Functional expression of bradykinin B1 and B2 receptors in neonatal rat trigeminal ganglion neurons

Aya Kawaguchi1, Masaki Sato2, Maki Kimura2, Takaki Yamazaki3, Hitoshi Yamamoto3, Masakazu Tazaki2, Tatsuya Ichinohe1 and Yoshiyuki Shibukawa2*

1 Department of Dental Anesthesiology, Tokyo Dental College, Tokyo, Japan, 2 Department of Physiology, Tokyo Dental College, Tokyo, Japan, 3 Department of Histology and Developmental Biology, Tokyo Dental College, Tokyo, Japan

Bradykinin (BK) and its receptors (B1 and B2 receptors) play important roles in inflammatory nociception. However, the patterns of expression and physiological/pathological functions of B1 and B2 receptors in trigeminal ganglion (TG) neurons remain to be fully elucidated. We investigated the functional expression of BK receptors in rat TG neurons. We observed intense immunoreactivity of B2 receptors in TG neurons, while B1 receptors showed weak immunoreactivity. Expression of the B2 receptor colocalized with immunoreactivities against the pan-neuronal marker, neurofilament H, substance P, isolectin B4, and tropomyosin receptor kinase A antibodies. Both in the presence and absence of extracellular Ca2+ ([Ca2+]o), BK application increased the concentration of intracellular free Ca2+ ([Ca2+]i). The amplitudes of BK-induced [Ca2+]i increase in the absence of [Ca2+]o were significantly smaller than those in the presence of Ca2+. In the absence of [Ca2+]o, BK-induced [Ca2+]i increases were sensitive to B2 receptor antagonists, but not to a B1 receptor antagonist. However, B1 receptor agonist, Lys-[Des-Arg9]BK, transiently increased [Ca2+]i in primary cultured TG neurons, and these increases were sensitive to a B1 receptor antagonist in the presence of [Ca2+]o. These results indicated that B2 receptors were constitutively expressed and their activation induced the mobilization of [Ca2+]i from intracellular stores with partial Ca2+ influx by BK. Although constitutive B1 receptor expression could not be clearly observed immunohistochemically in the TG cryosection, cultured TG neurons functionally expressed B1 receptors, suggesting that both B1 and B2 receptors involve pathological and physiological nociceptive functions.

Keywords: bradykinin, B1 receptor, B2 receptor, neuropathic pain, pain, trigeminal ganglion neuron, Ca2+ signaling

Introduction

Tissue damage results in an accumulation of endogenous chemical substances, such as bradykinin (BK), which are released by nociceptive afferents and/or non-neural cells in the injured area of the tissue (Julius and Basbaum, 2001; Basbaum et al., 2009). BK receptors, which are divided into two subtypes (B1 and B2), are plasma membrane G-protein-coupled receptors of the seven-transmembrane-domain family. The existence of B1 and B2 receptors has been confirmed by pharmacological and radioligand-binding studies, as well as by mRNA expression analyses, in a wide variety of cells (Hess et al., 1994; Pesquero et al., 1996; Hall, 1997).
Previous studies have indicated that B₂ receptors couple with the Gq protein. Activation of the Gq protein activates phospholipase C, which induces a number of intracellular second messenger systems, including 1, 2-diacylglycerol and inositol 1, 4, 5-trisphosphate, which activates protein kinase C and mobilizes intracellular Ca²⁺, respectively (Walker et al., 1995; Tiwari et al., 2005).

BK-induced changes in the chemical environment surrounding axons cause peripheral sensitization, which is associated with inflammatory responses (Basbaum et al., 2009). Neuropathic pain is also involved in peripheral and central sensitization, which increases chronic pain states (Cervero and Laird, 1996; Scholz and Woolf, 2002; Ochoa, 2009). Injury to trigeminal ganglion (TG) neurons, which occasionally induces neuropathic pain, has been reported to be mediated by both B₁ and B₂ receptors in the orofacial area. Formalin-induced orofacial pain responses in rats are occasionally induces neuropathic pain, has been reported to be mediated by both B₁ and B₂ receptors in the orofacial area. Thus, the functional role of BK receptors in TG neurons is still unclear and remain to be fully elucidated.

In the present study, we investigated the expression and localization, as well as physiological and pharmacological properties, of B₁ and B₂ receptors in primary cultured rat TG neurons.

**Materials and Methods**

**Ethical Approval**

All the animals used in our study were treated in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences, which was approved by the Council of the Physiological Society of Japan and the American Physiological Society. In addition, the study followed the guidelines that were established by the National Institutes of Health (USA) regarding the care and use of animals for experimental procedures. This study was approved by the Animal Research Ethics Committee of Tokyo Dental College (approval No. 252502).

**Cell Culture**

TG cells were isolated from neonatal Wistar rats (7 days old) (Kawaguchi et al., 2015) that were under pentobarbital sodium anesthesia (50 mg/kg) following the administration of isoflurane (3.0 Vol%). TG cells were dissociated by enzymatic treatment with Hank’s balanced salt solution (Life Technologies, Grand Island, NY, USA) containing 20 U/mL papain (Worthington Biochemical Corporation, Lakewood, NJ, USA) for 20 min at 37°C, which was followed by dissociation by trituration. After dissociation, the TG cells were plated on 35 mm-diameter dishes (Corning Incorporated Life Sciences, Tewksbury, MA, USA) and cultured for 48 h at 37°C (95% air and 5% CO₂). The primary cells were cultured in Leibovitz’s L-15 medium (Life Technologies) containing 10% fetal bovine serum, 1% penicillin-streptomycin (Life Technologies), 1% fungizone (Life Technologies), 26 mM NaHCO₃, and 30 mM glucose (pH 7.4). For the immunocytochemistry, TG cells were subjected to primary culture on poly-L-lysine-coated cover glasses (Matsunami Glass Ind., Ltd., Osaka, Japan).

**Immunofluorescence Analysis**

TGs isolated from neonatal Wistar rats (7 days old) were fixed in optimal cutting temperature compound and rapidly frozen in liquid nitrogen. Frozen tissues were cut at a thickness of 10 µm and placed on slides. After fixation by 50% ethanol and 50% acetone at −20°C for 30 min, primary cultured TG cells and cryosections were treated with 10% donkey serum at room temperature for 20 min and then incubated overnight at 4°C with primary antibodies (Kuroda et al., 2013). A cocktail of primary antibodies (Neuro-Chrom™ Pan Neuronal Marker, EMD Millipore, Billerica, MA, USA; 1:50 dilution), including mouse anti-Neuronal nuclei (NeuN), anti-microtubule-associated protein 2 (MAP2), anti-βIII tubulin, and anti-neurofilament H (NF-H) antibodies, was used as a neuronal marker. TG cells were also incubated with either mouse anti-NF-H (SantaCruz, CA, USA; 1:200 dilution) as an A-neuron marker, mouse anti-substance P (SP; R&D Systems, Minneapolis, MN, USA; 2.5 µg/100 µl dilution) as a peptidergic C-neuron marker, FITC-conjugated anti-isolectin B4 (IB4; Vector laboratories, CA, USA; 1:200 dilution) as a non-peptidergic C-neuron marker, goat anti-high-affinity nerve growth factor (NGF) receptor (a tropomyosin receptor kinase A (TrkA); R&D Systems; 1.5 µg/100 µl dilution) as an NGF-responsive nociceptor marker (Mantyh et al., 2011), and rabbit anti-B₂ receptor (Alomone Labs, Jerusalem, Israel; 1:50 dilution) and rabbit anti-B₂ receptor (Alomone Labs; 1:50 dilution) (Duehrkop et al., 2013; Dutra et al., 2013) antibodies. For negative controls, the sections were incubated with non-immune IgGs (Abcam, Cambridge, UK; 1:50; N = 4 from four rats) (Figure 2M). The cells and tissues were then washed and incubated with a secondary antibody at room temperature for 30 min. The secondary antibodies were Alexa Fluor 488 donkey anti-rabbit IgG, Alexa Fluor 568 donkey anti-mouse IgG, Alexa Fluor 568 donkey anti-rabbit IgG, and Alexa Fluor 568 donkey anti-goat IgG (1:50 dilution; Life Technologies) for the fluorescence staining and 4′, 6-diamino 2-phenylindole dihydrochloride (Life Technologies) for the nuclear staining (room temperature for 5 min). The cells and tissues were examined under fluorescence microscopes (Carl Zeiss AG, Jena, Germany; Keyence Corporation, Osaka, Japan).

**Solutions and Reagents**

A standard solution containing (in mM) 137 NaCl, 5.0 KCl, 2.0 CaCl₂, 0.5 MgCl₂, 0.44 KH₂PO₄, 0.34 Na₂HPO₄, 4.17 NaHCO₃, and 5.55 glucose (pH 7.4) was used as an extracellular solution. A high-K⁺ solution containing (in mM) 91 NaCl, 50 KCl, 2.0 CaCl₂, 0.5 MgCl₂, 0.44 KH₂PO₄, 0.34 Na₂HPO₄, 4.17 NaHCO₃, and 5.55 glucose (pH 7.4) was used to discern TG neurons from
glial cells by activation of depolarization-induced increases in intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}$]) in neurons. BK, a selective B$_2$ receptor antagonist (HOE140), a selective B$_3$ receptor antagonist (R715) and a highly selective B$_2$ receptor agonist (Lys-[Des-Arg$^9$]BK) were obtained from Tocris Bioscience (Bristol, UK). All the other reagents were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA), except where indicated.

**Measurement of [Ca$^{2+}$]$_i$**

Primary cultured TG cells were loaded for 90 min at 37°C in Hank's solution containing 10 µM of fur-2 acetoxyethyl ester (Dojindo Laboratories, Kumamoto Japan) and 0.1% (w/v) pluronic acid F-127 (Life Technologies). Cultured TG cells were then rinsed with fresh Hank's solution and mounted on a microscope stage (Olympus Corporation, Tokyo, Japan). Fura-2 fluorescence emission was measured at 510 nm in response to alternating excitation wavelengths of 340 nm (F340) and 380 nm (F380) with an Aquacosmos system and software (Hamamatsu Photonics K.K., Shizuoka, Japan), which controls the excitation wavelength selector and intensified charge-coupled device camera system (Hamamatsu Photonics K.K.). [Ca$^{2+}$]$_i$ was measured as the fluorescence ratio of F340 and F380 (R$_{340}$/F$_{380}$) and expressed as F/F$_0$ units. The R$_{340}$/F$_{380}$ value (F) was normalized to the resting value (F$_0$).

**Statistical and Offline Analysis**

The data were expressed as the mean ± standard error (S.E.) or standard deviation of the mean of N observations, where N represents the number of independent experiments or cells, respectively. The Kruskal–Wallis test, Dunn’s posthoc test, or Mann–Whitney test was used to determine the nonparametric statistical significance. P values less than 0.05 were considered significant. The statistical analysis was performed with GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

The dependence of the changes in [Ca$^{2+}$]$_i$ on each pharmacological agent was determined by fitting the data to the following function with Origin 8.5 (OriginLab Corporation, Northampton, MA, USA):

$$F/F_0 = [(F/F_{0\text{int}} - F/F_{0\text{fin}})/(1 + ([x]_0/K))] + F/F_{0\text{fin}}$$

where K is the equilibrium binding constant, [x]$_0$ indicates the applied concentration of the pharmacological agents, and F/F$_{0\text{int}}$ and F/F$_{0\text{fin}}$ are the initial and final F/F$_0$ responses, respectively.

**Results**

**Immunolocalization of BK Receptors in TG Neurons**

The cultured TG neurons showed positive immunoreactivity to a neuronal marker cocktail (Neuro-Chrom™ pan-neuronal marker), which contained mouse anti-NeuN, anti-MAP2, and anti-βIII tubulin antibodies (Figures 1A,D). Intense B$_2$ receptor immunoreactivity was observed in primary cultured TG neurons (Figure 1E), and it showed colocalization with the pan neuronal marker (Figure 1F) in somata, dendrites, axons, and perinuclear regions. Weak but positive B$_1$ receptor immunoreactivity was also observed in primary cultured TG cells (Figure 1B), and the immunoreactivity colocalized with the pan neuronal marker (Figure 1C).

In the TG cryosections, we could observe positive immunoreactivity against the neuronal marker cocktail (Figures 1G,J). These TG neurons in the cryosections showed positive immunoreactivity to the B$_2$ receptor antibody (Figure 1K), showing colocalization with the pan neuronal marker (Figure 1L) in somata, dendrites, axons, and perinuclear regions. However, the TG cryosections did not show B$_1$ receptor immunoreactivity (Figures 1H,I). Positive immunoreactivity was also observed with NF-H (an A-neuron marker; Figure 2A), SP (a peptidergic C-neuron marker; Figure 2D), IB4 (a non-peptidergic C-neuron marker; Figure 2G), and high-affinity NGF receptor (TrkA; an NGF-responsive nociceptor marker; Figure 2J) antibodies. These immunoreactivities against NF-H, SP, IB4, and TrkA antibodies showed colocalization with those against the B$_2$ receptor antibodies (Figures 2B,C,E,F,H,I,K,L).

**BK-Induced [Ca$^{2+}$]$_i$ Increases in TG Neurons**

We observed rapid and transient [Ca$^{2+}$]$_i$ increases in TG neurons following the administration of five different concentrations of BK (0.01, 0.1, 1.0, 10, and 100 nM) in the presence of external Ca$^{2+}$ (2.0 mM; Figure 3A). A semilogarithmic plot (Figure 3B) illustrates F/F$_0$ values as a function of the applied BK concentrations, and the equilibrium-binding constant was the half-maximal 50% effective concentration (EC$_{50}$) of 1.0 nM.

**HOE140, a B$_2$ Receptor Antagonist, Inhibited the BK-Induced [Ca$^{2+}$]$_i$ Increases in TG Neurons**

We examined the BK-induced [Ca$^{2+}$]$_i$ responses in both the presence and absence of external Ca$^{2+}$. The application of BK (1.0 nM) rapidly increased [Ca$^{2+}$]$_i$ to a peak F/F$_0$ value of 1.7 ± 0.03 F/F$_0$ units in the presence (2.0 mM) of external Ca$^{2+}$ and 1.4 ± 0.03 F/F$_0$ units in the absence (0 mM) of external Ca$^{2+}$ (Figures 4A-D). The amplitudes of the BK-induced [Ca$^{2+}$]$_i$ increases significantly differed between those in the presence and absence of extracellular Ca$^{2+}$. In the absence of extracellular Ca$^{2+}$, BK (1.0 nM)-induced [Ca$^{2+}$]$_i$ increases were significantly inhibited by a B$_2$ receptor antagonist (100 nM of HOE140) (Figures 4C,D) but not by a B$_1$ receptor antagonist (1.0 µM of R715) (Figures 4B,D).

The B$_1$ Receptor Antagonist R715 did not Affect the BK-Induced [Ca$^{2+}$]$_i$ Increases

The BK-induced (1.0 nM) increases in [Ca$^{2+}$]$_i$ were not significantly inhibited in TG neurons by the administration of four different concentrations of the B$_1$ receptor antagonist (0.001, 0.01, 0.1, and 1.0 µM of R715) in the presence (2.0 mM) of external Ca$^{2+}$ (Figures 5A,B).

**Pharmacological Identification of B$_1$ Receptors in TG Neurons**

We investigated the [Ca$^{2+}$]$_i$ increases during the administration of Lys-[Des-Arg$^9$]BK, which is an endogenous, potent, and
highly selective B₁ receptor agonist (Talbot et al., 2009; More et al., 2014). The increases in [Ca$^{2+}$]$_i$ in the TG neurons were induced by the administration of five different concentrations of Lys-[Des-Arg$^9$]BK (0.01, 0.1, 1, 10, and 100 nM) in the presence of extracellular Ca$^{2+}$ (2.0 mM) (Figure 5C). A semilogarithmic plot (Figure 5D) illustrates the $F/F_0$ values as a function of the applied concentration of Lys-[Des-Arg$^9$]BK with an equilibrium-binding constant of 0.4 nM. In the presence of extracellular...
FIGURE 2 | Immunolocalization of B2 receptors in the soma of TG neurons in cryosections. (A) Positive immunoreactivity to NF-H as an A-neuron marker in TG neurons (arrowhead). (B,E,H,K) B2 receptor immunoreactivity (arrowheads). (C) Triple immunofluorescence staining with antibodies against B2 receptors (green) and NF-H (red). Nuclei are shown in blue. (D) Positive immunoreactivity to SP as a peptidergic C-neuron marker in TG neurons (arrowheads). (F) Triple staining with antibodies against B2 receptors (green) and SP (red). Nuclei are shown in blue. (G) Positive immunoreactivity to IB4 as a non-peptidergic C-neuron marker in TG neurons (arrowheads). (I) Triple staining with antibodies against B2 receptors (red) and IB4 (green). Nuclei are shown in blue. (J) Positive immunoreactivity to TrkA as an nerve growth factor (NGF)-responsive nociceptor marker in TG neurons (arrowheads). (L) Triple staining with antibodies against B2 receptors (green) (Continued)
Ca<sup>2+</sup>, the Lys-[Des-Arg<sup>9</sup>]-BK-induced increase in [Ca<sup>2+</sup>]<sub>i</sub>, was significantly inhibited by a B<sub>1</sub> receptor antagonist (1.0 µM of R715) (Figures 5E,F).

**Discussion**

The present study demonstrated the functional expression of BK receptors (B<sub>1</sub> and B<sub>2</sub>) in TG neurons. B<sub>2</sub> receptors were present on axons and dendrites in A-neurons, non-peptidergic C-neurons, peptidergic C-neurons, and NGF-responsive nociceptors. While the localization pattern of the B<sub>1</sub> receptor was not clear in the TG cryosections, weak immunoreactivity for B<sub>1</sub> receptors was observed in the primary cultured TG neurons. The application of BK activated B<sub>2</sub> receptors and Lys-[Des-Arg<sup>9</sup>]-BK activated the B<sub>1</sub> receptors. B<sub>2</sub> receptor activation mobilized [Ca<sup>2+</sup>]<sub>i</sub> by releasing Ca<sup>2+</sup> from internal Ca<sup>2+</sup> stores with partial Ca<sup>2+</sup> influx from the extracellular medium.

B<sub>2</sub> receptors, which are expressed ubiquitously and constitutively in healthy tissues, are essential in the early stages of general pain generation (Hall, 1992). The constitutive expression of B<sub>2</sub> receptors in TG neurons has been studied by reverse transcription-polymerase chain reaction (RT-PCR) analyses (Ceruti et al., 2011) and immunocytochemical analyses in cultured TG neurons (Patwardhan et al., 2005). Although BK-induced [Ca<sup>2+</sup>]<sub>i</sub> increases have also been reported in TG neurons (Ceruti et al., 2008, 2011), precise functional expression patterns of B<sub>1</sub> and B<sub>2</sub> receptors in TG neurons remained unclear. The results of the present study showing the functional expression and localization of B<sub>2</sub> receptors in TG neurons were in line with the previous results. The results of this study were also in line with the pharmacological properties of BK, which is a potent and endogenous agonist for B<sub>2</sub> receptors and not B<sub>1</sub> receptors in the sympathetic neurons of the rat superior cervical ganglion (Babbedge et al., 1995) and in Chinese hamster ovary (CHO) cells stably expressing recombinant human B<sub>1</sub> or B<sub>2</sub> receptors (Simpson et al., 2000). Furthermore, BK has an affinity for B<sub>2</sub> receptors that is 500 times that for B<sub>1</sub> receptors (Simpson et al., 2000). Therefore, B<sub>2</sub> receptors are histologically and functionally expressed, and endogenous BK preferentially activates B<sub>2</sub> receptors in rat TG neurons.

The expression of the B<sub>1</sub> receptor, which is induced as a result of tissue damage and inflammation, is involved in chronic inflammation or tissue injury (Hall, 1992). The observations of the constitutive B<sub>1</sub> receptor expression in TG and dorsal root ganglion (DRG) neurons have been inconsistent. In DRG neurons, some immunohistochemical studies have reported constitutive B<sub>1</sub> receptor expression (Ma et al., 2000; Wotherspoon and Winter, 2000). In contrast, other studies have described that B<sub>1</sub> receptor activation-induced [Ca<sup>2+</sup>]<sub>i</sub> responses could not be observed in DRG neurons (Brand et al., 2001). In TG neurons, an immunohistochemical study has shown the constitutive expression of B<sub>1</sub> receptors (Ma et al., 2000). In contrast, RT-PCR analyses have demonstrated that B<sub>1</sub> receptor mRNA was barely expressed in intact tissue, while it was weakly
expressed in primary cultured TG neurons. In primary cultured TG neurons, the levels of expression of B1 receptor mRNA have been reported to depend on the length of the culture period (Ceruti et al., 2011). The present immunohistochemical and immunocytochemical results were similar to the previous RT-PCR results; B1 receptor immunoreactivity was weakly positive in cultured TG neurons and could not be detected in intact TG tissue. Although few report concerning B1 receptor-induced [Ca\(^{2+}\)]\(_i\) response in TG neurons exist, in the [Ca\(^{2+}\)]\(_i\) imaging in the present study, the B1 receptor agonist, Lys-[Des-Arg\(^9\)]BK which is a metabolite of endogenous BK in peripheral tissues (Regoli et al., 2001), dose-dependently increased [Ca\(^{2+}\)]\(_i\) in the presence of extracellular Ca\(^{2+}\), and this increase was suppressed by a B1 receptor-specific antagonist (Figures 5C–F). These results of B1 receptor expression in primary cultured TG neurons suggest that the expression of B1 receptors is induced in TG neurons by tissue damage and/or inflammation. However, further studies are required to evaluate the expression patterns of B1 receptors in native TG neurons.

BK-induced [Ca\(^{2+}\)]\(_i\) increases were observed in both the presence and absence of extracellular Ca\(^{2+}\). However, the amplitudes of the [Ca\(^{2+}\)]\(_i\) increases in the absence of extracellular Ca\(^{2+}\) were significantly smaller (84.9 ± 11.3%, N = 161) than those in the presence of Ca\(^{2+}\) (100%; Figures 4A,D). This indicated that the BK-induced [Ca\(^{2+}\)]\(_i\) mobilization (by B2 receptor activation) was mainly composed of Ca\(^{2+}\) release from internal stores with partial Ca\(^{2+}\) influx from the extracellular medium. Notably, BK has been reported to activate voltage-dependent Ca\(^{2+}\) channels in rat submucosal
plexus neurons (Avemary and Diener, 2010; Rehn et al., 2013), and transient receptor potential cation channel subfamily-V member-1 channels in rat DRG neurons (Ferreira et al., 2004; Mistry et al., 2014). However, BK-induced Ca$^{2+}$ currents could not be recorded in TG neurons (Kitakoga and Kuba, 1993). Although further studies are needed to clarify which Ca$^{2+}$...
influx pathways contribute to the BK-induced Ca\(^{2+}\) influx in TG neurons, the present results clearly indicate that BK mobilizes [Ca\(^{2+}\)]\(_{i}\) through both intracellular Ca\(^{2+}\) release and Ca\(^{2+}\) influx.

In addition, NGF-TrkA signaling plays important roles in not only the developmental processes of peptidergic nociceptive afferents, but also in the generation of acute and chronic pain state in adults. The signaling also up-regulates B\(_2\) receptor expression in peptidergic nociceptors (Mantyh et al., 2011). Thus, the results showing colocalization in TG neurons, the present results clearly indicate that BK regulates B\(_2\) nociceptive afferents, but also in the generation of acute pain in TG neurons through B\(_2\) and B\(_1\) receptor activation, respectively.

Acknowledgments

This research was supported by a Grant-in-Aid (23592751/26861559/25861762) for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

Avery, J., and Diener, M. (2010). Bradykinin-induced depolarisation and Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels in rat submucosal neurons. *Eur. J. Pharmacol.* 635, 87–95. doi: 10.1016/j.ejphar.2010.03.009

Babbedge, R., Dray, A., and Urban, L. (1995). Bradykinin depolarises the rat isolated superior cervical ganglion via B\(_2\) receptor activation. *Neurosci. Lett.* 193, 161–164. doi: 10.1016/0304-3940(95)11690-x

Basbaum, A. I., Bautista, D. M., Scherrer, G., and Julius, D. (2009). Cellular and Molecular Mechanisms of Pain. *Neuron* 63, 267–284. doi: 10.1016/j.neuron.2009.09.028

Brand, M., Klusch, A., Kurzai, O., Valdeolmillos, M., Schmidt, R. F., and Petersen, M. (2001). No evidence for bradykinin B\(_2\) receptors in rat dorsal root ganglion neurons. *Neuroreport* 12, 3165–3168. doi: 10.1097/00001756-200110080-00036

Ceruti, S., Fumagalli, M., Villa, G., Verderio, C., and Abbracchio, M. P. (2008). Purinoreceptor-mediated calcium signaling in primary neuron-glia trigeminal cultures. *Cell Calcium* 43, 576–590. doi: 10.1016/j.ceca.2007.10.003

Ceruti, S., Villa, G., Fumagalli, M., Colombo, L., Magni, G., Zanardelli, M., et al. (2011). Calcitonin gene-related peptide-mediated enhanced activation of purinergic neuron-glia communication by the allogenic factor bradykinin in mouse trigeminal ganglia from wild-type and R192Q Cav2.1 knock-in mice: implications for basic mechanisms of migraine pain. *J. Neurosci.* 31, 3638–3649. doi: 10.1523/JNEUROSCI.6440-10.2011

Cervero, F., and Laird, J. M. (1996). Mechanisms of touch-evoked pain (allodynia): a new model. *Pain* 68, 13–23. doi: 10.1016/0304-3959(96)03165-x

Dutra, R. C., Bento, A. F., Leite, D. F. P., Manjavachi, M. N., Marcon, R., Bicca, M. A., et al. (2011). The role of kinin B\(_1\) and B\(_2\) receptors in the persistent orofacial nociception in rats. *Br. J. Pharmacol.* 164, 1175–1184. doi: 10.1038/bj.2010.5724

Duehrkop, C., Banz, Y., Sprig, R., Miescher, S., Nolte, M. W., Spycher, M., et al. (2013). C1 esterase inhibitor reduces lower extremity ischemia/reperfusion injury and associated lung damage. *PLoS One* 8:e72059. doi: 10.1371/journal.pone.0072059

Dutra, A. C., Bento, A. F., Leite, D. F. P., Manjavachi, M. N., Marcon, R., Bicca, M. A., et al. (2013). The role of kinin B\(_1\) and B\(_2\) receptors in the persistent pain induced by experimental autoimmune encephalomyelitis (EAE) in mice: evidence for the involvement of astrocytes. *Neurobiol. Dis.* 54, 82–93. doi: 10.1016/j.nbd.2013.02.007

Ferreira, J., da Silva, G. L., and Calixto, J. B. (2004). Contribution of vanilloid receptors to the overt nociception induced by B\(_2\) kinin receptor activation in mice. *Br. J. Pharmacol.* 141, 787–794. doi: 10.1038/sj.bjp.0705546

Hall, J. M. (1992). Bradykinin receptors: pharmacological properties and biological roles. *Pharmacol. Ther.* 56, 131–190. doi: 10.1016/0163-7258(92)90016-s

Hall, J. M. (1997). Bradykinin receptors. *Gen. Pharmacol.* 28, 1–6. doi: 10.1016/S0306-3623(96)00174-7

Hall, J. M. (1997). Bradykinin B\(_1\) receptors. *Biochem. Biophys. Res. Commun.* 220, 219–225. doi: 10.1006/bbrc.1996.0384

In conclusion, B\(_2\) receptors were expressed constitutively, and their activation induced the mobilization of [Ca\(^{2+}\)]\(_{i}\), by releasing Ca\(^{2+}\) from intracellular stores with partial Ca\(^{2+}\) influx. In contrast, B\(_1\) receptor expression was faint in cultured TG neurons and absent in neurons in TG cryosections, although a metabolite of endogenous BK elicited [Ca\(^{2+}\)]\(_{i}\) increases. These results indicated that both BK and its metabolites activated [Ca\(^{2+}\)]\(_{i}\) mobilization in TG neurons through B\(_2\) and B\(_1\) receptor activation, respectively.
Regoli, D., Rizzi, A., Perron, S. I., and Gobeil, F. (2001). Classification of kinin receptors. *Biol. Chem.* 382, 31–35. doi: 10.1515/bc.2001.005

Rehn, M., Bader, S., Bell, A., and Diener, M. (2013). Distribution of voltage-dependent and intracellular Ca\(^{2+}\) channels in submucosal neurons from rat distal colon. *Cell Tissue Res.* 353, 355–366. doi: 10.1007/s00441-013-1643-5

Scholz, J., and Woolf, C. J. (2002). Can we conquer pain? *Nat. Neurosci.* 5(Suppl.), 1062–1067. doi: 10.1038/nn942

Simpson, P. B., Woollacott, A. J., Hill, R. G., and Seabrook, G. R. (2000). Functional characterization of bradykinin analogues on recombinant human bradykinin B\(_1\) and B\(_2\) receptors. *Eur. J. Pharmacol.* 392, 1–9. doi: 10.1016/s0014-2999(00)00046-7

Talbot, S., Théberge-Turmel, P., Liazoghli, D., Sénécal, J., Gaudreau, P., and Couture, R. (2009). Cellular localization of kinin B\(_1\) receptor in the spinal cord of streptozotocin-diabetic rats with a fluorescent [Nα-Bodipy]-des-Arg\(^9\)-bradykinin. *J. Neuroinflammation* 6:11. doi: 10.1186/1742-2094-6-11

Tiwari, M. M., Prather, P. L., and Mayeux, P. R. (2005). Mechanism of bradykinin-induced Ca\(^{2+}\) mobilization in murine proximal tubule epithelial cells. *J. Pharmacol. Exp. Ther.* 313, 798–805. doi: 10.1124/jpet.104.080408

Walker, K., Perkins, M., and Dray, A. (1995). Kinins and kinin receptors in the nervous system. *Neurochem. Int.* 26, 1–16; discussion 17–26. doi: 10.1016/0197-0186(94)00115-b

Wotherspoon, G., and Winter, J. (2000). Bradykinin B\(_1\) receptor is constitutively expressed in the rat sensory nervous system. *Neurosci. Lett.* 294, 175–178. doi: 10.1016/s0304-3940(00)01561-5

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2015 Kawaguchi, Sato, Kimura, Yamazaki, Yamamoto, Tazaki, Ichinohe and Shibukawa. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution and reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.