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The Discovery of Rat Sialorphin and Human Opiorphin: new modulators of opioid-dependent pathways

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Introduction

In mammals the Zinc metallo-ectopeptidases control the activities of numerous neuroendocrine peptide mediators which, in turn, co-ordinate the dynamics of the adaptive response of the organism to environmental changes. Enkephalins are very potent endogenous neuropeptides of the opoidergic pathway which are secreted in response to certain physical, physiological or psychological stress situations. *In vivo*, they are very rapidly inactivated by two metallo-ectopeptidases, NEP (Neutral-EndoPeptidase) and AP-N (AminoPeptidase-N).

Enkephalins play a crucial role in the dynamic control of neurotransmission pathways of pain and in the modulation of cerebral structure activity governing, among others, motivation and the adaptive equilibrium of emotional states [1, 2]. Their action is specifically transmitted, like that of morphine (an alkaloid opiate), via μ- and ∂- opioid membrane receptors. The identification of the mechanisms which control the upstream regulation of enkephalin signals is of fundamental significance to physiological and therapeutic studies because of the importance of the biological constants regulated by the endogenous opoid system [3].

The tale of the discovery of Opiorphin has its beginnings in the molecular characterization of a novel hormonal mediator in the rat, named sialorphin, and in the establishment of its significance and functional specificity *in vivo*. There followed the search and exciting discovery of a related human modulator, as described below.

Results and Discussion

Rat Sialorphin, the first natural regulator of enkephalin-inactivating NEP activity, identified in mammals - Sialorphin is a hormonal messenger of intercellular communication in the rat and was identified using a physio-pharmaco-chemical post-genomic approach. In a remarkable discovery, it was found that sialorphin is a physiological ligand of rat NEP and a competitive inhibitor of membrane-bound NEP, in both the nervous tissue (spinal cord) and in peripheral target-tissues (kidney, bone, teeth, placenta, prostate, submaxillary gland, intestine) [4, 5, 6]. *In vivo* in the adult male rat, using two models of the behavioral response to acute pain (Pin Pain Test and Formalin Test), sialorphin exerts a powerful anti-nociceptive activity, with a maximum effect at 100-200 μg/Kg, I.V. The analgesic effects induced by sialorphin require the specific activation of opioid receptor subtypes μ- and ∂- opioids. In addition, using a model of behavioral despair, the forced swim test, sialorphin exerts an anti-resignation or anti-depressive effect. Finally, in a model of socio-relational sexual response behavior, circulating physiological concentrations (1μg/Kg, I.V.) of sialorphin exert a stimulating effect on the behavioral parameters of motivation and/or arousal (frequency of socio-sexual interactions) and on certain parameters of sexual performance [7, 8].

Together these results led us to propose that *in vivo* by protecting endogenous enkephalins, liberated in response to environmental stimuli (pain, emotion, stress...), from inactivation by the ectopeptidases (NEP and AP-N), sialorphin positively regulates the adaptive physiological functions of enkephalins, in particular their anti-nociceptive and psycho-stimulating actions [1, 2, 9].

The discovery of human Opiorphin using a functional biochemistry approach - Because of the remarkable properties of sialorphin in the rat, we searched for its functional homologue in humans and, particularly, in human salivary secretions. A search of the literature suggested that there might be (non-characterized) substances which inhibit NEP in human saliva [10].

Using a clinical research protocol which was established with the Center for Biomedical Research at the Pasteur Institute (CRBm and ICARe), human saliva was collected from healthy individuals. The successive extraction and HPLC chromatographic procedures allowed the extraction and isolation of the major salivary component having the capacity to inhibit human NEP activity expressed by the LNCaP epithelial human cells. Analysis of the final purification product by micro-sequencing provided direct proof of the existence in humans of a pentapeptide with the sequence QRFSR, which is secreted in human saliva and whose function is similar to that of rat sialorphin pentapeptide (QHNPR-peptide). We named it Opiorphin. Genomic analysis *in silico* revealed that the QRFSR peptide potentially results from the selective maturation of the human PROL1 gene product, which is expressed in human salivary glands.

Using various molecular pharmacology models (including human recombinant NEP and AP-N) and of cellular pharmacology models (human cell membranes: cell line which constitutively expresses hNEP and a cell line transfected with cDNA-hNEP or cDNA-hAPN), we demonstrated that the Opiorphin peptide is a dual inhibitor

of both hNEP and hAP-N and that it protects circulating Met-enkephalin from degradation by these two enzymes *in vitro*.

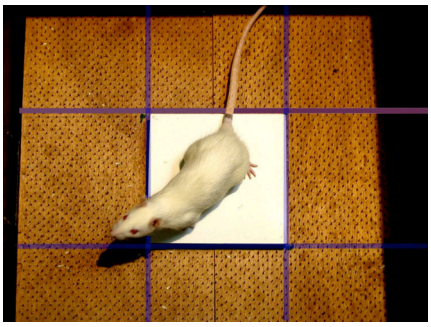

Figure 1. The Pin Pain Test, an ethopharmacological model for the analysis of the behavioral response to pain in the rat. The test is based on locomotor and exploratory analyses of the rat in an open-field with an aversive floor (mechanical type pain). The experimental device is composed of a compartment divided into 9 squares of equal size, 8 of which are peripheral and scattered with steel pins and one smooth central square. The test involves placing the rat initially in the central square and then quantifying its locomotor activity (horizontal and vertical) and exploratory activity in the peripheral aversive pin areas for 3 minutes. Model developed by ETAP-Applied Ethology.

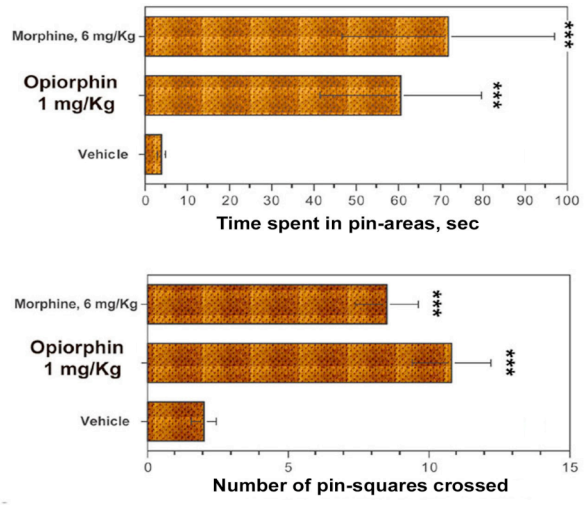

Figure 2. The pain-suppressive potency of Opiorphin at 1 mg/Kg I.V. *in the behavioral response to mechanical pain in the rat. In conditions without steel pins, the rat placed in the central area of the device moves quickly to the peripheral areas, where it spends 75% of its time exploring. As soon as the peripheral areas are made unattractive by placing a steel pin flooring, the rat makes a quick exploration in the periphery and then returns to the smooth central area where it spends 98% of its time during the 3 minute-test (Vehicle). Taken together, the behavioral parameters measured (time spent in pin areas and number of pin-squares crossed) demonstrate that Opiorphin, like morphine, significantly inhibits the perception of pain induced by the contact of the rat's feet with the steel pins. ***P ≤ 0.001 compared to the control rat group (vehicle) using the Mann-Whitney U test, (n=8-12 rats/group).*

In vivo, using a behavioral model of mechanical acute pain in the male rat, the Pin Pain Test (Figure 1), we have demonstrated that Opiorphin exerts a powerful anti-nociceptive activity at 1 mg/Kg, I.V., with similar effects to morphine at 6 mg/Kg, I.P. (Figure 2). In addition, the analgesic effect induced by the peptide is blocked in the presence of naloxone, an antagonist of opioid receptors. This indicates that the pain-suppressive action of Opiorphin is mediated *via* endogenous opioidergic pathways dependent on these receptors [11].

In conclusion, the converging data derived from a post-genomic approach and from biochemistry and pharmacology, allowed us to demonstrate, for the first time, the existence in humans of a physiological inhibitor of the activities of the enkephalin-inactivating metallo-ectopeptidases, NEP and AP-N. Remarkably, it was also found that Opiorphin is a powerful inhibitor of pain sensation *via* the activation of endogenous opioidergic pathways in the rat. In a physiological scenario, human Opiorphin would intervene in the process of adaptation, partly mediated by the enkephalins which are factors associated with pain and emotion, and particularly in the regulation of homeostatic equilibrium such as pronociception-antinociception and depression-motivation.

Genomic and functional biochemistry data lead us to believe that human Opiorphin has the same topological characteristics of synthesis and secretion as those identified for rat sialorphin. A clinical research program is in progress which aims to establish a secretion profile and a distribution profile for Opiorphin in humans. This project in physiological situation will be followed by a further approach in pathological situation aimed at identifying physiological situations and pathological states which regulate Opiorphin synthesis and secretion.

Moreover, because of its biological properties, Opiorphin might be a candidate to identify molecular pathways for use in the conception of new medical treatments. By inhibiting the destruction of endogenous opioids, especially enkephalins, Opiorphin represents a potential therapeutic area for pain therapy and in the treatment of socio-relational behavioral disorders associated with an emotional type unbalance. The main advantages is that pharmacological effects of Opiorphin or its derivatives might be induced only at opioid receptors tonically and dynamically stimulated by the natural effectors thus at least minimizing receptor overstimulation which is thought to be responsible for the major side effects of morphine.

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