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Original Reports

Acute Pain Increases Phosphorylation of DCLK-Long in the Edinger-Westphal Nucleus but not in the Hypothalamic Paraventricular Nucleus of the Rat

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Abstract: The doublecortin-like kinase (DCLK) gene is crucially involved in neuronal plasticity and microtubule-guided retrograde transport of signaling molecules. We have explored the possibility that DCLK is involved in pain-induced signaling events in adult male Wistar rats. Our results show that both DCLK-short and DCLK-long splice variants are present in the cell body and proximal dendrites of neurons in stress-related nuclei, ie, the paraventricular nucleus of the hypothalamus (PVN) and the non-preganglionic Edinger-Westphal nucleus (npEW) in the rostroventral periaqueductal grey. We found that DCLK-long but not DCLK-short is phosphorylated in its serine/proline-rich domain. Furthermore, we demonstrate that phosphorylation of DCLK-long in the npEW is increased by acute pain, whereas DCLK-long phosphorylation in the PVN remains unaffected. This is the first report revealing that DCLK isoforms in the PVN and npEW occur in the adult mammalian brain and that pain differentially affects DCLK-long-mediated neuronal plasticity in these 2 stress-sensitive brain centers.

***Perspective:** Pain is a burden for society and the individual, and although the mechanisms underlying pain are relatively well known, its treatment remains difficult and incomplete. Pain stress can lead to diseases like chronic pain and depression. The differential DCLK-phosphorylation in stress-sensitive brain areas is a potential novel therapeutic target in pain research.*

***Key words:** Non-preganglionic Edinger-Westphal nucleus, hypothalamic paraventricular nucleus, acute pain, doublecortin-like kinase, stress response.*

Pain is considered as an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage.[7] Pain is a burden for society and the individual, and although the mechanisms underlying pain are relatively well known, we know less about its treatment. It is known that acute pain triggers an organism to avoid harmful situations[36] and coincides with an activation of the hypothalamo-pituitary-adrenal (HPA)-axis, as revealed by increased blood titers of adrenocorticotropic hormone (ACTH) and cortisol or corticosterone.[23,34,52] Different pain conditions, such as acute pain stress (APS), lead to activation of mitogen-activated protein kinases (MAPKs).[27] This MAPK activation is a process that plays an important role in the induction and

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maintenance of neuronal plasticity, including peripheral and central sensitization underlying increased pain sensitivity after injuries.[26] MAPK activity phosphorylates proteins that have a specific proline-threonine-serine-proline (PTSP)-domain.[9,14,15,38] One family of proteins with a strongly conserved MAPK-phosphorylation site that recently gained particular interest as intracellular signaling molecules is the doublecortin-like kinase (DCLK) gene family.[6,17-19]

The *dclk* gene gives rise to short and long forms of doublecortin-like kinase, DCLK-short and DCLK-long, and to a doublecortin-like splice variant (DCL).[15,39,50] In the developing brain, DCL has been implicated in regulating microtubule dynamics.[16,17,60] The functions of DCL, DCLK-short and DCLK-long in the adult brain are largely unknown. Phosphorylation and dephosphorylation regulate the function and localization of doublecortin (DCX), a protein with a high homology to DCL, and result in plastic changes.[45] DCX phosphorylation lowers its affinity to microtubules in vitro, reduces its effect on polymerization, and displaces it from microtubules in cultured neurons.[4,51] Since all *dclk* products contain a conserved MAPK-PTSP phosphorylation site, and pain activates MAPK pathways, we raised the question whether acute pain changes the phosphorylation of the PTSP-motif of DCLK.

A frequently used animal paradigm to study the effects of acute pain on neuronal and neuroendocrine systems, which also activates the stress response, is subcutaneous injection of formalin into a rat's hind paw.[13,37,54,58,59] Activation of the stress response involves the secretion of corticotropin-releasing factor (CRF) from the paraventricular nucleus (PVN) of the hypothalamus.[10,11] The neuropeptide urocortin 1 (Ucn1), a member of the CRF peptide family, is also involved in stress adaptation. Its main expression site is the non-preganglionic Edinger-Westphal nucleus (npEW), located in the rostroventral periaqueductal grey. The activity of the npEW changes in response to acute and chronic stressors.[31,61] Both the PVN and npEW are activated by acute pain.[31,41] These nuclei show similar responses to acute stressors but opposite responses to long-lasting, chronic stressors.[32,61] This implies that, although both brain nuclei respond to a variety of stressors, different signaling cascades may lead to their eventual stress-mediated activation. Although the response of the PVN and the npEW to acute pain has been documented, the intracellular signaling pathways by which acute pain activates these stress-sensitive centers are unknown.

In this study, we have tested our hypothesis that acute pain changes the phosphorylation of the PTSP-motif of DCLK by determining; 1) which DCLK splice variants are present in the PVN and npEW; and 2) whether phosphorylation of the PTSP-motif is differentially regulated by acute pain. Our results reveal the presence of DCLK-short and DCLK-long isoforms in both brain centers, and show that acute pain leads to up-regulation of phosphorylation of the PTSP-motif of DCLK-long in the npEW but not in the PVN.

Methods

Animal Husbandry and Stress Paradigm

Albino male Wistar-R Amsterdam rats, bred in-house (Animal Facility of the Department of Anatomy, Pécs, Hungary), 12 to 14 weeks old, were housed in standard plastic cages (40 × 25 × 20 cm) in a temperature- and humidity-controlled environment. They were maintained on a 12 hour light/12 hour dark cycle (lights on at 6:00 AM, light intensity 200 lux) and were allowed ad libitum access to tap water and rodent chow throughout the experiment. Rats were acclimatized to these housing conditions for 1 week before starting the experiments.

APS animals (n = 5 per group) were given a subcutaneous injection in the left hind paw of 50 μL 4% paraformaldehyde in pyrogen-free saline (PFA; Sigma Chemical, St. Louis, MO). Immediately after injection, animals were put back to their home cages, and sacrificed 2 hours later by anesthetization and decapitation, as described below. The 2-hour time point was chosen since PVN and npEW show strong activation 2 hours after the initiation of an acute stressor.[20,31,57] Control animals (n = 5 per group) were treated in the same way, but had not been injected. Since this study concerns the effects of acute pain on 2 stress-responsive brain centers, no postoperative analgesia was applied.

All measures were taken to minimize the number of animals used and their suffering, and all procedures were conducted in accordance with the Declaration of Helsinki and the animal-use guidelines approved by the Medical Faculty Advisory Committee for Animal Resources of Pécs University, based on the law of 1998, XXVIII, for animal care and use in Hungary. Chemicals were obtained from Merck (Darmstadt, Germany) unless stated otherwise.

Antisera

Goat polyclonal antiserum against Ucn1 IgG (Santa Cruz Biotechnology, Santa Cruz, CA) had been generated against a peptide mapping at the C-terminus of rat Ucn1. Its high specificity has been reported by Bachtell et al.[2,3] Rabbit cFos antiserum (Santa Cruz Biotechnology) had been raised against the epitope corresponding to residues 3 to 16 of human cFos. The high serum specificities of these antisera have been previously confirmed by their preabsorption with the synthetic peptides to which they had been raised, which abolished staining in all cases.[21] Guinea pig polyclonal antiserum against CRF (Bachem Peninsula, San Carlos, CA) was collected from guinea pigs immunized with a synthetic peptide raised against rat CRF.[43] The high specificity of this serum was shown by comparing immunolabelings of CRF-wildtype and CRF-null mice.[55] Highly specific mouse candidate plasticity-related gene (CPG16) antiserum (BD Biosciences, San Jose, CA) had been generated against the C-terminal 330-424 kinase part of rat CPG16 and its specificity described before.[17] The rabbit polyclonal DCLK antiserum was produced by injection of a 55-amino acid-long synthetic peptide immunogen corresponding to the N-terminal domain of DCLK-short,