Vector-mediated release of GABA attenuates pain-related behaviors and reduces $\text{Na}_V 1.7$ 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 aminobutyric 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 ($\text{Na}_V 1.7$) protein that is characteristic of PDN. In vitro, infection of primary DRG neurons with vG prevented the increase in $\text{Na}_V 1.7$ resulting from exposure to hyperglycemia. The effect of vector-mediated GABA on $\text{Na}_V 1.7$ levels in vitro was blocked by phaclofen but not by bicuculline, a GABA receptor effect that was blocked by pertussis toxin (PTX) interference with $G_\alpha$ 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 $\text{Na}_V 1.7$ 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.

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 GABAA 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 ($\text{Na}_V 1.7$) 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 $\text{Na}_V 1.7$ protein in DRG neurons in vivo, coincident with a reduction in pain-related behaviors (Chattopadhyay et al., 2008). Because of
the substantial evidence that Na\textsubscript{v}1.7 in DRG may be a key contributor to the neuropathic pain phenotype (Dib-Hajj et al., 2007; Drenth and Waxman, 2007), the reduction of Na\textsubscript{v}1.7 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 Na\textsubscript{v}1.7 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 Q0ZHG 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; Sigma, St. Louis, MO); phaclofen, (EC\textsubscript{50}: 229–500 \textmu M (Robinson et al., 1989; Shibuya et al., 1992)); Biomol, Plymouth Meeting, PA); baclofen, (EC\textsubscript{50}: 2.8–10 \textmu M (Ong et al., 1990; Robinson et al., 1989); Sigma, St. Louis, MO); pertussis toxin (PTX; Tocris, Ellisville, MO); choloral hydrate (CTX; Sigma, St. Louis, MO).

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 algometer (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 collage-nase (0.25%, Sigma, St Louis, MO, USA) for 1 h at 37 °C and then dissociated with 2.5% trypsin (Sigma) in 1 mmol/l ethylene-diaminetetraacetic acid (EDTA; Sigma) for 30 min at 37 °C before being plated on laminin, poly-D-lysine-covered coverslips at 10\textsuperscript{6} cells per well in a 24-well plate in 500 \mu 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 7.05 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, (EC\textsubscript{50}: 10–100 \textmu M (Rho et al., 1996); Sigma, St. Louis, MO); phaclofen, (EC\textsubscript{50}: 229–500 \textmu M (Robinson et al., 1989; Shibuya et al., 1992)); Biomol, Plymouth Meeting, PA); baclofen, (EC\textsubscript{50}: 2.8–10 \textmu M (Ong et al., 1990; Robinson et al., 1989); Sigma, St. Louis, MO); pertussis toxin (PTX; Tocris, Ellisville, MO); choloral hydrate (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 Na\textsubscript{v}1.7 (1:400, Millipore, Bedford, MA); pPKC\textsubscript{ab} (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-Na\textsubscript{v} antibody for Na\textsubscript{v}1.7 was confirmed in primary neurons in culture using an HSV vector expressing an shRNA specific for Na\textsubscript{v}1.7 to selectively knock-down Na\textsubscript{v}1.7 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 Na\textsubscript{v},1,7 (1:500; Neumonab; UC Davis, CA) and GABA\textsubscript{AR} (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 ± 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 NaV1.7 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 NaV1.7 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 NaV1.7 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 NaV1.7 by glucose in adult DRG neurons in vitro

In order to study the relationship of the amount of NaV1.7 in DRG neurons to hyperglycemia we compared the adult DRG culture with primary E17 DRG neurons in vitro. The adult neurons constitutively express NaV1.7 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 NaV1.7 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 = naive control; D = diabetic; DvG = diabetic inoculated with vG; DvZ = diabetic inoculated with vZ; all data presented as mean ± SEM, n = 6–8 per group. #P < 0.0001 or ***P < 0.001 vs. untreated diabetic animals or DvZ.](attachment:fig1.png)
3.5. Continuous release of GABA by vG prevents the increase in Na\textsubscript{V1.7} 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\textsubscript{V1.7} resulting from exposure 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. 5a).

3.6. The effect of vector-mediated release of GABA on Na\textsubscript{V1.7} is reversed by phaclofen but not by bicuculline

In order to assess the relative contribution of GABA\textsubscript{A} and GABA\textsubscript{B} receptors in mediating the effects of vector-produced GABA, DRG
neurons were transfected with vG at 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 had no effect on the decrease in NaV1.7 resulting from transfection with vG (Fig. 6a). In contrast, the effect of transfection with vG in preventing the increase in NaV1.7 was completely blocked by the addition of 500 μM phaclofen (Fig. 6b). This finding was confirmed by experiments using the GABAaR agonist baclofen. Addition of 10 μM baclofen (Fig. 6c) to primary DRG neurons exposed to hyperglycemia blocked the increase in NaV1.7 resulting from hyperglycemia. Colocalization of GABAaR and NaV1.7 in individual DRG neurons in vitro was confirmed by double-label immunocytochemistry (Supplementary Fig. 3).

3.7. GABAa receptor-mediated regulation of voltage-gated sodium channel NaV1.7 involves PKC in DRG neurons in vitro

We previously found that inhibition of PKC activation by addition of 10 μM myristolated PKC inhibitor 20–28 blocks the increase in NaV1.7 in primary DRG neurons exposed to hyperglycemia (Chattopadhyay et al., 2008). To test whether the effect of GABA receptor activation proceeds through PKC, cells were transfected
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 pPKC \( \text{ab} \) (Fig. 7a and b). These results support that the vector mediated effect through the GABABR involves a block in phosphorylation of PKC downstream of GABABR activation.

3.8. Continuous activation of the GABABR by vector in reduction of NaV 1.7 is mediated through activation of Ga(i/o) 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 GABABR activation on the amount of NaV1.7, but treatment with CTX (250 ng/ml overnight) did not result in any change in amount of NaV1.7 (Fig. 8a and b). These results indicate that the effect of GABA on NaV1.7 levels is mediated through Gi/o 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 NaV1.7 in primary sensory afferents that is characteristic of diabetic neuropathy through a GABABR 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; McLemore 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 (McLemore and Tomlinson, 1998).

GABA acting through the GABA\(\text{A}\) 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 (Jolivalt et al., 2007) and intrathecal administration of baclofen produces an antinociceptive effect in acute pain (Dirig and Yaksh, 1995; McLemore 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 (McLemore and Tomlinson, 1998).

4.2. NaV1.7 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 Na\textsubscript{v}1.7 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 Na\textsubscript{v}1.7 leads to conditions characterized by spontaneous pain including primary erythermalgia and paroxysmal extreme pain disorder (Dib-Hajj et al., 2007; Dreyfus 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 Na\textsubscript{v}1.7 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 Na\textsubscript{v} isoform Na\textsubscript{v}1.3 is also increased in DRG of diabetic animals (Hong et al., 2004), but the total amount of Na\textsubscript{v}1.3 in DRG neurons is very low, and unlikely to contribute significantly to the pain phenotype. Other isoforms present in DRG neurons, such as Na\textsubscript{v}1.8, 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 Na\textsubscript{v}1.7 will require selective knock-down of Na\textsubscript{v}1.7 or selective interference with Na\textsubscript{v}1.7 function, taken together, the evidence form inherited forms of neuropathic pain and the observations in animals with PDN strongly implicate increases in Na\textsubscript{v}1.7 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. GABA\textsubscript{R} activation and Na\textsubscript{v} protein levels

The observation that GABA prevents the increase in Na\textsubscript{v}1.7 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 Na\textsubscript{v}1.7 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 GABA\textsubscript{A} receptor had no effect on vector-mediated GABA release reduction in Na\textsubscript{v}1.7 protein in DRG neurons exposed to hypoglycemic conditions. While this does not entirely exclude the possibility of a GABA\textsubscript{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 GABA\textsubscript{A} receptor on regulating Na\textsubscript{v}1.7 levels.

4.4. Implications of the regulation of Na\textsubscript{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 Na\textsubscript{v}1.7 in DRG neurons. Whether there are other changes in DRG phenotype produced by continuous GABA\textsubscript{A} or GABA\textsubscript{B} 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 Na\textsubscript{v}1.7 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.

References

Castro-Lopes JM, Malcangio M, Pan BH, Bowery NG. Complex changes of GABA and GABA receptor binding in the spinal cord dorsal horn following peripheral inflammation or neuroectomy. Brain Res 1995;679:289–97.
Chattopadhyay M, Mata M, Fink DJ. Continuous delta opioid receptor activation reduces neuronal voltage gated sodium channel (Na\textsubscript{v}1.7) levels through activation of protein kinase C in painful diabetic neuropathy. J Neurosci 2008;28:6652–8.
Engle MP, Gassman M, Sykes KT, Bettler B, Hammond DL. Spinal nerve ligation does not alter the expression or function of GABA(B) receptors in spinal cord and dorsal root ganglia of the rat. Neuroscience 2006;138:1277–87.


