Downregulation of GIRK2 Mediated by PPARγ Inhibition in DRG Contributes to the Neuropathic Pain Induced by Spare Nerve Injury

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Research

**Keywords:** Spared nerve injury, Neuropathic pain, GIRK2, PPARγ, DRG

**DOI:** https://doi.org/10.21203/rs.3.rs-115886/v1

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Abstract

Neuropathic pain, as the most common chronic and intractable neurological disorder, seriously endangers the health and even life of patients. Due to the unclear mechanism, there is no effective treatment for neuropathic pain at present. Here, we used spared nerve injury (SNI) rat model to investigate the underlying mechanism involved in neuropathic pain. We found that SNI significantly decreased the expression of G protein-coupled inwardly rectifying potassium channel subunit 2 (GIRK2) and peroxisome proliferation-activated receptor gamma (PPARγ) in dorsal root ganglion (DRG). Activation of GIRK2 by intrathecal injection of activators-ML-297 or overexpression of GIRK2 by intrathecal injection of adenovirus associated virus (AAVs)-AAV-GIRK2-EGFP remarkably attenuated the mechanical allodynia induced by SNI in rats. Similarly, activation or overexpression of PPARγ also relieved the SNI-induced mechanical alldynia. We further found that the expression of PPARγ was co-localized with GIRK2-positive neurons, and overexpression of PPARγ rescued the down-regulation of GIRK2 induced by SNI. The results of chromatin immunoprecipitation (ChIP) assays further showed that PPARγ was bound to the potential binding site in the promoter region of GIRK2, and overexpression of PPARγ recovered the binding in GIRK2 promoter region in DRG, which was decreased by SNI. Altogether, our results suggested that the reduction of PPARγ induced downregulation of GIRK2 in DRG, which was involved in SNI-induced mechanical alldynia.

Introduction

Neuropathic pain is one of serious health problems afflicting about 10% of the general population, which is generally accompanied by a lesion or disorder of the somatosensory system. It is characterized by abnormal pain responses and negative affective experiences.¹-³ However, the treatment of neuropathic pain was seriously restricted by the inadequate understanding of intrinsic mechanisms.

Peripheral nerve injury is one of the most common causes of neuropathic pain, of which the pathological process was well replicated in various animal models for behavioral and mechanism studies.⁴ For instance, the spared nerve injury (SNI) model exhibited the prolonged (at least until 6 months) changes in mechanical sensitivity in rats.⁵ As reported previously, peripheral nerve injury decreased the expression of inwardly rectifying potassium channels (Kir), which sufficiently induced the ectopic hyperactivity of afferent neurons in dorsal root ganglions (DRGs), because Kir channels play an important role to maintain the resting potential and regulate the cell excitability of sensory neurons in the setting of acute or chronic pain.⁶,⁷ G protein-coupled inwardly rectifying potassium channel (GIRK, also known as Kir3), one of seven members of the Kir family, is extensively expressed in nociceptors and plays an important role in regulating neuronal activity and signal propagation throughout the nervous system.⁸⁻¹¹ GIRK are homo- or heterotramers formed by GIRK1–4 subunits, while only GIRK2 is able to assemble functional homotetramers. Studies showed that the spinal GIRK2 was linked to pain sensitivity and tolerance, as well as analgesic effectiveness.¹²,¹³ However, it still remains unclear whether the GIRK2 in DRG is involved in the neuropathic pain.
Peroxisome proliferation-activated receptors γ (PPARγ), as an important member of nuclear hormone receptor superfamily, is a ligand-activated transcription factor to mediate the expression of various genes.\textsuperscript{14,15} It plays a vital role in regulating metabolism of glucose and lipid.\textsuperscript{16} Increasing evidence also shows that PPARγ is involved in the occurrence and development of many diseases.\textsuperscript{16,17} For example, PPARγ participates in chronic and acute pain through regulating the activity of astrocytes in spinal cord.\textsuperscript{18,19} Activation of PPARγ in brain can alleviate peripheral neuropathic pain, perhaps due to their anti-inflammatory effects\textsuperscript{20}. Furthermore, studies showed that PPARγ can regulated the expression of G-protein coupled receptors such as opioid µ-receptor.\textsuperscript{21} However, whether PPARγ can regulate the expression GIRK and its involvement in neuropathic pain remain unknown.

**Methods**

**Experimental Animals**

Male Sprague-Dawley rats weighing 200 to 250 g were purchased from the Institute of Experimental Animals of Sun Yat-Sen University. All animals were housed in separated cages, and the room was kept at 24 °C temperature and 50–60% humidity, under a 12/12 h light/dark cycle with *ad libitum* access to food and water. All experimental procedures were approved by the Local Animal Care Committee and were carried out in accordance with the guidelines of the National Institutes of Health on animal care and the ethical guidelines.

**Surgery and drug application**

The SNI surgery was performed as described previously.\textsuperscript{5} Briefly, the rats were anesthetized with isoflurane, and three peripheral branches of the sciatic nerve of the left hind limb were exposed. The common peroneal and the tibial nerves were ligated and cut (2 mm sections removed), but the sural nerve was carefully kept intact. The surgical incision was sutured in two layers.

Intrathecal injection was performed according to the method described previously.\textsuperscript{22} In short, the L5 vertebra laminectomy was performed after isoflurane gas anesthesia. A polyethylene-10 catheter was implanted into the L5/L6 intervertebral subarachnoid space, and the tip of the catheter was located between the levels of L4-L6 spinal segmental. The rats with laminectomy were recovered for 7 days. Animals, exhibiting hind limb paresis or paralysis after laminectomy were excluded from the study.

GIRK1/2 activator ML297 (20mg/kg, i.p., once a day for 14 days; MedChemExpress) or PPARγ endogenous ligand 15d-PGJ2 (100 µg, i.t., twice a day for 14 days; MedChemExpress) was used to activate GIRK1/2 and PPARγ. Recombinant adeno-associated virus (AAV) encoding GIRK2 or PPARγ (OBi0) was intrathecally injected into the subarachnoid space of L4-L6 spinal cord in naïve rats to overexpression of GIRK2 or PPARγ.

**Behavioral tests**
Pain sensitivity testing was performed before and after surgery or drug administration. The animals were habituated for 15-20 min in separate transparent Plexiglas chambers before tests. Mechanical sensitivity was tested with the up-down method using a set of von Frey hairs (0.16–15 g, Stoelting) as described earlier. Briefly, each von Frey hair application was kept at 6-8s on the sural nerve innervation area in the hind paw. A quick withdrawal or licking paw indicated a positive response. The decrease in paw withdrawal thresholds (PWTs) was considered mechanical allodynia.

**RNA extraction and quantitative polymerase chain reaction**

Trizol was used to extract total RNA of DRG tissues. The reverse transcription was performed with oligo-dT primer and M-MLV reverse transcriptase (Promega, USA) according to the manufacturer's protocol. The sequences of specific primers for the examined mRNA and the internal standard for PCR reactions were listed in Table 1.

**Table 1. Specific Primer Sequences for qPCR**

| GENE     | PRIMER | SEQUENCE                        |
|----------|--------|---------------------------------|
| GIRK2 (Rat) | Forward | 5’ - CTGGCGGAACCTTGCTACCTGTG-3’  |
|          | Reverse | 5’ - GCTTGGCGTCTCTGGATGCTG-3’    |
| PPARγ (Rat) | Forward | 5’ - AACCTGAGCCCTCCACCTTG-3’       |
|          | Reverse | 5’ - TGCCTGTGTCTTAGCCAGTGTTTC-3’  |
| β-actin (Rat) | Forward | 5’ - AGGGAAATCGTGCGTGACAT-3’       |
|          | Reverse | 5’ - GAACCGCTCTTGCCGATAG-3’       |

The reactions were set up based on the manufacturer's protocol. PCR reactions conditions were incubation at 95 °C for 3 min followed by 40 cycles of thermal cycling (10 s at 95 °C, 20 s at 58 °C, and 10 s at 72 °C). The relative expression ratio of mRNA in the DRG tissues was quantified by the $2^{-\Delta\Delta CT}$ method.

**Western blot**

The rats were deeply anesthetized using sodium pentobarbital at 50 mg/kg dose (i.p.) at various time points. L4-L6 DRGs were immediately removed and frozen at -80°C until use. The sample tissues were homogenized in RIPA Lysis Buffer containing the inhibitors of proteinase and phosphatase on ice. The protein sample was separated by SDS-PAGE and then transferred onto a PVDF membrane. The PVDF membrane was incubated with primary antibodies against GIRK2 (1:1000; Abcam), PPARγ (1:1000; Abcam) or GAPDH (1:1000; CST) after blocking with the block buffer for 1 hour at RT. Then, horseradish peroxidase-conjugated secondary antibodies were used to incubate the blots, and the immune complex was detected using enhanced chemiluminescence (Pierce). The intensity of bands was examined by NIH Image J.

**Immunohistochemistry**
Perfusion was performed through the ascending aorta with 4% paraformaldehyde after application of sodium pentobarbital (50 mg/kg dose, i.p.). The removed DRGs were placed into 4% paraformaldehyde for post fixation overnight. Cryostat sections of 16μm thickness were obtained, and immunohistochemistry was performed with primary antibodies for GIRK2 (1:100; Abcam), PPARγ (1:200; Abcam), IB4 (1:50; SigmaAldrich), NF200 (1:200; Chemicon), and GFAP (1:400; CST). After overnight incubation at 4°C, the sections were incubated with secondary antibodies, which conjugated with cy3 or Alex488-fluorescein isothiocyanate for 1 hour at room temperature. A Nikon (FCK-50C, Japan) microscopy was used to examine the fluorescence of tissue slices.

Chromatin immunoprecipitation assays

Kit (Thermo) was used to perform this assay. The L4-L6 DRGs of rat was isolated and placed in 1% formaldehyde for 2 minutes. Sonication was applied to fragment the DNA, which was subsequently digested by micrococcal nuclease. A small portion of samples (100 μl) were saved as input after the addition of ChIP dilution buffer. PPARγ antibody (5 μl) was added to 500 μL for preclearing, and then the samples were incubated overnight. Immunoprecipitation with control rabbit IgG (Sigma) was performed as negative control. The DNA was purified from the DNA/antibody complexes through elution and reversion. qPCR was applied on 5 μL of precipitated DNA samples. Primers 5'-GAAGCTTACGTACCAAGAGA-3' and 5'-CTGGATAAAAGCGGGCGCAGCG-3' were projected to amplify a fragment with the sequence of localized on the GIRK2 promoter (-540/-385, which containing the PPARγ binding site) in rats. The ChIP/input ratio was then calculated.

Statistical analysis

All data were expressed as means ± SEM, and analyzed with SPSS 25.0 (SPSS, USA). Statistical significance between two groups was calculated using the two independent-samples t-test. Statistical significance among three or more groups was calculated using the one-way or two-way ANOVA followed by Tukey post hoc test. The criterion for statistical significance was $P < 0.05$. While no power analysis was performed, the sample size was determined according to our and peers' previous publications in behavior and pertinent molecular studies.

Results

3.1 The expression of GIRK2 in SNI-induced neuropathic pain in DRG

Consistent with the peer's results, SNI significantly decreased the mechanical withdrawal threshold on day 3, which lasted to day 14 (the end of the experiment) (Figure. 1A). Next, PCR analysis showed that the expression of GIRK2 mRNA was significantly decreased on day 3, 7 and 14 in DRG following SNI (Figure. 1B). Consistently, the level of GIRK2 protein was downregulated at the same time points after SNI treatment (Figure. 1C). The decreased expression of GIRK2 was also confirmed by immunofluorescence staining (Figure.1D). Double immunofluorescence staining showed that GIRK2 was co-localized with IB4
(a marker for C-type neuron) and NF200 (a marker for A-type neuron) but not with GFAP (a marker for satellite glial cells) (Figure. 1E).

3.2 The upregulation of GIRK2 mediated the SNI-induced neuropathic pain in rats DRG

Next, we explored the role of GIRK2 in SNI-induced mechanical allodynia in rats. A GIRK1/2 activator ML297 was intraperitoneally administrated for consecutive 14 days (20mg/kg, once daily), which significantly recovered the decrease of mechanical threshold caused by SNI (Figure. 2A). Furthermore, we designed and synthesized AAV-GIRK2-EGFP to accurately overexpress GIRK2 in vivo. 21 days after intrathecal injection of the recombinant virus, the marked green fluoresces (Figure. 2B) and the elevated GIRK2 protein (Figure. 2C) and mRNA (Figure. 2D) in DRG suggested a high efficiency of transfection. Importantly, mechanical allodynia was greatly attenuated in AAV-GIRK2-EGFP injected rats with nerve injury, when compared to that in AAV-EGFP injected rats (Figure. 2E).

3.3 SNI increased the expression of PPARγ in DRG

Recent studies have shown that PPARγ in spinal dorsal horn was involved in the development of inflammatory pain and neuropathic pain. To determine whether the DRG PPARγ participated in SNI-induced painful neuropathy, we assessed the level of PPARγ mRNA and protein in DRG of rats. qPCR and western blot results showed that the expression of PPARγ was significantly down-regulated on day 3 after SNI and continued until day 14 (Figure. 3A and B). Immunostaining results also confirmed that the level of PPARγ expression was significantly down-regulated on day 14 after SNI compared to the sham group (Figure. 3C). Double immunostaining results indicated that PPARγ was co-localized with IB4 (a marker for C-type neuron) and NF200 (a marker for A-type neuron) but not with GFAP (a marker for satellite glial cells) (Figure. 3D).

3.4 The GIRK2 upregulation is involved in the SNI-induced neuropathic pain in rats DRG

To further explore the role of PPARγ in DRG in SNI-induced neuropathic pain, we investigated the behavioral response to the artificial manipulation of PPARγ function in DRG. The results showed that intrathecal injection of an endogenous ligand of PPARγ-15d-PGJ2 (15-deoxy-(12,14)-prostaglandin J2) at dose of 100 µg twice per day for consecutive 14 days obviously attenuated the mechanical allodynia induced by SNI (Figure. 4A). Meanwhile, we specifically up-regulated the expression of PPARγ in rats by intrathecal administration of AAV-PPARγ-EGFP. The overexpression efficiency of AAV-PPARγ-EGFP was validated by qPCR and western blot (Figure. 4B and 4C). The pain behavior test was performed after 21 days. Compared with the AAV-EGFP injected group, the injection of AAV-PPARγ-EGFP significantly relieved the mechanical allodynia induced by SNI in rats (Figure. 4D).

3.5 PPARγ regulated the expression of GIRK2 following SNI

As a robust nuclear transcription factor, PPARγ can translocate to the nucleus and regulate gene expression by binding to the gene promoter. In this study, we found that PPARγ was co-localized with
GIRK2-positive cells (Figure. 5A). Furthermore, the results of qPCR showed that the overexpression of PPARγ by intrathecal injection of AAV-PPARγ-EGFP significantly prevented the down-regulation of DRG GIRK2 mRNA and protein induced by SNI (Figure. 4B and C). However, AAV-GIRK2-EGFP injection did not relieve the decrease of DRG PPARγ mRNA or protein induced by SNI (Figure 5D and E). These results suggested that the down-regulation of GIRK2 was attributed to the reduction of PPARγ following SNI.

It is well known that transcription factors bind to the specific sites of gene promoter, by which they regulate the expression of target protein. To further elucidate the mechanism underlying the expression of GIRK2 mediated by PPARγ, we first predicted the binding sites of PPARγ at GIRK2 promoter through TFSEARCH and JASPAR databases. *In silico* analysis revealed that DNA fragment spanning from -472 to -453 (5'-flanking region relative to the transcription start site) in the GIRK2 promoter is a potential binding site of PPARγ. To further confirm whether the predicted binding site plays a key role in the expression of GIRK2 following SNI, we performed a ChIP-PCR assay in DRG tissue. The DNA, which was precipitated by the PPARγ antibody, was subjected to qPCR to amplify a 156-bp fragment (-540/-385) of the GIRK2 promoter. The results of qPCR analysis revealed that the binding of PPARγ to GIRK2 promoter in DRG was obviously reduced on day 14 after SNI (Figure. 5F). In addition, the decreased recruitment on -472 to -453 binding site in DRG tissue was remarkably recovered by AAV-PPARγ-EGFP application prior to SNI in the rats (Figure. 5F). Altogether, these results confirmed that SNI treatment decreased binding of PPARγ in promoter region of GIRK2 and attenuated the activity of GIRK2 gene promoter, thus leading to the reduction of GIRK2 expression in DRG.

**Discussion**

In the present study, we found that SNI surgery markedly down-regulated the expression of GIRK2 mRNA and protein in DRG, and activation or overexpression of GIRK2 by using ML297 or AAV-GIRK2-EGFP attenuated SNI-induced mechanical allodynia in rats, respectively. In addition, the expression of PPARγ mRNA and protein also showed significant down-regulation on day 3 to day 14 after SNI surgery. Furthermore, the time course of GIRK2 upregulation was consistent with that of PPARγ, and GIRK2 was expressed on the PPARγ-positive cells. Overexpression of PPARγ in DRG rescued the down-regulated expression of GIRK2 and relieved the mechanical allodynia induced by SNI. Importantly, CHIP assay further revealed that SNI obviously decreased the recruitment of PPARγ on GIRK2 promoter in DRG. Taken together, these results suggested that down-regulation of PPARγ, through decreasing its binding to GIRK2 promoter, induced the downregulation of GIRK2 in DRG, and contributed to SNI-induced neuropathic pain.

G protein-coupled inwardly rectifying potassium channel (GIRK/Kir3.x) is one of seven members of the Kir family, which demonstrates pivotal role to regulate neuronal activity and signal propagation throughout the nervous system including peripheral sensory neurons.8-11 Accumulating evidence have shown that GIRK channels are activated by a large number of G protein-coupled receptors (GPCRs) and regulate the electrical activity of neurons.25 GIRK channels have been functionally associated with drug addiction, pain perception, and other disorders, in particular as major mediators for opioid-induced function in both central and periphery nervous system.26-28 The four subunits of GIRK (GIRK1-4) function by forming
various heterotetrameric and homotetrameric channels in different mammalian tissues. In neurons, GIRK1/GIRK2 or GIRK1/GIRK3 hereotetramer and GIRK2/GIRK2 homotetramer are generally formed. For example, in a number of brain regions, including the hippocampus, cerebellum, and ventral tegmental area, the composition of GIRK channels are mostly hereotetramers which are composed of GIRK1, GIRK2 and GIRK3, while the compositions of GIRK channels are mostly hereotetramer which composed of GIRK1 and GIRK2 in the superficial layers of the spinal dorsal horn and DRG. DRG neurons are the primary afferent neurons in somatic and visceral tissues. Hence, we focused on the role of GIRK2 in DRG tissues following nerve injury. The results revealed that GIRK2 was primarily co-localized with IB4 and NF200-positive cells, but not with GFAP-positive cells. In addition, SNI significantly reduced the expression of GIRK2 mRNA and protein in rats, and activation or overexpression of GIRK2 remarkably recovered the threshold of mechanical allodynia in rats with SNI. Study showed that activation of GIRK channel decreases neuronal firing, which leading to inhibition at the neuronal network level. In the present study, reduction of GIRK2 in DRG potentially enhanced the neuronal activity and mediated the neuropathic pain. The results were also consistent with the previous report that GIRK2 knock-out mice exhibited hyperalgesia in the tail-flick test of thermal nociception. Together, the current data suggested that the functional adaptation of GIRK2 may be substantially involved in the development of neuropathic pain induced by nerve injury.

PPARs are a family of ligand-dependent nuclear hormone transcription factors which contain two transcription activation domains and a large ligand binding domain. Studies showed that PPARs are widely expressed in tissues in mammalian, including nervous systems. Among them, PPARγ is activated by endogenous lipids or by thiazolidinediones, such as 15d-PGJ2, rosiglitazone and pioglitazone. It has been reported that PPARγ activation plays an important role in the occurrence and development of nervous system diseases. For example, PPARγ activation through suppressing neuro-inflammatory responses contributed to the pathogenesis of Alzheimer's disease. In the present study, we found that the expression of PPARγ was decreased in the DRG following SNI surgery, and cellular distribution of PPARγ generally overlaid with that of GIRK2 (co-localized with IB4 and NF200, but not with GFAP). Moreover, activation or overexpression of PPARγ by injection of 15d-PGJ2 or AAV-PPARγ-EGFP alleviated the hand paw mechanical hypersensitivity in rats. The results were consistent with the peer’s reports that PPARγ was involved in the neuropathic pain induced by diabetes or trigeminal inflammatory. Studies showed that transcription factor PPARγ regulated the transcription of TNF-α and IL-6 in spinal cord in the setting of chronic pain. In the current study, immunofluorescence results found that PPARγ and GIRK2 were highly co-stained in DRG neurons. Furthermore, CHIP assays further demonstrated that PPARγ was indeed bound to the potential binding site in the promoter region of GIRK2 in the rats with SNI, which was alleviated by overexpression of PPARγ.

In summary, this study dissected the molecular mechanism underlying PPARγ-mediated regulation of GIRK2 in DRG and its functional involvement in the pathogenesis of neuropathic pain induced by nerve injury. These may provide new insights and potential targets for the treatment of painful neuropathy induced by peripheral nerve injury.
Abbreviations

SNI: spared nerve injury.; GIRK2: G protein-coupled inwardly rectifying potassium channel subunit 2.; PPARγ: peroxisome proliferation-activated receptor gamma.; DRG: dorsal root ganglion.; ChIP: chromatin immunoprecipitation.; Kir: inwardly rectifying potassium channels.; GIRK, also known as Kir3: G protein-coupled inwardly rectifying potassium channel.; PWTs: paw withdrawal thresholds.; GPCRs: G protein-coupled receptors.

Declarations

Ethics approval for use of animals

All experimental procedures were approved by the Local Animal Care Committee and were conducted in accordance with the guidelines of the National Institutes of Health (NIH) on animal care and with the ethical guidelines.

Consent for publication

Not applicable

Funding

This work is supported by natural science foundation of Guangdong Province China General Program (Grant No. 2019A1515011447).

Authors' contributions

All authors reviewed the manuscript. The authors read and approved the final manuscript.

Competing interests

The authors declare that the research was conducted in the absence of any commercial.

Availability of data and materials

Please contact author for data requests.

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