*Neuroscience* 269 (2014) 318–330

# CHRONIC TOOTH PULP INFLAMMATION INDUCES PERSISTENT EXPRESSION OF PHOSPHORYLATED ERK (PERK) AND PHOSPHORYLATED P38 (PP38) IN TRIGEMINAL SUBNUCLEUS CAUDALIS

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**Abstract—*Background:* Extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase are transiently phosphorylated (activated) in the spinal cord and trigeminal nucleus by acute noxious stimuli. Acute stimulation of dental pulp induces short-lived ERK activation in trigeminal subnucleus caudalis (Vc), and p38 inhibition attenuates short-term sensitization in Vc induced by acute pulpal stimulation. We have developed a model to study central changes following chronic inflammation of dental pulp that induces long-term sensitization. Here, we examine the effects of chronic inflammation and acute stimulation on the expression of phosphorylated ERK (pERK), phosphorylated p38 (pp38) and Fos in Vc.**

***Results:* Chronic inflammation alone induced bilateral expression of pERK and pp38 in Vc, but did not induce Fos expression. Stimulation of both non-inflamed and inflamed pulps significantly increased pERK and pp38 bilaterally; expression was greatest in inflamed, stimulated animals, and was similar following 10-min and 60-min stimulation. Stimulation for 60 min, but not 10 min, induced Fos in ipsilateral Vc; Fos expression was significantly greater in inflamed, stimulated animals. pERK was present in both neurons and astrocytes; pp38 was present in neurons and other non-neuronal, non-astrocytic cell types.**

***Conclusions:* This study provides the first demonstration that chronic inflammation of tooth pulp induces persistent bilateral activation of ERK and p38 within Vc, and that this activation is further increased by acute stimulation. This altered activity in intracellular signaling is likely to be linked to the sensitization that is seen in our animal model and in patients with pulpitis. Our data indicate that pERK and pp38 are more accurate markers of central change than Fos expression. In our model, localization of pERK and pp38 within specific cell types differs from that seen following acute stimulation. This may indicate specific roles for different cell types in the induction and maintenance of pulpitic and other types of pain.** 

**Key words: pERK, pp38, MAPK, chronic inflammation, pain, trigeminal nucleus.**

## INTRODUCTION

Evidence indicates that altered activity in intracellular signaling cascades plays a significant role in altered excitability and sensitization in the spinal cord and trigeminal nucleus, and this activity has been implicated in the development of pain. The mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinase (ERK) and p38 MAPK are phosphorylated (activated) in the spinal cord and trigeminal nucleus by noxious stimulation. It is also well established that the proto-oncogene c-fos and its protein product Fos can be rapidly induced in these regions by peripheral noxious stimulation. Thus, these molecules have been widely used as correlates of activity related to nociception.

Although Fos, phosphorylated ERK (pERK) and phosphorylated p38 (pp38) are induced by a range of peripheral stimuli, the time course of expression of these molecules varies widely according to the stimulus. For example a number of studies have described relatively short-term increases (peak 2–20 min, returning to baseline levels at ~2 h) in levels of pERK following the application of a number of acute noxious stimuli including electrical, thermal, mechanical, and chemical stimulation (mustard oil, capsaicin, and carrageenan) (Ji et al., 1999, 2002; Galan et al., 2002; Pezet et al., 2002). A study in the trigeminal system (Huang et al., 2000) describes increased numbers of pERK-immunoreactive (IR) neurons in ipsilateral trigeminal subnucleus caudalis (Vc) 1 h after formalin injection into the peri-oral skin. However, some studies in models of chronic inflammation have found that increased pERK levels are maintained for long periods under these conditions. For

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*Abbreviations:* ANOVA, analysis of variance; AOI, area of interest; CFA, complete Freund's adjuvant; Cy3, indocarbocyanine; EMG, electromyogram; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; GFAP, glial fibrillary acidic protein; JOR, Jaw-opening reflex; MAPK, mitogen-activated protein kinase; NDS, normal donkey serum; NeuN, neuron-specific nuclear protein; NGS, normal goat serum; p38, p38 MAPK; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing Triton-X; pERK, phosphorylated ERK; pp38, phosphorylated p38; Vc, trigeminal subnucleus caudalis.

http://dx.doi.org/10.1016/j.neuroscience.2014.03.056

example, increased levels of pERK still persist 7 days after injection of complete Freund's adjuvant (CFA) into the hind paw (Adwanikar et al., 2004), whereas a study in a neuritis model (Kominato et al., 2003) demonstrated that pERK (and Fos) was not increased in animals with neuritis alone but, following noxious stimulation (pinch) 3 and 7 days after induction of neuritis, levels of pERK (and Fos) were greater in animals with neuritis than in control animals.

The MAPK p38 is also activated by a variety of acute stimuli including injection of intraplantar formalin and intrathecal substance P (Svensson et al., 2003). This increase in activity is short lived (present at 5 min, returning to baseline by 20 min). More persistent activation of p38 has also been reported in other models, for example ligation of L5 spinal nerve results in activation of p38 starting within 1 day of the lesion which persists for longer than 3 weeks (Jin et al., 2003). Unlike the increases in pERK, p38 activation has generally been reported in glia rather than neurons (Ji et al., 2009). This increase in p38 activity has also been linked to neuronal Fos expression as p38 MAPK inhibition prevented Fos expression (and attenuated hyperalgesia) following intraplantar formalin injection (Svensson et al., 2003).

We have developed a model to study central changes following chronic inflammation of the dental pulp in the ferret and have examined Fos expression in the trigeminal nucleus following electrical tooth pulp stimulation in animals with normal and inflamed tooth pulps (Oakden and Boissonade, 1998; Worsley et al., 2007, 2008). In this model, unlike that reported in models of acute pulpal inflammation (e.g. stimulation with lipopolysaccharide (Chattipakorn et al., 2002)), we do not see Fos protein expressed under conditions of inflammation alone. However when the inflamed pulp is electrically stimulated 5 days post induction of inflammation, Fos expression is significantly greater than that seen following similar stimulation of the non-inflamed pulp (Worsley et al., 2007, 2008). This indicates that in this model although the inflammation alone does not induce Fos expression, it does induce long-term sensitization. Previous studies have shown that acute stimulation of the dental pulp induces short-lived ERK activation in the trigeminal nucleus (Shimizu et al., 2006), but this was shown to return to baseline levels 2 h post stimulus. It has also been demonstrated that inhibition of p38 can attenuate central sensitization in Vc nociceptive neurons induced by acute noxious stimulation of the tooth pulp with mustard oil (Xie et al., 2007). However it is not yet established whether these molecules are activated for longer time periods in chronic pulpal inflammation, such as that which occurs in patients with pulpal inflammatory pain.

Persistent activation of these molecules in our model of chronic pulpal inflammation could contribute to the increased Fos expression seen following stimulation of inflamed tooth pulp. Thus our model provides an excellent opportunity to identify central changes in MAPK activation that may play a role in central sensitization within the trigeminal system.

The aim of this study was to determine the effect of chronic inflammation on expression of pERK, pp38 and Fos. We compared expression of pERK, pp38 and Fos in animals with non-inflamed and chronically inflamed pulps, and examined their expression following electrical stimulation (for 10 or 60 min) of the tooth pulp in both groups of animals. In some sections, we carried out dual labeling to examine colocalization of pERK and pp38 with the neuronal marker neuron-specific nuclear protein (NeuN) and the astrocyte marker glial fibrillary acidic protein (GFAP).

This paper reports that chronic inflammation of the tooth pulp induces significant and persistent bilateral expression of pERK and pp38 in Vc. The pERK is present in neurons and astrocytes, while the pp38 is seen in neurons and other non-neuronal, non-astrocytic cell types.

## EXPERIMENTAL PROCEDURES

Experiments were carried out in 32 adult female ferrets (5–8 months old; Highgate Farm, UK), under UK Home Office Licence regulation and approval. The ferrets were prepared under anesthesia (ketamine, Fort Dodge, Southampton, UK; 25 mg/kg; xylazine, Bayer, Newbury, UK; 2 mg/kg; intramuscularly) in order to allow tooth pulp inflammation and stimulation of the upper and lower left canine teeth, and an electromyogram (EMG) to be recorded from the digastric muscle during a subsequent experiment. Briefly, a connector block composed of a mini 9-way socket and an intravenous injection port was attached to the skull. Leads from this block were passed subcutaneously to insulated Ag–AgCl fillings in the left canine teeth for stimulation, and to the left digastric muscle for recording its EMG. A cannula from the injection port was inserted into the left jugular vein. Following cannula insertion anesthesia was maintained intravenously, using alfaxalone (Vétoquinol; Buckingham, UK) at 6 mg/kg/h. These procedures have been described in greater detail previously (Oakden and Boissonade, 1998; Worsley et al., 2007). In 16 of these animals, pulpal inflammation was induced by the introduction of human caries into deep buccal cavities in the upper and lower left canine teeth as described previously (Worsley et al., 2007).

Five days later the animals were anesthetized with alfaxalone (4 mg/kg) via the indwelling cannula. Light anesthesia was then maintained by continuous infusion of alfaxalone (4–14 mg/kg/h), adjusted to allow a withdrawal reflex to be seen following a paw squeeze. Body temperature was maintained at 38 ± 0.5 °C. In 16 animals (non-inflamed $n = 8$, inflamed $n = 8$) the tooth pulps were electrically stimulated via the tooth electrodes (a train of three 0.5-ms duration stimuli at 200 Hz) and the amplitude of the stimulus required to produce the jaw-opening reflex (JOR) was determined for each tooth. The tooth pulps were then stimulated at ten times the JOR threshold, once per second for either 10 min (non-inflamed $n = 4$, inflamed $n = 4$) or 60 min (non-inflamed $n = 4$, inflamed $n = 4$). The remaining 16 animals were kept under anesthesia for either 10 min