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Research Paper

Redox-guided axonal regrowth requires cyclic GMP dependent protein kinase 1: Implication for neuropathic pain

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ABSTRACT

Cyclic GMP-dependent protein kinase 1 (PKG1) mediates presynaptic nociceptive long-term potentiation (LTP) in the spinal cord and contributes to inflammatory pain in rodents but the present study revealed opposite effects in the context of neuropathic pain. We used a set of loss-of-function models for *in vivo* and *in vitro* studies to address this controversy: peripheral neuron specific deletion (SNS-PKG1$^{-/-}$), inducible deletion in subsets of neurons (SLICK-PKG1$^{-/-}$) and redox-dead PKG1 mutants. In contrast to inflammatory pain, SNS-PKG1$^{-/-}$ mice developed stronger neuropathic hyperalgesia associated with an impairment of nerve regeneration, suggesting specific repair functions of PKG1. Although PKG1 accumulated at the site of injury, its activity was lost in the proximal nerve due to a reduction of oxidation-dependent dimerization, which was a consequence of mitochondrial damage in injured axons. *In vitro*, PKG1 deficiency or its redox-insensitivity resulted in enhanced outgrowth and reduction of growth cone collapse in response to redox signals, which presented as oxidative hotspots in growing cones. At the molecular level, PKG1 deficiency caused a depletion of phosphorylated cofilin, which is essential for growth cone collapse and guidance. Hence, redox-mediated guidance required PKG1 and consequently, its deficiency *in vivo* resulted in defective repair and enhanced neuropathic pain after nerve injury. PKG1-dependent repair functions will outweigh its signaling functions in spinal nociceptive LTP, so that inhibition of PKG1 is no option for neuropathic pain.

1. Introduction

Peripheral nerve injury is a frequent cause of persistent neuropathic pain that may be interpreted as the result of multi-faceted adaptive processes gone wrong. Peripheral neurons have the inherent capacity to regrow and eventually re-innervate the target if the correct path is not lost, a prerequisite that depends on a complex network of guidance cues and intact myelin sheaths [1,2]. It is believed that successful re-innervation leads to pain resolution [3] whereas aberrant growth and sprouting increases pathological spontaneous activity and hyperexcitability [4,5]. But even on structural restitution, pain may persist as a consequence of functional or structural synaptic potentiation [6–8].

The induction of spinal long-term potentiation involves cGMP [9–11]. The process starts with activation of the NMDA receptor, subsequent activation of neuronal nitric oxide synthase (nNOS), nitric oxide (NO) production and activation of soluble guanylyl cyclase (sGC), finally producing cyclic GMP. cGMP also originates from natriuretic peptide/Npr signaling and regulates multiple cellular targets, including diverse cGMP-gated ion channels [12,13], the cGMP-dependent protein kinases, PKG1 and PKG2 [14,15], phosphodiesterases and cysteine rich protein 2 [16]. Nearly all of these molecular targets of cGMP are expressed in nociceptive pathways and potentially contribute to the key role of cGMP in synaptic scaling in the spinal cord [9–11].

Among these targets, PKG1 has emerged as a key mediator of

Abbreviations: ATF3, Activating transcription factor-3; ADF, Actin depolymerizing factor; cGMP, cyclic guonosine monophosphate; Cre, Cre recombinase; DRG, Dorsal root ganglia; EYFP, Enhanced yellow fluorescent protein; GAP43, Growth associated protein 43; IP3, Inositol trisphosphate; IRAK, Inositol trisphosphate receptor-associated cGMP-kinase substrate; H2O2, Hydrogen peroxide; NF200, Neurofilament of 200 kDa; NO, nitric oxide; NOS, nitric oxide synthase; Npr, Natriuretic peptide receptor; OXPHOS, Oxydative phosphorylation; PKG1, cGMP dependent protein kinase 1 (prkg1); ROS, Reactive oxygen species; sGC, soluble guanylyl cyclase; SLICK, Single neuron labeling with inducible cre-mediated knockout; SNI, Spared nerve injury; SNS, Tetrodotoxin-resistant sodium channel, Nav1.8; TAM, tamoxifen; TRPA1, Transient receptor potential channel subfamily A, type 1; TRPV1, Transient receptor potential channel subfamily V, type 1; Vasp, Vasodilator-stimulated phosphoprotein

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nociceptive signaling in inflammatory conditions [9,16–18]. The α-isoform of PKG1 is highly expressed in primary sensory neurons in the dorsal root ganglia (DRG) and in several regions in the brain and the spinal cord [16,17,19] and pharmacological and genetic studies have linked PKG1 to the development of nociceptive hypersensitivity and spinal mechanisms of inflammatory hyperalgesia [17].

PKG1 activation does not entirely depend on cGMP but also ensues on oxidative cysteine modification resulting in its dimerization and auto-activation [20,21]. This redox switch is particularly important in nociceptive neurons that mostly do not express sGC [16] and therefore may depend on oxidation-mediated PKG1 activation to maintain its function. PKG1 phosphorylates key regulators of intracellular calcium stores, namely the inositol 3-phosphate receptor [9] and its substrate (IRAG) [22], as well as effector molecules, which regulate the dynamics of actin and myosin [9,23,24]. These mechanisms are crucial for synaptic vesicle cycling and hence glutamate and neuropeptide release. As a consequence, PKG1 promotes inflammatory hyperalgesia and a specific loss of PKG1 in presynaptic compartments of the nociceptive synapse abolishes C-fiber-evoked long-lasting potentiation [9]. PKG1-mediated regulations of actin and myosin dynamics may also critically impact on regrowth and guidance of peripheral axons after injury, and injury-evoked redox imbalances may interfere with PKG1 dimerization and activity. As PKG1 has been suggested as a target for pain control, we asked if and how the complex functions of PKG1 affect neuropathic pain and repair after sciatic nerve injury.

2. Methods

2.1. Generation of neuron-specific PKG1 deficient mice

Mice lacking PKG1 specifically in primary nociceptive neurons were generated via Cre-loxP-mediated recombination by mating mice carrying the floxed *prkg1* allele ($PKG1^{fl/fl}$) [19] with a mouse line expressing Cre recombinase under control of the Nav1.8 promoter (SNScre) [25,26]. SNScre mice enable gene recombination commencing at birth selectively in Nav1.8-expressing sensory neurons of the dorsal root ganglia and trigeminal ganglia, without affecting gene expression at other sites [26,27]. We have shown that SNS-$PKG1^{-/-}$ have a specific deletion of PKG1 in the dorsal root ganglia but not in the spinal cord or brain [9]. Unlike global $PKG1^{-/-}$ mice [17], SNS-$PKG1^{-/-}$ mice show normal lamination of the spinal cord and have no developmental deficits [9]. To generate tamoxifen inducible PKG1-deficient mice in subsets of neurons we crossed the $PKG1^{fl/fl}$ mice with SLICK-X mice [28], which carry a double-headed Thy1-promoter, one head driving tamoxifen-inducible creERT expression and the other driving EYFP expression. Successful Cre-mediated excision of the *prkg1* allele was confirmed by PCR-based genotyping as described [19]. Genotyping for cre-recombinase used the primer: left: gaa agc agc cat gtc caa ttt act gac cgt ac; right: gcg cgc ctg aag ata tag aag a) and was done as described [29].

2.2. Mouse strains

Male and female 8–16 weeks old SNS-$PKG1^{-/-}$ and their $PKG1^{fl/fl}$ littermates were used for behavioral experiments, generation of primary DRG neuron cultures, western blotting and immunofluorescence studies. Tamoxifen versus vehicle treated SLICK-X-PKG1 mice were used to assess regrowth in vivo. The tamoxifen protocol consisted in once daily i.p. injection of 0.15 mg/gram of body weight for 5 consecutive days in 9:1 corn oil/ethanol followed by a free interval of 14 days before induction of the sciatic nerve lesion. The controls were littermates treated with vehicle (9:1 corn oil/ethanol). For analysis of redox sensitive growth cone collapse we used the DRG neurons of redox-dead Cys42Ser PKGI-alpha knock-in mice, in which the redox sensitive cysteine Cys42 has been replaced with a serine [20]. Mice had free access to food and water and were maintained in climate controlled rooms with a 12 h light-dark cycle. Behavioral experiments were performed between 10 am and 3 pm. The experiments were approved by the local Ethics Committee for animal research (Darmstadt, Germany), adhered to the guidelines for pain research in conscious animals of the International Association for the Study of PAIN (IASP) and those of the Society of Laboratory Animals (GV-SOLAS) and were in line with the European and German regulations for animal research.

2.3. Injury of the sciatic nerve

Surgery was performed under 1.5–2% isoflurane anesthesia plus local anesthesia of the skin. For the spared nerve injury (SNI) model of neuropathic pain, two of the three peripheral branches of the sciatic nerve, the common peroneal and the tibial nerves, were ligated and distally transected, leaving the sural nerve intact [30], which eventually sprout into denervated skin areas [5]. For the crush injury the exposed sciatic nerve was crushed for 30 s with a blunt, finely serrated 2 mm tip needle holder. The injury spares the myelin sheaths and allows for regrowth and re-innervation. Mechanical, heat and cold withdrawal latencies were recorded before the injuries and up to 4 weeks thereafter.

2.4. Behavioral experiments

The tests were performed by an investigator who was unaware of the mouse genotype and included 8–10 mice per group. Mice were habituated to the room and test chambers before the baseline measurement. The latency of paw withdrawal on pointy mechanical stimulation was assessed using a Dynamic Plantar Aesthesiometer (Ugo Basile, Comerio, Italy). The steel rod was pushed against the plantar paw with ascending force (0–5 g, over 10 s, 0.2 g/s) and then maintained at 5 g until the paw was withdrawn. The paw withdrawal latency was the mean of three consecutive trials with at least 30 s intervals.

To assess cold allodynia a drop of acetone was applied to the plantar ipsi-lateral hindpaw and the withdrawal response including paw lifting, shaking and licking was recorded with a stop watch for 90 s starting right after acetone application. The sensitivity towards cold was also assessed with a cold plate kept at 4 °C. Mice were put onto the plate and the number of withdrawal reactions including paw licking, lifting or shaking or jumping were counted during an observation period of 90 s, after which mice were returned to their home cage. Cold tests were done once per day.

The sensitivity to painful heat stimuli was assessed by recording the paw withdrawal latency with a Hot Plate (52 °C surface, Föhr Medical Instruments, Germany) or with the Hargreaves test (IITC Life Sciences). For the latter, a heating lamp was placed with a mirror system underneath the respective hind paw. By pressing the start button the lamp starts to emit a heat-beam until the paw is withdrawn, which stops the lamp. The mean paw withdrawal latency of three tests with at least 10 min intervals was used for statistical analysis.

Motor function was assessed with the accelerating RotaRod test (15–30 rpm, ramp 3 rpm/min, cut-off 5 min). Habituation encompassed 2–3 test runs. The fall-off latency was averaged from 2 tests. Behavioral tests were performed at baseline and 1, 3, 7, 14, 21 and 28 days after sciatic nerve injury. Exact time courses are shown in the respective figures.

2.5. Quantitative RT-PCR (QRT-PCR)

Total RNA was extracted from homogenized tissue according to the