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## Acute Tramadol Administration Induces the Expression of pERK1/2 in Different Limbic and Pain Processing Structures

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### Abstract

Tramadol is a painkiller with some abuse potentials. The current study aimed to investigate rat cerebral structures that were activated by acute intraperitoneal administration of tramadol (10 mg/kg). The expression of pERK1/2 was used as a molecular tool for tramadol-induced neuronal activation in the brain. Tramadol induced a differential pERK1/2 labeling expression in the brain. A robust pERK1/2 expression was present in limbic, motor, and pain processing structures when compared to others. pERK1/2 labeling was observed in somatosensory, motor, insular cingulate cortex, hippocampus, amygdala, thalamus, habenula, and striatum. Descending pain-processing structures such as periventricular hypothalamus nucleus, periaqueductal grey, dorsal raphe, and rostral ventromedial medulla presented also a high pERK1/2 expression. In medullary and spinal dorsal horns, pERK1/2 was highly expressed principally in superficial laminae (outer lamina II and lamina I) which processes pain. These results are following the reinforcing, motor, and pain effects of tramadol.

**Keywords:** Pain; Addiction; Tramadol, pERK1/2; Neuronal activation

### Introduction

Tramadol an opioid agonist commonly used alone or as co-medication for chronic pain. It constitutes one of the most chosen analgesic opioids in many pharmacopeias [1]. It provides analgesia through a dual mechanism of action: one through the activation of opioid receptors and a second through its inhibition of central monoaminergic reuptake, which increases brain levels of monoaminergic neurotransmitters [2,3]. In this way, Tramadol has also an antidepressant action [4]. Tramadol acts on different receptors and ion channels (GABAA, glycine, NMDA, adrenergic, nicotinic acetylcholine, sodium channels) [3-8].

Different clinical and preclinical studies revealed an abuse effect of tramadol [9-28].

The Extracellular Signal-Regulated Kinase (ERK) is a component of the Mitogen-Activated Protein Kinase (MAPK) cascade. They are involved in relaying extracellular signals into intracellular responses. They play different functions in the brain especially in synaptic plasticity, learning, and memory, addiction, and pain [29-31]. The phosphorylation of ERK1/2, which constitutes their activation, plays an important role in pain [31].

This phosphorylation is induced in different peripheral and central brain structures as a response of noxious stimuli or in inflammatory and neuropathic pain situations. Alternatively, exposure to a variety of substances (alcohol, amphetamine, and cocaine, nicotine), with abuse potential induced the phosphorylation of ERK1/2 in the different brain structures [32-36]. The phosphorylation of ERK1/2 may promote the drug's rewarding effects.

In light of the above, the current study aimed to investigate the expression of the phosphorylated pERK1/2 in the brain upon acute peritoneal administration of tramadol in the rat. Specific interest was given to addiction and pain processing structures. This is the first study evaluating pERK1/2 expression in brain regions of tramadol-treated rats. The immunohistochemical method was used to achieve this goal.

### Materials and Methods

#### Animals

Twelve adult Sprague Dawley rats (Charles Rivers, L'Arbresle, France) were used. Rats were maintained in a controlled environment (lights on 07:00-19:00 h, 22°C) with *ad libitum* access to food and water. The experiments followed the ethical guidelines of the animal ethics committee of the University of Auvergne (APAFIS#19965-20190325052285).

Tramadol dissolved in saline (10 mg/kg) was intraperitoneally administered to the rats (n=6). A control group (n=6) was injected with saline. Four minutes after tramadol or saline injections. Rats were deeply anesthetized with 1% ketamine and Xylazine. All rats (control and tramadol injected) were perfused through the heart with saline followed by 4% paraformaldehyde in Phosphate-Buffered Saline (PBS). Brains were then removed and placed in 30% sucrose and 0.05% sodium azide solution overnight at 4°C. Coronal sections (30 µm) were obtained using a freezing microtome and collected in 0.05 M Tris-Buffered Saline (TBS). Free-floating sections were placed in 1% normal goat serum for 1 h before overnight incubation in primary polyclonal antibody solution (anti-pERK1/2 antibody, 1:500, rabbit, Ozyme, France). After several rinses, sections were incubated with a secondary antibody (1:400 for goat anti-rabbit Cy3; Vector Lab, France) for 3 h at room temperature. All antibodies were diluted in TBS containing 0.25% bovine serum albumin and 0.3% TritonX-100. The sections were finally rinsed in TBS, mounted onto gelatin-coated slides, dehydrated in alcohol, cleared in xylene, and cover-slipped with distyrene-plasticizer-xylene. The specificity of the immunostaining was assessed by pERK1/2 protein pre-adsorbed antibody (0.25 µg/µl, Proteintech, United Kingdom; 24 hours before use), which resulted in the absence or a reduced signal. Some control experiments were done by substitution of the primary antibody with rabbit or mouse serum resulting in the absence of staining. For double staining, sections were incubated simultaneously with primary antibodies anti-pERK1/2 and anti-PKCg antibody (1:5000 mouse anti-PKCg, Sigma-Aldrich, France) and corresponding Cy2 rabbit anti-mouse secondary

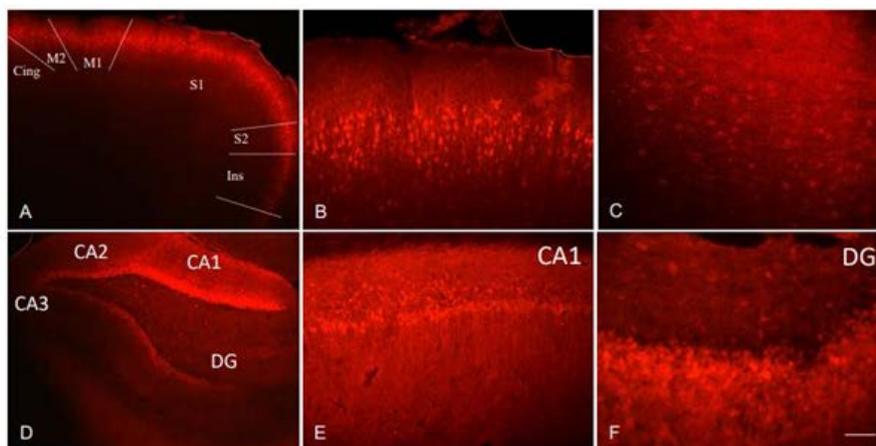
antibody (Victor lab, France) as described previously [37,38]. Photomicrographs of immuno-stained sections were captured and image intensity analysis was completed using ImageJ software (ImageJ v1.41, National Institute of Health, USA).

## Results

The current study focused on structures having robust staining and that are implicated in addiction and pain. Thus, differential expression of pERK1/2 was observed in the brain with some structures having robust labeling while others presenting a less prominent one.

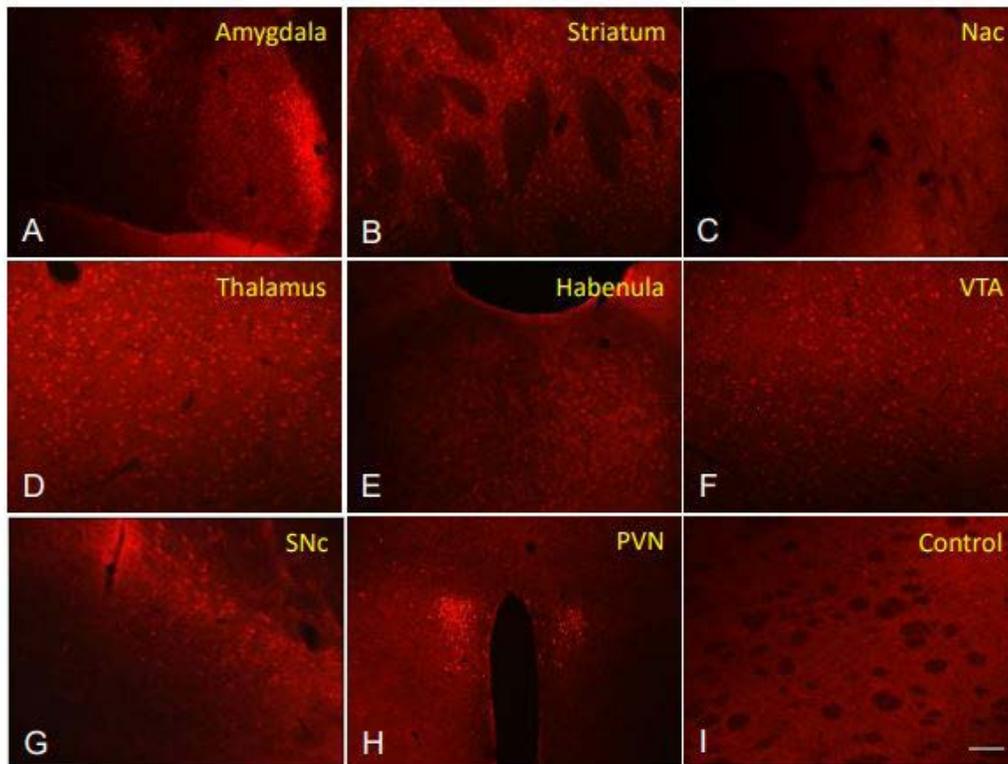
A differential pERK1/2 staining was observed within the cerebral cortex (Figure 1A) with some cortices having robust staining while others presenting a less intense one. Intense labeling is highlighted by an increase in the labeling signal and its presence in the dendritic processes (Figure 1B). Less intense labeling corresponded to a lower intensity of the labeling that was mostly restricted to cell somata (Figure 1C). Intense pERK1/2 labeling was observed in cortices that included primary and secondary motor cortex, somatosensory cortex, insular cortex, primary and secondary visual cortex, anterior cingulate cortex, primary and secondary auditory cortex, parietal association cortex, temporal association cortex, perirhinal cortex, and entorhinal cortex.

In the hippocampus, intense pERK1/2 labeling was observed in all subdivisions (CA1, CA2, CA3, CA4, and DG dentate gyrus). Intense pERK1/2 was observed in granule cells of the DG (Figure 1D) and in virtually all pyramidal cells of CA1, CA2, and CA3 (Figure 1E) in addition to another neuronal cell type.



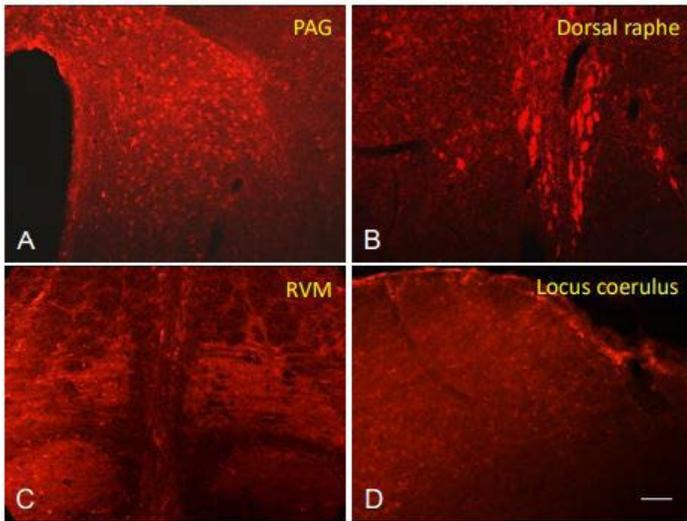
**Figure 1:** A Micrograph showing the expression of pERK1/2 labeling in the cortex and the hippocampus. Intense pERK1/2 is observed in the cortex (A). At high magnification, very intense labeling is present within neuronal cell bodies and processes. Some of pERK1/2 positive cells are pyramidal cells (B). The labeling is located within the cell body and dendritic processes. In other cerebral cortices, a weak pERK1/2 labeling is present in cell somata only (C). In the hippocampus, intense pERK1/2 labeling is observed in all hippocampal subdivisions. Intense pERK1/2 is located in virtually all pyramidal cells of CA1, CA2, and CA3 (Figure 1D) and in granule cells of the dentate gyrus (DG) (Figure 1F). Cing: cingulate cortex, M1/M2 primary, and secondary motor cortex, S1/2: primary and secondary somatosensory cortex, Ins: insular cortex. The bar represents 200 µm in A and D, 120 µm in B and E, and 100 µm in C and F.

Within the amygdala (Figure 2A) intense pERK1/2 labeling was observed in the central nucleus. A robust pERK1/2 labeling is present in the lateral bed nucleus of the stria terminalis. Within the striatum pERK1/2 labeling was present in virtually all neurons (Figure 2B). In the nucleus accumbens, an intense pERK1/2 labeling was observed in some neurons of the shell subdivision while the core subdivision had a very low or no pERK1/2 labeling (Figure 2C). Intense pERK1/2 staining was present in the thalamus (Figure 2D), the habenula (Figure 2E), the ventral tegmental area (Figure 2F), the substantia nigra pars compacta (Figure 2G), and the periventricular nucleus of the hypothalamus (Figure 2H). Negative or very few cell staining was present in the control brain section (Figure 2I).



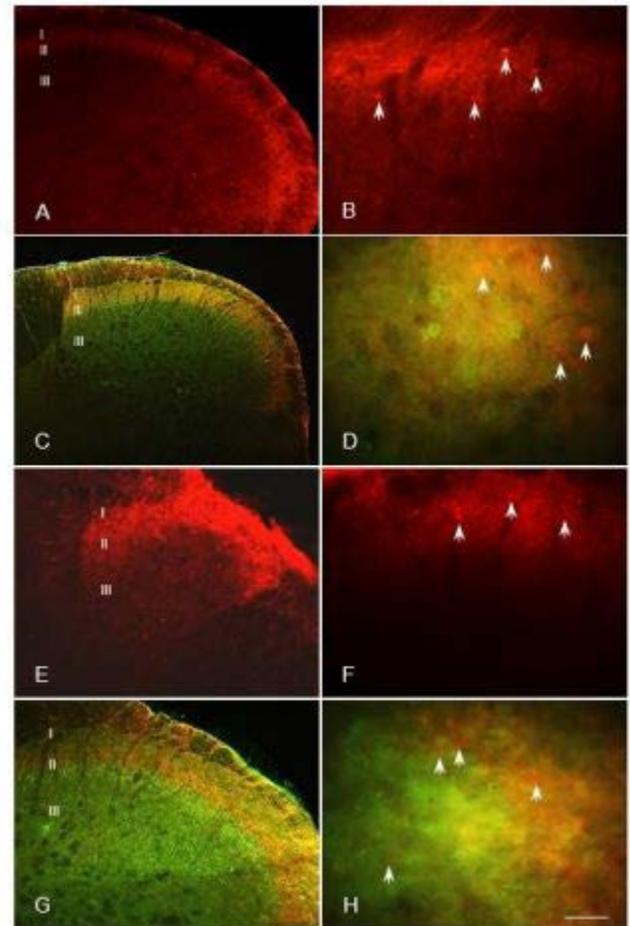
**Figure 2:** A micrograph showing the expression of pERK1/2 in different brain structures. Within the amygdala (A) intense pERK1/2 labeling is observed in the central nucleus. Robust pERK1/2 staining is also observed in the lateral bed nucleus of the stria terminalis. Most cells of the striatum expressed pERK1/2 labeling (B). In the nucleus accumbens intense pERK1/2 labeling is present only in some neurons of the shell subdivision while the core subdivision is almost devoided to labeling (C). Intense pERK1/2 staining is located in neurons in the thalamus (D), the habenula (E), the ventral tegmental area (2F), the substantia nigra compacta (G), and the periventricular nucleus of the hypothalamus (H). Control section is presented in (I) where a very few pERK1/2 stained cells are present. Nac: nucleus accumbens, SNc: substantia nigra pars compacta, PVN: periventricular hypothalamic nucleus, VTA: ventral tegmental area. The bar represents 150µm in A, 120µm in B-G and I, and 200µm in H.

Within descending pain processing structures intense pERK1/2 labeling is present in neurons of all periaqueductal grey subdivisions (Figure 3A). In the dorsal raphe (Figure 3B) intense staining was observed in large neuronal cell bodies while a less intense one was present in other cell subtypes. A less intense pERK1/2 labeling was located in neurons within the rostral ventromedial medulla (Figure 3C) and in neurons in the locus coeruleus (Figure 3D).



**Figure 3:** A micrograph showing the expression of pERK1/2 labeling in different descending pain processing structures. Intense pERK1/2 labeling is located in neurons of periaqueductal grey subdivisions (A). In the dorsal raphe (B), intense staining is present in large neuronal cell bodies while a less intense one is located in other cell subtypes. A less intense pERK1/2 labeling is located in neurons in the rostral ventromedial medulla (C) and the locus coeruleus (D). PAG: periaqueductal grey, RVM: rostral ventromedial medulla. The bar represents 120  $\mu\text{m}$  in A, B, and D, 150  $\mu\text{m}$  in C.

In the medullary dorsal horn pERK1/2 labeling was present within superficial laminae I and II (Figure 4A). At high magnification pERK1/2 positive neurons located within superficial lamina I-IIo and in some neurons within deeper lamina III (Figure 4B). pERK1/2 expression was also explored with the PKC $\gamma$  a specific marker of excitatory interneurons localized especially within internal lamina Ii and III [38-40]. PKC $\gamma$  presence delimited lamina Ii and separate it from outer lamina II. pERK1/2 was present in lamina I and Ii, while PKC $\gamma$  was located in lamina Ii and III mostly (Figure 4C). At high magnification (Figure 4D), there was no match between pERK1/2 and PKC $\gamma$  positive cells.



**Figure 4:** A micrograph showing the expression of pERK1/2 in medullary (A-D) and spinal (E-H) dorsal horns. In the medullary dorsal horn pERK1/2 labeling is present within superficial laminae I and II (A). pERK1/2 positive neurons are located within superficial lamina I-IIo and in some neurons within deeper lamina III (B). Double labeling using pERK1/2 and PKC $\gamma$  reveals a different pattern of labeling. pERK1/2 is present in lamina I and Ii, while PKC $\gamma$  was located in lamina Ii and III mostly (C). At high magnification (D), there was no match between pERK1/2 and PKC $\gamma$  positive cells. In the spinal dorsal horns pERK1/2 labeling is present within superficial laminae I and II (E). At high magnification pERK1/2 positive cells were observed in cells located within superficial laminae Ii and I (F) and some cells within lamina III. Double labeling using pERK1/2 and PKC $\gamma$  revealed the presence of pERK1/2 in lamina I and Ii, while PKC $\gamma$  was located in lamina Ii and III (G). At high magnification, there was no match between pERK1/2 and PKC $\gamma$  positive cells (H). Ii: internal lamina II, Ii: outer lamina II. The bar represents 250  $\mu\text{m}$  in A, C, E, G, 60  $\mu\text{m}$  in D and H, 90 $\mu\text{m}$  in B and F.

In the spinal dorsal horns pERK1/2 labeling was present within superficial laminae I and II (Figure 4E). At high magnification pERK1/2 positive cells were observed in a few cells within superficial laminae (Figure 4F) and some cells within lamina III. Double labeling using pERK1/2 and PKCg revealed the presence of pERK1/2 in lamina I and IIo, while PKCg was located in lamina IIo and III (Figure 4G). At high magnification, there was no match between pERK1/2 and PKCg positive cells (Figure 4H). The pERK1/2 intensity of labeling in different brain structures is highlighted in Figure 5.

Structure	Labelling intensity
Cingular cortex (primary and secondary)	+++
Motor cortex (primary and secondary)	+++
Auditory cortex (primary and secondary)	+++
Visual cortex (primary and secondary)	+++
Insular cortex (dorsal and ventral)	+++
Parietal associative cortex	+++
Hippocampus	+++
Striatum	++
Amygdala (central nucleus)	++
lateral bed nucleus of the stria terminalis	+++
Habenula	++
Thalamus	++
Hypothalamus (PVN)	+++
Dorsal raphe nucleus	+++
Interpolaris nucleus	+
Substantia nigra pars compacta	++
Substantia nigra pars reticula	+
Ventral tegmental area	++
Periaqueductal grey	++
Locus coeruleus	++
Rostral ventromedial medulla	++
Medullary dorsal horn	+++
Spinal dorsal horn	+++

**Figure 5:** The table represents pERK1/2 labeling intensity in different brain structures. +++ robust staining that can extend to dendritic processes; ++ intense staining; + weak staining.

## Discussion

The main results of the current study are that acute administration of tramadol induces intense pERK1/2 expression in different brain structures. Most of them are involved in addiction and pain regulation. Tramadol activated pERK1/2 expression in structures such as cortex, amygdala, hippocampus, striatum, ventral tegmental area, nucleus accumbens, which are implicated in the regulation of addiction. Besides, tramadol activated hypothalamic periventricular nucleus, periaqueductal gray, dorsal raphe, rostroventral medulla, locus coeruleus, medullary, and spinal dorsal horns that are structured important in the regulation of pain. Therefore, the current results are following the dual reinforcing and pain effects of tramadol.

Acute tramadol administration induced a robust pERK1/2 expression in different cerebral structures involved in addiction (nucleus accumbens, lateral bed nucleus of the stria terminalis, central amygdala, and deep prefrontal cortex, hippocampus, habenula, thalamus, VTA, SNc,) [41]. The expression of pERK1/2 in the current study is in accord with previous results showing that all drug of abuse administration induced the phosphorylation of ERK1/2 in nucleus accumbens, lateral bed nucleus of the stria terminalis, central amygdala and deep layers of the prefrontal cortex [32]. Conversely, the inhibition of ERK1/2 phosphorylation prevented conditioned place preference induced by cocaine, THC or MDMA [42-44]. This demonstrates an essential role of pERK1/2 in behavioral conditioning. Mice knockout in ERK1 are more sensitive to the rewarding properties of morphine, and this hypersensitivity correlated with a stimulus-dependent increase in pERK2 [45]. Last, tramadol-induced pERK1/2 was observed in structures that expressed high levels of MOR opioid receptor [46-50]. These regions included cerebral cortex, hippocampus, amygdala, thalamus, striatum, periaqueductal gray, locus coeruleus, raphe magnus, medullary and spinal dorsal horns. Activation of ERK1/2 participates to opioid addiction [45,51,52]. Although in the current study acute tramadol administration was used, pERK1/2 expression had a similar brain distribution as chronic morphine exposure with an intense signal in the locus coeruleus, caudate/putamen [45], nucleus accumbens [53] and ventral tegmental area [51]. Tramadol effect could also be due to the activation of non-opioid receptors (GABAA, glycine, NMDA, adrenergic, nicotinic acetylcholine) or monoaminergic reuptake [4-8].

Pain sensation is regulated by two main ascending (lateral and medial) and descending pathways [54]. The lateral is responsible for the sensory aspects of pain and the medial for the emotional aspects of pain. Tramadol induced pERK1/2 expression in many pain-processing structures linked to both pain processing ascending and descending pathways. A robust pERK1/2 labeling was observed in somatosensory cortices, insular cortex, anterior cingulate cortex, hippocampus, lateral bed nucleus of the stria terminalis, amygdala, hypothalamus PVN, PAG, locus coeruleus, and RVM. While a less intense pERK1/2 staining was observed in other pain processing structures. ERK signaling is very important in the pain process [31]. ERK activation is exclusively induced by noxious stimuli but not by innocuous stimuli [55]. Furthermore, pERK1/2 constitutes a biomarker for activated cells involved in pain signaling and nocifensive reflex [37,40,56]. In the case of tramadol, pERK1/2 activated cells might constitute cell subtypes that are involved in the inhibition of pain mechanism.

Interestingly, tramadol administration induced the expression of pERK1/2 in structures that have a high expression of MOR-1 (somatosensory cortex, hippocampus, amygdala, lateral bed nucleus of the stria terminalis, thalamus, hypothalamus PVN, the periaqueductal gray, the locus coeruleus, and raphe magnus,

medullary and spinal dorsal horns), all of which are implicated in pain [54]. Morphine is known to modulate pain processing in both medial and lateral pain pathways.

At the level of medullary and spinal dorsal horns, tramadol induced a robust pERK1/2 expression especially in superficial lamina II especially outer region Ilo. This lamina is known to regulate the pain process since it receives both ascending and descending pain projections [57]. Besides, lamina II contains also a high expression of MOR receptors [46-50]. pERK1/2 did not colocalize in PKCg excitatory cells that play an important role in allodynia [37,38]. The activation of ERK1/2 in medullary and dorsal horns constitutes a pain biomarker [37,56] and participates in the generation and maintenance of pain. The phosphorylation of ERK1/2 constitutes a marker for neurons activated by nociceptive stimuli. The inhibition of ERK1/2 activation blocked or alleviated pain [58-60]. Therefore, tramadol induced pERK1/2 expression in cell subtype within lamina Ilo that are involved in pain inhibition.

## Conclusion

Tramadol induced the expression of pERK1/2 in cerebral structures that participate to both pain process and addiction which is following its painkiller and reinforcing effect.

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