



## Vector-mediated release of GABA attenuates pain-related behaviors and reduces Nav1.7 in DRG neurons

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### ABSTRACT

Pain is a common and debilitating accompaniment of neuropathy that occurs as a complication of diabetes. In the current study, we examined the effect of continuous release of gamma amino butyric acid (GABA), achieved by gene transfer of glutamic acid decarboxylase (GAD67) to dorsal root ganglia (DRG) *in vivo* using a non-replicating herpes simplex virus (HSV)-based vector (vG) in a rat model of painful diabetic neuropathy (PDN). Subcutaneous inoculation of vG reduced mechanical hyperalgesia, thermal hyperalgesia and cold allodynia in rats with PDN. Continuous release of GABA from vector transduced cells *in vivo* prevented the increase in the voltage-gated sodium channel isoform 1.7 (Nav1.7) protein that is characteristic of PDN. *In vitro*, infection of primary DRG neurons with vG prevented the increase in Nav1.7 resulting from exposure to hyperglycemia. The effect of vector-mediated GABA on Nav1.7 levels *in vitro* was blocked by phaclofen but not by bicuculline, a GABA<sub>B</sub> receptor effect that was blocked by pertussis toxin-(PTX) interference with G $\alpha_{(i/o)}$  function. Taken in conjunction with our previous observation that continuous activation of delta opioid receptors by vector-mediated release of enkephalin also prevents the increase in Nav1.7 in DRG exposed to hyperglycemia *in vitro* or *in vivo*, the observations in this report suggest a novel common mechanism through which activation of G protein coupled receptors (GPCR) in DRG neurons regulate the phenotype of the primary afferent.

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### 1. Introduction

Neuropathy is a major complication of diabetes mellitus, and a common cause of neuropathic pain (Tavakoli et al., 2008). Like other forms of chronic neuropathic pain, painful diabetic neuropathy (PDN) is difficult to treat (Finnerup et al., 2010). In an attempt to directly target delivery of analgesic peptides to nociceptive pathways (Mata et al., 2008) we have developed a series of non-replicating herpes simplex virus (HSV)-based vectors that efficiently target gene delivery to dorsal root ganglia (DRG) from skin inoculation (Glorioso and Fink, 2004; Wolfe et al., 1999). In previous studies we have demonstrated the efficacy of HSV vectors designed to release enkephalin, endomorphin-2 or gamma aminobutyric acid (GABA) in models of inflammatory pain, central neuropathic pain, and pain caused by cancer (Goss et al., 2002, 2001; Hao et al., 2003, 2009; Liu et al., 2004; Wolfe et al., 2007). The first of these HSV-based vectors, a non-replicating HSV vector expressing preproenkephalin is currently in clinical trial in patients with intractable pain from cancer (Wolfe et al., 2009).

The role of spinal GABA in neuropathic pain syndromes is uncertain. Several recent studies have suggested that in models of chronic pain induced by inflammation or peripheral nerve injury, a reduction in the expression of the potassium chloride cotransporter 2 (KCC2) in neurons of the superficial spinal dorsal horn may induce a shift in the effect of GABA on the GABA<sub>A</sub> receptor from inhibitory to excitatory (Coull et al., 2003; Zhang et al., 2008) and thus contribute to the neuropathic pain phenotype (Mantyh and Hunt, 2004; Zhang et al., 2008). Despite these observations, we previously found that inoculation of an HSV vector encoding glutamic acid decarboxylase (GAD) that results in release of GABA in the dorsal horn is effective in reducing pain-related behaviors in the spinal nerve ligation model of neuropathic pain (Hao et al., 2005).

The rodent model of PDN in rats with streptozotocin (STZ)-induced diabetes is characterized by increased amounts of the voltage-gated sodium channel  $\alpha$  subunit isoform 1.7 (Nav1.7) in DRG (Hong et al., 2004; Hong and Wiley, 2006), an increase that occurs as a result of a post-transcriptional process mediated by protein kinase C (PKC) (Chattopadhyay et al., 2008). We previously observed that HSV-mediated expression of enkephalin in DRG of diabetic animals results in a reduction in the amount of Nav1.7 protein in DRG neurons *in vivo*, coincident with a reduction in pain-related behaviors (Chattopadhyay et al., 2008). Because of

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the substantial evidence that  $\text{Na}_v1.7$  in DRG may be a key contributor to the neuropathic pain phenotype (Dib-Hajj et al., 2007; Drenth and Waxman, 2007), the reduction of  $\text{Na}_v1.7$  produced by delta opioid receptor (DOR) activation represents an additional effect, beyond the well-recognized effects of enkephalin as an inhibitory neurotransmitter, through which enkephalin might act to reduce neuropathic pain in PDN.

The current study was undertaken to answer two questions: (1) would HSV-mediated release of GABA from primary afferents reduce neuropathic pain in rats with PDN; and (2) would HSV-mediated release of GABA reduce  $\text{Na}_v1.7$  protein levels in the DRG of rats with PDN?

## 2. Materials and methods

### 2.1. Vector construct

The non-replicating HSV vector vG is defective in expression of the HSV immediate early (IE) genes ICP4, ICP22, ICP2 and ICP47 and contains the human GAD67 gene under the control of the human cytomegalovirus immediate early promoter (HCMV IEP) in the UL41 locus. Construction and characterization of this vector has been previously described (referred to as QHGAD67 in (Liu et al., 2004)). Control vector vZ is identical to vG except that it contains the *E. coli lacZ* reporter gene under the control of the HCMV IEP in the HSV tk locus (referred to as QOZHG in (Liu et al., 2004)).

### 2.2. Animal studies

All the experiments were conducted on young adult male Sprague Dawley rats weighing 200–250 g (Charles River; Portage, MI, USA) at the start of the experiment, and were in compliance with approved institutional animal care and use protocols and in conformance with the ethical standards of the AAALAC and IASP. Rats were rendered diabetic by injection of streptozotocin (STZ; 50 mg/kg, ip; Sigma, St Louis, MO, USA). The average blood glucose levels of animals in the study were  $522 \pm 12/9$  mg/dl in the diabetic group and  $140 \pm 9$  mg/dl in the control group. At 2 weeks after injection of STZ, groups of 10 rats were injected with  $30 \mu\text{l}$  containing  $1 \times 10^7$  plaque forming units (pfu) of either of vG, vZ, or a similar volume of vehicle alone subcutaneously into the plantar surface of both hind feet. Ten animals that did not receive STZ served as normal controls. All analyses were carried out by an observer blinded to the treatment group 4 weeks after vector inoculation (at 6 weeks of diabetes). A separate group of rats were injected in a similar fashion with vG and sacrificed by perfusion 7 days later for the studies of transgene expression *in vivo*.

### 2.3. Behavioral studies

#### 2.3.1. Thermal hyperalgesia

The latency to hind-paw withdrawal from a thermal stimulus was determined by exposing the plantar surface of the hind paw to radiant heat using a modified Hargreaves thermal testing device (Hargreaves et al., 1988) as described (Chattopadhyay et al., 2008). **Mechanical hyperalgesia.** Mechanical nociceptive threshold was assessed using an analgesimeter (Ugo Basile, Comerio, VA, Italy) (Chattopadhyay et al., 2008; Randall and Sellito, 1957). **Cold allodynia.** Cold allodynia was assessed by withdrawal from acetone spray, as described (Chattopadhyay et al., 2008).

### 2.4. Adult DRG culture

DRG were removed from adult female rats, treated with collagenase (0.25%, Sigma, St Louis, MO, USA) for 1 h at  $37^\circ\text{C}$  and then

dissociated with 2.5% trypsin (Sigma) in 1 mmol/l ethylenediaminetetraacetic acid (EDTA; Sigma) for 30 min at  $37^\circ\text{C}$  before being plated on laminin, poly-D-lysine-coated coverslips at  $10^5$  cells per well in a 24-well plate in 500  $\mu\text{l}$  of defined Neurobasal medium containing B27, Glutamax I, Albumax II and penicillin/streptomycin (Gibco-BRL, Carlsbad, CA, USA), supplemented with 100 ng/ml of 7.0S NGF per ml (Sigma, St. Louis, MO, USA). To model hyperglycemic conditions, 20 mM glucose was added to medium (45 mM total glucose) that did not contain B27.

### 2.5. Embryonic DRG culture

DRG from 17-day rat embryos were cultured as previously described (Chattopadhyay et al., 2008). At 17 days *in vitro* the medium was changed to 45 mM glucose (20 mM glucose in addition to the basal 25 mM glucose) with Glutamax I, Albumax II, penicillin/streptomycin and NGF, but without B27. Control cells were exposed to identical medium lacking B27 with a total of 25 mM glucose.

### 2.6. Reagents

Bicuculline, ( $\text{EC}_{50}$ : 10–100  $\mu\text{M}$  (Rho et al., 1996); Sigma, St. Louis, MO); phaclofen, ( $\text{EC}_{50}$ : 229–500  $\mu\text{M}$  (Robinson et al., 1989; Shibuya et al., 1992); Biomol, Plymouth Meeting, PA); baclofen, ( $\text{EC}_{50}$ : 2.8–10  $\mu\text{M}$  (Ong et al., 1990; Robinson et al., 1989); Sigma, St. Louis, MO); pertussis toxin (PTX; Tocris, Ellisville, MO); cholera toxin (CTX; Sigma, St. Louis, MO).

### 2.7. Western blot

Western blot was performed as previously described (Chattopadhyay et al., 2008) using the primary antibodies against  $\text{Na}_v1.7$  (1:400; Millipore, Bedford, MA); pPKC $\alpha\beta$  (1:400 Cell Signaling, Beverly, MA); GAD67 (1:500; Millipore);  $\beta$ -actin (1:2000; Sigma) and secondary horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (1:5000 Amersham, Piscataway, NJ) visualized with ECL (Pierce, Rockford, IL). The intensity of each band was determined by quantitative chemiluminescence using a PC-based image analysis system (ChemiDoc XRS System, Bio-Rad Laboratories) and normalized to the respective level of  $\beta$ -actin. The specificity of the commercially obtained anti- $\text{Na}_v$  antibody for  $\text{Na}_v1.7$  was confirmed in primary neurons in culture using an HSV vector expressing an shRNA specific for  $\text{Na}_v1.7$  to selectively knock-down  $\text{Na}_v1.7$  expression (Supplementary Fig. 1).

### 2.8. Immunocytochemistry

Immunocytochemistry was performed as previously described (Chattopadhyay et al., 2008) using the primary antibodies anti-GAD67 (1:1000; Chemicon, Temecula, CA, USA) or  $\text{Na}_v1.7$  (1:500; Neuromab; UC Davis, CA) and GABA $_B$ R (1:400; Santa Cruz Biotechnology, Santa Cruz, CA) and the secondary fluorescent antibody, Alexa Fluor 594 goat anti-rabbit IgG (1:1000, Molecular Probes, Eugene, OR).

### 2.9. Statistical analysis

The statistical significance of the difference between groups was determined by ANOVA (Systat 9) using Bonferroni's correction for the multiple post hoc analyses. All results are expressed as mean  $\pm$  SEM. All the tissue culture experiments were repeated three times. The animal experiments, with 8–10 animals per group, were repeated twice.

### 3. Results

#### 3.1. HSV vector vG expresses GAD67 in DRG neurons *in vitro* and *in vivo*

Primary DRG neurons transduced at a multiplicity of infection (MOI) of 1 by vG showed a substantial increase in GAD67 expression compared to control or vZ-infected cells *in vitro* by Western blot and immunocytochemistry (Supplementary Fig. 2). Following subcutaneous inoculation into the hind foot, rats inoculated with the vector vG showed increased GAD67 expression in lumbar DRG by Western blot and immunocytochemistry (7 days after vector inoculation; Supplementary Fig. 2). In previous studies we have reported that infection of DRG with this non-replicating vector results in constitutive release of GABA from primary afferent terminals in the dorsal horn of spinal cord (Liu et al., 2006, 2004).

#### 3.2. Continuous release of GABA reduces thermal hyperalgesia, mechanical hyperalgesia and cold allodynia in diabetic animals 4 weeks after inoculation of the vector vG

Diabetic animals demonstrated thermal hyperalgesia manifested by a decrease in withdrawal latency in response to noxious thermal stimuli (Fig. 1a). Four weeks after inoculation of vG (6 weeks after diabetes) there was a statistically significant increase in thermal latency in vG treated animals compared to diabetic and control vector vZ-inoculated diabetic animals (Fig. 1a). Diabetic rats also developed mechanical hyperalgesia 6 weeks after diabetes, and inoculation of vG significantly increased hind-paw withdrawal threshold compared to diabetic animals (Fig. 1b) measured 4 week after inoculation. Diabetic animals inoculated with vZ showed no difference in mechanical threshold compared to diabetic animals (Fig. 1b). Diabetic rats also developed cold allodynia 6 weeks after diabetes, with the latency to withdraw from the cold

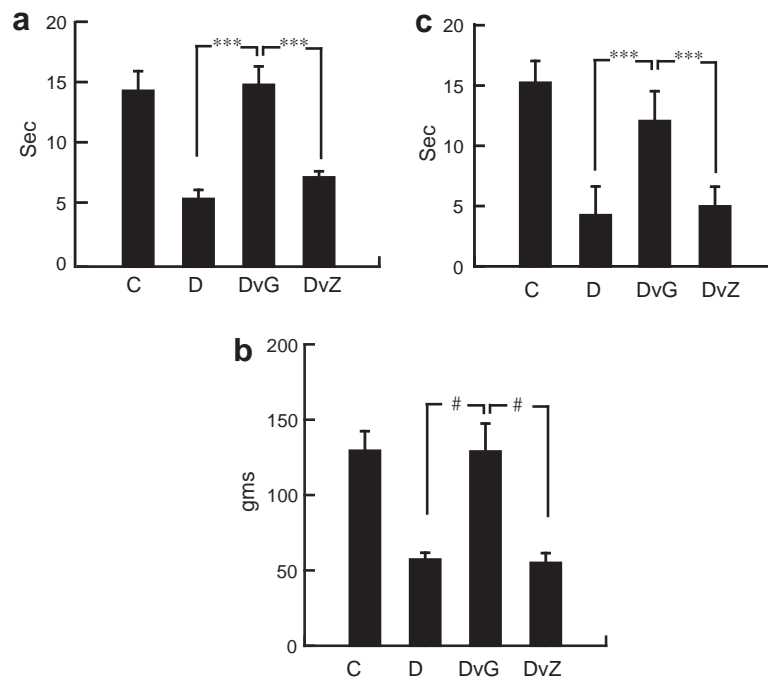
stimulus significantly decreased in diabetic compared to control animals (Fig. 1c). Diabetic animals inoculated with vG showed a normal latency to withdraw from this stimulus (Fig. 1c).

#### 3.3. Vector-mediated release of GABA prevents the increase in $Na_v1.7$ and pPKC $\alpha\beta$ in diabetic DRG *in vivo*

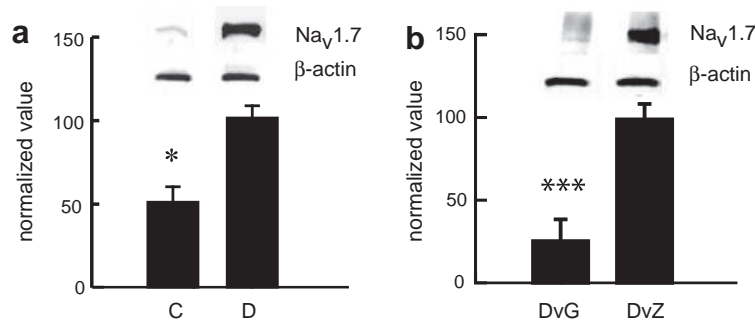
As reported previously (Chattopadhyay et al., 2008; Hong et al., 2004) there was a significant increase in  $Na_v1.7$  protein in diabetic compared to control DRG 6 weeks after diabetes (Fig. 2a). Animals inoculated with vG 2 weeks after induction of diabetes by STZ showed a substantial and significant reduction in the amount of  $Na_v1.7$  in DRG at 4 weeks after vector inoculation compared to animals inoculated with vZ (Fig. 2b). Similarly, the amount of pPKC $\alpha\beta$  was increased significantly in diabetic DRG compared to control, but in animals inoculated with vG 2 weeks after induction of diabetes by STZ there was a substantial and significant reduction in the amount of pPKC $\alpha\beta$  in DRG at 4 weeks after vector inoculation (Fig. 3a and b).

#### 3.4. Modulation of $Na_v1.7$ by glucose in adult DRG neurons *in vitro*

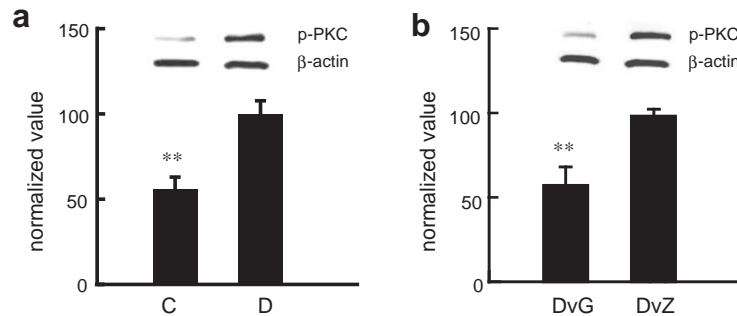
In order to study the relationship of the amount of  $Na_v1.7$  in DRG neurons to hyperglycemia we compared the adult DRG culture with primary E17 DRG neurons *in vitro*. The adult neurons constitutively express  $Na_v1.7$  similar to primary embryonic DRG. After 18 h exposure to culture medium containing 45 mM glucose there was a substantial increase in the amount of  $Na_v1.7$  protein in the adult DRG (Fig. 4) that was similar to that seen in embryonic DRG in culture; the subsequent experiments were conducted using embryonic DRG neurons in order to minimize the number of animals that would need to be employed for these studies.



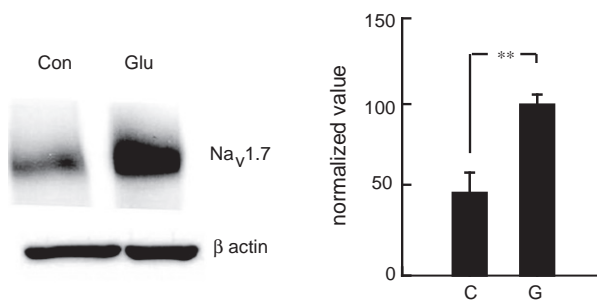
**Fig. 1.** Vector mediated expression of GABA reverses pain-related behaviors in animals with PDN. (a) Thermal withdrawal latency (Hargreave's test) in seconds. (b) Mechanical hyperalgesia (Randall Selitto test) threshold in grams. (c) Cold allodynia in seconds. C – naïve control; D – diabetic; DvG – diabetic inoculated with vG; DvZ – diabetic inoculated with vZ; all data presented as mean  $\pm$  SEM,  $n = 6-8$  per group. # $P < 0.0001$  or \*\*\* $P < 0.001$  vs. untreated diabetic animals or DvZ.



**Fig. 2.** vG mediated GABA expression attenuates the increase in Na<sub>v</sub>1.7 in DRG *in vivo* characteristic of PDN. Amount of Na<sub>v</sub>1.7 determined by Western blot, normalized to D (a) or DvZ (b). C – control; D – diabetic; DvG – diabetic inoculated with vG; DvZ – diabetic inoculated with vZ; \*\*\**P* < 0.001 or \**P* < 0.01; *n* = 5 animals per group. Representative Western blots showing one sample from each group are presented above each graph.



**Fig. 3.** Inoculation of vG prevents phosphorylation of PKC in diabetic DRG *in vivo*. Amount of pPKC $\alpha\beta$  determined by Western blot, normalized to D (a) or DvZ (b). \*\**P* < 0.005; *n* = 5 animals per group. C – Control; D – diabetic; DvG – diabetic inoculated with vG; DvZ – diabetic inoculated with vZ. Representative Western blots showing one sample from each group are presented above each graph.



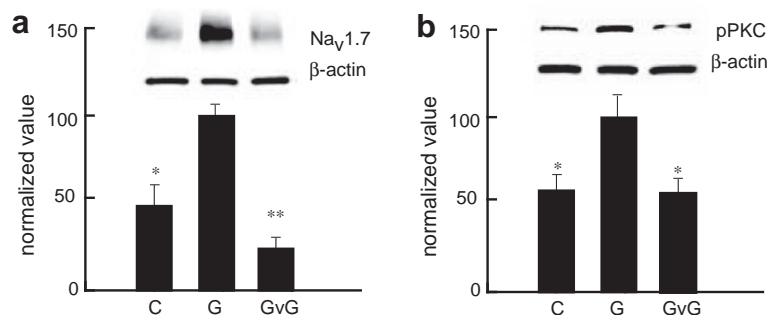
**Fig. 4.** Na<sub>v</sub>1.7 levels are increased in adult DRG neurons in culture exposed to hyperglycemic conditions. Adult DRG neurons in culture were exposed to medium containing 45 mM glucose for 18 h, and the amount of Na<sub>v</sub>1.7 determined by Western blot.

### 3.5. Continuous release of GABA by vG prevents the increase in Na<sub>v</sub>1.7 expression and phosphorylation of PKC in primary DRG neurons *in vitro* exposed to hyperglycemia

Transfection of cells exposed to hyperglycemia with vG at an MOI of 1 blocked the increase in Na<sub>v</sub>1.7 resulting from exposure to glucose (Fig. 5a). There was a substantial increase in the amount of pPKC $\alpha\beta$  in the primary DRG neurons exposed to hyperglycemic conditions. Transfection of cells exposed to hyperglycemia with vG at an MOI of 1 blocked the increase in phosphorylation resulting from exposure to hyperglycemic conditions *in vitro* (Fig. 5b).

### 3.6. The effect of vector-mediated release of GABA on Na<sub>v</sub>1.7 is reversed by phaclofen but not by bicuculline

In order to assess the relative contribution of GABA<sub>A</sub> and GABA<sub>B</sub> receptors in mediating the effects of vector-produced GABA, DRG



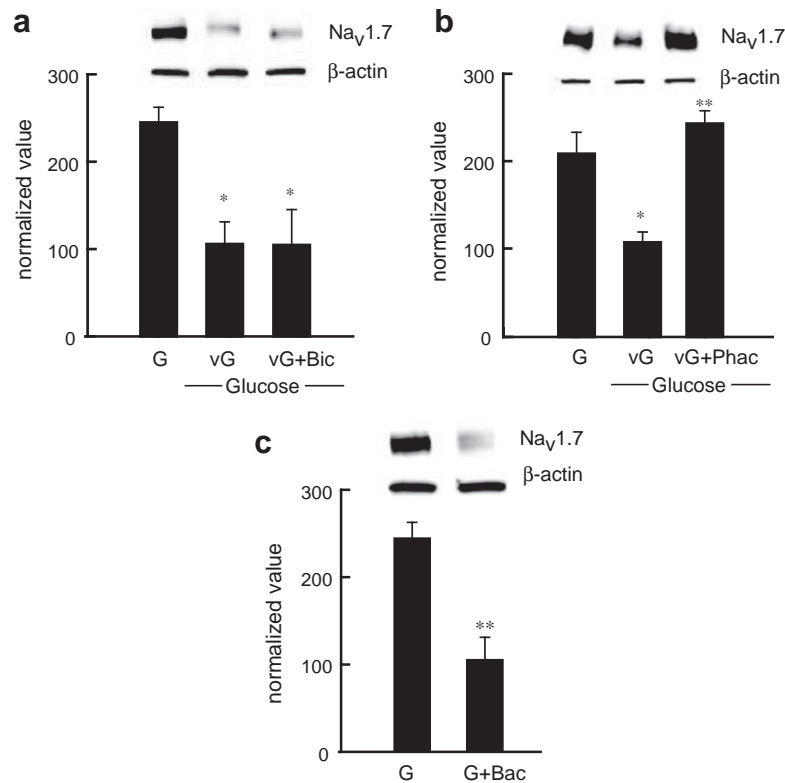
**Fig. 5.** Transgene-mediated expression of GABA prevents the increase in Na<sub>v</sub>1.7 in primary DRG neurons exposed to hyperglycemic conditions *in vitro*. (a) Significant reduction in the amount of Na<sub>v</sub>1.7 in hyperglycemic neurons treated with vG as determined by Western blot, normalized to G. (b) Amount of pPKC $\alpha\beta$  determined by Western blot, normalized to C. \*\**P* < 0.005 or \**P* < 0.01; C – control; G – hyperglycemia; GvG – hyperglycemia, transfected with vG. Representative Western blots showing one sample from each group are presented above each graph.

neurons were transfected with vG at MOI of 1 and 24 h after transfection, exposed to 45 mM glucose along with either 50  $\mu$ M of bicuculline or 500  $\mu$ M phaclofen. Addition of bicuculline had no effect on the decrease in  $\text{Na}_v1.7$  resulting from transfection with vG (Fig. 6a). In contrast, the effect of transfection with vG in preventing the increase in  $\text{Na}_v1.7$  was completely blocked by the addition of 500  $\mu$ M phaclofen (Fig. 6b). This finding was confirmed by experiments using the  $\text{GABA}_B$  agonist baclofen. Addition of 10  $\mu$ M baclofen (Fig. 6c) to primary DRG neurons exposed to hyperglycemia blocked the increase in  $\text{Na}_v1.7$  resulting from hyperglycemia. Colocalization of  $\text{GABA}_B$ R and  $\text{Na}_v1.7$  in individual

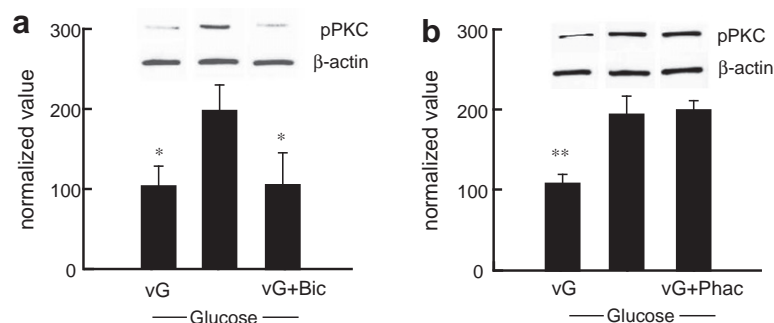
DRG neurons *in vitro* was confirmed by double-label immunocytochemistry (Supplementary Fig. 3).

### 3.7. $\text{GABA}_B$ receptor-mediated regulation of voltage-gated sodium channel $\text{Na}_v1.7$ involves PKC in DRG neurons *in vitro*

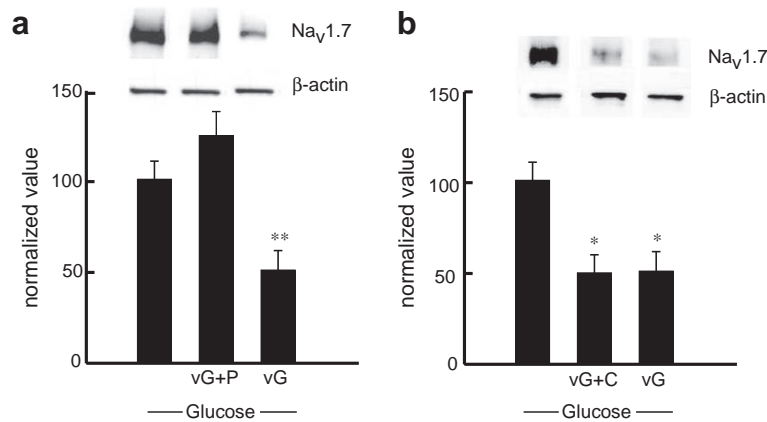
We previously found that inhibition of PKC activation by addition of 10  $\mu$ M myristoylated PKC inhibitor 20–28 blocks the increase in  $\text{Na}_v1.7$  in primary DRG neurons exposed to hyperglycemia (Chattopadhyay et al., 2008). To test whether the effect of  $\text{GABA}$  receptor activation proceeds through PKC, cells were transfected



**Fig. 6.** Phaclofen but not bicuculline blocks the effect of vG on the amount  $\text{Na}_v1.7$  in DRG neurons exposed to hyperglycemic conditions *in vitro*. (a) No substantial changes in the amount of  $\text{Na}_v1.7$  in primary DRG neurons exposed to hyperglycemia, transfected with vG in the presence of 50  $\mu$ M of bicuculline (vG + bic). (b) Significant changes in the amount of  $\text{Na}_v1.7$  in primary DRG neurons exposed to hyperglycemia and transfected with vG in the presence of 500  $\mu$ M of phaclofen (vG + phac), normalized to vG; (c) Substantial changes in the amount of  $\text{Na}_v1.7$  in primary DRG neurons exposed to hyperglycemia in the presence of 10  $\mu$ M of baclofen (G + bac). G – hyperglycemia; vG – cells transfected with vector; vG + phac – cells transfected with vG in presence of phaclofen; vG + bic – cells transfected with vG in presence of bicuculline, G + bac – hyperglycemia, in presence of baclofen. \* $P < 0.01$ , \*\* $P < 0.005$ . Representative Western blots showing one sample from each group are presented above each graph.



**Fig. 7.** Phosphorylation of PKC in hyperglycemic DRG neurons is blocked by a  $\text{GABA}_B$  antagonist. (a) No changes in pPKC $\alpha\beta$  in primary DRG neurons exposed to 45 mM glucose and transfected with vG in the presence of 50  $\mu$ M bicuculline (vG + bic) compared to vG treated neurons. (b) There were significant changes in the amount of pPKC $\alpha\beta$  in primary DRG neurons exposed to 45 mM glucose and transfected with vG in the presence of 500  $\mu$ M phaclofen (vG + phac) compared to vG treated neurons; \* $P < 0.01$ , \*\* $P < 0.005$ ;  $n = 3$  wells per group. Representative Western blots showing one sample from each group are presented above each graph.



**Fig. 8.** Effect of continuous activation of GABA<sub>B</sub>R by vG on levels of Na<sub>v</sub>1.7 is mediated through G<sub>i/o</sub> proteins. (a) Treatment with vG-infected neurons exposed to hyperglycemic conditions with pertussis toxin (PTX; 250 ng/ml) reversed the effect of GABA<sub>B</sub>R activation on the amount of Na<sub>v</sub>1.7 compared to vG-infected neurons exposed to hyperglycemic conditions \*\**P* < 0.005. (b) Treatment with cholera toxin (CTX) in similar conditions did not result in any change in amount of Na<sub>v</sub>1.7 in vG infected DRG neurons \**P* < 0.01.

with vG at an MOI of 1, and 24 h after transfection exposed to 45 mM glucose along with either 50 μM of bicuculline or 500 μM phaclofen. Addition of bicuculline to vG-transfected cells exposed to hyperglycemic conditions resulted in no change in pPKCαβ, while the addition of phaclofen to vG-transfected cells prevented the vG effect of pPKCαβ (Fig. 7a and b). These results support that the vector mediated effect through the GABA<sub>B</sub>R involves a block in phosphorylation of PKC downstream of GABA<sub>B</sub>R activation.

### 3.8. Continuous activation of the GABA<sub>B</sub>R by vector in reduction of Na<sub>v</sub>1.7 is mediated through activation of G<sub>i/o</sub> proteins

G proteins are the key signal transducing molecules for G-protein coupled receptors (GPCRs). Treatment of primary DRG neurons exposed to hyperglycemia with PTX (250 ng/ml overnight) blocked the effect of GABA<sub>B</sub>R activation on the amount of Na<sub>v</sub>1.7, but treatment with CTX (250 ng/ml overnight) did not result in any change in amount of Na<sub>v</sub>1.7 (Fig. 8a and b). These results indicate that the effect of GABA on Na<sub>v</sub>1.7 levels is mediated through G<sub>i/o</sub> proteins.

## 4. Discussion

There are two important observations in this study: (1) spinal release of GABA effected by transfection of DRG neurons *in vivo* with a GAD expressing vector reduces pain-related behaviors in rats with PDN; and (2) continuous exposure to GABA reverses the increase in Na<sub>v</sub>1.7 in primary sensory afferents that is characteristic of diabetic neuropathy through a GABA<sub>B</sub>R mediated pathway.

### 4.1. HSV-mediated release of GABA reduces neuropathic pain-related behaviors in rats with PDN

In previous reports we have demonstrated that subcutaneous inoculation of non-replicating HSV-based vectors results in transfection of DRG neurons *in vivo* (Chattopadhyay et al., 2003; Goss et al., 2001), and that infection of DRG neurons with the HSV vector expressing GAD67 results in constitutive release of GABA (Liu et al., 2006, 2004). Transfection of DRG neurons *in vivo* with the HSV vector expressing GAD67 reduces pain-related behaviors in the lateral hemisection model of central neuropathic pain (Liu et al., 2004) and in the selective spinal nerve ligation model of peripheral neuropathic pain (Hao et al., 2005). The results of the current study

extend these findings to a model of the clinically prevalent neuropathic pain condition caused by diabetes.

Tonic spinal GABAergic inhibition plays an important role in the modulation of basal pain sensation. Intrathecal bicuculline in normal animals produces hyperalgesia (Hwang and Yaksh, 1997), and reductions in both GAD and GABA in dorsal horn have been reported in models of pain created by peripheral nerve injury (Castro-Lopes et al., 1993; Eaton et al., 1998; Ibuki et al., 1997; Moore et al., 2002). In addition to our studies with HSV mediated gene transfer of GAD, direct injection of an adeno-associated virus-based vector expressing GAD65 into DRG neurons reduces pain related symptoms in animals with neuropathic pain (Lee et al., 2007) and intrathecal administration of baclofen produces an antinociceptive effect in acute pain (Dirig and Yaksh, 1995; Malcangio and Bowery, 1995; Smith et al., 1994), in animal models of pain following nerve injury (Castro-Lopes et al., 1995; Engle et al., 2006; Smith et al., 1994; Yang, 2004) and in rats with PDN (Malcangio and Tomlinson, 1998).

GABA acting through the GABA<sub>A</sub> receptor may also function as an excitatory neurotransmitter as it does during development or in conditions characterized by a reduction in the expression of KCC2 (Coull et al., 2003; Zhang et al., 2008) including PDN (Jolivald et al., 2008). How might we reconcile the differences between the evidence that GABA may function as an excitatory neurotransmitter in PDN with the current studies demonstrating that transgene-mediated release of GABA in the dorsal horn results in a reduction in pain-related behaviors? One possibility regards the distinct but overlapping roles played by GABA<sub>A</sub> and GABA<sub>B</sub> receptors. While changes in KCC2 will result in a change the reversal potential of the GABA<sub>A</sub>R this change would not be expected to have an effect on GABA<sub>B</sub>R function. The observations in our current study, indicating that vector-mediated release of GABA results in a reduction in pain-related behaviors in the rat likely reflects presynaptic GABA<sub>B</sub>R effects, since the effect of GABA in preventing the increase in Na<sub>v</sub>1.7 in the DRG of diabetic rats is clearly presynaptic, though the possible additional role of postsynaptic effects cannot be excluded.

### 4.2. Na<sub>v</sub>1.7 in DRG neurons plays an important role in neuropathic pain

Neuropathic pain results from hyperexcitability of primary afferent nociceptors that may result from electrical discharges at the site of axonal injury or ectopic/spontaneous activity in DRG neurons (Baron, 2000; Devor, 2006; Sukhotinsky et al., 2004).

Voltage-gated sodium channel isoforms expressed in nociceptive neurons participate in fine tuning the firing properties of nociceptors (Devor, 2006; Lai et al., 2003), and among these isoforms there is evidence that  $\text{Na}_v1.7$  in DRG neurons, plays a critical role in the pathogenesis of neuropathic pain (Black et al., 2004; Cummins et al., 2007; Ekberg and Adams, 2006; Wilson-Gerwing et al., 2008). Gain of function mutations in the *SCN9A* gene coding for  $\text{Na}_v1.7$  leads to conditions characterized by spontaneous pain including primary erythralgia and paroxysmal extreme pain disorder (Dib-Hajj et al., 2007; Drenth and Waxman, 2007); loss of function mutations in *SCN9A* results in an inherited channelopathy characterized by congenital insensitivity to pain (Cox et al., 2006); and hyperexcitability of primary afferents in human PDN is associated with increased inter-nodal sodium currents (Misawa et al., 2009).

In rats with STZ-induced diabetes the amount of  $\text{Na}_v1.7$  protein in DRG neurons is increased (Chattopadhyay et al., 2008; Hong et al., 2004), and alterations in physiologic properties of the DRG neurons from diabetic rats with PDN concordant with this increase have been defined (Hong et al., 2004). The embryonic  $\text{Na}_v$  isoform  $\text{Na}_v1.3$  is also increased in DRG of diabetic animals (Hong et al., 2004), but the total amount of  $\text{Na}_v1.3$  in DRG neurons is very low, and unlikely to contribute significantly to the pain phenotype. Other isoforms present in DRG neurons, such as  $\text{Na}_v1.8$ , are reduced in animals with PDN and unlikely to play an important role in the pathogenesis of pain in these animals. While final proof that neuropathic pain in diabetic animals results from the increase in  $\text{Na}_v1.7$  will require selective knock-down of  $\text{Na}_v1.7$  or selective interference with  $\text{Na}_v1.7$  function, taken together, the evidence from inherited forms of neuropathic pain and the observations in animals with PDN strongly implicate increases in  $\text{Na}_v1.7$  protein in pathogenesis of neuropathic pain in this condition. The results presented here do not exclude the possibility that alterations in the biophysical properties of voltage-gated sodium channels resulting from phosphorylation or other post-translational modifications may also play a role in increasing electrical excitability of peripheral afferents in diabetic animals, but experiments to investigate these effects are beyond the scope of the current investigation.

#### 4.3. $\text{GABA}_B$ activation and $\text{Na}_v$ protein levels

The observation that GABA prevents the increase in  $\text{Na}_v1.7$  in the DRG of diabetic animals provides a second means through which GABA may be reducing neuropathic pain in these animals. Considered in conjunction with our previous study showing that continuous release of the inhibitory neurotransmitter enkephalin in diabetic animals reduces pain coincident with a reduction in  $\text{Na}_v1.7$  levels in DRG neurons through activation of the DOR (Chattopadhyay et al., 2008), the current results suggests the existence of a novel common mechanism of presynaptic GPCR regulation of voltage-gated channel protein levels. Bicuculline block of the  $\text{GABA}_A$  receptor had no effect on vector-mediated GABA release reduction in  $\text{Na}_v1.7$  protein in DRG neurons exposed to hyperglycemic conditions. While this does not entirely exclude the possibility of a  $\text{GABA}_A$  receptor-mediated effect that might be observed using other doses of the inhibitor, taken together the results of the *in vitro* experiments clearly support a role for GABA acting through the  $\text{GABA}_B$  receptor on regulating  $\text{Na}_v1.7$  levels.

#### 4.4. Implications of the regulation of $\text{Na}_v 1.7$ by GPCR

The data in the current report indicate that vector-mediated release of GABA may provide an analgesic effect in PDN through at least two distinct mechanisms: actions as an inhibitory neurotransmitter as well as distinct effects to reduce the amount of

$\text{Na}_v1.7$  in DRG neurons. Whether there are other changes in DRG phenotype produced by continuous  $\text{GABA}_B$  or  $\text{GABA}_A$  receptor activation is beyond the scope of the current experiments, but may be interesting to explore in the future. However, the findings that reveal a previously unrecognized relationship between this presynaptic GPCR and the level of the voltage-gated channel in the primary sensory neuron have potential implications for the regulation of these channels in normal homeostasis. Taken together with our previous findings of a similar relationship between continuous activation of the DOR GPCR and  $\text{Na}_v1.7$  levels in DRG neurons, the results suggest that it is possible that continuous activation of GPCR *in vivo* by release of endogenous neurotransmitters may regulate the steady state levels of voltage gated ion channels, and in addition provide insight that may be useful in designing therapies to treat painful diabetic neuropathy.

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#### Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpain.2011.03.007.

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