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Downregulation of the spinal dorsal horn clock gene *Per1* expression leads to mechanical hypersensitivity via c-jun N-terminal kinase and CCL2 production in mice

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ABSTRACT

Disturbances of circadian rhythm and dysregulation of clock gene expression are involved in the induction of various neurological disorder states, including chronic pain. However, the relationship between the CNS circadian-clock gene system and nociception remains poorly defined. Significant circadian oscillations of *Period* (*Per1*, *Per2*), *Bmal1* and *Cryptochrome 1* (*Cry1*) mRNA expression have been observed in the lumbar spinal dorsal horn of naïve mice. The current study examined the expression of clock genes in the lumbar spinal dorsal horn of mice with neuropathic pain due to a partial sciatic nerve ligation (PSNL). Seven days after PSNL, the mice displayed a robust unilateral hind paw mechanical hypersensitivity. The normal circadian oscillations of *Per1*, *Per2* and *Cry1*, but not *Bmal1*, mRNA expression were significantly suppressed in the ipsilateral lumbar spinal dorsal horn of PSNL mice 7 days following surgery. The circadian expression of PER1 protein, in particular, was also significantly suppressed in the ipsilateral spinal dorsal horn of PSNL mice. Double-labeling immunohistochemistry revealed downregulation of PER1 in neurons and astrocytes, but not microglia. Knockdown of *Per1* expression by intrathecal treatment with Per1 siRNA also induced mechanical hypersensitivity, phosphorylation of c-jun N-terminal kinase (JNK) and the upregulation of chemokine (C–C motif) ligand 2 (CCL2) production in the lumbar spinal dorsal horn. Per1 siRNA-induced mechanical hypersensitivity was attenuated with intrathecal treatment of either the JNK inhibitor SP600125 or the selective CCL2 receptor (CCR2) antagonist RS504393, indicating that these intracellular messengers are crucial in mediating the mechanical hypersensitivity following the downregulation of PER1 expression. These results suggest that the downregulation of the spinal dorsal horn clock genes such as *Per1* expressed could be crucial in the induction of neuropathic pain following peripheral nerve injury. Modulating clock gene *Per1* expression could be a novel therapeutic strategy in alleviating neuropathic pain.

1. Introduction

Clock genes, such as *Period* (*Per1*, *Per2* and *Per3*), *Bmal1*, *Clock* or *Cryptochrome* (*Cry1* and *Cry2*), have pivotal roles in the regulation of circadian rhythm. The expression of each of these genes is synchronized by transcription–translation feedback loops, which create the oscillating nature of gene expression (Reppert and Weaver, 2002). The master pacemaker of rhythm regulation resides in the suprachiasmatic nucleus (SCN). However, other parts of the CNS, such as the cortex, hippocampus, striatum and spinal cord (Falcon et al., 2013; Morioka et al., 2012; Musiek et al., 2013), and peripheral organs such as the heart, liver and immune cells have been shown to express circadian rhythm (Chen et al., 2000; Peirson et al., 2006).

A number of changes in physiological functioning of the spinal dorsal horn neurons, astrocytes and microglia mediate the development and maintenance of chronic pain following injury to the peripheral tissue (Basbaum et al., 2009; Gao and Ji, 2010). Clinical and animal studies suggest that one important change in functioning could be due to a change in circadian rhythm (Kusunose et al., 2010; Odrcich et al., 2006; Zhang et al., 2012), although the mechanism by which this change could mediate chronic pain has not been entirely elaborated.

Spinal astrocytes express several clock genes, which show circadian rhythmicity (Prolo et al., 2005; Sugimoto et al., 2011) and a number of genes modulate nociception, which also show circadian rhythmicity. For example, astrocyte inhibitor fluorocitrate attenuated circadian

Abbreviations: CCL2, chemokine (C-C motif) ligand 2; CCR2, CCL2 receptor; Cry, cryptochrome; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; HVJ, hemagglutinating virus of Japan; 5-HT, 5-hydroxytriptamine; JNK, c-jun N-terminal kinase; MAP, mitogen-activated protein; MCP-3, monocyte chemotactic protein; NA, noradrenaline; PMSF, phenylmethylsulfonyl fluoride; PSNL, partial sciatic nerve ligation; Per, period; SCN, suprachiasmatic nucleus; SDS, sodium dodecyl sulfate; siRNA, small interfering RNA.

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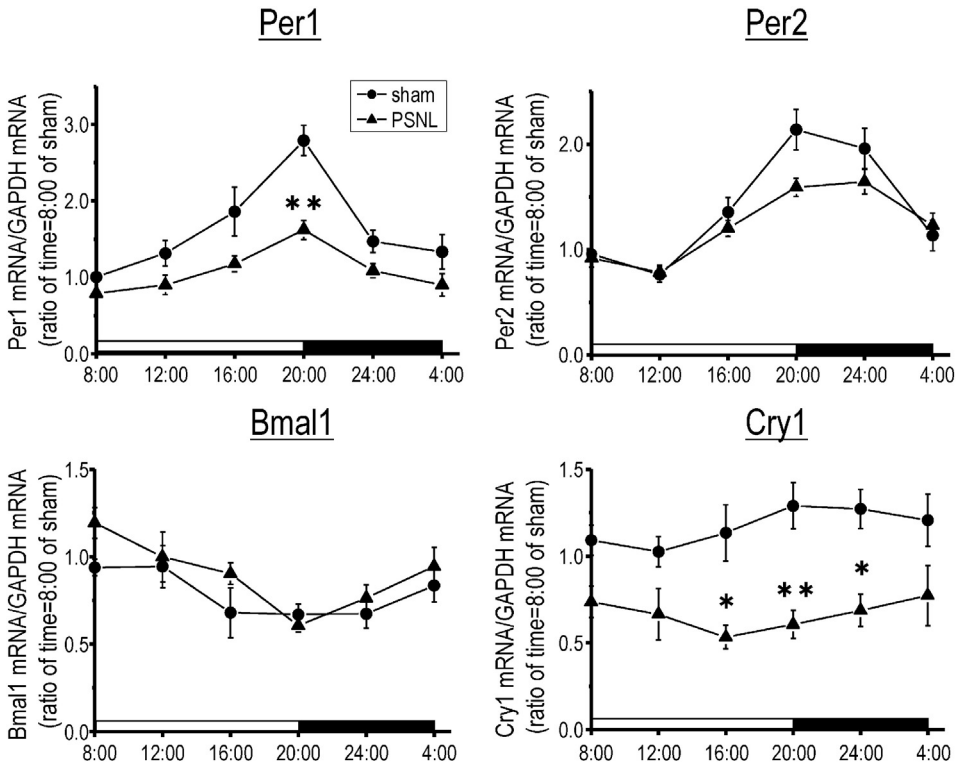

Fig. 1. Circadian variation in mRNA expression of clock genes in the ipsilateral lumbar spinal dorsal horn of sham and partial sciatic nerve ligation (PSNL) mice under light–dark conditions. The mRNA expression levels of clock genes in the ipsilateral lumbar spinal dorsal horn from sham-operated (circle) and PSNL-operated (triangle) mice 7 days after surgery are shown. Each value is expressed relative to levels at 8:00 in sham-operated mice. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01 vs. sham mice at the corresponding time point. n = 7–13/group.

oscillation and rhythmicity of mRNA expression of glutamine synthase and cyclooxygenase-1 in the spinal dorsal horn (Morioka et al., 2012). An interaction between clock genes and non-clock genes could be important in mediating nociception. Astrocytic clock gene *Per1* has crucial roles in controlling the expression of other clock genes and the production of proinflammatory molecules such as chemokine (C–C motif) ligand 2 (CCL2) and interleukin-6 via the regulation of mitogen-activated protein (MAP) kinases and NF-κB (Sugimoto et al., 2014). However, it is yet unclear how clock genes expressed in the spinal dorsal horn specifically contribute to the development and maintenance of chronic pain.

Absolute levels of clock gene expression are modulated under pathological conditions such as inflammatory arthritis, cancer, traumatic brain injury, and drug addiction (Bang et al., 2012; Boone et al., 2012; Falcon et al., 2013; Hwang-Verslues et al., 2013). It is possible that clock gene expression in the spinal dorsal horn could be changed following a peripheral nerve injury and an interaction between clock genes and nociceptive transmission at the spinal level.

The current study investigated changes in circadian mRNA expression of clock genes in the lumbar spinal dorsal horn of mice following a partial sciatic nerve ligation (PSNL). Rhythmicity of clock gene expression was significantly disrupted and decreased clock gene expressions were observed following PSNL. The current study focused primarily on the role of PER1 in the lumbar spinal dorsal horn in neuropathic pain and elaborated the cellular mechanism related to *Per1* using selective inhibition of *Per1* expression by small interfering RNA (siRNA).

2. Materials and methods

2.1. Animals

Male ICR mice, 5 weeks of age, were used for the experiments (Japan SLC, Inc., Shizuoka, Japan). The mice were maintained at a room temperature of 22 ± 2 °C with a 12 h light/dark cycle (lights on at 8:00 AM), and given access to food and water available ad libitum during the experimental period. All the experiments using animals were conducted in accordance with the "Guidelines for the Care and Use of Laboratory Animals" established by Hiroshima University, and the procedures of all animal experiments were reviewed and approved by the Committee of Research Facilities for Laboratory Animal Science of Hiroshima University.

2.2. Partial sciatic nerve ligation (PSNL) in mice

A unilateral peripheral neuropathy of the sciatic nerve, which leads to significant ipsilateral hind paw cutaneous hypersensitivity, was induced in the mice as previously described (Nakamura et al., 2013). Under sodium pentobarbital (50 mg/kg, i.p.) anesthesia, a tight ligation of approximately one-third to one-half of the diameter of the left sciatic nerve (ipsilateral) was performed with 8–0 silk suture. In sham-operated mice, the sciatic nerve was exposed without ligation.

Measurement of hind paw sensitivity to tactile stimulation using von Frey monofilaments was described previously (Nakamura et al., 2013, 2015).

2.3. Real-time PCR analysis

Seven days after either a sham or PSNL surgery, the mice were decapitated under ether anesthesia at 8:00, 12:00, 16:00, 20:00, 24:00, and 4:00 and the lumbar (L4–L6) spinal cord was removed. Total RNA from the lumbar spinal cord was prepared as previously described (Chomczynski and Sacchi, 1987) and used to synthesize cDNA with MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA) and a random hexamer primer. Real-time PCR was performed with specific primers and EXPRESS SYBR® GreenER™ qPCR SuperMix (Invitrogen, Carlsbad, CA, USA). The sequences of the primers are described in our previous report (Morioka et al., 2012). Real-time PCR assays were conducted using a DNA engine Opticon 2 real-time PCR