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# Abstract

Extracellular signal-regulated kinase (ERK), is a member of the mitogen-activated protein kinase (MAPK) family of serine/threonine protein kinases. Functioning as intracellular signaling molecules, they are responsible for multiple extracellular signals transduction into diverse intracellular responses through transcriptional and post-translational regulations. ERK is proposed to be a molecular substrate linked to several nociceptive-related processes such as central sensitization, corresponding to a drastical increase on synaptic efficacy of neurons involved in nociceptive-transmission, their threshold reduction, pain signals enhancement, thus contributing to various chronic pain syndromes. Regulation of those pathways occurs primarily by MAPKs through central and peripheral mechanisms, whereby inhibition could provide significant anti-nociceptive effects. Although neurobiological mechanisms underlying pain pathways are extensively studied, many details remain unclear.

Herein, an immunohistochemical study was designed to investigate whether phosphorylated extracellular signal-regulated kinase (pERK) is found among neurons in nociceptive-related brain areas following mechanical noxious stimuli (pinch) applied to the tail and right hindpaw of three male Swiss-Webster B6 mice. Administration of ketamine was used for anesthetic purposes, while only one subject was viable for further experiments. Coronal sections were incubated on primary antibody rabbit anti-phospho-p44/42 ERK; 1:250, and immunostained using biotinylated donkey anti-rabbit secondary antibody; 1:500. Confocal immunofluorescence microscopy revealed that phospho-ERK immunopositive cells were activated and expressed in integral amygdala regions of one brain sample, with higher intensity on the laterocapsular division of central amygdala. Accumulating evidences suggests pERK expression in amygdala to be responsible for pain modulation and emotional aspects. However, absence of control subjects leads to paucity of evidences, where no definitive conclusion can be drawn. Molecular mechanisms of pain and modulation by the amygdala associated with pERK require further elucidation.



*Keywords:* Amygdala, Extracellular signal-regulated kinase (ERK), Mitogen-activated protein kinase (MAPK), Nociceptive, Pain

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# Abbreviations

BDNF – Brain derived neurotrophic factor
CALCRL – Calcitonin receptor-like receptor
CeA – Central nucleus of the amygdala
CeLC – Laterocapsular division of the central nucleus of the amygdala
CGRP – Calcitonin gene-related peptide
CREB – cAMP response element-binding protein
ERK – Extracellular signal-regulated kinase
MAPKs – Mitogen-activated protein kinases
NK1 – Neurokinin-1 receptor
PAG – Periaqueductal grey
RVM – Rostral ventromedial medulla
SCDH – Spinal cord dorsal horn neurons
SP – Substance P

# 1 Introduction

Nociception is a term used to describe the ability of an organism that possess a central and peripheral nerve system, to feel pain. The nociceptive system is defined to have four intrinsically steps; Transduction, transmission, modulation and perception. Despite the fact that there is not an isolated brain area defined to be unique for pain roles, each step occurs in polymorphous regions of the body and is critical for pain pathways. Thus, pain results from a combination of scattered group of structures, like the somatosensory cortex, associated with the sensory-discriminative properties (location and intensity) and others, like the anterior cingulate gyrus, insular cortex and amygdala, related to the emotional aspects of pain. (Apkarian et al., 2005).

The nervous system has evolved to sense a broad range of stimuli that could propose danger, developing in this manner a defense mechanism against harmful agents. Nociceptors are key points in this system, as they enable detection of noxious stimuli linked to distinct receptors and react in response to those substances. Nonetheless, when this system does not work properly, it may enhance pain signals (pain sensitization), resulting in unbearable discomfort. Neuropathic and chronic pain occurs when physical damage or diseases arises in peripheral nerves eliciting symptoms such as allodynia, when a normal innocuous stimuli is perceived as very painful, or such as hyperalgesia, when a not very painful stimuli, is sensed with higher intensity. (Apkarian et al., 2005).

"Pain is personal and subjective, is affected by mood and psychosocial factors, and demonstrates tremendous individual variation" (National Institutes of Health 2001). Worldwide, pain is considered a problematic health issue estimated to affect at least 20% of global population in adults suffering from a series of pain conditions with 10% newly diagnosed cases each year. (National Institutes of Health 2001)

## 1.1 MAPK/ERK

The mitogen-activated protein kinase (MAPK) is a highly conserved family of serine/threonine protein kinases, characterized as intracellular signaling molecules, responsible for the transduction of multiple extracellular signals (neurotransmitters, hormones, growth factors, etc.) into varied intracellular responses through transcriptional and post-translational regulations. (Widmann et al., 1999) To date, three dominant members of this family were identified; Extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. All members have being defined to have distinct signaling cascades that contributes to pain sensitization (Seger., et al 1995).

The first member identified, was extracellular signal-regulated kinase (ERK), described to prosecute essential cellular functions such as; differentiation, proliferation, apoptosis, stress and inflammatory responses, among others (Widmann et al., 1999). Activation of ERK occurs with an extensive influx of calcium ions, membrane depolarization and phosphorylation of Thr and Tyr regulatory residues through specific upstream MAPK/ERK kinase (MEK). (Seger., et al 1995). In this manner, pERK is strongly related to nociceptive mechanisms, but seems to be highly involved in neuronal plasticity, analogous to long-term potentiation, sharing similarities with learning and memory processes (Ji et al., 2003).

## 1.2 ERK and pain

Phosphorylation of extracellular signal-regulated kinase (pERK) is tightly connected to noxious stimuli (thermal/mechanical), as well as it's expression is increased through activation of high-threshold C-fiber and Aδ-fiber, where intensity and duration are determinant factors. (Baba., et al 1999; Zhuang., et al 2015). Recent experiments using distinct stimulation methods such as; electrical (Fukui., et al 2007), mechanical (Huma., et al 2015), and itch (Jiang., et al 2015), have shown pERK immunopositive cells activation in regions such as; Cerebral cortex (Takamura., et al 2008), RVM and locus coerulus (Imbe., et al 2004), microglia and astrocytes (Zhuang., et al 2015), demonstrating correlation with nociceptive-related processes, like peripheral sensitization (Dai., et al 2002), peripheral inflammation (Imbe., et al 2005) (Imbe., et al 2008), allodynia/neuropathic pain (Zhuang., et al 2015) and hypersensitivity (Obata., et al 2004) (Baba., et al 1999).

When primary afferent nociceptors are stimulated and reach threshold, they form what is called a "glutamatergic synapse" thereby glutamate, an excitatory neurotransmitter, is released from presynaptic neurons, binding on postsynaptic neurons to specialized receptors; Ionotropic (NMDA/AMPA/Kainate) (Krapivinsky et al., 2003) and metabotropic (mGlur) (Karim et al., 2001). Subsequent studies have proved that NMDA receptors are the main target of protein kinases playing two-sided roles, as an upstream activator, coupling straightforward or diffusely through PKA and PKC to ERK activation, and as a downstream target, due to the fact that ERK, PKA and PKC are able to phosphorylate NMDA receptors enhancing current flow and accelerating it's kinetics (Krapivinsky et al., 2003). Specifically to amygdala, protein kinases involved in nociceptive events are PKA and ERK but not PKC. (Fu et al., 2008).

Neuropeptides and their respective receptors; Substance P (SP) (Choi et al., 2005) and receptor G-protein coupled neurokinin-1 (NK1) (Polgár et al., 2007), calcitonin gene-related peptide (CGRP) and calcitonin receptor-like receptor (CALCRL) (Han et al., 2005), brain derived neurotrophic factor (BDNF) and receptor tropomyosin receptor kinase B (TrkB) (Pezet et al., 2002), are also critical for nociceptive transmission, well-established to potentiate nociceptive effects by playing critical roles in multiple pathways responsible for ERK activation. Phosphorylated ERK (pERK) there-

upon travels from the cytoplasm to the nucleus, activating Rsk2, consequently phosphorylating the transcription factor CREB on Serine 133 (Xing., et al 1996) resulting in gene transcription and synthesis of pro-inflammatory and pro-nociceptive mediators that enhance and prolong pain in a transcriptional regulation manner, implicating in the induction and maintenance of inflammatory pain related to late effects of central sensitization. Though, other factors contributes to early and acute central sensitization in a post-translational regulation manner, as upregulation of aforesaid glutamatergic receptors and downregulation of Kv4.2 potassium channels (Hu et al., 2006).

Inhibition of MAPK/ERK pathways resulted in neuropathic pain relief in several animal models (Weiya et al., 2005).

## 1.3 ERK expression in amygdala

The amygdala is localized in the medial temporal lobe with an almond shape structure, is part of the limbic system and plays an essential role in emotions derived from sensory stimulus, learning and memory. Previous studies have elicited the amygdala to be responsible for linking external stimuli to defense responses and to play a fundamental role in affective disorders such as depression and anxiety (LeDoux, 2003).

Composed of different nuclei such as; lateral, basolateral, accessory basal, medial and central nucleus, is well known for being a neural center for pain modulation, especially the laterocapsular division that has been characterized as the nociceptive amygdala (Neugebauer et al., 2004). The amygdala receives nociceptive inputs in a direct manner from spinal cord and brainstem, in an indirect manner via the lateral-basolateral amygdala circuitry from thalamus and cortex, where highly processed polymodal information is gathered, a mechanism hypothesized to be involved in how the amygdala attaches emotional significance to painful events (LeDoux, 2003). The amygdala appears to be involved in dual nociceptive pathways, ascending via the spino-parabrachio-amygdaloid pathway, and descending via amygdala-PAG-RVM–spinal cord, where it plays two-sided roles such as inhibitory and facilitatory (pain enhancement/diminishment). (Neugebauer et al., 2004) The question whether pERK activation in amygdala modulates either one of them or both, needs to be further elucidated.

Using the formalin pain model, a previous study has confirmed ERK activation in the CeLC. (Carrasquillo et al., 2007) Inhibition of this pathway exhibited successful antinociceptive effects. (Cruz et al., 2005). Distinct signaling pathways such as PKA and PKC were suggested to act in concert in order to phosphorylate and activate ERK in amygdala neurons (Ji., et al 2003) However it was proved that in the amygdala, PKA, that is activated by neuropeptide receptors, and ERK, activated by multiple glutamate receptors, respectively, are responsible for pain transmission and modulation. (Fu et al., 2008) Nonetheless, how ERK contributes to synaptic transmission and plasticity in the CeLC remains unknown.

# 2 Materials and Methods

## 2.1 Animals

Experiments were performed in 3 adult (2-4 months old) Male Swiss-Webster B6 mice (23-25g) (Jax Stock 008069) kept in a 12-h light-dark cycle, at 22 ± 0.5°C. Food and water were available ad libitum.

All animal procedures were performed in accordance with the guidelines and approved by the local Swedish ethical committee (C248/11, C157/11, C366/12 Uppsala Animal Ethics Committee, Jordbruksverket).

## 2.2 Nociceptive stimulation

All subjects were transferred from the animal facility and allowed 30 min for acclimation to the laboratory. The type of noxious stimulus chosen was the mechanical (pinch) according to a previous study (Polgár et al., 2007) in which the most numerous pERK positive cells were obtained.

Repeated pinching was applied to the right hindpaw and to the tail for one minute period interval. Immediately after the stimulus, animals were deeply anesthetized with ketamine (0.025 ml/g body weight), the time from onset of anesthesia to perfusion was approximately 5 minutes in all individuals. Mice were then perfused through the ascending aorta on the left ventricle, with physiological saline (PBS) followed by ice-cold phosphate-buffered 4% paraformaldehyde (0.1M, pH 7.4). A peristaltic perfusion pump was set for 15min at a flow rate of 5-6 ml/min.

Whole brains were dissected and post-fixed for cryoprotection in 30% sucrose for 48 h at 4°C. Frozen coronal sections were cut 35µm-thick on a cryostat (CRYOCUT 1800, Reichert-Jung).

## 2.3 Immunohistochemistry procedure

Coronal sections were collected as one set and processed for immunohistochemistry procedure through washing with phosphate-buffered saline (PBS, 0.1M) 3 x 5 min, then blocked in 1.5% goat serum and 0.1% Triton X-100 with 0.1M PBS for 45 min. Sections were then incubated with the primary antibody (rabbit anti-phospho-p44/42 ERK; 1:250; Catalog number 9101S, Cell Signaling Technology) for a period of 48 hours at 4°C.

Sections were rinsed 3 x 5 min (PBS, 0.1M) following incubation with biotinylated donkey anti-rabbit secondary antibody (1:500; Catalog number R37118, Alexa Fluor 488) for 2 hours at room temperature. During incubation periods, sections were continuously agitated. After rinsing, sections were mounted on glass slides and coverslipped with glycerol mounting medium.

# 3 Results

The present results are from one individual only, as after anesthesia administration, the other two subjects turned unviable for further experiment steps. Using specific antibodies for pERK, coronal sections from the stimulated animal were immunostained and showed that the pinch applied to the right hindpaw and to the tail evoked ERK phosphorylation visualized by confocal immunofluorescence microscopy (Olympus, BX61WI) that revealed phospho-ERK immunopositive cells activation and expression in amygdala, more intensely on laterocapsular division, followed by mechanical noxious (pinch) stimuli. (Figure 1)

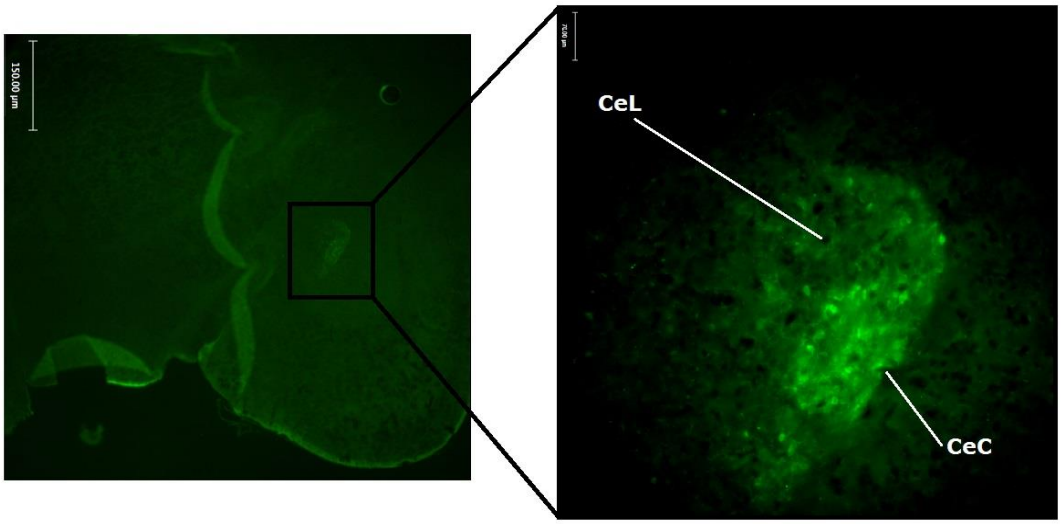


***Figure 1:*** Coronal sections from subject where mechanical noxious stimulus (pinch) evoked ERK phosphorylation in integral amygdala regions, with a clearly greater intensity on laterocapsular division. 150µm and 70µm respectively. CeL: Lateral subdivision of central nucleus. CeC: Capsular subdivision of central nucleus.

# 4 Discussion

Ketamine was administered for anesthetic purposes (Klein., et al 2007). Previous studies have elucidated the interference of anesthesia effects on immunohistochemical detection (Takamura., et al 2008) and ERK activation (Tochiki., et al 2015). The results obtained confirmed those facts, as two subjects turned unviable for further experiments, while only one individual proceeded to the next steps of immunohistochemistry analyses, where confocal immunofluorescence microscopy (Olympus, BX61WI), revealed that phospho-ERK immunopositive cells were activated and expressed in amygdala, (more intensely on laterocapsular division) followed by mechanical noxious (pinch) stimuli. (Figure 1).

# 5 Conclusion

The present study indicated ERK characterization with incisive pathways. Results confirmed pERK expression in the amygdala and elucidated it's involvement in nociceptive behavior. However, absence of control subjects leads to paucity of evidences, where no definitive conclusion can be drawn.

To further clarify the molecular mechanisms of pain and modulation by the amygdala associated with phosphorylated extracellular signal-regulated kinase (pERK), supplementary studies using modern techniques are desirable.

# 6 Future perspectives

Optogenetics is a modern technique more suitable for such experiment, due to advantages of directly stimulating and light-controlling genes in specific neurons of interest, leading to more clear results where more precisely conclusions can be drawn.

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