord segments were included in each of the groups. (B) Confocal images showing effects of i.t. EphB1-Fc (in the same protocols as in A) on activation of GFAP and IBA1 (both in green). Magnification: 200x. Twenty sections from four spinal cord segments were included in each of the groups. (C) Western blot analysis showing effects of repetitive i.t. EphB1-Fc (5 μg, daily for three consecutive days on 10–12 days after STZ injection) on expression of GFAP and IBA1. Tissues were taken on the 12th day, 6 h after the last injection. Four spinal cord segments were included in each of the groups. (A-C) Left: representative bands or confocal images. Right: data summary. One-way ANOVA, $^{**}p < 0.01$ versus control without STZ and EphB1-Fc. $^{\#\#}p < 0.01$ versus STZ 28 days (A, B), or STZ 12 days (C).

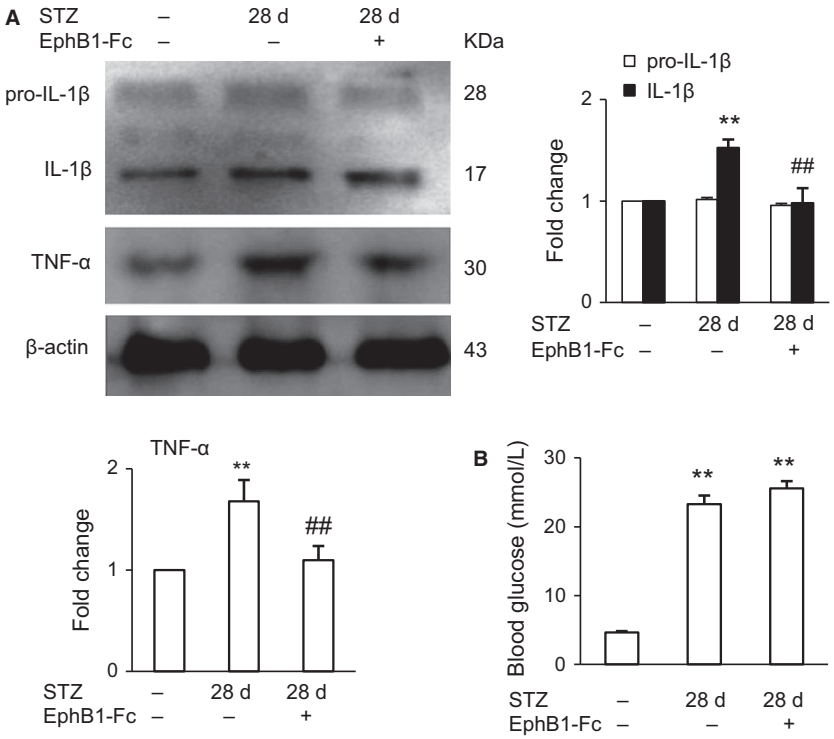

Figure 5 Spinal administration of EphB1-Fc inhibits STZ-induced activity of IL-1β and TNF-α in the spinal cord. (A) Western blotting analysis showing effects of repetitive administration of EphB1-Fc (5 μg, daily for consecutive 3 days on 26–28 days after STZ injection) on expression of IL-1β and TNF-α in spinal cord after STZ injection. Tissues were collected 6 h after the last injection of EphB1-Fc). Top left: representative Western blot bands. Histograms: data summary. Four samples (each included one lumbar spinal cord segment) were included in each of the groups. (B) Blood glucose levels measured at 6 h after injection of EphB1-Fc on the 28 days after injection of STZ. Twelve rats were included in each group. One-way ANOVA, $^{**}p < 0.01$ versus control without STZ and EphB1-Fc. $^{\#\#}p < 0.01$ versus STZ 28 days.

binding of Src homology (domains) 2 and 3, phosphotyrosine domain, or post-synaptic density protein-95 domain-containing downstream signalling molecules and activate corresponding signalling pathways (Himanen and Nikolov, 2003; Pasquale, 2005; Himanen et al., 2007; Coulthard et al., 2012).

In addition to providing further mechanism underlying the pathogenesis of DNP, this study also demonstrates that the neural mechanisms underlying induction of DNP are completely different from that underlying induction of pain caused by direct peripheral nerve injury or bone cancer. There is a need to further investigate the cellular and molecular mechanisms that may underlie such differences among the painful conditions of diabetes, peripheral nerve injury and bone cancer.

Inflammation plays important roles in diabetic neuropathy (Hotamisligil, 2006; Averill and Bornfeldt, 2009; Kaul et al., 2010). Activation of astrocytes and microglial cells and the pro-inflammatory cytokines are important in pathogenesis of DNP (Gonzalez-Clemente et al., 2005; Ledeboer et al., 2005; Devaraj et al., 2007; Uceyler et al., 2007; King, 2008; Averill and Bornfeldt, 2009; Ortmann and Chattopadhyay, 2014) and other forms of painful conditions, such as bone cancer pain (Liu et al., 2011, 2013). The present study shows that activation of the astrocytes and the cytokines TNF-α and IL-1β in the spinal cord associated with DNP can be greatly suppressed by spinal blocking EphB1 receptor activation. This suggests that activation of EphB1 receptor may in certain way be responsible for activation of astrocytes and production of the pro-inflammatory cytokines including TNF-α and IL-1β, which cause spinal central sensitization and further behavioural signs of DNP such as mechanical allodynia in this study.

In summary, this study reveals a role of spinal EphB1 receptor signalling in the pathogenesis of DNP. Activation of EphB1 receptor is critical for the maintenance of DNP. Blockade of EphB1 receptor in the spinal cord results in a great reduction in the established mechanical allodynia as well as the accompanying activation of the astrocytes and the

pro-inflammatory cytokines in rats with DNP. This study suggests that targeting EphB1 receptor may relieve chronic pain due to diabetic neuropathy.

Acknowledgements

We thank P. Zhao, B. Zheng, and H. Ma for their assistance in conducting the behavioural experiments.

Author contributions

X.T.D. and X.J.S. designed the project. X.T.D., M.Z.W., P.C.M. and N.X. conducted the experiments. X.T.D. analysed the data and performed statistical analysis. X.J.S. with assistance of X.T.D. wrote the manuscript. All authors read and approved the final manuscript.

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