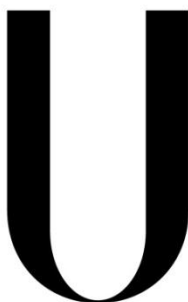


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**Sara Alexandra Matos dos Santos**

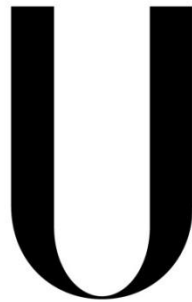
Doctorate in Biomedical Sciences

**Specialty in Medical Biochemistry**

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**Sara Alexandra Matos dos Santos**

Thesis supervised by Prof. Doctor Miguel Augusto Rico Botas Castanho

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# Preface

---

My PhD was devoted to the study of the possible relationship of pain and Alzheimer disease (AD) using the analgesic Kyotorphin as a bridge.

I became very interested in the area of chronic pain still on the faculty of Medicine. Later, I started my hospital rotations and this area changed from a theoretical to a practical subject, since pain is one of the main complaints that drive patients to hospitals. The study and treatment of chronic pain were some of the reasons why I chose the residency in Anesthesiology.

I always found laboratory work and basic research fascinating, so the possibility of working at the Institute of Molecular Medicine - Faculty of Medicine was a great opportunity to develop my PhD and link basic and clinical research.

I was fortunate enough to know Professor Miguel Castanho and his group, who proposed me a translational project involving the study of the peptide kyotorphin in the relation between pain and neurodegeneration.

This project conducted to pain evaluation in patients with the diagnosis of Alzheimer disease using validated scales. Later, the quantification of kyotorphin in the cerebrospinal fluid of AD patients was determined. To achieve this, we collaborated with the laboratory of metabolopathies of the Hospital of Santiago Compostela, with the development of mass spectrometry techniques to detect KTP in biological fluids. The results were promising enough to advance further to the testing of kyotorphin derived peptides in an animal model of Alzheimer disease.

During this path I worked with people specialized in the various fields the work involved, namely pain, neurosciences and biochemistry, which was a very enriching experience.

My PhD work originated the following papers, most of them already published:

## I. Biomedical applications of di- and tri-peptides

**Santos SM**, Torcato I, Castanho M

Biopolymers Peptide Science (2012) Volume 98, Issue 4, pages 288–293 (DOI: 10.1002/bip.22067)

**II.** The use of visual analogue scales to compare pain between patients with Alzheimer' disease and patients without any known neurodegenerative disease and their caregivers.

**Santos SM**, Castanho M

Am J Alzheimer's Dis and Other Demen (2013) (DOI: 10.1177/1533317513517046)  
[Epub ahead of print]

**III.** Neuropeptide kyotorphin (tyrosyl-arginine) has decreased levels in the cerebrospinal fluid of Alzheimer's disease patients: potential diagnostic and pharmacological implications.

**Santos SM**; Garcia-Nimo L, Sá Santos S, Tavares I, Cocho JA, Castanho MARB.

Frontiers in Aging Neuroscience (2013) Volume 5, Article 68 (DOI: 10.3389/fnagi.2013.00068)

**IV.** Amidated and ibuprofen conjugated kyotorphins are neuroprotective in the hippocampus and improve spatial working memory after bilateral carotid occlusion in female rats

*In preparation*

**V.** Side effects of analgesic Kyotorphin derivatives: advantages over clinical opioid drugs

Ribeiro MM, Santos SS, Sousa DS, Oliveira M, **Santos SM**, Heras M, Bardaji E, Tavares I, Castanho M

Amino Acids (2013) Volume 45, Issue 1, pages 171-178 (DOI: 10.1007/s00726-013-1484-2)

This dissertation is divided into 5 sections. In the first section the reader can find an introduction to the complexity of pain physiological pathways and to the complexity of endogenous opioid peptides as well as the potential of peptides to treat pain conditions, with a particular focus on kyotorphin. In this chapter the reader can also

find a brief approach to the pathophysiology and actual therapeutic options for Alzheimer's disease.

In sections 2 and 3 different studies carried out within the context of this thesis are presented, in the form of scientific papers. A general conclusion of all results is presented in Chapter 4. There were two key papers seminal to this work: the first (Ribeiro, 2011a) is a paper on the subject of the inhibition of nociceptive responses after systemic administration of amidated kyotorphin and the second (Ribeiro, 2011b) is a paper showing that the chemical conjugation of Kyotorphin and Ibuprofen enhances brain targeting and analgesia. Both papers originated from the same investigation group and support the later work, condensed in this dissertation.

In this dissertation, two distinct referencing methods were followed. The References cited along the thesis (excluding articles) are listed in section 5, sorted alphabetically by first author's last name. Within each paper published, the format of the References cited comply with the guidelines defined by the respective journal and are listed at the end of the articles.

# Abbreviations and Symbols

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$\delta$ -OR – delta opioid receptor  
 $\kappa$ -OR – kappa opioid receptor  
 $\mu$ -OR – mu opioid receptor  
BBB – blood-brain barrier  
CNS – central nervous system  
i.c.v. - intracerebroventricular  
i.p. – intra-peritoneal  
IASP – International Association for the Study of Pain  
Ib – ibuprofen  
IbKTP – kyotorphin linked to ibuprofen  
IbKTP-NH<sub>2</sub> – kyotorphin-amide linked to ibuprofen  
KTP – kyotorphin  
KTP-NH<sub>2</sub> – kyotorphin amide  
KTPr – kyotorphin specific receptor  
NO – nitric oxide  
NOP – nociceptin/orphanin receptor  
NSAIDs – non-steroidal anti-inflammatory drugs  
DRG - dorsal root ganglia  
AD – Alzheimer’s Disease  
Ach – Acetylcholine  
MCI - mild cognitive impairment  
CSF - cerebrospinal fluid

# Resumo

---

O aumento da esperança média de vida tem provocado um aumento na incidência de doenças relacionadas com o processo de envelhecimento. Entre estas, destacam-se as doenças neurodegenerativas, nomeadamente a Doença de Alzheimer (DA), e outras entidades associadas a fenómenos de dor crónica.

A possível correlação entre duas entidades – dor e doença de Alzheimer - com uma carga epidemiológica tão grande afigura-se um passo relevante. A dor, hoje considerada como 5º sinal vital e presente numa larga camada da população, encontra-se ainda de grande modo sub-tratada, sendo necessários novos fármacos que actuem em alvos diferentes e que, ao mesmo tempo, tenham uma menor panóplia de efeitos adversos. Por outro lado, a doença de Alzheimer é uma patologia largamente disseminada na nossa população, afectando primariamente as camadas idosas mas também e cada vez mais uma população mais jovem. O desconhecimento, a complexidade dos processos bioquímicos e fisiológicos envolvidos e a progressiva perda cognitiva dos pacientes resultam em terapêuticas ainda pouco eficazes. O aumento do conhecimento científico sobre a doença aos vários níveis é a via obrigatória para assegurar uma melhoria na prestação de cuidados de Saúde apropriados e aumentar a possibilidade de intervir em estadios precoces da doença, inclusivamente ao nível da prevenção e diagnóstico. As consequências do diagnóstico e do evoluir desta patologia são bem conhecidas: alteração do ambiente familiar/emocional do doente e sobrecarga para os prestadores de cuidados. Por isso, uma estratégia visando a correlação entre estas duas entidades e possíveis alvos terapêuticos comuns a um único agente farmacológico é promissora.

Como já foi referido, o projecto proposto foca dois temas de extrema importância clínica e forte impacto social: doença de Alzheimer e Dor. O aspecto mais inovador prende-se com a hipótese de ambos os problemas poderem estar correlacionados e poderem, portanto, ser abordados numa abordagem terapêutica única. Esta hipótese é sustentada por trabalho laboratorial *in vitro*, partindo-se agora para uma outra fase, com experimentação animal em modelos de doenças e investigação clínica.

Neste trabalho pretendeu-se avaliar uma molécula, a quiotorfina, como um elo molecular de ligação dos mecanismos envolvidos nos dois casos.

A Quiotorfina (KTP), descoberta em 1979, foi descrita como um dipéptido analgésico endógeno actuante no encéfalo. Com uma actividade analgésica cerca de 4 vezes superior a outros péptidos endógenos como a met-enkefalina, este dipéptido apresenta características muito interessantes e pensa-se que actua através de mecanismos opióides. Moléculas analgésicas derivadas da quiotorfina tinham sido previamente testadas para o seu efeito simultaneamente analgésico *in vivo*, em animais modelo, e neuroprotector *in vitro*, após administração periférica.

O presente projecto traz estes resultados até à investigação clínica e os seus principais objectivos foram: 1) perceber se existe uma correlação entre doença de Alzheimer e Dor, 2) a hipótese de péptidos analgésicos derivados da quiotorfina serem fármacos capazes de neuromodulação e 3) o potencial da quiotorfina como marcador molecular na doença de Alzheimer.

Para atingir esses objectivos, combinou-se investigação clínica e básica, utilizando técnicas diversificadas como questionários e escalas de dor, técnicas analíticas sofisticadas de espectrometria de massa e experimentação animal.

Numa primeira fase avaliou-se a percepção dolorosa em doentes com Alzheimer e seus cuidadores, através da utilização de escalas de dor validadas. Este estudo levou à conclusão de que, em consonância com os achados na literatura, a dor em doentes com Alzheimer é frequentemente sub-avaliada e conseqüentemente, subtratada. Este fenómeno acontece porque provavelmente estes doentes são incapazes de valorizar e/ou expressar o seu sofrimento, mesmo em estadios moderados da doença.

A investigação clínica envolveu outro componente: a colheita de amostras de líquido céfalo-raquidiano em doentes com DA para determinação dos níveis de quiotorfina, e sua comparação com indivíduos sem doenças neurodegenerativas conhecidas; verificou-se que os níveis de quiotorfina, por si um neuropéptido endógeno, diminuem com a progressão da doença de Alzheimer.

Este achado abre novas possibilidades, nomeadamente o uso da quiorfina como possível marcador de neurodegenerescência, e que este neuropéptido tenha acções neuromoduladoras.

Posteriormente estudou-se o efeito de neuropéptidos derivados da quiorfina capazes de atravessar a barreira hemáto-encefálica - IbKTP-NH<sub>2</sub> e KTP-NH<sub>2</sub> - em modelos animais, com dois objectivos: perceber se os fármacos apresentavam efeitos secundários significativos por comparação a opióides de referência usados na prática clínica, e qual o seu efeito num modelo animal de neurodegenerescência.

Estes derivados amidados KTP-NH<sub>2</sub> e IbKTP-NH<sub>2</sub>, ao contrário da morfina e do tramadol (dois fármacos utilizados na prática clínica), não provocaram os principais efeitos secundários associados aos opióides, o que constitui mais uma indicação de que os mecanismos de acção destes péptidos e dos opióides não coincide totalmente. Por outro lado, os animais modelo de neurodegenerescência aos quais estes compostos foram administrados de forma crónica revelaram uma melhoria no padrão comportamental relativamente aos animais com lesões neurológicas aos quais não tinha sido administrado nenhum dos compostos.

Globalmente os nossos resultados apontam a quiorfina como um possível biomarcador na doença de Alzheimer, e os seus derivados IbKTP-NH<sub>2</sub> e KTP-NH<sub>2</sub>, capazes de neuromodulação/neuroprotecção, além moléculas analgésicas eficazes com efeitos secundários reduzidos.

# Abstract

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The increased lifespan has brought about an increase in the incidence of diseases related to the aging process. Among these, there are the neurodegenerative diseases, including Alzheimer's disease (AD) and other entities associated with the phenomena of chronic pain.

The possible correlation between two entities - pain and Alzheimer's disease - with such a large epidemiological burden is of enormous importance. Nowadays pain is considered the fifth vital sign and is highly prevalent, still largely under-treated. New drugs acting on different targets with a smaller range of adverse effects are needed. Moreover, Alzheimer's disease is widely spread in our population, primarily affecting the elderly but also a younger population. The ignorance of the complexity of the biochemical and physiological processes involved and progressive cognitive impairment of patients still result in ineffective treatment. The increase in scientific knowledge about the disease at various levels is mandatory in order to ensure an improvement in the provision of appropriate health care and the chance of intervening in the early stages of the disease, including at the level of prevention and diagnosis. The consequences of diagnosis and evolution of this pathology are well known: change in family / emotional environment of the patient and burden on caregivers. Therefore, a strategy for the correlation between these two entities and possible common therapeutic targets involving a single pharmacological agent is promising.

As already mentioned, this project focuses on two issues of utmost clinical importance and strong social impact: Pain and Alzheimer's disease. The most innovative aspect relates to the hypothesis that both problems can be related and may, therefore, be addressed in a single therapeutic approach. This hypothesis is supported by previous experimental work in vitro, evolving now to another phase, with animal experimentation in disease models and clinical research.

This thesis intended to evaluate a molecule, kyotorphin, as a molecular link in the mechanisms involved in both cases. Kyotorphin (KTP), discovered in 1979, was described as an endogenous analgesic dipeptide acting in the brain. With an

analgesic activity about 4 times higher than other endogenous peptides such as met-enkephalin, this dipeptide has very interesting features and is thought to act via opioid mechanisms. Analgesic derived molecules of kyotorphin had previously been tested for their analgesic effect *in vivo* in animal models and for *in vitro* neuroprotective effects after peripheral administration.

This project brings these results to clinical research and its main objectives were: 1) to understand if there is a correlation between Alzheimer's disease and pain, 2) if analgesic peptides derived from kyotorphin are capable of neuromodulation and 3) evaluate kyotorphin as a potential molecular marker in Alzheimer's disease. To achieve these objectives, clinical and basic research were combined using diverse techniques such as questionnaires and pain scales, sophisticated analytical techniques of mass spectrometry and animal experimentation.

In a first stage we assessed pain perception in Alzheimer patients and their caregivers, using validated pain scales. This study concluded that, in line with the findings in the literature, pain in Alzheimer patients is often under-evaluated and therefore undertreated. This phenomenon probably occurs because these patients are unable to value and/or express their suffering, even in moderate stages of the disease. Clinical research involved another component: the collection of cerebrospinal fluid samples of patients with AD for determination of Kyotorphin levels and its comparison with individuals without known neurodegenerative diseases; it was found that the levels of kyotorphin, a neuropeptide endogenous *per se*, decreases with the progression of Alzheimer's disease. This finding opens new possibilities, including the use of kyotorphin as a possible marker of neurodegenerescence and that this neuropeptide has neuromodulatory actions. Subsequently, we studied the effect of neuropeptide kyotorphin derivatives capable of crossing the blood brain barrier - IbKTP-NH<sub>2</sub> e KTP-NH<sub>2</sub> – in animal models with two objectives: realize if these drugs showed significant side effects compared to reference opioids used in clinical practice, and what were their effects in an animal model of neurodegenerescence. These amidated derivatives IbKTP-NH<sub>2</sub> e KTP-NH<sub>2</sub>, unlike morphine and tramadol (two largely used drugs in clinical practice), caused no major side effects associated with opioids, which is a further indication that the mechanism of action of these peptides and opioids not fully

coincide. On the other hand, the neurodegeneration animal model to which these compounds were chronically administered revealed an improvement in the behavioral pattern in comparison with animals with neurological lesions to which none of the compounds had been administered.

Overall, our results indicate kyotorphin as a possible biomarker for Alzheimer's disease, and its derivatives IbKTP-NH<sub>2</sub> and KTP-NH<sub>2</sub> capable of neuromodulation/neuroprotection, in addition to effective analgesic molecules with reduced side effects.

# Keywords

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Neuropeptides

Kyotorphin

Alzheimer

Neurodegeneration

Pain

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Section 1

# Introduction

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# 1. Pain

Pain is defined by the International Association for the Study of Pain as “an unpleasant sensory and emotional experience related to actual or potential tissue damage, or described in terms of such damage.” Pain is an unpleasant subjective experience that is the net result of a complex interaction of the ascending and descending nervous systems involving organic chemistry, physiological, psychological, and neocortical processes. Pain can have an effect on all areas of a person’s life, including sleep, thought, emotion, and activities of daily living. Since there are not any reliable, objective markers for pain, the patients and their caregivers are the sole ones to describe the intensity and quality of their pain (Porter, 2008).

Pain is the most common symptom prompting patients to search for medical attention and is reported by over 80% of people who visit their primary care provider (Mularski, 2006). Despite the frequency of pain symptoms, individuals often do not get satisfactory relief of pain which has led to initiatives in health care to designate pain as the fifth vital sign, therefore making pain assessment equally vital as getting a patient’s temperature, pulse, pressure level, and respiratory rate (Pasero, 1997).

## 1.1 Epidemiology And Etiology

### Prevalence of Pain

Most people experience pain at some time in their lives, and pain is a symptom of a variety of diseases. For some, pain may be mild to moderate, intermittent, easily managed, and have minimal effect on daily activities. For others, pain may be chronic, severe or disabling, all consuming, and be treatment resistant. Thus, identifying the exact prevalence of pain is a difficult task. According to the American Pain Foundation, more than 50 million people in the United States of America (U.S.A.) suffer from

chronic pain and an additional 25 million experience acute pain from injury or surgery. About 20% of adults, mostly women and the elderly, experience chronic pain such as back pain, headache, and joint pain (Gallagher, 2006). Overall, older people are more likely to have painful pathology due to the increased incidence of chronic medical conditions, particularly of rheumatology (osteoporosis, osteoarthritis) and oncology fields; pain represents 25-50% of the complaints in outpatient and 45-80% of the complaints in institutionalized patients (Scherder, 2008). Studies suggest that 25% to 50% of community-dwelling elderly suffer pain. Pain is also quite common among nursing home residents: it is estimated that pain in 45% to 80% of nursing home patients contributes to functional impairment and a decreased quality of life (Landi, 2001; Reyes-Gibby, 2002).

Prevalence rates for a variety of different types of pain have been described. The annual incidence of moderate-intensity back pain is 10% to 15% of the adult population with a point prevalence of 15% to 30% (Andersson, 1999). Cancer is commonly associated with both acute and chronic pain, and about 70% of those diagnosed with cancer will experience significant pain (Burton, 2007).

The prevalence of neuropathic pain is largely unknown because of the lack of epidemiological studies. Current estimates suggest that it affects approximately 4 million people in the USA each year. It is associated with many diseases, including diabetic peripheral neuropathy, post-herpetic neuralgia, human immunodeficiency virus-related disorders, and chronic radiculopathy. Central neuropathic pain is estimated to occur in 2% to 8% of all stroke patients (Chen, 2004).

The impact of pain on economies is enormous, with some studies showing that the cost of back pain alone is equivalent to more than a fifth of one country's total health expenditure and 1.5% of its annual gross domestic product, or that it represents three-times the total cost of all types of cancer (Phillips, 2006). Every year, pain costs 34

billion € in Europe and in the USA 80 billion € are spent in direct health care expenditure and lost work time (Melnikova, 2010).

Despite the growing emphasis on pain management, pain often remains undertreated and continues to be a problem in hospitals, long-term care facilities, and the community. In one series of reports, 50% of seriously ill hospitalized patients reported pain; however, 15% were dissatisfied with pain control, and some remained in pain after hospitalization (Desbiens 1996; Desbiens 2006).

## **1.2 Pathophysiology**

The pathophysiology of pain involves a complex array of neural networks in the brain that are acted on by afferent stimuli to produce the experience we know as pain. In acute pain, this modulation is short-lived, but in some situations, the changes may persist, and chronic pain develops (Loeser, 1999; Woolf, 2004).

### **1.2.1 Types of Pain**

#### **Classification of Pain**

Pain has always been described as a symptom. However, recent advances in the understanding of neural mechanisms have demonstrated that unrelieved pain may lead to changes in the nervous system known as neural plasticity. Since these changes reflect a process that influences a physiologic response, pain, particularly chronic pain, may be considered a disease itself.

A primary distinction lies in its duration and divides pain in acute (transient) and chronic (persistent). Chronic pain has been defined temporally as a pain that persists

for more than 3 to 6 months or that remains in the absence of its causes (Sandkuller, 2009). Acute pain is also referred to as adaptive pain since it serves to protect the individual from further injury or promote healing. However, chronic pain has been called maladaptive, a pathologic function of the nervous system or pain as a disease.

Several distinct types of pain have been described: nociceptive, inflammatory, neuropathic, and functional (Costigan, 2009). Nociceptive pain is a transient pain in response to a noxious stimulus at nociceptors that are located in cutaneous tissue, bone, muscle, connective tissue, vessels, and viscera. Nociceptors may be classified as thermal, chemical, or mechanical. The nociceptive system extends from the receptors in the periphery to the spinal cord, brain stem, and to the cerebral cortex where pain sensation is perceived. This system is a key physiologic function that prevents further tissue damage due to the body's autonomic withdrawal reflex. When tissue damage occurs despite the nociceptive defense system, inflammatory pain ensues. The body now changes focus from protecting against painful stimuli to protecting the injured tissue. The inflammatory response contributes to pain hypersensitivity that serves to prevent contact or movement of the injured part until healing is complete, thus reducing further damage (Passero, 1999).

Neuropathic pain, caused by dysfunction or damage to the peripheral or central nervous system, is typified by the symptoms described by patients with painful diabetic neuropathy, post-herpetic neuralgia and central poststroke pain. All these conditions are more common in the elderly. Neuropathic pain has long been recognized as one of the more difficult types of pain to treat; however, with the current emphasis on providing a multidisciplinary assessment and approach to management, more patients will be offered relief of their symptoms and an improved quality of life (Ahmad, 2002).

Functional pain, a relatively newer concept, is pain sensitivity due to an abnormal processing or function of the central nervous system in response to normal stimuli. Several conditions considered to have this abnormal sensitivity or hyperresponsiveness include fibromyalgia and irritable bowel syndrome (Bennett, 1998).

### **1.2.2 Mechanisms of Pain**

#### **Pain Transmission**

The mechanisms of nociceptive pain are well-defined and provide a foundation for the understanding of other types of pain.

Nociception is the process by which intense thermal, mechanical or chemical stimuli are detected by a subpopulation of peripheral nerve fibers, called nociceptors (Basbaum and Jessell, 2000). The cell bodies of nociceptors are located in the dorsal root ganglia (DRG) for the body and the trigeminal ganglion for the face, and have both a peripheral and central axonal branch that innervates their target organ and the spinal cord, respectively. Nociceptors are excited only when stimulus intensities reach the noxious range, suggesting that they possess biophysical and molecular properties that enable them to selectively detect and respond to potentially injurious stimuli. Nociceptors can be divided into two general types: A-fibers and C-fibers.

A-fiber nociceptors have lightly myelinated axons, conduct action potentials rapidly, and have medium to large-diameter cell bodies. A-fibers mediate the fast, pricking quality of pain. These include medium diameter myelinated ( $A\delta$ ) afferents that mediate acute, well-localized “first” or fast pain. These myelinated afferents differ considerably from the larger diameter and rapidly conducting  $A\beta$  fibers that respond to innocuous mechanical stimulation (i.e. light touch). Electrophysiological studies have further subdivided  $A\delta$  nociceptors into two main classes, type I (HTM: high threshold

mechanical nociceptors - respond to both mechanical and chemical stimuli, but have relatively high heat thresholds -  $>50^{\circ}\text{C}$ ) and type II (much lower heat threshold, but a very high mechanical threshold) (Basbaum, 2009).

C-fibers have unmyelinated axons, conduct action potentials slowly, and have small-diameter cell bodies. C-fibers mediate the slower, burning quality of pain. C-fibers comprise around 70% of all nociceptors. Two classes of C-fibers have been identified. One class contains a variety of neuropeptides, including substance P and calcitonin gene-related peptide, and expresses trkA receptors, the high-affinity receptor for nerve growth factor (Averill, 1995). These neurons project to the outermost region of the spinal dorsal horn (lamina I and outer lamina II) and terminate largely on spinal neurons that project to higher-order pain centers in the brain (Stucky, 2001). The other class contains few neuropeptides but expresses a surface carbohydrate group that selectively binds to a plant lectin called isolectin B<sub>4</sub> (IB<sub>4</sub>). This subpopulation of neurons is supported by glial-derived neurotrophic factor during early postnatal development (Molliver, 1997). The IB<sub>4</sub>-binding neurons project to a different region of the spinal dorsal horn (inner lamina II) that contains primarily local spinal interneurons. Important questions are whether these two types of nociceptors have different functional responses to painful stimuli and whether they have distinct roles in specific types of pain.

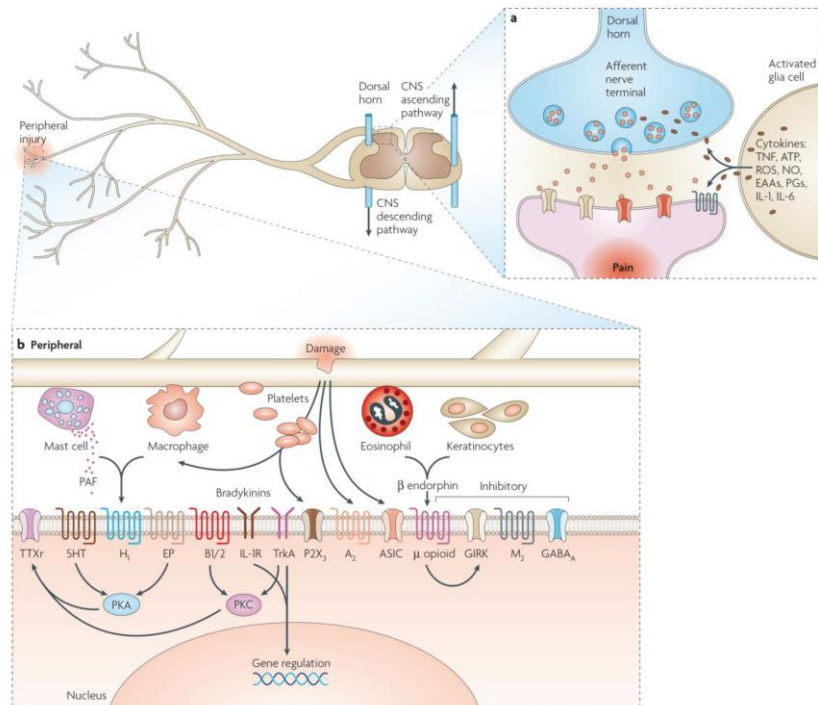
Nociceptors		
A $\beta$ fibers	A $\delta$ fibers	C fibers
myelinated	Lightly myelinated	unmyelinated
Large diameter	Medium diameter	small diameter
Fast signal conduction	Fast signal conduction	Slow signal conduction
innocuous mechanical stimuli	Acute localized first pain	Delayed difused second pain
	Polymodal	Polymodal
	<b>Type I</b>	<b>Type II</b>
	differential in heat responsiveness	
	~53°C threshold	~43°C threshold
		Noxious thermal, mechanical and chemical stimuli
		<b>Peptidergic</b> <b>Nonpeptidergic</b>

**Figure 1** – Nociceptors diversity. Nociceptors can be distinguished in A $\beta$ , A $\delta$  and C fibers that differ in physiological and functional aspects to detect and conduct different types of pain. Mechanical, thermal and chemical stimuli are perceived and transduced.

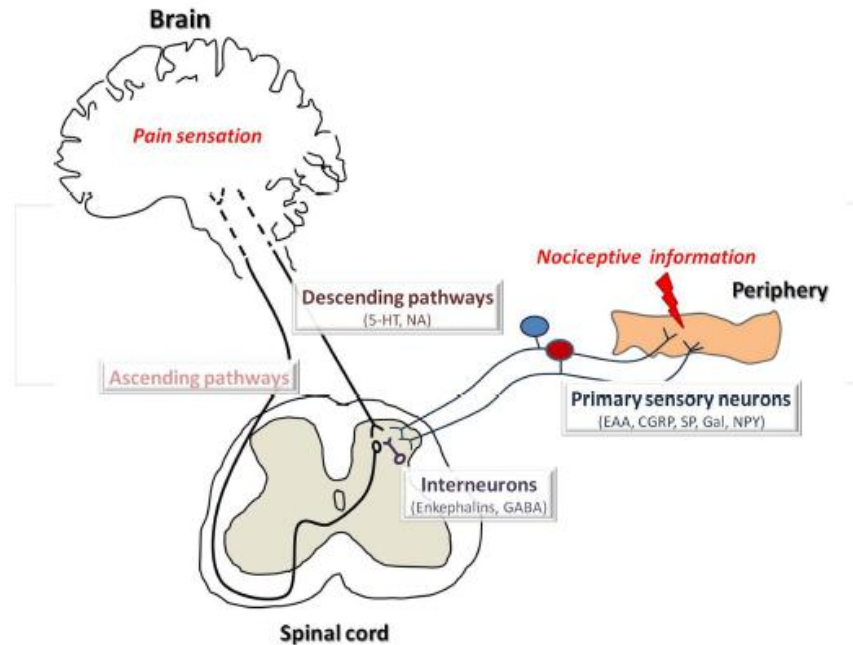
Neurobiological research in the pain field provided solid information regarding the transmission and modulation of nociceptive information from the periphery to the brain, where a pain sensation is produced (Figure 2 and 3). Nociceptive signals are conveyed by primary afferent fibers from peripheral organs, like the bladder or muscles, to the spinal cord. This is the first relay station involved in the modulation of nociceptive information, namely by local inhibitory interneurons that use opioid peptides or aminoacids ( $\gamma$ -amminobutiric acid-GABA and glycine). Nociceptive information is then transmitted supraspinally, namely to the thalamus, and to several brainstem areas, where additional modulation of the nociceptive signal occurs. The thalamo-cortical pathway ensures that the nociceptive information reaches the somato-sensory and prefrontal cortices, where the nociceptive signal is finally perceived as a pain sensation. Some brain areas which directly or indirectly receive nociceptive information from the spinal cord are also involved in descending pain

modulation (Tavares, 2013). The brainstem concentrates all regions directly modulating the ascending nociceptive transmission from the spinal dorsal horn and mediates the pain control orders triggered by the forebrain areas responsible for coordination of the final pain perception. Moreover, the brainstem (and the medial thalamus) is also the main recipient of the spinal nociceptive information concerning the motivational-affective dimension of pain (Almeida, 2006).

Both inhibition and facilitation may occur and chronic pain may derive from a reduction of the former and enhancement of the latter (Tavares, 2013).



**Figure 2** – Pain signaling and pathways. Nociceptors are activated in the periphery by noxious stimuli and send afferent information to the dorsal horn of the spinal cord. **a)** Synaptic transmission in the dorsal horn is modulated by several neurochemicals. **b)** Peripheral mediators of pain transduction after tissue injury. Inflammation leads to the release of numerous chemicals from mast cells, macrophages and injured cells that act directly or indirectly to alter the sensitivity of receptors and ion channels on peripheral nerve terminals (from Woodcock, 2007).



**Figure 3** - Schematic diagram of pain pathways involved in pain transmission and modulation. Nociceptive information is transmitted from the periphery to the spinal dorsal horn by primary sensory neurons. At the spinal level, these neurons transmit nociceptive information to second order neurons (Ascending pathways) through the release of neurotransmitters like the excitatory amino acids (EAA) glutamate and aspartate, calcitonin gene-related peptide (CGRP), substance P (SP) galanin (Gal) and neuropeptide Y (NPY). In the brain, the nociceptive information is then perceived as a pain sensation. The transmission of nociceptive information at the spinal level is modulated by interneurons (mainly inhibitory) through the release of opioid peptides and GABA and also by supraspinal descending neurons (descending pathways) through the release of serotonin (5-HT) and noradrenaline (NA). Descending pathways may inhibit or enhance nociceptive transmission from the spinal cord (from Tavares, 2013).

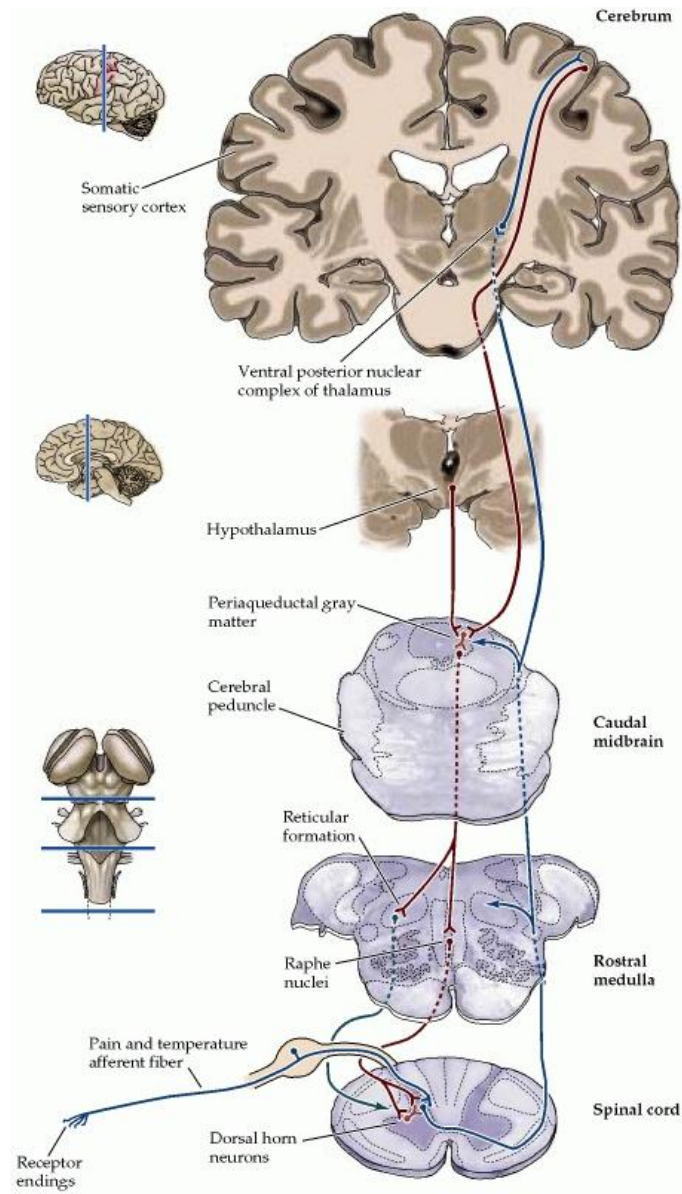
### 1.2.3 Pain Modulation

Understanding the central modulation of pain perception was greatly advanced by the finding that electrical or pharmacological stimulation of certain regions of the midbrain produces relief of pain. This analgesic effect arises from activation of descending pain-modulating pathways that project, via the medulla, to neurons in the dorsal horn—particularly in Rexed's lamina II—that control the ascending information in the nociceptive system. The major brainstem regions that produce this effect are located in poorly defined nuclei in the periaqueductal gray matter and the rostral medulla. Electrical stimulation at each of these sites in experimental animals not only produces

analgesia by behavioral criteria, but also demonstrably inhibits the activity of nociceptive projection neurons in the dorsal horn of the spinal cord.

A quite ordinary example of the modulation of painful stimuli is the ability to reduce the sensation of sharp pain by activating low-threshold mechanoreceptors: a reaction is to vigorously rub the site of an injury for a minute or two. Even though further investigation led to modification of some of the original propositions in Melzack and Wall's gate theory of pain, the idea stimulated a great deal of work on pain modulation: that the flow of nociceptive information through the spinal cord is modulated by concomitant activation of the large myelinated fibers associated with low-threshold mechanoreceptors.

Pain modulation may result through several other complex processes. One of the most exciting advances in this long-standing effort has been the discovery of endogenous opioids. For centuries it had been apparent that opium derivatives such as morphine are powerful analgesics—indeed, they remain a mainstay of analgesic therapy today. Modern animal studies have shown that a variety of brain regions are susceptible to the action of opiate drugs, particularly—and significantly—the periaqueductal gray matter and the rostral ventral medulla. There are, in addition, opiate-sensitive regions at the level of the spinal cord. In other words, the areas that produce analgesia when stimulated are also responsive to exogenously administered opiates. It seems likely, then, that opiate drugs act at most or all of the sites shown in Figure 4 in producing their dramatic pain-relieving effects (Purves D. et al, 2001b).



**Figure 4** - The descending systems that modulate the transmission of ascending pain signals. These modulatory systems originate in the somatic sensory cortex, the hypothalamus, the periaqueductal gray matter of the midbrain, the raphe nuclei, and other nuclei of the rostral ventral medulla. Complex modulatory effects occur at each of these sites, as well as in the dorsal horn (from: Purves et al, 2001a).

The analgesic action of opiates implied the existence of specific brain and spinal cord receptors for these drugs long before the receptors were actually found during the 1960s and 1970s (Table 1). Since such receptors are unlikely to exist for the purpose of responding to the administration of opium and its derivatives, the conviction grew that

there must be *endogenous* compounds for which these receptors had evolved. Several categories of endogenous opioids have now been isolated from the brain and intensively studied (Table 2). These agents are found in the same regions that are involved in the modulation of nociceptive afferents, although each of the families of endogenous opioid peptides has a somewhat different distribution. All three of the major groups (enkephalins, endorphins, and dynorphins) are present in the periaqueductal gray matter. The enkephalins and dynorphins have also been found in the rostral ventral medulla and in the spinal cord regions involved in the modulation of pain (Purves D. et al, 2001b).

**Table 1 – Opioid receptors: location and response on activation**

Receptor	CNS location	Response on activation
$\mu$	Brain (laminae III and IV of the cortex, thalamus, periaqueductal gray), spinal cord (substantia gelatinosa)	$\mu$ 1: supraspinal analgesia, physical dependence; $\mu$ 2: respiratory depression, miosis, euphoria, reduced gastrointestinal motility, physical dependence
$\kappa$	Brain (hypothalamus, periaqueductal gray, claustrum), spinal cord (substantia gelatinosa)	Spinal analgesia, sedation, miosis, inhibition of antidiuretic hormone release
$\delta$	Brain (pontine nucleus, amygdala, olfactory bulbs, deep cortex)	Analgesia, euphoria, physical dependence

**Table 2** – Classes of opioid peptides with representative examples of peptides, their sequence and preference binding to opioid receptors. Orange and blue colours highlight the different amino acid residues inside each class.

Class	Peptide	Ligand to opioid receptor	Sequence
Endomorphins	Endomorphin-1	Highest affinity for $\mu$ -receptors	H-Tyr-Pro-Trp-Phe-NH <sub>2</sub>
	Endomorphin-2		H-Tyr-Pro-Phe-Phe-NH <sub>2</sub>
Dermorphins	Dermorphin	Preferential interaction with $\mu$ -receptors	H-Tyr-DAla-Phe-Gly-Tyr-Pro-Ser-NH <sub>2</sub>
Enkephalins	Met-enkephalin	Endogenous ligands for $\delta$ -receptors, also binds to $\mu$	H-Tyr-Gly-Gly-Phe-Met-OH
	Leu-enkephalin		H-Tyr-Gly-Gly-Phe-Leu-OH
Dynorphins	Dynorphin-A	Endogenous ligands for $\kappa$ -receptors	H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH
	Dynorphin-B		H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Asp-Phe-Lys-Val-Val-Thr-OH
Endorphins	$\beta$ -Endorphin	Endogenous ligands for $\mu$ -receptors	H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH

## 2. Alzheimer Disease

### 2.1 Introduction

Alzheimer's disease (AD) was described in the early 20th century. It has rapidly emerged as a major public health issue throughout the world. It is estimated to be by far the most common form of dementia in the United States, currently afflicting over 5 million people, mainly elderly individuals, with an associated healthcare cost in excess of US\$100 billion annually (Alzheimer's Association, 2009). It is characterized most notably by memory loss, and increasing age is its single most important risk factor (Bird, 2007). Memory loss and dementia, in general, are progressive and irreversible, though the rate of progression is highly variable and impossible to predict (Bird, 2007).

### 2.2 Epidemiology and Genetics

Advancing age is the single most major risk factor for AD, with the prevalence doubling every 5 years between the ages of 65 and 95 years and increasing from 2% at 65 years of age to 40% at over 85 years of age (Gao, 1998). While people do experience minor changes in their memory and thinking as they age, these changes do not affect daily functioning or the ability to live independently. Although the illness has been reported to occur in exceedingly rare patients in their 20s and 30s, onset of clinical symptoms in this illness is uncommon until the 50s (Duijn, 1991). The second major risk factor for AD is family history, with a threefold to fourfold higher risk among individuals having a single first-degree relative with AD and a nearly eightfold higher risk among individuals with two or more first-degree relatives with AD (Duijn, 1991). In contrast late-onset AD, early-onset AD is relatively rare, affecting only 5% of AD patients and developing in individuals of 30-60 years of age (Duijn, 1991). Some cases of early-onset AD, termed familial AD, are inherited in an autosomal dominant manner, with genetic mutations on chromosomes 21, 14 and 1, resulting in the formation of abnormal precursor proteins, presenilin 1 (PS-1) and presenilin 2 (PS-2). The presenilins have been found to operate in a complex that acts functionally as  $\gamma$ -secretase (Brunkan, 2005). Specifically,

a few dozen families have mutations in the amyloid precursor protein (APP) gene, usually in the region of the gene that codes for the  $\beta$ -amyloid proteins (Goedert, 2006). Increased levels of  $\beta$ -amyloid have been found in AD patients with PS-1 or PS-2 mutations (Scheuner, 1996). Apolipoprotein E (ApoE) gene status on chromosome 19 appears to be a major genetic susceptibility risk factor for the development of typical late-onset AD (Tsai, 1994). Other possible risk factors for AD include gender, education, head trauma, memory deficit with severity of any extent, and small hippocampal volume. The very large Women's Health Initiative Memory Study of estrogen in elderly women has shown that estrogen replacement may increase, rather than decrease, the risk for AD (Rapp, 2003). Several studies indicate that lack of education is also a risk factor for AD, or alternatively, education may impart a "cognitive reserve" that delays the onset of clinical manifestations of AD (Schmand, 1997). Studies have been muddled by wide differences in reported series in the criteria applied to define significant head trauma history. Further, ApoE e4 patients have been demonstrated to recover less well from head trauma, so the greater manifestations of trauma may be a pseudo-marker for ApoE e4 inheritance, which is a risk factor for AD (Jellinger, 2004). A wealth of data from various studies has suggested that a strong association between the metabolic syndrome and vascular risk factors appears to increase the risk for AD (Martins, 2006). Specifically, diabetes mellitus, insulin resistance, high cholesterol, hypertension, reduced exercise, and obesity are all risk factors with some association for AD (Sidera, 2005; Moreira, 2007).

### **2.3 Neuropathology and Pathophysiology**

Dementia is causally associated with disruption of cerebral neuronal circuits, with the amount and location of neuronal loss resulting in its characteristic symptomatology. Loss of larger neurons of the superficial cortex is a consistent feature of AD, as are synaptic alterations such as reduction of pre-synaptic terminal density (Cummings,

1998). The neurotransmitter acetylcholine (Ach) appears to be particularly important for memory, and loss of cholinergic neurons may underlie memory loss in AD (Francis, 1999). Anatomically, AD begins in the entorhinal cortex and progresses to the hippocampus and the posterior temporal and parietal neocortex, ultimately resulting in diffuse degeneration throughout the cerebral cortex. Grossly, AD is characterized by diffuse atrophy of the cerebral cortex, reflecting loss and shrinkage of neurons, with resulting enlargement of the ventricles. In particular, the hippocampus, part of the mesial temporal lobe memory system, is damaged and atrophied in AD, even at the earliest stages of the disease (Braque, 1991; Squirre, 1991). Microscopically, the two identifying features of AD are amyloid plaques and neurofibrillary tangles. In addition to amyloid plaques, wispy accumulations of an intracellular proteomous material called neurofibrillary tangles (NFTs) are present. These are the cardinal features originally described by Alois Alzheimer. The so-called "amyloid hypothesis", which ascribes a causative role in AD to abnormal amyloid processing and deposits, remains one of the prevailing models regarding AD causation (Hardy, 2002). As AD progresses, glutaminergic, noradrenergic, and serotonergic system deficiencies develop and have been associated with further cognitive deterioration and/or behavioral abnormalities. Therapeutic efforts during the last decades have largely focused on correcting these neurotransmitter deficits, and some modest success in improving symptoms has been achieved.

## **2.4 Presentation and Natural History**

Memory loss, particularly short-term memory loss, is also the most common presenting symptom of AD. Longer-term memory is initially preserved but will eventually deteriorate as well with disease progression. This is referred to as Ribot's law of memory, but this is only relatively true, as it is difficult to check the accuracy of ancient memories (Wixted, 2004). Impairment of cognitive function that slightly interferes with the functions of daily living is characterized as mild cognitive

impairment (MCI), and many individuals with MCI will progress to AD dementia; the progression rate is about 12% per year, (Dujin, 1991) with faster progression in some subgroups, e.g. those with severe memory deficits especially when additional cognitive impairment is also present. Behavioral changes and psychiatric symptoms are not uncommon in AD, especially in the more advanced stages of the disease (Yaari, 2007). These include agitation, paranoia, psychosis, delusions, anxiety and insomnia. Frequently reported sleep disturbances include nighttime awakening, early morning awakening; excessive daytime sleepiness and on rare occasions, a diurnal reversal of sleep-wake cycle with the main sleep period occurring in the daytime (Yesavage, 2002). AD is progressive and remains incurable and ultimately it is fatal, with death typically occurring 4-6 years after initial diagnosis.

## **2.5 Clinical, pathological and radiological evaluation**

The most commonly used clinical criteria for the diagnosis of AD are those of the Diagnostic Manual of Mental Disorders, Fourth Edition (DSM-IV) (American Psychiatric Association, 1994) and those developed in 1984 by a joint task force on the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) (McKhann, 1984). Normally, a neuropsychological examination explores in depth an individual's performance in a wide range of functional domains. Various screening tests and batteries have been developed during the last years, but the Mini Mental State Examination (MMSE) is still the most widely used, despite its weakness when it comes to detecting mild dementia.

The general neurologic examination may often be normal in the demented patient with AD. Continuing gait problems can occur in the late stages of AD, leading to substantially increased risk for falls. There is currently no laboratory test to confirm the

diagnosis of AD. The prevailing neuropathologic criteria for AD are those promulgated by the National Institute on Aging (NIA) and National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) (Mirra, 1993). These criteria include minimal neocortical plaque densities that are age-adjusted but do not specify either the plaque type or the neocortical region involved. Both DSM-IV-Text Revision (DSM-IV-TR) and NINCDS-ADRDA criteria rely heavily on history and the neurologic examination, and recent evidence suggests that both have fallen behind due to the recent dramatic advances in our scientific knowledge of AD, with reliable biomarkers available now being based on structural Magnetic Resonance Imaging (MRI), molecular imaging with Positron Emission Tomography (PET), and cerebrospinal fluid (CSF) analyses (DuBois, 2007). Although the revised NINCDS-ADRDA criteria remain focused on a clinical determination of memory impairment, they also stipulate that there must also be at least one abnormal biomarker among structural neuroimaging with MRI, molecular neuroimaging with PET, and CSF analysis of  $\beta$ -amyloid or Tau proteins (DuBois, 2007). Structural MRI in patients with AD or MCI shows atrophy in the entorhinal cortex and hippocampus, predictive of future cognitive decline and conversion to AD among individuals with MCI. It has been suggested, therefore, that MRI volumetry may be a useful imaging adjunct in the diagnosis of AD and may even exceed the diagnostic accuracy of clinical evaluation (Duara, 2008; Desikan, 2009). PET-based imaging includes measurement of regional cerebral glucose metabolism (rCMR<sub>glc</sub>) using the partially metabolized glucose analog fluorine-18 (<sup>18</sup>F)-labeled 2-fluoro-2-deoxy-d-glucose (FDG). FDG-PET brain images in AD are characterized by significant regional hypometabolism. A reduction of glucose metabolism in the bilateral temporal, parietal and posterior cingulate region is currently the most commonly described diagnostic criterion for AD (Herholz, 2007).

## 2.6 Treatment

Over a hundred years after its discovery, AD remains incurable and its progression inevitable, with the primary focus of treatment on mitigation of associated behavioral and neurologic problems. Currently no therapy has been proven to delay biological progression of disease. The development of drugs that will delay disease progression in affected individuals or primarily prevent its onset in normal older subjects remains a crucial, but far elusive goal (Roberson, 2006). The currently available symptomatic therapies for AD mildly improve defects in cognitive function, activities of daily living (ADLs) and global functioning, as well as delay onset of or slightly improve behavioral symptoms (Mangialasche, 2010). The role of family members or other caregivers is critical, and any benefits need to be weighed against adverse effects that may occur in determining an appropriate dose or deciding whether to continue therapy with a particular drug (Haley, 1997). Memory aids such as notebooks and posted daily reminders may be helpful in the early stages of the disease. The patient's home, especially the kitchen and bathrooms, must be made as safe as possible, and eventually patients must stop driving and can no longer be responsible for their finances and other personal affairs.

A number of drugs have been approved for treatment of AD, albeit they are not curative. The current pharmacologic therapies for AD can be broadly divided into two categories: (1) symptomatic approaches based on enhancement of neurotransmitter systems and (2) neuroprotective strategies using antioxidants such as vitamin E. Many AD patients also are prescribed antipsychotics or antidepressants to manage psychiatric and behavioral symptoms, but with an apparently increased risk of mortality (Yaari, 2007).

The most effective medications for AD to date are the acetylcholinesterase (AChE) inhibitors, which reduce the enzymatic degradation of the neurotransmitter Ach, deficient in the AD brain, and thus enhance the cholinergic system. The three AChE

inhibitors approved by the United States Food and Drug Administration (FDA) for treatment of AD, donepezil, galantamine and rivastigmine, have been demonstrated to improve cognition, function in ADL, and behavior in patients with AD in double-blind, placebo-controlled trials (Roberson, 2006; Geldmacher , 2004). Despite the perception among clinicians of limited therapeutic efficacy and cost-effectiveness of AChE inhibitors, this class of drugs is actually highly effective in early (i.e. mild to moderate) AD in terms of symptomatic control and delay of its long-term adverse effects (Geldmacher,2004; Geldmacher,2008).

Memantine is an N-methyl-d-aspartate (NMDA) receptor antagonist also approved for use in AD and was the first drug approved for treatment of moderate to severe AD (Witt, 2004). Although its mechanism of action is not entirely understood, it works by antagonizing glutamate at the NMDA receptor, potentially improving signal transmission, and by preventing excess calcium to rush into the neurons with glutamate stimulation, and may therefore protect against toxic damage to cholinergic neurons. In a study, patients with moderate to severe AD treated with memantine alone showed significant improvement in cognitive function and ADLs in a placebo-controlled trial (Atri, 2008). In another clinical study of patients with moderate to severe AD, memantine in combination with the AChE inhibitors (donepezil, galantamine, or rivastigmine) significantly slowed deterioration in both cognitive function and ADLs compared to patients treated with placebo or AChE inhibitors alone (Atri, 2008).

## **2.7 Conclusion**

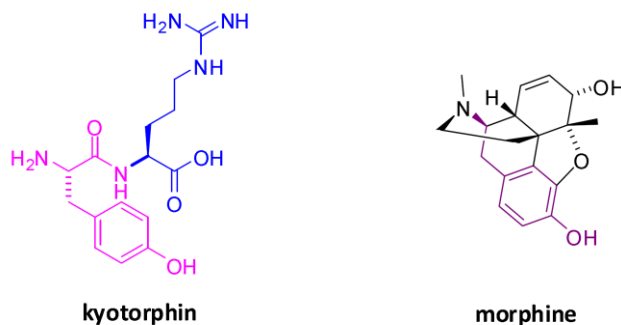
There is a compelling need to establish novel treatments for AD and research. AD therapy has been at least partly successful in terms of developing symptomatic treatments, but has also had several failures in terms of developing disease modifying therapies. While progress has been frustratingly slow in the development of effective

treatments for AD, understanding of its underlying biology continues to advance as well as the number of promising therapies.

### 3. Kyotorphin

Kyotorphin (KTP) is an endogenous peptide, with only two amino acid residues in its structure: L- Tyr-L-Arg. First isolated from bovine brain in 1979 (Takagi et al., 1979), it was subsequently found in the brains of mice and rats (Ueda et al., 1980), guinea pigs, rabbits, squirrels (Svirayev V et al., 1992) and in human cerebrospinal fluid (Nishimura et al., 1991). KTP acts as a neurotransmitter/neuromodulator in nociceptive responses in the CNS (Inoue et al., 1999), having an analgesic effect approximately 4.2 fold higher than met-enkephalin (Shiomi et al., 1981).

Kyotorphin owes its name to the city when it was discovered – Kyoto - and to its morphine-like effect. The similarities with opioid molecules go beyond the activity: structurally, both display a phenolic ring, considered essential for the interaction of morphine with receptors (Figure 4).



**Figure 4** – Chemical structures of kyotorphin and morphine. The structural resemblances are evident: the phenolic hydroxyl in close proximity to a positive charge (colored in pink and purple for KTP and morphine, respectively).

Kyotorphin is synthesized in specific brain regions where it may modulate synaptic transmission. Opioid systems may have mediated the effects of kyotorphin. The majority of research associated with kyotorphin relates to modulation of pain mechanisms via its ability to directly excite cortical neurons, and indirectly exert  $\mu$ - and

$\delta$ -opioid receptors to produce potent naloxone-reversible and long-lasting analgesia via releasing methionine-enkephalin (Met-Enk) and  $\beta$ -endorphins (Takagi, 1979; Kawabata, 1992). However, KTP showed a wide dynamic range of bell-shaped dose-response curves in peripheral pain experiments (Ueda, 1999).

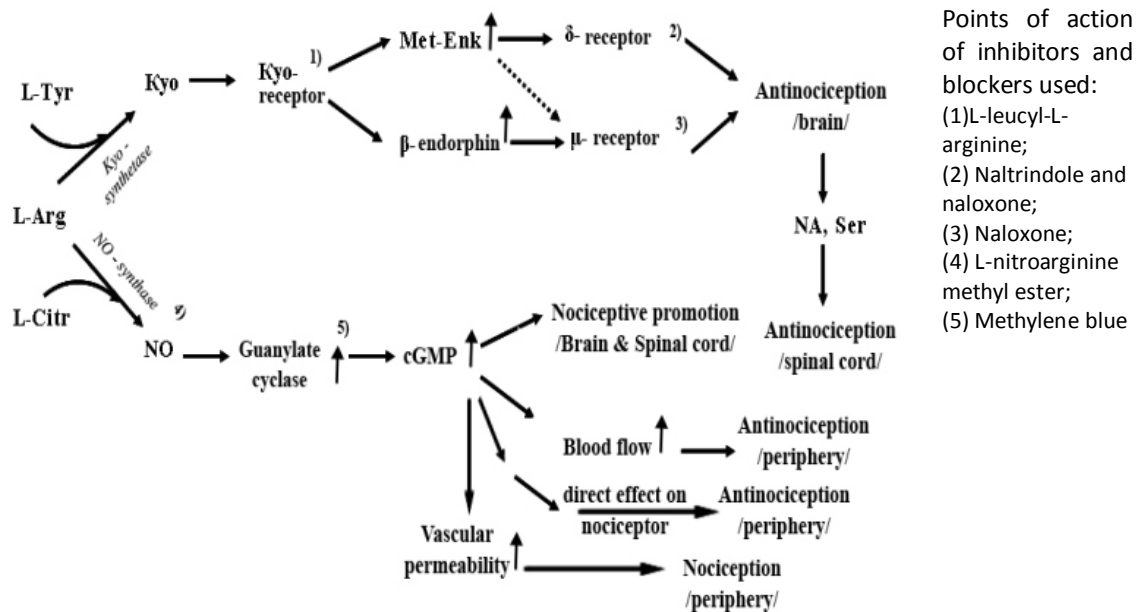
The exact mechanism of action of KTP remains an unsolved issue. While some authors defend that KTP binding to its specific receptor induces met-enkephalin release followed by activation of G-protein and Phospholipase C (Ueda, 1987); others report that KTP suffers a fast degradation, originating L-Arg, a substrate of nitric oxide (NO) synthase. This would induce the formation of NO, leading to the release of met-enkephalin (Arima, 1996; Arima, 1997). Whatever the mechanism, a release of Met-enkephalin is acknowledged by all the authors.

Figure 5 shows possible mechanisms for KTP and L-arginine nociceptive modulation in the CNS and periphery. L-Arginine, a semi-essential amino acid, localized predominantly in glial cells (Aoki, 1991), is a substrate for enzymes in the central nervous system, two of which are nitric oxide synthase (NOS) required for the production of nitric oxide (Moncada, 1992) and KTP synthetase, which catalyzes the formation of KTP. KTP is also metabolized to L-Arg – a possible substrate for inducible and neuronal NOS (Arima, 1996). Some studies revealed that KTP may be able to indirectly stimulate the release of endogenous noradrenaline and 5-HT from nerve terminals of the descending monoaminergic neurons, mediated by activating the  $\delta$ -opioid receptors, resulting in transmission blockade of the nociceptive information in the spinal cord (Ochi, 2002).

KTP-induced nociception is abolished by i.p. injection with NK1 antagonists, by local pretreatment with capsaicin to deplete substance P (SP) from nociceptor endings, or in mice with targeted disruption of the tachykinin 1 gene. In this way, KTP induced nociception through a SP release from SP-containing neurons (Inoue, 1999; Ueda, 2000).

At the same time, it has been found that the action of KTP on integrative brain functions in animals, particularly the exploratory activity in an open field, is not blocked by naloxone (Dzambazova, 2010). This fact indicates that such types of brain activity do not depend on the enkephalin-releasing mechanism of KTP effects. The non-analgesic mechanisms of KTP action remain poorly investigated. In addition to anti-nociceptive activity, this di-peptide was also reported to have several other activities, namely, inhibiting cell proliferation (Bronnikov et al., 1997), anti-hibernating regulation (Ignat'ev et al., 1998) and even an epilepsy seizure protection effect (Godlevsky et al., 1995). In the peripheral nervous system, KTP has a non-opioid activity (Inoue et al., 1997). Sakurada and collaborators, following observation of a hypothermic response in mice after i.c.v. injection of KTP, defined that KTP displays a thermoregulatory activity (Sakurada et al., 1983). Other authors reported that KTP attenuates vasopressin liberation and when in excess mimics the stress response with an increase of oxytocin and activation of sympathetic nervous system, with a consequent increase of blood pressure (Summy-Long et al., 1998). Regarding locomotor behavior, studies with gold fish and i.p. administration in doses starting from 200 µg revealed reduction in orientation as result of olfactory and sensorial stimuli (Kolaeva et al., 2000).

KTP is unequally distributed in the different brain regions, being more concentrated in mid brain, pons and medulla oblongata and spinal cord, which overlap with the brain regions more sensitive to morphine microinjection (Ueda et al., 1980). In humans, a study using cerebrospinal fluid samples revealed that in patients with persistent pain, KTP content is lower, which suggests that kyotorphin acts as a putative neuromediator and/or an endogenous pain modulator in the human brain (Nishimura et al., 1991).



**Figure 5** - Possible mechanisms for L-arginine nociceptive modulation in the CNS and periphery, and points of action of the inhibitors and blockers employed. KTP, kyotorphin; Met-Enk, methionine-enkephalin (from Dzambazova, 2010).

KTP mechanisms of action and the wide range of biological effects make possible the assumption that this peptide is a potent neuromodulator and that it can become a valuable and marketable drug if modified to have an enhanced BBB-crossing ability, while preserving most of its structure and chemistry so that the end result can remain effective and nontoxic. Most findings emphasize the effects on the CNS, including the spinal cord as well as the brain, but these effects also concern the peripheral body and communication across the BBB. Although fifty percent of the total KTP amount is found in the cerebral cortex, an area where the contents of opiate receptors and enkephalins is low and its physiological effects are opioid and non-opioid mediated, KTP actions are far more extensive, hopefully stimulating consideration of possible therapeutic applications.

## 4. Peptides as Drugs Candidates

There has been a rapid expansion in the use of peptides as drugs over the last decade, and this is likely to continue. Peptides regulate most physiological processes, acting at some sites as endocrine or paracrine signals and at others as neurotransmitters or growth factors. They are already being used therapeutically in such diverse areas as neurology, endocrinology and hematology.

Most peptides cannot be administered orally as they are rapidly inactivated by gastrointestinal enzymes, so that subcutaneous or intravenous administration is required. Therefore, research is focusing on alternative routes of delivery, including inhaled, bucal, intranasal and transdermal routes, as well as novel delivery systems such as the use of protective liposomes. Neuropeptide systems in the brain are being examined as potential targets for therapeutics, providing an exciting future development area. The dual problems of local targeted delivery and the blood-brain barrier prevent administered peptides from readily gaining access to the required site of action. (Edwards, 1999)

Usually, peptides act by binding to specific cell surface receptors. The perfect therapeutic agent would be a small-molecular-mass chemical mimic of the receptor ligand, which would be cheap to manufacture and could get to the site of action after oral administration. However, receptors are large with many binding sites, and peptides have a complex tertiary structure, both of which improve specificity as well as affording protection from simple invading molecules, like bacterial toxins. Consequently, production of successful peptide mimics using chemical libraries is a difficult task and we still largely rely on the native peptide for therapeutics (Edwards, 1999).

## **Significance and the Increasing Market**

Peptide drugs have been successfully applied in treating certain human diseases. For instance, Goserelin (a synthetic gonadotropin-releasing hormone analog) is applied to treat breast cancer and prostate cancer. Glatiramer acetate (a synthetic peptide with four amino acids) is used for multiple sclerosis and Exenatide (a synthetic glucagon-like peptide-1 analog) for type 2 diabetes. The synthetic somatostatin analogs such as octreotide and lanreotide are the most common drugs used in treating neuroendocrine tumors while conventional chemotherapy and radiotherapy have very limited effects. In the commercial market, there is no lack of blockbuster peptide drugs generating more than \$1 billion in annual sales. However, the annual sales of all the approved peptide drugs are only about 20 billion US dollars. This is just a small amount (approximately 2%) of the huge drug market. However, the approval rate for peptide drugs may be twice as high as that for small molecules. The peptide drug market is also growing twice as fast in the worldwide drug market. Currently, there are around 60-70 approved peptide drugs in the global market, with 100-200 more in clinical trials, 400-600 more in pre-clinical studies and possibly hundreds to thousands more on the laboratory bench (Sun, 2013). In table 3 we can see some of the peptide based drugs marketed in the past 10-15 years and the huge investment made.

**Table 3** – Examples of marketed peptide-based drugs (from Thayer, 2011)

GENERIC NAME	TRADE NAME	DISEASE TARGET	COMPANY	2010 SALES (\$ MILLIONS)
Teriparatide	Forteo	Osteoporosis	Eli Lilly & Co.	\$830
Exenatide	Byetta	Type 2 diabetes	Amylin/Lilly	710
Liraglutide	Victoza	Type 2 diabetes	Novo Nordisk	416
Lanreotide	Somatuline	Growth disorders	Ipsen	228
Pramlintide	Symlin	Diabetes	Amylin	92
Enfuvirtide	Fuzeon	HIV	Roche/ Trimeris	88
Ziconotide	Prialt	Pain	Elan/Azur Pharma	20 <sup>a</sup>
Icatibant	Firazyr	Angioedema	Shire	11
Ecallantide	Kalbitor	Angioedema	Dyax	9
Tesamorelin	Egrifta	Lipodystrophy	EMD Serono	na
Degarelix	Firmagon	Prostate cancer	Ferring	na
Mifamurtide	Mepact	Bone cancer	Takeda	na
Nesiritide	Natrecor	Heart failure	Johnson & Johnson	na

**NOTE:** Includes U.S. and non-U.S. approvals. **a** C&EN estimate. **na** = not available.

With the increase of approved peptide-based drugs and the advance in peptide-associated technologies, peptide-based drug therapeutics will become more significant and will open up more commercial opportunities for treating human diseases (Castanho, 2011).

## **ARTICLE 1**

# **Biomedical Applications of di and tri-peptides**

*Biopolymers Peptide Science, 2012*

*I, Sara Matos Santos, declare that the bibliographic research was conducted by me and Inês Torcato under guidance of Prof Miguel A.R.B. Castanho. This manuscript was written by me under the advice and guidance of my supervisor Prof. Miguel A.R.B. Castanho.*

## Review

# Biomedical Applications of Dipeptides and Tripeptides

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### ABSTRACT:

Peptides regulate many physiological processes, acting at some sites as endocrine or paracrine signals and at others as neurotransmitters or growth factors, for instance.

These molecules represent a major evolution in medical and industrial fields, as it is becoming mandatory to design and exploit molecules that do not necessarily fit the description of classical drug classes. The list of peptides with potential biomedical applications is huge and is growing each year. These biomedical applications range from uses as drugs to flavor-active peptides as ingredients in natural health products, nutraceuticals and functional foods. Among the peptide family, dipeptides and tripeptides are very appealing for drug discovery and development because of their cost-effectiveness, possibility of oral administration, and simplicity to perform molecular structural and quantitative structure-activity studies. Our objective is to review different actual and future uses of dipeptides and tripeptides as well as the major advances and obstacles in this growing area.

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**Keywords:** dipeptides; tripeptides; drug development; dipeptides/tripeptides transporters; biomedical applications

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### INTRODUCTION

Peptides regulate many physiological processes, acting at some sites as endocrine or paracrine signals and at others as neurotransmitters or growth factors, for instance. They are being used therapeutically in diverse areas such as neurology, endocrinology and hematology.<sup>1</sup> As many of the molecules that play crucial roles in disease processes cannot easily be modulated by small molecular weight inhibitors that recognize particular binding pockets, it is becoming mandatory to design and exploit molecules that do not necessarily fit on the description of classical drug classes, such as peptides, which have the potential to fill part of this gap.<sup>2</sup> The list of peptides with potential biomedical applications is huge and grows each year. These biomedical applications range from uses as drugs to flavor-active peptides as ingredients in natural health products, nutraceuticals, and functional foods.<sup>3</sup>

Most peptides act by binding to specific cell receptors. The perfect therapeutic agent would be a small-molecular-mass chemical mimic of the receptor ligand, cheap to manufacture, and available after oral intake. This is not easy to obtain with peptides but significant steps are being taken toward that purpose, such as encapsulation of peptides in liposomes or polymers allowing protection from digestion. The peptides recently used with a good cost-effectiveness are within the domain of nutraceuticals, such as aspartame; those with medical interest are still few with higher production costs.<sup>3</sup>

Dipeptides and tripeptides are very appealing for drug discovery and development because they combine: (i) cost-effective synthesis; (ii) possibility of oral administration<sup>4</sup>; (iii) low-molecular weight and (iv) simplicity to perform

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Table I Selected Examples of Dipeptides and Tripeptides of Biomedical Interest

	Peptide Example	Function	Origin
Nutraceuticals	Asp-Phe <sup>3</sup>	Low-calorie sweetener	Synthetic
	Ile-Tyr <sup>14</sup>	Blood pressure lowering effect	Extracts or hydrolysates of fish meat, seaweed, mushrooms
	Ile-Trp <sup>14</sup>		
	Lys-Trp <sup>14</sup>		
	Soy peptides <sup>14</sup>	Decrease muscle damage after exercise Antiobesity effects	Soy protein
Medical applications	Lys-Glu <sup>15</sup>	Antitumor activity	Synthetic
	Leu-Ile <sup>15</sup>	Neuroprotective effect	Synthetic
	Tyr-Gly <sup>16</sup>	Proliferation of peripheral blood lymphocytes	Synthetic
	Tyroservatide <sup>17</sup>	Antitumor effects on human hepatocellular carcinoma	Synthetic
	C-capped dipeptides <sup>18</sup>	Inhibitor of RND-family efflux pumps in Gram-negative bacteria	Synthetic
	Kyotorphin <sup>19</sup>	Endogenous analgesic properties	Cerebrospinal fluid

molecular structural and quantitative structure-activity studies. Crystallographic,<sup>5</sup> spectroscopic,<sup>6,7</sup> and molecular simulation<sup>8,9</sup> techniques can be used to obtain very detailed information on the conformation of dipeptides, which is important for protein docking or membrane interaction.<sup>10</sup> Both these actions are not independent.<sup>11–13</sup>

Small peptides are easily transformed into derivatives with improved pharmacological properties (specific examples are shown in Table I) or they can be used as building blocks for combinatorial libraries.<sup>20</sup> Among the chemical transformations of dipeptides, for instance, cyclization is particularly relevant.<sup>21–23</sup> Cyclization may improve both the pharmacodynamics and pharmacokinetic properties of peptides. Moreover, interaction with peptide transporters and hepatic cytochrome P450 is maintained.<sup>24,25</sup> Hydroxylation,<sup>26,27</sup> amidation,<sup>28</sup> acylation,<sup>29</sup> and binding to hydrophobic groups others than fatty acids<sup>30,31</sup> are among the other chemical transformations that may improve short peptides.

### THE LIMITATIONS OF USING PEPTIDES AS DRUGS

Peptides have attracted much attention as potential drugs due to their high selectivity, efficient activity, and low toxicity.<sup>32,33</sup> They are constituted by a short sequence of amino acid residues that can be altered to increase specificity and activity or to decrease toxicity. In addition, due to the small number of amino acid residues that constitutes them, their synthesis is less demanding than other pharmacological molecules.<sup>32</sup> Moreover, they have low toxicity as they are constituted by amino acid residues that are responsible for the regulation of numerous body processes.<sup>32</sup> Nowadays, a wide range of peptide drugs is produced on a commercial scale as result of advances in the biotechnology field representing an

annual market of \$300–500 millions and a growth rate until 25% per year.<sup>32</sup> However, the use of peptides as drugs has disadvantages. Their activity can be impaired by pH value and salt concentrations existent in physiological conditions, the synthesis process cost can be elevated, and they may have poor bioavailability due to the impermeability of some epithelial barriers and to peptides' susceptibility to peptidases.<sup>32–34</sup> In addition to important improvements in synthetic strategies, the enhancement of peptides' stability and lipophilicity is very much needed.<sup>32,33</sup>

### ADVANCES IN ORAL ADMINISTRATION OF PEPTIDE DRUGS

The most desired route of drugs administration is the oral route, but the development of peptide drugs that can be administered by this route is still a challenge.<sup>33,35</sup> To overcome the action of presystemic enzymatic degradation and poor penetration of the intestinal membrane diverse strategies have been proposed. Tight junctions' permeability modulation to increase paracellular transport of drug molecules as well as using protease inhibitors as additives are successful *in vitro* strategies that can unfortunately bring undesired side effects *in vivo*.<sup>33,36,37</sup> The use of enzyme inhibitors in long-term therapy can cause absorption of unwanted proteins, disturbance of the digestion of nutritive proteins, and stimulation of protease secretion as a result of feedback regulation.<sup>33,38</sup> To circumvent these side effects, other strategies that do not alter the normal behavior of the human organism can be used: (i) optimization of the pharmacokinetic properties of the peptides (e.g., enhancing lipophilicity and decreasing enzymatic susceptibility); (ii) conjugation with molecules recognized by membrane transport systems; and (iii) the use of a delivery carrier system.<sup>33,39</sup>

### Optimization of the Pharmacokinetic Properties of Peptides

The production of prodrugs that are only active after suffering a transformation is a strategy that can be used both to enhance lipophilicity and stability.<sup>33,39</sup> Conjugation with polyethylene glycol can be used to enhance stability because, by improving peptide solubility, it offers protection from enzymatic degradation.<sup>33</sup> Furthermore, it is possible to increase permeability by conjugating the peptide with a hydrophobic compound in a covalent (lipidization) or noncovalent manner.<sup>40</sup> Nevertheless, these alterations must not impair the biological activity of these peptides. To overcome the activity impairment caused by lipidization, a new reversible lipidization technique has been developed in which the regeneration of active polypeptides from their lipid conjugates occurs following oral absorption.<sup>41</sup>

### Conjugation with Molecules Recognized by Membrane Transport Systems

By attaching pharmacological peptides to specific ligands that are recognized by membrane transporters or by receptors that mediate endocytosis, it is possible to enhance membrane permeability.<sup>33</sup> However, it is necessary to take into account the size of the peptide because membrane transporters are usually limited to transport relatively small drugs whereas receptor-mediated endocytic systems do not seem to be limited by this factor.<sup>33</sup> In addition, cell-penetrating peptides (CPPs) have been recognized as a promising delivery system.<sup>42</sup> CPPs are chemically hybridized with target materials and cross membranes by directly perturbing the lipid bilayer structure of the cell membrane or by endocytosis. They can deliver small molecules, macromolecules, liposomes, and nanoparticles into cells or tissues without detectable undesirable side effects *in vivo*.<sup>33,42</sup>

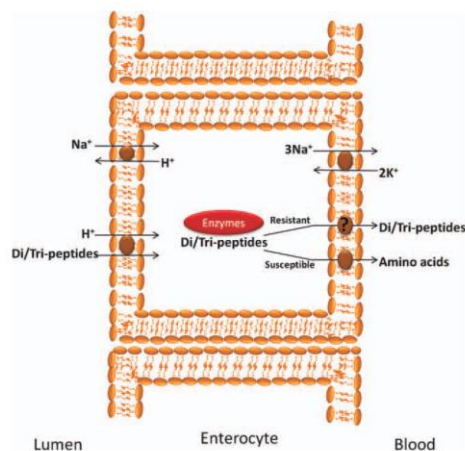
### Delivery Carrier Systems

Particulate delivery carrier systems based on hydrogels, microspheres, nanoparticles and lipid-based drug delivery systems, protect fragile peptides against enzymatic degradation and, in some cases, can be taken up by epithelial cells or by lymphoid tissues in Peyer's patches without the need of additional penetration enhancers.<sup>33</sup> Although lipid-based carrier systems (e.g., microemulsions and liposomes) have been enhanced by the application coating with mucoadhesive polymers, for example, they still have some disadvantages, like the poor stability and low efficiency in entrapping peptide drugs.<sup>33</sup> Recently, nanoparticles have attracted much attention but issues like precise control of drug release and of particle aggregation have to be optimized to develop successful oral drug delivery systems.<sup>33,43</sup>

### THE ABSORPTION OF DIPEPTIDES AND TRIPEPTIDES

The difficulty in the oral administration of peptides turns pharmacological dipeptides and tripeptides in an advantageous pharmacological possibility due to the existence of specific intestinal membrane transporters for these peptides.<sup>44</sup> Two human dipeptide and tripeptide transporters have been identified and extensively functionally characterized: human peptide transporter 1 (hPepT1) and 2 (hPepT2).<sup>35,44–46</sup> hPepT1 is expressed in the small intestine, proximal tubules of the kidney, lysosomes of liver, pancreatic, and renal cells, whereas hPepT2 is expressed in the proximal tubules, brain, lungs and in the mammary gland.<sup>45</sup> In the lung and in the brain, hPepT2 probably removes dipeptides and tripeptides arising from the activity of ectopeptidases and dipeptides and tripeptides that can have the function of neurotransmitters, respectively.<sup>44</sup> hPepT1 is constituted by 707–710 whereas hPepT2 is constituted by 729 amino acid residues. Both have several putative glycosylation phosphorylation sites, and it has been suggested that these transporters are constituted by 12 transmembrane domains, with both the C and N-terminal localized inside the cell.<sup>46</sup> Amino acid identity between these two receptors is ~50%, being the amino acid sequence more divergent in the intracellular or in the extracellular loops than in the putative transmembrane regions.<sup>46</sup> In general, PepT2 accepts the same substrates as PepT1 but with higher affinity and lower maximal uptake rate being considered as a “high affinity-low capacity” uptake system.<sup>44</sup> Small peptides are therefore prone to be systemically distributed after oral intake and reach specific tissues and organs such as the brain by using hPepT1 and hPepT2.

PepT1 and PepT2 transport dipeptides and tripeptides across epithelial membranes in an electrogenic proton-dependent manner and therefore belong to the proton-dependent oligopeptide transporter family.<sup>44,45</sup> The peptide/proton stoichiometry appears to be 1:1 for PepT1 and 1:2 for PepT2, regardless of the charge of the transported substrate.<sup>44</sup> The luminal peptide transport leads to intracellular acidification and to the activation of the apical sodium proton exchanger, whereby protons are exchanged for sodium ions. This exchanger seems to be required for optimal luminal transport of dipeptides and tripeptides across the intestinal lumen.<sup>44</sup> After entering the cells the dipeptides and tripeptides experience rapid enzymatic degradation to amino acids that are transported across the serosal membrane by a variety of specific amino acid transporters. On the other hand, dipeptides/tripeptides and peptidomimetic drug molecules resistant to enzymatic degradation in the cytosol are suggested to be transported by a saturable transport system that has not been yet identified.<sup>44,47</sup> Drug transport via this peptide system is a



**FIGURE 1** PepT1 peptide transport pathway. Dipeptides and tripeptides transport across the intestinal lumen causes intracellular acidification and consequently activation of the apical sodium proton exchanger. Inside the cell, dipeptides and tripeptides can be hydrolyzed by cellular enzymes and the resultant amino acids are transported across the serosal membrane by various specific amino acid transporters. However, dipeptides and tripeptides resistant to enzymatic hydrolysis are suggested to be transported by a saturable transport system yet to be identified.

saturable and a concentration dependent phenomenon which is characterized by the Michaelis–Menten constant.<sup>35</sup> The PepT1 transport system is schematically represented in Figure 1.

The discovery of the intestinal peptide transport system and of its broad substrate specificity has led to the development and synthesis of small peptide analogs that are metabolically stable and recognizable by the intestinal peptide transport system.<sup>35</sup> In fact, common drug classes such as  $\beta$ -lactam antibiotics, ACE-inhibitors, renin inhibitors, thrombin inhibitors, and the dipeptide-like antineoplastic drug betastin have a high bioavailability because they are recognized by these transporters.<sup>35,44</sup> Recently, the necessary structural characteristics for substrate binding to these transporters have been identified: (i) a free amine terminal is necessary as it is responsible for directing the rest of the molecule in the binding site; (ii) substrates with large, hydrophobic areas and a c-terminal proline residue are the substrates with higher affinity toward hPepT1; (iii) a peptide bond is not mandatory; and (iv) the introduction of D-amino acids decreases binding.<sup>48</sup>

A second type of dipeptides and tripeptide transporters, the dipeptides and tripeptide histidine transporter PHT1,

was identified in rat brain.<sup>44,49,50</sup> In human's hPHT1 and a sequence homolog, hPHT2, have been inferred by bioinformatic analysis.<sup>44</sup> Moreover, the expression of PHT1 mRNA has been observed in Caco-2 cells suggesting that it may participate in the intestinal absorption of peptide based agents.<sup>49</sup> Nevertheless, the roles of these transporters are not fully defined.<sup>50</sup>

## BIOACTIVE PEPTIDES AS FUNCTIONAL FOODS AND NUTRACEUTICALS

The growing demand for nutraceuticals and functional foods in our diet to promote health and reduce risk of disease has led to a plethora of research on amino acids, peptides, proteins, and protein hydrolysates with specific biological and physiological functions beyond basic nutritional contributions.

The taste of dipeptides has also been studied for a long time.<sup>3</sup> The research into the taste of synthetic dipeptides revealed that most of them appeared to be bitter, and the relationship between their physicochemical properties and bitterness has attracted the attention of the researchers. If we consider commercial application, aspartame is the only dipeptide of outstanding importance. Today almost 20,000 tons of aspartame, which is hundreds of times sweeter than sugar, is consumed annually throughout the globe as a low-calorie sweetener. The impact of sugar replacements on human health is tremendous.<sup>51</sup>

An interesting observation that captured the researchers' attention was the antihypertensive effect of peptides. Extracts or hydrolysates of fish meat, seaweed, or mushrooms were found out to exert a blood pressure lowering effect, with the active agents being identified as a few kinds of dipeptides like Ile-Tyr, Ile-Trp, and Lys-Trp. Such effects of the dipeptides in question were considered to be derived from their inhibitory effect on angiotensin-I-converting enzyme. As a result, in Japan, the extracts or hydrolysates with such dipeptides were approved as foods for specified health uses.<sup>3,14</sup>

Another example is the use of soy peptides<sup>14</sup> from soy protein that could well serve as a nutritional supplement as well as decreasing muscle damage after exercise and having antiobesity effects.

Aside from the peptides mostly applied in industry, there are a number of others that are not used practically, but their functions are still well-known. For example, kyotorphin (KTP) was isolated from bovine brain to feature analgesic effects; a synthetic dipeptide Lys-Glu was found to show anti-tumor activity, whereas Leu-Ile appeared to have a neuroprotective effect, and Tyr-Gly is known for enhancing proliferation of peripheral blood lymphocytes. The study of this and

other peptide functions and modes of action will bring new insights to this area and promote the synthesis of new peptide products with biomedical applications.<sup>15,16</sup>

### MEDICAL APPLICATIONS

Three examples have been selected to illustrate how dipeptides and tripeptides can be important for medicine.

Tyroservatide (YSV) is a bioactive tripeptide that has been shown to have antitumor effects on human hepatocellular carcinoma BEL-7402 cells *in vitro* and *in vivo*. In a study by Shi et al.,<sup>17</sup> to enhance the chemosensitivity of tumor cells, attention has been focused on multidrug resistance (MDR) modulators. These MDR modulators represent a major obstacle to the success of cancer chemotherapy. These authors evaluated the reversal effect of YSV on MDR, and explored its mechanism of action *in vitro*. Administration of YSV reversed the MDR of human hepatocellular carcinoma BEL-7402/5-FU cells significantly. The intracellular accumulation of doxorubicin and Rhodamine-123 (Rh123) were increased, which implied that the function of the P-glycoprotein (P-gp) efflux pump was inhibited by YSV. Moreover, the mRNA and protein expression of MDR gene (MDR1) were also decreased by YSV. They evaluated the reversal effect of YSV on MDR, and explored its mechanism of action *in vitro*. Administration of YSV reversed the MDR of human hepatocellular carcinoma BEL-7402/5-FU cells significantly. The intracellular accumulation of doxorubicin and Rhodamine-123 (Rh123) were increased, which implied that the function of the P-glycoprotein (P-gp) efflux pump was inhibited by YSV. Moreover, the mRNA and protein expression of MDR gene (MDR1) were also decreased by YSV. In a study by Zheng et al.,<sup>52</sup> YSV was also able to inhibit tumor growth and metastasis in lung cancer. This highlights this tripeptide as one extra future weapon in the field of Oncology.

Inhibitors of drug efflux pumps have great potential as pharmacological agents that restore the drug susceptibility of MDR bacterial pathogens. Attention has been focused on the discovery of small molecules that inhibit the resistance nodulation division (RND) family drug efflux pumps in Gram-negative bacteria. The prototypical inhibitor of RND-family efflux pumps in Gram-negative bacteria is MC-207,110 (Phe-Arg- $\beta$ -naphthylamide), a C-capped dipeptide.<sup>18</sup> These compounds act by competitively binding the substrate interacting sites of RND-family efflux pumps; indeed, they are recognized and exported by RND pumps, and constitutes a promising class of new drugs in the field of Infectiology.

KTP is a dipeptide (Tyr-Arg) with endogenous analgesic properties, first described by Takagi et al.,<sup>53</sup> isolated from bovine brain. It was subsequently isolated from other sources,

including brains of mice and rats,<sup>19</sup> guinea pig, rabbits, squirrels, and detected in the cerebrospinal fluid in humans.<sup>54</sup> Despite being about four times more analgesic than endogenous opioids,<sup>19</sup> KTP effect is only induced after direct injection in the brain. This limited capacity to cross the blood-brain barrier prevents its pharmacological applications.

KTP is synthesized in nerve terminals and released by depolarizing stimuli. It may modulate the synaptic transmission and directly excite cortical neurons. Indirectly, it exerts opioid-like actions, producing analgesia through release of met-enkephalin.<sup>55</sup> There is evidence that the peptide has neuroprotective and neuromodulating properties,<sup>55</sup> also acting as a neuroleptic and inhibiting calcium-dependent currents in the postsynaptic membrane.<sup>56</sup> Other studies, by means of animal models, have revealed the neuroprotective actions of KTP in the hippocampus and cerebellum. It is also clear the anticonvulsant effect reported in animal models of epilepsy.<sup>57</sup> A nonopioid activity was also described in the peripheral nervous system and other organs like the heart. It was shown that the KTP, due to its L-arginine group, could act as substrate for nNOS (nitric oxide synthase located in neurons).<sup>56</sup>

Recently, we discovered and characterized a new kyotorphin derivative, KTP-amide. This new peptide, KTP-NH<sub>2</sub>, is analgesic upon systemic administration to animal models, which, combined with an inhibition of nociceptive responses of spinal neurons, suggests a central effect of the drug.<sup>28</sup>

### CONCLUSIONS

In this work, some of the different uses for peptides and future developments have been described. This is an area of actual significance as new peptides with biomedical applications are intensively pursued, as well as new ways of delivery, which involve the parenteral and nonparenteral route. In the future, it is expectable that peptides may thrive as drugs to fight common diseases and that their industrial uses as functional foods and nutraceuticals will also increase.

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## 5. Main Goals of the Project

This project aims to unravel the relationship between the mechanisms of pain and Alzheimer's disease (AD). Specifically, we will evaluate the potential of the peptide kyotorphin as the link of both diseases. Additionally, 1) its possible use as a marker in the diagnosis of Alzheimer's disease will be addressed and 2) the therapeutic effect of recently discovered analgesic kyotorphin derivatives in an animal model of Alzheimer's disease will be evaluated. With the use of human samples and duly supported by studies in animal models, the project will extend the knowledge in the area of new pain drugs, diagnosis and treatment of Alzheimer's disease, aiming at important implications for patients suffering from these conditions.

The main objective of this project is to supplement the gaps of knowledge in a largely overlooked area and on which the available information is contradictory:

- 1) Relationship between pain and Alzheimer's Disease
- 2) Potential for kyotorphin derivatives as analgesic drugs capable of neuromodulation, and
- 3) Potential of endogenous kyotorphin as a biomarker in Alzheimer's Disease.

To achieve these goals we used a multitarget approach: *in vivo* evaluation of analgesic potency, tolerance and side effects of previously synthesized KTP derived peptides, pain evaluation of Alzheimer's disease patients, KTP determination levels in cerebrospinal fluid samples of AD patients and testing of KTP effects in an animal model of AD.

In the following sections, published results are presented in the paper format. A brief motivation for each study was included.

Section 2 is dedicated to the theme of pain evaluation in Alzheimer's disease and Section 3 gathers three articles relating to the potential of KTP as an analgesic capable of neuromodulation. In section 4, the final conclusions of the thesis are presented.



Section 2

# Pain in Alzheimer's Disease

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## **ARTICLE 2**

# **The Use of Visual Analog Scales to Compare Pain between Patients with Alzheimer's Disease and Patients Without Any Known Neurodegenerative Disease and Their Caregivers**

***American Journal of Alzheimer's Disease and Other Dementias, 2013***

*I, Sara Matos Santos, declare that the experimental design, the data collection, the data analysis and discussion were carried on by me under supervision of Prof. Miguel A.R.B. Castanho. The manuscript was written by me under the guidance of my supervisor Prof. Miguel A.R.B. Castanho.*

## Neurologic Diseases and Pain

Clinical neuroscience studies suggest that chronic pain is dependent on brain function. Recent advances in pain research, with the aid of neuroimaging studies, have engendered a transformation in our understanding of how pain affects the brain. As a result, the notion that changes in sensory systems are the predominant process in chronic pain has been replaced by a conceptualization of chronic pain as a very complex central nervous system (CNS) state in which patterns of sensory system activation are integrated apparently with activity in other brain systems, including emotional, cognitive and modulator processes (Borsook, 2012).

There are causes such as peripheral nerve injury-induced pain (neuropathic pain) that affect a large number of brain regions with a wide range of other functions such as the anterior cingulate cortex, insular cortex, ventrolateral orbitofrontal area, amygdala, striatum, thalamus, hypothalamus, rostral ventromedial medulla, periaqueductal grey, pons (locus coeruleus), red nucleus, medulla oblongata and other less obvious causes of pain including those associated with primary depression where there is no injury or prior pain condition (Borsook, 2012). In many cases, such as in Parkinson's disease, chronic pain is a direct result of the neurological disease, or may even be considered an integral part of the underlying disease (Ford, 2010).

There are both sensory and affective (behavioral) dimensions in pain, and the sensation is often accompanied by desires to terminate, reduce, or escape its presence. Pain is shaped by the situation in which it emerges, by an individual anticipation and emotional feelings. These contextual and cognitive factors are partly the result of the fact that pain often occurs in threatening contexts, such as physical trauma or disease (Price, 2002). There is a complex interaction between pain effects on brain and emotional processing: depression with symptoms such as low energy and sleep disturbances, are commonly found in patients with co-morbid pain, whereas the opposite is true for symptoms such as guilt and loneliness. Increasingly, major depression is seen as being composed of psychological, somatic and painful physical symptoms (Lepine and Briley, 2004).

Part of the affective dimension of pain is the moment-by-moment unpleasantness of pain, with feelings such as annoyance, fear, or distress. Pain-unpleasantness is often, though not always, closely linked to both the intensity and unique qualities of the painful sensation. Another component of pain affect, "secondary pain affect", includes emotional feelings directed toward long-term implications of having pain (e.g., "suffering"). These pain dimensions and their interactions relate to ascending spinal

pathways and a central network of brain structures that process nociceptive information both in series and in parallel (Price, 2002).

### **Alzheimer's disease (AD) patients and pain**

Progressive dementia encompasses a variety of diseases, with Alzheimer's being the most prevalent (Cacabelos, 2012). This disease leads to a deterioration of intellectual faculties, such as memory, concentration, and judgment, resulting from an organic disease or a disorder of the brain. It is sometimes accompanied by emotional disturbance and personality changes. Its course is gradual and results in significant impairment of social and occupational functioning (Am Psy Ass, 1994).

Overall, older people are more likely to have painful pathology due to the increased incidence of chronic medical conditions, particularly of rheumatologic (osteoporosis, osteoarthritis) and oncology fields; pain represents 25-50% of the complaints in outpatient and 45-80% of the complaints in institutionalized patients (Scherder, 2008). Insufficient use of analgesics for treating nursing home residents with pain was frequently reported, especially in those with a low cognitive status (Takai, 2010). Several epidemiological studies have shown that in many cases, pain in the elderly is not recognized and therefore not treated (Frampton, 2003), which can alter their quality of life, increasing phenomena such as depression, aggression, social withdrawal and decreased function.

In the case of AD, studies show that sensory-discriminative components of pain are preserved even in advanced stages of the disease (Benedetti, 2004; Schmidt, 2010), while pain tolerance increases with disease severity (Benedetti, 1999). However a recent study (Jensen-Dahm, 2014) showed no decrease in pain tolerance for mild to moderate AD patients. A study by Cole (2006), using brain imaging (fMRI) reports that pain perception and processing are not diminished in AD.

The motor and cognitive impairment in AD patients is accompanied by a reduction in the ability to communicate, which makes it difficult to detect pain in these patients. By failing to obtain adequate pain treatment, structural and irreversible changes may occur in central systems structures involved in the transmission/modulation of nociceptive information, which accounts to chronic pain installation (Borsook, 2012). Curiously, the two components of the pain response are differentially affected in AD patients (Benedetti, 1999; Farrell, 1996): whereas the sensory-discriminative

component is preserved, pain tolerance, associated with the affective-emotional aspect, largely increases.

These apparent discrepancies appear to have a neurobiological explanation since the somatosensory cortex and thalamic nuclei involved in sensory-discriminating component of pain response appear to be preserved in AD, while the neuronal loss was detected in the prefrontal and limbic structures, with obvious implications for affective-emotional pain-related reactions (Scherder, 2008).

Alzheimer's disease is a double-edged sword when it comes to pain assessment. Pain affects cognitive function (Moriarty, 2011) and cognitive function also affects pain assessment and pain treatment because the primary method for pain assessment is still patient reporting (Licht, 2009).

The evaluation of pain, with no specific test or tracers and large individual variability, is always complex. To worsen matters, in the case of dementias one third of the patients are in later stages of the disease, therefore unable to complete a proper evaluation (Krulwich, 2000). According to the 2002 "American Geriatrics Society Panel on persistent pain in older persons", the assessment of pain is extremely important in patients suffering dementia and should be performed using validated scales. There is evidence that the administration of pain questionnaires can be reliable in mild and moderate cognitively impaired people (Rastogi, 2012), although there are fears about the expressive and receptive language abilities, which deteriorate during the course of AD (Martine, 1983). As children under 7 years old also have problems with language, use of visual analogue scales (VAS) developed for them can be reliably administered in early/moderate patients with AD (Martine, 1983; Scherder, 2000). A variety of instruments are available to measure pain intensity. Psychometric evaluation of pain intensity scales suggests that variations of the numeric rating scales (NRS), verbal descriptor scale (VDS), faces pain scales (FPS), and visual analogue scale (VAS) are appropriate for use with older adults. As mentioned earlier, a prerequisite for selecting an appropriate pain measurement scale involves determining the individual's ability to learn, and understand the directions for completing the tool (Herr, 2001).

Many times, the health professional evaluating patients with AD relies on the family caregiver testimony, which accuracy may be impacted by behavioral changes of the patient and be in potential disagreement with that of the patients themselves (Karp, 2008; Miaskowski, 1997; Arons, 2013). In the case of AD, studies comparing self-report and family caregiver pain perception are scarce. However, many actuation protocols in

the field of pain are based on caregiver report. Moreover, accurate pain and comfort assessment relieves patient and caregiver associated stress (Lim, 2004).

### **Outline and main findings of the experimental work**

The objectives of this study were 1) to compare the pain intensity and to a lesser degree pain affect both in AD and in cognitively normal patients and 2) to analyze the difference in pain perception between patients and their family caregivers. We evaluated the pain intensity report of 121 patients with chronic osteoarticular pain, 60 with mild to moderate AD and 61 without any know neurodegenerative disease, using the colored pain scale/faces pain scale (CAS/FPS), and the caregiver's perception.

Self-report is a valuable tool to assess pain intensity because it is simple to use in the daily setting and permits to evaluate the variation of pain in the same individual during time (Herr, 2001). It was also shown that elders without cognitive impairment and those in early stages of disease can report their pain degree in a reliable way (Pautex, 2006). However, studies also point to AD patients reporting less pain as their disease progresses (Scherder, 2008; Farrell, 1996; Herr, 2001).

In our study, both in the case of CAS and FPS, AD patients reported significantly less pain in comparison with normal (N) patients. Furthermore, the discrepancy between FPS and CAS was higher in AD than in N group. These results suggest that even in mild to moderate cases of the disease, pain perception can be affected, with demented patients reporting less pain. Also, the discrepancy between FPS and CAS could also suggest that the affective component of pain in AD is compromised relatively to normal patients, and/or that AD patients do not value their complaints even in mild/moderate stages of the disease.

We also found that, in contrast to other caregiver pain reports, namely in the oncology field (Yeager, 1995; Arons, 2013), family caregiver considered the pain degree of their relative substantially lower in the case of AD patients. This is important since in most cases surrogates are the primary source of information regarding the well-being of AD patients, and health professionals rely on the caregiver information to add or alter prescribed drugs such as painkillers.

As mentioned earlier, most researchers agree that in patients with dementia, particularly of the Alzheimer type, sensitivity to pain is kept, although the emotional component, dependent on higher brain structures is reduced or even abolished. This

means that AD patients present less pain complaints which appear to decrease as the disease progresses. Our study supports this: we found that in patients with prior osteoarticular pathology, with and without Alzheimer's, AD group reported less subjective pain, and that their pain was not as perceived by their family caregiver as in normal patients.

This study also points to the need of, when evaluating AD patients, always measure their pain level using appropriate scales instead of solely depending on the information provided by their primary care provider.

# American Journal of Alzheimer's Disease and Other Dementias

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## **The Use of Visual Analog Scales to Compare Pain Between Patients With Alzheimer's Disease and Patients Without Any Known Neurodegenerative Disease and Their Caregivers**

Sara Santos and Miguel Castanho

*AM J ALZHEIMERS DIS OTHER DEMEN* published online 26 December 2013

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# The Use of Visual Analog Scales to Compare Pain Between Patients With Alzheimer's Disease and Patients Without Any Known Neurodegenerative Disease and Their Caregivers

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## Abstract

Patients diagnosed with Alzheimer's disease (AD) tend to underestimate their pain degree as disease progresses. Their caregivers are the most important source of information by providing regular pain evaluation. Our objectives were to compare pain intensity and affective pain between patients with AD and cognitively normal individuals (N) and to evaluate differences in pain perception between their caregivers. We evaluated pain scores of 121 patients with chronic osteoarticular pain, 60 AD, and 61 N using the colored pain scale/faces pain scale and the caregiver's perception. Data were analyzed using one and two-tailed paired t tests ( $P < .05$ ). We found that the AD group reported less pain intensity and that their pain was less perceived by their caregiver. This study also points to the need of, when evaluating patients with AD always measure their pain degree using appropriate scales, instead of relying only on the caregiver.

## Keywords

Alzheimer, pain, scales, caregiver

## Introduction

Progressive dementia encompasses a variety of diseases, with Alzheimer's disease (AD) being the most prevalent.<sup>1</sup> This disease leads to a deterioration of intellectual faculties, such as memory, concentration, and judgment, resulting from an organic disease or a disorder of the brain. It is sometimes accompanied by emotional disturbance and personality changes. Its course is gradual and results in significant impairment of social and occupational functioning.<sup>2</sup>

Overall, older people are more likely to have painful pathology due to the increased incidence of chronic medical conditions, particularly of rheumatologic osteoporosis, osteoarthritis, and oncology fields; pain represents 25% to 50% of the complaints in outpatient and 45% to 80% of the complaints in institutionalized patients.<sup>3</sup> Insufficient use of analgesics for treating nursing home residents with pain was frequently reported, especially in those with a low cognitive status.<sup>4</sup> Several epidemiological studies have shown that in many cases pain in the elderly patients is not recognized and therefore not treated,<sup>5</sup> which can alter their quality of life, increasing phenomena such as depression, aggression, social withdrawal, and decreased function.

There are several studies pointing out that in AD, perception and processing of pain are not decreased, in contrary to what was

usually accepted.<sup>6,7</sup> The motor and cognitive impairment in patients with AD is accompanied by a reduction in the ability to communicate, which makes it difficult to detect pain in these patients. By failing to obtain adequate pain treatment, structural and irreversible changes may occur in the structures of the central nervous system involved in the transmission/modulation of nociceptive information, which accounts to chronic pain installation.<sup>8</sup> Curiously, the 2 components of pain response are differentially affected in patients with AD.<sup>7,9</sup> Although the sensory-discriminative component is preserved, pain tolerance, associated with the affective-emotional aspect, largely increases.

These apparent discrepancies appear to have a neurobiological explanation since the somatosensory cortex and thalamic nuclei involved in sensory-discriminating component of pain response appear to be preserved in AD while the neuronal loss was detected in the prefrontal and limbic structures, with

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**Table 1.** Demographic Characteristics of the Controls (N Group) and Patients With AD (AD Group).

	Controls	Patients With AD
Sex	69% female; 31% male	72% female; 28% male
Age	68.8 (SD 7.2)	72.8 (SD 6.8)
Educational level (% of the patients)	Elementary school not finished: 24.5; elementary school: 54.09; lower secondary school: 13.11; higher secondary school: 4.91; higher vocational training for 18+/university: 3.27; (average: 2.1)	Elementary school not finished: 50; elementary school: 43.33; lower secondary school: 5; higher secondary school: 1.67; higher vocational training for 18+/university: 0; (average: 1.6)

Abbreviations: AD, Alzheimer's disease; SD, standard deviation; N, cognitively normal individuals.

obvious implications for affective-emotional pain-related reactions.<sup>3</sup> Although psychophysical and neuroimaging techniques have evolved over the recent years in order to unravel the structural and functional changes in the brain of patients with AD, it remains challenging interpreting the relationship between these tools and pain reports.<sup>10</sup>

The evaluation of pain, with no specific test or tracers and large individual variability, is always complex. To worsen matters, in the case of dementias, one-third of the patients are in later stages of the disease therefore unable to complete a proper evaluation.<sup>11</sup> According to the 2002 "American Geriatrics Society Panel on persistent pain in older persons,"<sup>12</sup> the assessment of pain is extremely important in patients having dementia and should be performed using validated scales. There is evidence that the administration of pain questionnaires can be reliable in people with mild and moderate cognitive impairment,<sup>13</sup> although there are fears about the expressive and receptive language abilities that deteriorate during the course of AD.<sup>14</sup> As children less than 7 years old also have problems with language, use of visual analog scales (VASs) developed for them can be reliably administered in early/moderate patients with AD.<sup>14,15</sup> A variety of instruments are available to measure pain intensity. Psychometric evaluation of pain intensity scales suggests that variations in the numeric rating scales, verbal descriptor scale, faces pain scales (FPSs), and VAS are appropriate for use with older adults. As mentioned earlier, a prerequisite for selecting an appropriate pain measurement scale involves determining the individual's ability to learn and understand the directions for completing the tool.<sup>16</sup>

Frequently, the health professional relies on the family caregiver testimony when evaluating patients with AD. However, the accuracy of this testimony may be impacted by behavioral changes of the patient and be in potential disagreement with that of the patients themselves.<sup>17-19</sup> In the case of AD, studies comparing self-report and family caregiver pain perception are scarce. However, many actuation protocols in the field of pain are based on caregiver report. Moreover, accurate pain and comfort assessment relieves patient and caregiver-associated stress.<sup>20</sup>

The objectives of this study were (1) to compare the pain intensity and to a lesser degree the affective component of pain in both patients with AD and N and (2) to analyze the difference in pain perception between patients and their family caregivers.

## Methods

Patients and their caregivers were informed about the aim and procedure of this study and gave their informed consent. A local medical ethical committee approved the study.

### Patients

The sample consisted of 138 patients with chronic osteoarticular pain (selected from a Portuguese hospital database), 75 of them with the diagnosis of AD (AD group) and 63 without any known cognitive impairment (N group). Patients who had a history of cerebral injury, transient ischemic attack, neoplasm, epilepsy, disturbances of consciousness or focal brain disorders, or were incapable of communication were excluded from participation. After exclusion criteria, the final sample consisted of a total of 121 patients, 60 in the AD group and 61 in the N group. In the AD group, 72% of the individuals were female, and the mean age was 72.8 (standard deviation [SD] 6.8) years old. The mean time of diagnosis and follow-up in neurology consultation was of 14.8 months. In the N group, the individuals had similar population characteristics: 69% female and an average age of 68.8 (SD 7.2) years old. They did not have any known cognitive impairment and were subjected to the same evaluation. The AD and the N groups were also compared for education using 5 categories (Table 1): elementary school not finished, elementary school, lower secondary school, higher secondary school, and higher vocational training for 18+/university. Scoring these 5 categories 1 to 5 from the lowest to the highest academic level, the mean values for AD and N groups were 1.6 and 2.1, respectively.

All patients with AD met the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria for the clinical diagnosis of probable AD.<sup>21</sup> Mini-Mental State Examination (MMSE) in patients with AD was obtained in a previous psychological consultation (comprehensive evaluation of cognitive, behavioral, and emotional functioning performed using standardized tests and procedures such as MMSE, Mini-cog, and mood valuation). They presented MMSE values of 19.4 (SD 2.1), meaning mild to moderate cognitive impairment.

The patients in the AD group were selected based on the following criteria:

- moderate AD with signs of moderate cognitive impairment (difficulty in concentrating, decreased memory of recent events, and difficulties in managing finances, or traveling alone to new locations; some patients in these stages have trouble in completing complex tasks efficiently or accurately and may be in denial about their symptoms) assessed at Neurology and Psychology consults (ie, stages 4-5 of the Global Deterioration Scale)<sup>22</sup>;
- previous complaints of osteoarticular pain due to arthritis/arthrosis for more than 3 months, ability to perform simple tasks (meaning these patients still remember significant details about themselves and their family and require no assistance with eating or using the toilet), and recognize patterns.

Patients with AD were compared to individuals without any known neurologic disease but with a similar history of osteoarticular pain.

### Characteristics of Painful Conditions

A prerequisite for participation in the survey was that the participants had complaints of chronic osteoarticular pain at rest for more than 3 months before the diagnosis of AD, determined by review of the medical records. These painful conditions included arthritis/arthrosis, status post recent fractures (in the last year) or miscellaneous conditions (tendinitis and neuropathy), and the number of painful conditions did not differ between the AD and the N groups ( $\chi^2 = 0.5$ ,  $df = 2$ ,  $P > .05$ ). Other comorbidities such as congestive heart failure, pulmonary disease, diabetes mellitus, chronic renal disease, hypertension, and tumors were also evaluated and their incidence did not differ between the groups.

### Pain Evaluation

There is evidence that the patient's self-report is an accurate and reliable evidence of the existence of pain and its intensity<sup>16,23</sup> using appropriate scales, such as VAS.

The colored analog scale (CAS; Figure 1) was initially developed to assess the intensity of pain in children in a non-verbal manner. Selecting the appropriate scale position takes place by sliding a horizontal marker from the bottom (no pain) to the top (maximum pain). The patient's score is the numerical value on the back of the scale, which matches the selected scale position.<sup>24</sup>

The FPS (Figure 2) primarily measures the intensity of pain and, to a lesser extent, its affective components. This scale consists of 6 design faces lined with a neutral face, and the other 5 corresponding to a series of progressively distressed facial expressions/increasing pain sensations. The faces are ranked 0 to 10, from left to right. Individuals can set their sensations as "no pain" (score 0) to "most severe pain" (score 10).<sup>25</sup>

Psychometric evaluations of the FPS suggest that it is an authentic and a valid alternative for measuring pain intensity in cognitively intact elders and elders with mild to moderate

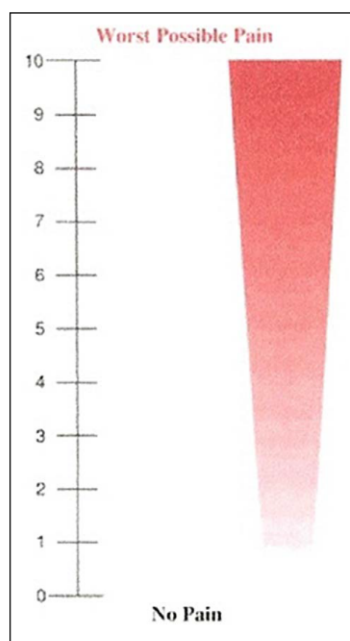


Figure 1. The Colored Pain Scale

impairment.<sup>25</sup> Preliminary evaluation of the FPS with 39 cognitively intact and impaired African American older adults suggests that the FPS may actually measure a broader pain construct that includes affective and sensory components.<sup>26,27</sup> The FPS is also advantageous for older adults with limited education, low literacy levels, or dyslexia.<sup>16</sup>

**Administration of scales.** Evaluation of patients took place once in the morning at a quiet hospital office. During evaluation, patients omitted their usual analgesic medication. Family caregivers (people living with the patients or caring for them on a daily basis) completed the tools at the same time but in a different setting to avoid knowing of each other's results.

First, we analyzed whether patients understood the concept of the scale. In the case of FPS, they were asked to indicate which face showed most pain and which face showed least pain. For the CAS, they were asked to indicate at what level the marker should be positioned when a person had the most severe pain (top of the scale) or no pain at all (bottom of the scale). They were then asked to paint on the CAS where the marker should be to match their own pain and on the FPS to paint the face which best reflected their pain. The CAS was also shown to the family caregiver who was asked to indicate which level of pain intensity would be appropriate for describing their relative's degree of pain.

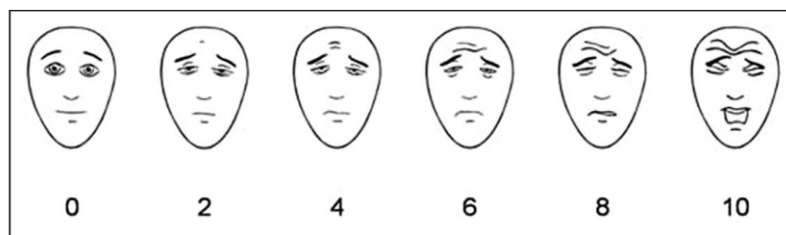


Figure 2. The Faces Pain Scale

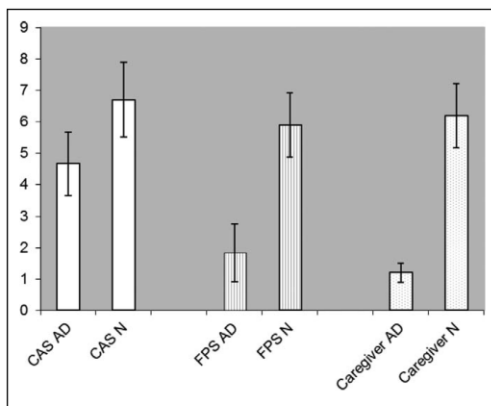


Figure 3. Difference in pain report between AD group and N group.

### Data Analysis

The SPSS-PC program was used for statistical analyses, including 1-tailed and 2-tailed paired *t* tests for comparing pain intensity. The significance level was accepted for a  $P < .05$ . The normality of data distribution was analyzed using the Kolmogorov-Smirnov test ( $P > .05$ ).

### Results

Data were collected and analyzed for the:

1. Patients with AD and N report on pain using CAS and FPS
2. Family caregivers report on pain of patients with AD (FAD) or N (FN) using CAS and FPS

The SPSS-PC program was used for statistical analyses, including 1-tailed and 2-tailed paired *t* tests for comparing pain intensity. The significance level was accepted for a  $P < .05$ .

### Colored Analog Scale/FPS

Report on pain intensity (Figure 3):

1. Regarding CAS, we found a statistically significant difference ( $P < .05$ ) between the AD and the N groups: 4.67 (SD 1.01) versus 6.7 (SD 1.19), respectively (Table 2).
2. Regarding FPS, we found a statistically significant difference ( $P < .05$ ) between the AD and the N groups: 1.83 (SD 0.92) versus 5.9 (SD 1.02), respectively (Table 3).
3. The family caregivers were asked about the degree of pain of his or her relative using CAS. The results differ ( $P < .05$ ) between FAD and FN scores: 1.2 (SD 0.3) versus 6.2 (SD 1.12), respectively (Table 2).

### Discussion and Conclusions

In this study, we evaluated the pain intensity report in patients with mild to moderate AD using 2 self-report scales: CAS and FPS. Self-report is a valuable tool to assess pain intensity because it is simple to use in the daily setting and permits to evaluate the variation of pain in the same individual during time.<sup>16</sup> It was also shown that elders without cognitive impairment and those in early stages of disease can report their pain degree in a reliable way.<sup>23</sup> However, studies also point to patients with AD reporting less pain as their disease progresses.<sup>3,9,16</sup>

In our study, both in the case of CAS and FPS, patients with AD reported significantly less pain in comparison with normal individuals. Furthermore, the discrepancy between FPS and CAS was higher in the AD group than in the N group. These results suggest that even in mild to moderate cases of the disease, pain perception can be affected, with patients with dementia reporting less pain. Also, the discrepancy between FPS and CAS could also suggest that the affective component of pain in AD is compromised relatively to normal individuals, and/or that patients with AD do not value their complaints even in mild/moderate stages of the disease.

We also found that, in contrast to other caregiver pain reports,<sup>19</sup> the family caregiver considered the pain degree of their relative substantially lower in the case of patients with AD. This is important since in most cases they are the primary source of information regarding the well-being of patients with AD, and health professionals rely on the caregiver information

**Table 2.** Difference in Pain Intensity Report Between the AD and N Groups Using CAS, and Family Caregiver's Pain Report.

	Controls		Patients With AD		Student t Test
	Mean	SD	Mean	SD	P
CAS	6.7	1.19	4.67	1.01	<.05
Family caregiver report	6.2	1.12	1.2	0.3	<.05

Abbreviations: AD, Alzheimer's disease; CAS, colored analog scale; SD, standard deviation.

**Table 3.** Difference in Pain Intensity Report Between the AD and N Groups Using FPS.

	Controls		Patients With AD		Student t Test
	Mean	SD	Mean	SD	P
FPS	5.9	1.02	1.83	0.92	<.05

Abbreviations: AD, Alzheimer's disease; SD, standard deviation; FPS, faces pain scale.

to add or alter prescribed drugs such as painkillers. The FN group result matches the CAS N and FPS N results, which is self-consistent and a sign that the method is robust.

As mentioned earlier, most researchers agree that in patients with dementia, particularly of the Alzheimer type, sensitivity to pain is observed, although the emotional component, dependent on higher brain structures, is reduced or even abolished. This means that patients with AD present less pain complaints, which appear to decrease as the disease progresses. Our study supports that in patients with prior osteoarticular pathology, with and without AD, the AD group reported less subjective pain, and that their pain was not as perceived by their family caregiver as in normal individuals.

This study also points to the need of, when evaluating patients with AD, always measure their pain level using appropriate scales instead of solely depending on the information provided by their primary care provider.

### Limitations

The most significant limitations of this study are:

- Data are not a demonstration that there is less pain in patients with AD because only specific brain areas are affected, which could be evaluated measuring responses to determined pain stimuli;
- Pain reports were for a point in time; assessment on various days would be valuable, as pain report is dependent on several physical and psychological factors that can vary over days;
- Our patients were in a mild/moderate stage of the disease. We do not know how their behavior and their caregiver perception would change with the evolution of disease.

For that, we suggest a study including groups from different stages of the disease or following the same patients during an enlarged period of time.

### Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## Section 3

# Kyotorphin and derivatives in Alzheimer's Disease

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### **ARTICLE 3**

## **Neuropeptide kyotorphin (tyrosyl-arginine) has decreased levels in the cerebro-spinal fluid of Alzheimer's disease patients: potential diagnostic and pharmacological implications**

***Frontiers in Aging Neuroscience, 2013***

*I, Sara Matos Santos, declare that the experimental design, the sample collection and the data analysis and discussion were carried out by me under supervision of Prof. Miguel A.R.B. Castanho. The manuscript was written by me under the guidance of my supervisor Prof. Miguel A.R.B. Castanho. Laura Garcia-Nimo and Prof. José Cocho developed the mass spectrometry techniques capable of quantifying KTP in CSF. Sónia Sá Santos and Prof. Isaura Tavares collaborated in the results, discussion and preparation of the manuscript.*

## **Kyotorphin as a possible biomarker in Alzheimer's Disease**

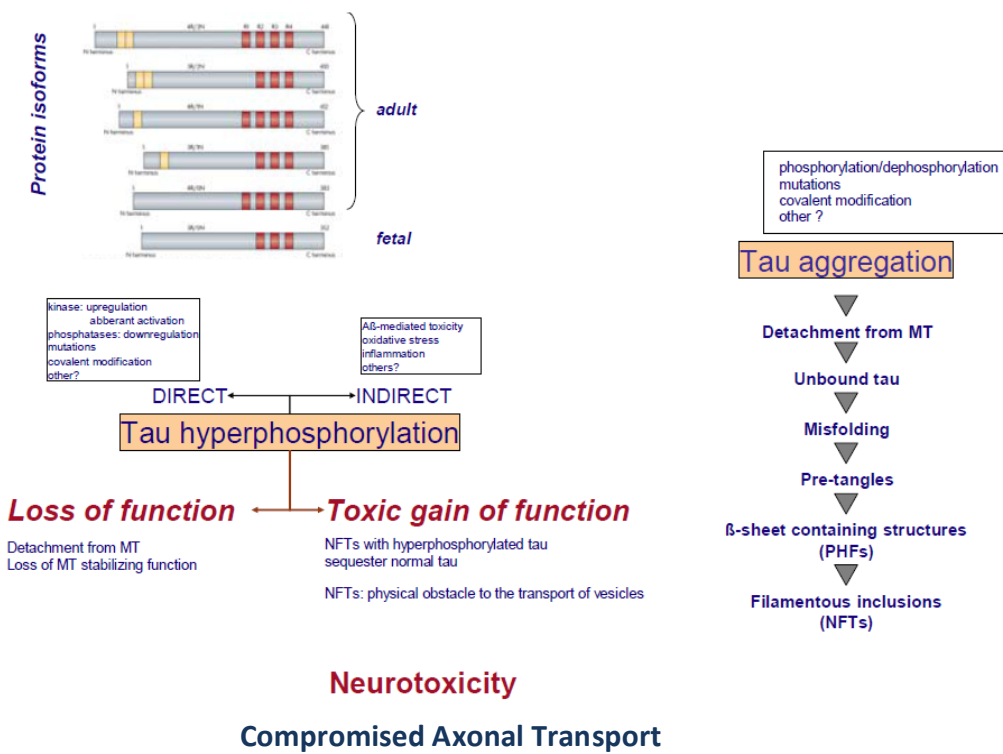
As shown in previous studies – see introduction and article 2 - underestimation of pain is frequent in Alzheimer's disease, which leads to two undesired clinical settings: 1) Unrelieved suffering of the patients due to inadequate treatment, and 2) Pain itself is a factor that contributes to the aggravation of neurodegeneration. The benefits of finding a biomolecular marker for pain is two-fold: i) To have an objective material parameter to estimate pain, regardless of the state of the cognitive impairment of the patient, and ii) The biomarker molecule itself may be involved in the mechanism of pain/nociception and therefore constitute a drug lead worth developing. The quest for a biomarker of pain, which would additionally be able to potentially be used as anti-nociceptive drug, is an endeavor worth pursuing. Our work is a contribution towards this end.

Despite KTP efficient analgesic activity, which is about 4 times higher than the endogenous opioids (Shimoni, 1981), it has been suggested that KTP has neuromodulating and neuroprotective (in hippocampus and cerebellum) properties (Bocheva, 2004; Nazarenko, 1999), as well as an antiepileptic effect (Ribeiro, 2011). This data led us to the hypothesis that KTP-NH<sub>2</sub> may be simultaneously an analgesic and neuroprotective drug by interfering with cellular pathways that are common to AD progression and analgesia. It was shown by others that KTP, due to its L-arginine group, could act as substrate for nNOS (nitric oxide synthase located in neurons) (Arima, 1997). Indirectly, this NO release causes opioid-like actions, producing analgesia through the release of met-enkephalin (Bocheva, 2004). Moreover, in recent years, there has been accumulating evidence that AD may be primarily a vascular disease with neurodegenerative consequences (de la Torre, 2004). The convergence of two key factors (i.e. aging and decreased cerebral perfusion) for AD to develop, results in a critically attained threshold of cerebral hypoperfusion (CATCH), which promotes distortion of brain capillary structure and impairment of NO homeostasis. Tissues that do not maintain the basal level of NO are more predisposed to an amendment of their regulation, extending the excitatory state. Thus, the advanced ageing associated with a vascular risk factor may contribute to endotheliopathies involving basal NO deficit to such a degree that it could initiate the neurodegenerative changes of AD (de la Torre, 2000). Brain cell death may in turn cause a decreased level of endogenous KTP in the brain, which further impacts on chronic pain.

## Outline and main findings of the experimental work

The above mentioned data led us to the hypothesis that kyotorphin is a key-molecule linking molecular mechanisms of pain and neurodegeneration. More specifically, we were prompted to test the hypothesis that decreased levels of kyotorphin in AD patients would contribute to chronic pain in these patients. Should this hypothesis proven true, the resulting chronic pain is a contribution to neurodegeneration and a detrimental cycle is created: pain worsens AD and AD worsens pain.

Kyotorphin levels in the cerebro-spinal fluid (CSF) of AD patients were correlated to phosphorylated protein Tau levels, which is a validated biomarker for AD (Prvulovic, 2011). Levels of total Tau/phosphorylated Tau (p-Tau181P) reflect tangle formation. The origin, structure, and function of these proteins are shown in Figure 6 for Tau isoforms. Well established ELISA-based testing methods are available for assessment of candidate CSF biomarkers such as CSF p-Tau181P or p-Tau231P. In contrast, the detection and quantification of kyotorphin levels in CSF is very far from being trivial. Detecting nanomolar or sub nanomolar analytes in a biological matrix such as CSF is a demanding analytical and instrumental challenge. This serious limitation made us team-up with collaborators in the laboratory of metabolopathies of the Clinical Hospital of Santiago Compostela, Spain, whom are specialists in the application of Mass Spectrometry to detect biomarkers in biological fluids. It was necessary to resort to advanced analytical techniques, namely electrospray ionization tandem mass spectrometry (ESI-MS/MS). Calibration solutions were prepared with different additions of KTP in a CSF matrix, were at concentrations from 0.625 to 10nM. We obtained a detection limit of 0.8nM. We used an API4000 triple quadrupole mass spectrometry (SciexAppliedBiosystems) equipped with an electrospray source with the turbo gas temperature set at 750°C. The equipment was operated in positive ionization polarity at a potential of 5400V. After fine tuning the sophisticated instrumental setup and the methodologies, the difficulties were overcome and we succeeded in determining with high precision in the CSF the levels of kyotorphin, which were later correlated to p-Tau levels in CSF.



**Figure 6** - The tau protein. The domain structure of the tau isoforms (tau gene location: 17q21) that are expressed in the human brain are shown. The isoforms differ (1) in the number of tubulin-binding domains (three or four repeats located in the C-terminal half of the protein; referred to as 3R or 4R tau isoforms) and (2) in the presence or absence of either one or two 29-amino-acid-long, highly acidic inserts at the N-terminal portion of the protein (the projection domain). The flow towards neurotoxicity is shown. (MT = microtubule; NFT = neurofibrillary tangles; PHF = paired helical filaments). (Vanderstichele, 2008)

Having gathered CSF samples of twenty five AD patients in different stages of the disease (i.e. a wide range of p-Tau levels), we found decreasing levels of kytorphin with AD progression. The negative correlation between KTP levels and p-Tau is clear (Figure 2 in the paper). The hypothesis of KTP being a molecular link between pain and neurodegeneration is thus valid and new avenues are open in clinical research in the largely unexplored domain of pain in patients with cognitive impairment. It is equally important to pursue the stimulating hypothesis that KTP derivatives of pharmacological interest to fight pain may also have in itself a neuroprotective effect, unifying in a single strategy the amelioration of pain and neurodegeneration.



## Neuropeptide kyotorphin (tyrosyl-arginine) has decreased levels in the cerebro-spinal fluid of Alzheimer's disease patients: potential diagnostic and pharmacological implications

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In Alzheimer's disease (AD), besides the characteristic deterioration of memory, studies also point to a higher pain tolerance in spite of sensibility preservation. A change in the normal tau protein phosphorylation is also characteristic of AD, which contributes to the pathogenesis of the disease and is useful in early diagnosis. Kyotorphin (KTP) is an endogenous analgesic dipeptide (Tyr-Arg) for which there is evidence of eventual neuroprotective and neuromodulatory properties. The objective of this work was to study the possible correlation between KTP and phosphorylated tau protein (p-tau) levels in cerebro-spinal fluid (CSF) samples of AD patients. CSF samples were collected from 25 AD patients and 13 age-matched controls (N), where p-tau and KTP levels were measured. We found a statistically significant difference between p-tau/KTP values in AD and N groups with an inverse correlation between p-tau and KTP values in AD samples. These results suggest that in the future KTP may be a candidate biomarker for neurodegeneration and may be a lead compound to be used pharmacologically for neuroprotection.

**Keywords:** kyotorphin, pain, Alzheimer, drug, cerebro-spinal fluid, neuroprotection

### INTRODUCTION

Medical and pharmacological developments in recent years have allowed a significant increase in aging of the population. Concomitant with the aging of the population the number of people with dementia has increased. Alzheimer's Disease (AD) is the most prevalent neurodegenerative disease associated with dementia. It is a progressive debilitating disease with no known effective cure. Symptoms of memory loss begin at a variable age, usually between 40 and 65 years of age, progressing with over the years. It is expected that the current number of cases doubles by 2030, with a huge social and economic impact (Knickman and Snell, 2002).

Patients suffering from AD show a progressive deterioration of memory, orientation, emotional stability, speech, abstract thinking, motor skills, and ultimately self-care. The motor and cognitive deterioration in AD patients is accompanied by a reduction in the ability to communicate, which makes it difficult to detect pain in these patients. By failing to receive adequate pain treatment, structural and irreversible changes may occur in central systems structures involved in the transmission/modulation of nociceptive information, which accounts to chronic pain installation (Borsook, 2012). Curiously, the two components of the pain response are differentially affected in AD patients (Farrell et al., 1996; Benedetti et al., 1999). Whereas the sensory-discriminative component is preserved, pain tolerance, associated with the affective-emotional aspect, largely increases. These apparent discrepancies

appear to have a neurobiological explanation since the somatosensory cortex and thalamic nuclei involved in sensory-discriminating component of pain response appear to be preserved in AD, while the neuronal loss was detected in the prefrontal and limbic structures, with obvious implications for and affective-emotional pain-related reactions (Benedetti et al., 1999; Borsook, 2012).

In pathophysiological terms, AD is characterized by cognitive losses, associated cholinergic deficit in the frontal brain area and extensive neuronal loss as well as synaptic changes in the neuronal pattern at the cortex and hippocampus. There is also evidence of neuronal tangle formation, made up of tau protein. Tau proteins are a group of neuronal microtubule-associated proteins, having a role in the regulation of neurite outgrowth, microtubule dynamics and axonal transport. There is strong evidence that in AD there is a change in the normal tau phosphorylation, which contributes to the pathogenesis of the disease. In fact, the abnormal phosphorylation leads firstly to a loss of function with decreased microtubule binding and, secondly, an increase of tau-tau interactions with negative effects on the neuronal stability (Johnson and Stoothoff, 2004). It has been well studied and commonly accepted that abnormally phosphorylated tau (p-tau) is the major protein subunit of Alzheimer's paired helical filaments (PHFs), being useful in the early diagnosis of this pathology (Kurtz et al., 1998). Tau is normally an intracellular protein, and the amount found in cerebro-spinal fluid (CSF) is low. The slow neurodegenerative

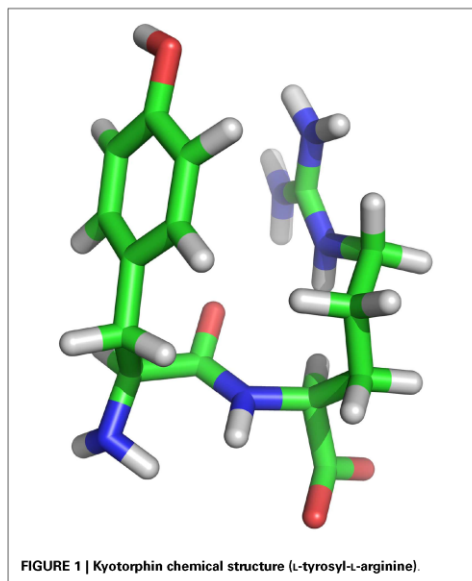
process that occurs in AD leads to increased neuronal loss which may give rise to increased tau levels in CSF. CSF samples of AD patients show high p-tau levels compared to normal subjects, correlating with hyperphosphorylated tau in cortical brain biopsies (Seppälä et al., 2012).

The other neuropathological hallmark of the AD is the formation of amyloid plaques, made of amyloid- $\beta$  ( $A\beta$ ) peptide, derived from the amyloid precursor protein (APP). The "amyloid cascade hypothesis" states that an imbalance between the production and clearance of the  $A\beta$  is the initiating event in AD, with the increase in  $A\beta$  ultimately leading to tau pathology, neuronal degeneration, and dementia (Blennow et al., 2010).  $A\beta$  is produced during normal cell metabolism and secreted into the CSF and can also be used as a biomarker for AD, specifically its most abundant species –  $A\beta_{1-42}$ , with AD patients consistently exhibiting a decrease of  $A\beta_{1-42}$  in CSF (Blennow, 2004; Blennow et al., 2010).

Kyotorphin (KTP) is a dipeptide (Tyr-Arg; **Figure 1**) with endogenous analgesic properties, first described by Takagi et al. (1979), isolated from bovine brain. It was subsequently isolated from other sources, including the brains of mice and rats (Ueda et al., 1980), guinea pig, rabbits, and squirrels (Ignat'ev et al., 1998) and detected in the CSF in humans (Nishimura et al., 1991). Despite being about fourfold more analgesic than endogenous opioids (Shiomi et al., 1981a), KTP effect is only induced after direct injection in the brain. This limited capacity to cross the blood-brain barrier prevents its pharmacological applications. KTP derivatives however have recently been demonstrated to be analgesic after systemic administration in several pain models which indicates the ability of KTP derivatives to cross the blood-brain barrier (Ribeiro et al., 2011a,b).

Kyotorphin is synthesized in nerve terminals and released by depolarizing stimuli (Shiomi et al., 1981b). It may modulate the synaptic transmission and directly excite cortical neurons. It was proposed that it exerts indirect opioid-like actions, producing analgesia through the release of met-enkephalin (Ribeiro et al., 2011b). Other studies in animal models have revealed the neuroprotective actions of KTP in the hippocampus and cerebellum (Nazarenko et al., 1999; Bocheva and Dzambazova-Maximova, 2004). In addition, there is further evidence that the peptide has neuroprotective and neuromodulating properties (Santalova et al., 2004; Dzambazova and Bocheva, 2010), also acting as a neuroleptic and inhibiting calcium-dependent currents in the post-synaptic membrane (Gorenkova et al., 2002; Santalova et al., 2004). There is also an anticonvulsant effect reported in animal models of epilepsy (Godlevsky et al., 1995; Shandra, 1999).

Although there is no immunohistochemical mapping of kyotorphin-containing neurons in the brain, rat brain homogenates show that the highest levels are found in the mid-brain, pons, and medulla oblongata, correlating with the areas most sensitive to morphine or electrical stimulation-induced analgesia. However when the results are expressed as a percentage of the total KTP found in different sections, the cortex seems to contain around 50%, an area where enkephalins receptors are low, which suggests that KTP non-opioid actions are important in its neurochemical action (Ueda et al., 1982; Dzambazova and Bocheva, 2010). For KTP, a non-opioid activity was also described in the



**FIGURE 1 |** Kyotorphin chemical structure (L-tyrosyl-L-arginine).

peripheral nervous system and other organs like the heart (Inoue et al., 1997; Li et al., 2006). It was shown that the KTP, due to its L-Arginine residue, could act as substrate for nNOS [nitric oxide synthase (NOS), located in neurons] (Arima et al., 1996, 1997). Moreover, in recent years, there is increasing evidence that AD may be primarily a vascular disease with neurodegenerative consequences, rather than a neurodegenerative disorder with vascular consequences (de la Torre and Stefano, 2000). Two key factors for the development of AD have been proposed: aging and decreased cerebral perfusion. The convergence of them will result in the so-called "critically attained threshold of cerebral hypoperfusion" (CATCH). It was proposed that the CATCH leads to a distortion of the architecture of the brain capillary, involving impairment of the release of nitric oxide (NO), which will affect the signaling between the immune, nervous, and cardiovascular systems. Tissues with decreased levels of NO may be severely deregulated. The concomitance of advanced age and risk factors may contribute to vascular lesions involving eNOS (located in the endothelium), causing a deficit of NO to such a degree that this could initiate the neurodegenerative changes characteristic of AD (de la Torre and Stefano, 2000).

So it seems that KTP could function in two separate but correlated domains: pain and neuromodulation. This assumption would make us pose another question: when neuronal death occurs, will there be a loss of KTP production that could worsen neurodegeneration and with concomitant alteration of pain threshold in patients? This would place KTP as a central player in the amplification of the continuous degradation of the clinical condition of AD patients. The goal of the present work was

to study the possible correlation between KTP and p-tau levels in CSF samples of Alzheimer patients to contribute to a better understanding of KTP mechanisms of action and open new horizons in this area of research.

### MATERIALS AND METHODS

All participants and caregivers were informed about the aim and procedure of this work and gave their oral and written informed consent prior to the commencement of the study.

We evaluated 25 patients with the diagnosis of AD, followed in Neurology consultation at a Portuguese Hospital with moderate cognitive impairment (stage 5 of the Global Deterioration Scale), and 13 age and sex-matched controls. The control group comprised healthy patients, who underwent lumbar puncture because of suspected (but not confirmed) subarachnoid hemorrhage or other indications in the usual neurological survey. Subjects with a history of cerebral trauma, transient ischemic attack, neoplasm, epilepsy, disturbances of consciousness, or focal brain disorders were excluded from participation in this study. Traumatic spinal punctures (i.e., with evidence of blood contamination) were also excluded from the study.

After lumbar puncture, the CSF samples were collected in polyethylene tubes, protected from light exposure with aluminum foil and stored at  $-80^{\circ}\text{C}$  until analysis (without additional freeze-thaw cycles). Appropriate evaluation of the degree of patient's dementia was performed by quantifying p-tau in CSF with a commercial tau-specific sandwich ELISA kit (Innogenetics, Ghent, Belgium).

Due to low levels of Kyotorphin that are estimated to be present in the CSF ( $10^{-9}\text{M}$ ) (Nishimura et al., 1991) it was necessary to resort to advanced analytical techniques namely electrospray ionization tandem mass spectrometry (ESI – MS/MS). The specimens of CSF were dried under a stream of nitrogen, derivatized with HCl-butanol and reconstituted with a solution containing  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  7:3 and 15 mL/L formic acid. This procedure it is similar to the routine method is used in our laboratory for metabolite profiles in urine samples (Rebollido-Fernandez et al., 2012). The solution was shaken on a vortex-mix system for 15 min at room temperature, and 20  $\mu\text{L}$  solution was injected into the mass spectrometer. Calibration solutions were prepared with different additions of KTP in a CSF matrix, were at concentrations from 0.625 to 10 nM (Figure S1 in Supplementary Material). We obtained a detection limit of 0.8 nM. The recovery ranges between were 60.7 and 100.9%, at the working levels of 2.5 nM was 85.4%. The precision ranges between were 1.9 and 14.8%. We used an API 4000 triple quadrupole mass spectrometry (Sciex Applied Biosystems) equipped with an electrospray source with the turbo gas temperature set at  $750^{\circ}\text{C}$ . The equipment was operated in positive ionization polarity at a potential of 5400 V. We performed multiple reaction monitoring (MRM) measurements, with Q1 394.3 and Q3 136.1 (Figure S2 in Supplementary Material).

The results are expressed as mean  $\pm$  SD unless otherwise stated; parametric data were analyzed with the Student's *t*-test and  $p < 0.05$  was considered statistically significant. Statistical analysis was performed using the SPSS software version 11.5.1 (2002, SPSS base 11.0 for Windows SPSS Inc., Chicago, IL, USA).

### RESULTS

In the AD group 63% of individuals were female and 37% male, with an average age of  $70.1 \pm 8$  years. The mean time of diagnosis and follow-up in neurology consultation was of 16 months. These patients were classified in clinical grounds as having AD in moderate stage, with mini-mental state examination (MMSE) values of  $14.3 \pm 6$ . In the N group, patients had the same population characteristics but without any known neurodegenerative disease (Table 1).

We found a statistically significant difference ( $p < 0.01$ ) between p-tau/KTP values in AD and N groups (Table 1). Regarding p-tau in AD group the values were  $403.2 \pm 157.6$  pg/mL whereas in the N group the values were  $68.8 \pm 18.3$  pg/mL ( $p < 0.01$ ). Regarding KTP in AD and N groups the values were  $1.8 \pm 0.62$  and  $3.4 \pm 1.2$  nM, respectively.

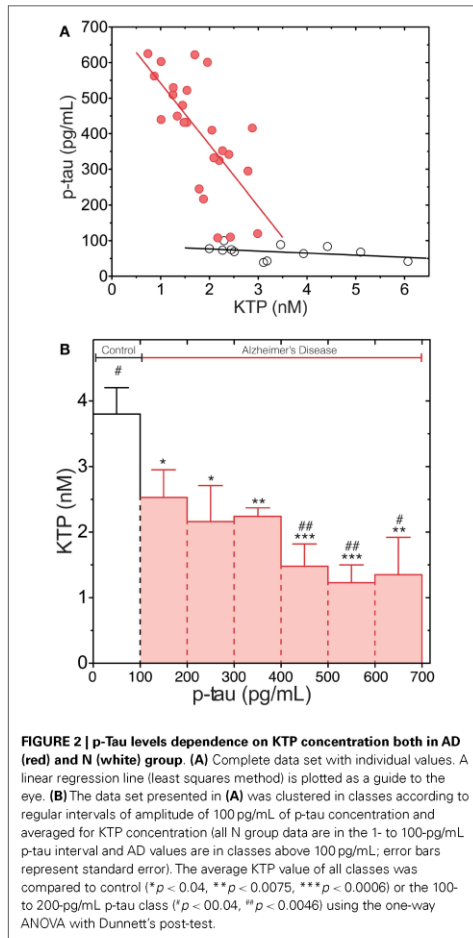
Figure 2A shows a plot of the p-tau and KTP concentrations in the CSF of AD patients and control group (N). There is a clear inverse correlation between the levels of p-tau and KTP in the AD group (Pearson correlation coefficient of  $-0.69$ ) in contrast with the N group (Pearson correlation coefficient of  $-0.38$ ). This is corroborated by the application of a linear regression model to fit the data: the slope is not significantly different from zero in the N group ( $p = 0.22$ ). The data was also clustered and the average KTP level was plotted against the concentration of p-tau for different classes (Figure 2B). This plot confirms that all data points in N are in the lowest class of p-tau level while simultaneously having the highest average KTP level. All AD classes have significantly lower levels of KTP than the control group, N. In AD patients decreasing average KTP levels are concomitant with increasing p-tau levels. The statistical analysis shows that above 400 pg/mL all AD classes have significantly lower KTP levels than the class 100–200 pg/mL. This result further supports an inverse relationship between KTP and tau in the CSF of AD patients.

### DISCUSSION

Our study is in agreement with previous studies aiming at other neuromolecules (Sulkava et al., 1985; Blennow et al., 2010) that have shown that in AD and vascular dementia, as the disease progresses several neuropeptides levels fall. Here we also found a statistically significant difference in KTP levels between AD patients and N individuals, being lower in the first. This can be explained by to a disease-specific cortical thinning and hippocampal volume loss, with an acceleration phase during the early stages of the AD (Sabuncu et al., 2011), meaning less cortical mass, which possibly results in less KTP production capability and KTP dropping levels in the CSF of these patients (Figure 2). Moreover, there

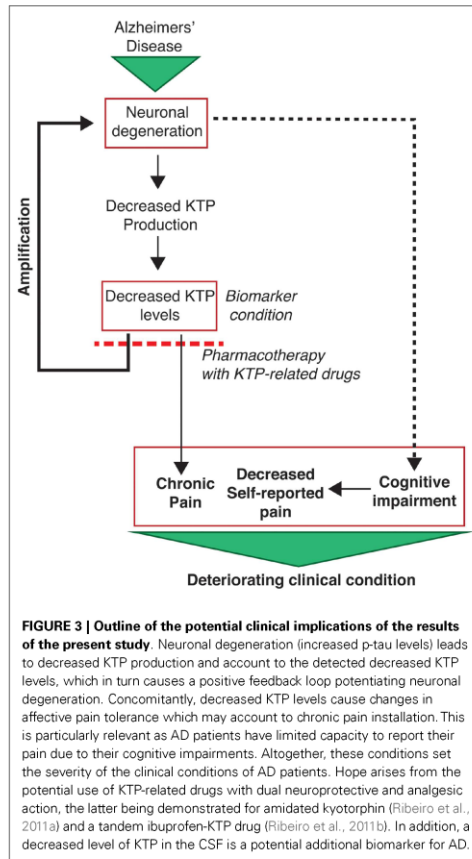
**Table 1 | Alzheimer's disease patient characterization and CSF concentration results (mean  $\pm$  SD; SE, standard error).**

	AD group	N group	p-Value
Male/female (%)	63/37	56/44	–
Age (years)	$70 \pm 8$	$68 \pm 6$	$>0.01$
KTP (nM)	$1.8 \pm 0.6$ (SE 0.1)	$3.4 \pm 1.2$ (SE 0.3)	$<0.01$
p-Tau (pg/mL)	$403.2 \pm 157.6$ (SE 31.5)	$68.8 \pm 18.3$ (SE 5.2)	$<0.01$



is an inverse correlation between p-tau and KTP in CSF in AD patients. p-Tau in CSF acts as a marker of neurodegeneration, being released from senescent neurons. KTP is also produced in neuronal cells, its levels naturally falling as neurons die. As more neuronal cells are destroyed in the course of the disease p-tau is released and KTP production is impaired (Figure 2).

Lower levels of a molecule with analgesic properties such as KTP may help to explain why AD patient population is believed to have an increased incidence of hidden chronic pain (see Introduction); in agreement, previous studies (Nishimura et al., 1991) have shown that KTP levels decrease in chronic pain conditions. Another potential implication of these results stems from the



correlation between KTP and NO production in AD patients as lower levels of KTP might lead to a decreased NOS activity with consequent fall of NO levels. This NO deficit and consequent cerebral hypoperfusion (see Introduction) compromise further or even help to initiate neurodegenerative deregulations characteristic of AD, with increased both neuronal loss and levels of p-tau.

#### POTENTIAL IMPLICATIONS

One important implication of the results here presented is that KTP is a candidate biomarker for neurodegeneration. Several studies in animal models (Takagi et al., 1979; Nazarenko et al., 1999; Santalova et al., 2004; Ribeiro et al., 2011b) have shown that KTP functions not only as a potent endogenous analgesic molecule but also as neuroprotector and neuromodulator. We suggest further studies in animal models in order to unveil if exogenous administration of KTP (with increase of its CSF levels) can result

in neuroprotection. There is evidence of the KTP role in integrative functions in animals, particularly in the field of exploratory activity (Kolaeva et al., 2000), which are not inhibited by naloxone, therefore independent of its analgesic effects. It is also important to recall that amidated KTP and other KTP-related molecules have important analgesic efficacy, as shown in previous studies (Ribeiro et al., 2011a,b). A dual neuroprotective analgesic action in a single drug would be of utmost importance.

In summary, this study points to KTP as a possible but overlooked biomarker with potential clinical dual importance in the context of pain and neurodegeneration in Alzheimer disease (Figure 3).

#### ACKNOWLEDGMENTS

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#### SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at [http://www.frontiersin.org/Aging\\_Neuroscience/10.3389/fgni.2013.00068/abstract](http://www.frontiersin.org/Aging_Neuroscience/10.3389/fgni.2013.00068/abstract)

##### Figure S1 | Calibration curve of KTP (kyotorphin) in a CSF matrix (0.625–10 nM).

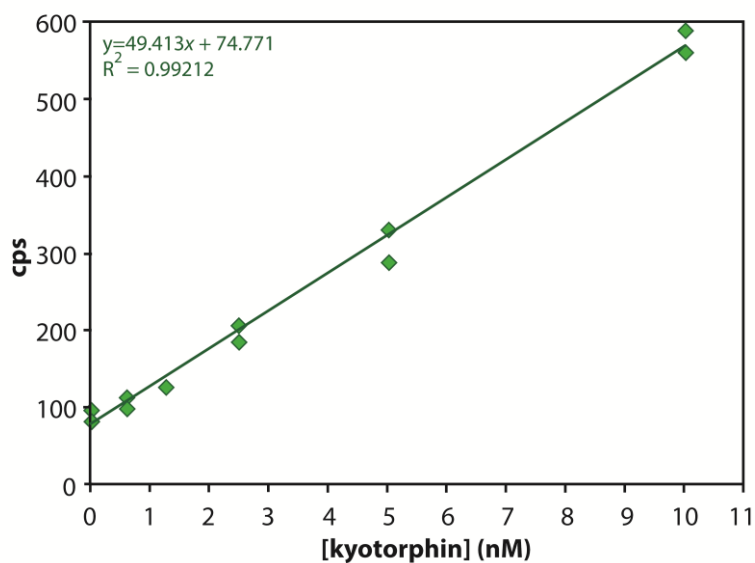
##### Figure S2 | Multiple reaction monitoring (MRM) measurements, with Q1 394.3 and Q3 136.1, using an API 4000 triple quadrupole mass spectrometry (Sciex Applied Biosystems) equipped with an electrospray source.

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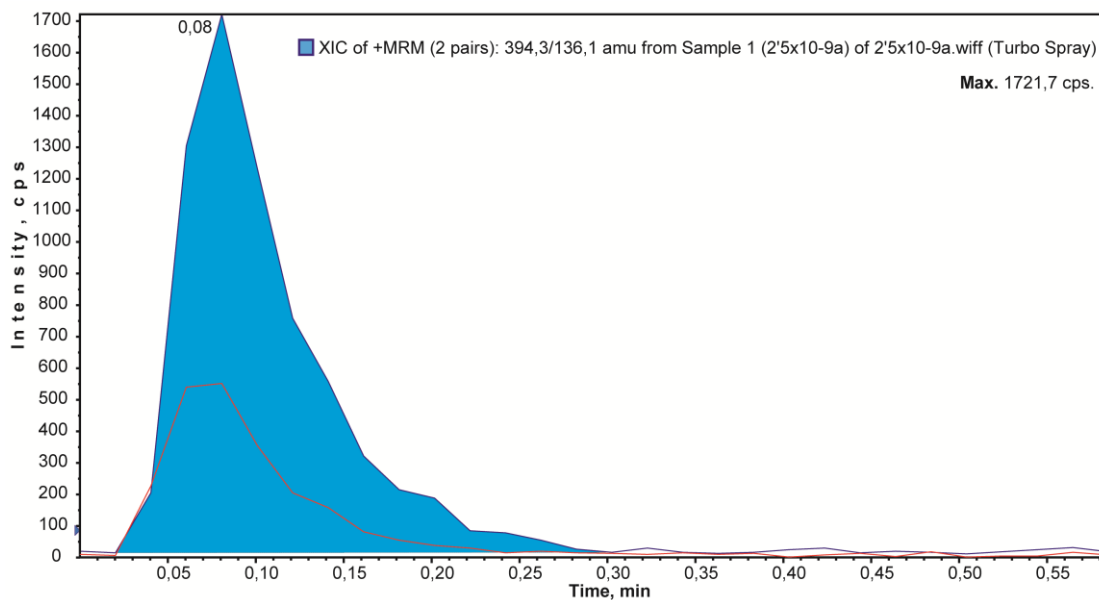
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- This article was submitted to the journal *Frontiers in Aging Neuroscience*. Copyright © 2013 Santos, Garcia-Nimo, Sá Santos, Tavares, Cocho and Castanho. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

## Supplementary Material to Section 3 (Article 3)



**Figure S1.** Calibration curve of KTP (kyotorphin) in a CSF matrix (0.625–10 nM).



**Figure S2.** Multiple reaction monitoring (MRM) measurements, with Q1 394.3 and Q3 136.1, using an API 4000 triple quadrupole mass spectrometry (Sciex Applied Biosystems) equipped with an electrospray source.



## **ARTICLE 4**

Amidated and ibuprofen-conjugated kyotorphins  
are neuroprotective in the hippocampus and  
improve spatial working memory after bilateral  
carotid occlusion in female rats

***In preparation for submission***

*I, Sara Matos Santos, declare that the experimental design, the animal experimentation, data collection, data analysis, and conclusions were carried by me under supervision of Prof. Miguel Castanho and with the collaboration of Sónia Sá Santos.*

*Antónia R.T. Pinto helped in animal experimentation.*

*Synthesis of Kyotorphin and Ibuprofen-Kyotorphin were performed by Prof Montesserat Heras and Prof. Eduard Bardají.*

*A manuscript for publication will be prepared by Prof Isaura Tavares, Prof Miguel Castanho, Sónia Sá Santos and I.*

## **Outline and main findings of the experimental work**

Following the work presented in previous chapters, it was imperative to evaluate the efficacy of kyotorphin derived peptides in an animal model of AD. In recent years, investigators have increasingly tended to accept that AD may be primarily a vascular disease with neurodegenerative consequences (de la Torre, 2000), rather than a neurodegenerative disorder with vascular consequences, and that ischemic states associated with aging like carotid insufficiency can lead to a chronic disruption of cerebral blood flow, which induce neurological deficits and dementia, such as AD (Bennet, 1998).

With that premise in mind and duly supported by previous work from other groups (Farkas, 2007), we conducted bilateral carotid occlusion in a group of rats in order to mimic the neurodegeneration observed in humans due to shortage of cerebral blood flow. This model is particularly interesting because there is neuronal death in key memory areas such as the hippocampus (Farkas, 2007).

In this study, KTP-NH<sub>2</sub> and IbuprofenKTP-NH<sub>2</sub>, which had already shown pronounced analgesic activity after systemic administration, were neuroprotective in the hippocampus and improved spatial working memory in the rat model of neurodegeneration. This finding is encouraging and opens the window to new studies to evaluate if these peptides can be a weapon to fight conditions such as AD.

# **Amidated and ibuprofen-conjugated kyotorphins are neuroprotective in the hippocampus and improve spatial working memory after bilateral carotid occlusion in female rats**

## **Abbreviations used:**

2VO, two-vessel occlusion; AD, Alzheimer's disease; BBB, Blood-Brain Barrier; CATCH, critically attained threshold of cerebral hypoperfusion; CNS, Central Nervous System; CSF, cerebrospinal fluid; DMSO, Dimethyl sulfoxide; GFAP, glial fibrillary acidic protein; H&E, hematoxylin and eosin; i.p., intraperitoneal; KTP, kyotorphin; KTP-NH<sub>2</sub>, kyotorphin-amide; IbKTP-NH<sub>2</sub>, KTP-NH<sub>2</sub> linked to Ibuprofen; NFL, neurofilament-L protein; OFT, open field test; PBS, phosphate buffered saline; SEM, standard error of the mean.

**Running Title:** Neuro-modulating effects of KTP derivatives

## ABSTRACT

Chronic brain ischemia is a prominent risk factor for neurological dysfunction and progression for dementias, including Alzheimer's disease (AD). In recent years, there has been accumulating evidence that AD may be primarily a vascular disease with neurodegenerative consequences, rather than a neurodegenerative disorder with vascular consequences.

In rats, permanent bilateral ligation of the common carotid arteries (two-vessel occlusion, 2VO) causes progressive and irreversible cognitive impairment with Alzheimer's phenotype: learning difficulties and memory loss, failure of neuronal signaling, neuropathological damage in the hippocampus and cerebral cortex within a variable time frame since occlusion.

Kyotorphin (KTP) is an endogenous dipeptide (L-Tyr-L-Arg) that plays an important role in pain regulation at the central nervous system (CNS). It has also been suggested that KTP has neuromodulating and neuroprotective properties. We have recently succeeded in designing two new KTP derivatives, KTP-amide (KTP-NH<sub>2</sub>) and KTP-NH<sub>2</sub> linked to ibuprofen (IbKTP-NH<sub>2</sub>), with antinociceptive action following systemic administration.

The objective of our work was to investigate the effects of KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub> on motor function and spatial recognition memory and histopathological alterations at the hippocampus of female rats in chronic cerebral hypoperfusion (2VO-rat model).

Overall, our experimental findings show that KTP-derivatives, mainly IbKTP-NH<sub>2</sub>, improved cognitive impairment and prevented neuronal damage in hippocampal CA1 subfield, induced by chronic cerebral hypoperfusion. This means that these compounds, besides its already known analgesic efficacy, are also capable of acting in key brain areas as positive neuromodulators or slowing neurodegeneration, being potentially useful in the treatment of dementia.

**Keywords:** kyotorphin derivatives, analgesic drugs, chronic cerebral hypoperfusion, 2VO rat model, cognitive impairment, locomotor function, dementia

## INTRODUCTION

Current estimates indicate that 35.6 million people worldwide are living with dementia, a number that is expected to nearly double every 20 years [1]. Chronic brain ischemia is a prominent risk factor for neurological dysfunction and progression for dementia including Alzheimer's disease (AD) [2-4]. In fact, AD is the most prevalent neurodegenerative disease in the elderly, responsible for about 50-70% of cases of dementia [5]. This irreversible disease is characterized by progressive deterioration of cognitive and memory functions, formation of amyloid plaques and neurofibrillary tangles as well as profound alteration in glial responses, cholinergic dysfunction, extensive synaptic and neuronal loss [6-8]. Moreover, in recent years, there has been accumulating evidence that AD may be primarily a vascular disease with neurodegenerative consequences, rather than a neurodegenerative disorder with vascular consequences [7]. The convergence of two key factors (aging and decreased cerebral perfusion) for AD to develop, results in a "critically attained threshold of cerebral hypoperfusion" (CATCH), which promotes distortion of brain capillary structure and impairment of nitric oxide (NO) release [9].

Experimental animal models have been improved to investigate circulation-dependent behavioral deficits resultant of chronic cerebrovascular insufficiency as it occurs in human aging and AD [3]. In rats, permanent bilateral ligation of the common carotid arteries (two-vessel occlusion, 2VO) causes progressive and irreversible cognitive impairment with Alzheimer's phenotype: learning difficulties and memory loss, failure of neuronal signaling, neuropathological damage in the hippocampus and cerebral cortex within a variable time frame since occlusion [3]. In the 2VO-model there is neurodegeneration of various cerebral structures, particularly in the CA1 pyramidal cell layer of the hippocampus, a brain region known to be highly implicated in spatial learning and memory [10], and also susceptible to postischemic inflammatory phenomena and  $\beta$ -amyloid peptide accumulation [11,12].

Kyotorphin (KTP) is an endogenous dipeptide (L-Tyr-L-Arg) that plays an important role in pain regulation at the central nervous system (CNS) [13-15]. It was first isolated from bovine brain by Takagi and colleagues in 1979 [14, 15], and subsequently found in the brains of other mammals, including in human cerebrospinal fluid (CSF) [16]. The remarkable analgesic activity of KTP in animal models was observed only when the molecule was directly injected into the brain [17], which is a consequence of its reduced ability to cross the blood-brain barrier (BBB).

To improve KTP delivery to CNS, we have recently succeeded in designing two new KTP-derivatives: KTP–amide (KTP–NH<sub>2</sub>) and KTP–NH<sub>2</sub> linked to ibuprofen (IbKTP–NH<sub>2</sub>) [18, 19] (**Figure 1**). Both derivatives proved to induce strong analgesic activity following systemic administration to model animals [18, 19], with the absence of the major side-effects when compared to clinically relevant opioids [20].

In addition to analgesic activity, it has been suggested that KTP has neuromodulating and neuroprotective (in hippocampus and cerebellum) properties [21, 22], as well as an antiepileptic effect [23].

KTP is synthesized in nerve terminals and released by depolarizing stimuli [24]. It may modulate the synaptic transmission and directly excite cortical neurons. It was proposed that KTP, due to its L-arginine group, could act as substrate for nNOS (nitric oxide synthase located in neurons), with subsequently formation of NO which would then induce analgesia via met-enkephalin release [25]. Moreover, it has been proposed that disruption of NO homeostasis may hasten the development of AD. In fact, the prolonged brain hypoperfusion brought on by CATCH seems to promote regional endotheliopathies due to basal deficit of NO that over time, can evolve to such a degree that lead to AD symptoms and to progressive neurodegeneration [9]. Additionally, when neuronal death occurs it may in turn cause a decreased level of endogenous KTP in brain which further impacts on chronic pain and impairment of NO production.

Our recent clinical studies support there is a link between AD, pain and KTP in humans. In fact, not only we observe that pain is underestimated in AD patients [26] but also that KTP has decreased levels in the CSF of AD patients [27]. Moreover, there was an inverse correlation between levels of phosphorylated-tau protein (a molecular marker of AD progression) and of KTP [27].

In brief, the analgesic KTP and its derivatives have shown important properties that may influence the course of AD: neuromodulation and neuroprotection, substrate for nNOS, role as a neuroleptic agent inhibiting calcium-dependent currents in the postsynaptic membrane.

The present study was conducted to investigate the effects of chronic treatment with KTP-derivatives (KTP–NH<sub>2</sub> and IbKTP–NH<sub>2</sub>) on memory impairment, motor function, and hippocampal injury in a 2VO rat model.

## **MATERIALS AND METHODS**

### **Ethics Statement**

All described procedures were conducted in compliance with the European Community legislation (Directive 2010/63/EU), and were approved by the Ethical Committee for Animal Research of the Faculty of Medicine, University of Lisbon, and the Portuguese Competent Authority for Animal Welfare (DGV).

### **Compounds**

The peptides KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub> were synthesized as described elsewhere [18, 19]. Anesthetic and analgesic drugs used during and post-operative procedure: Imalgene<sup>®</sup> 1000 (Ketamine 100 mg/mL; Merial, France); Domitor<sup>®</sup> (Medetomidine hydrochloride 1 mg/mL; Pfizer, Orion Pharma, Finland); Antisedan<sup>®</sup> (Atipamezole hydrochloride 5 mg/mL; Pfizer, Orion Pharma, Finland); Budale<sup>®</sup> (Buprenodale 0.3mg/mL; Dechra, UK).

### **Animals and housing**

Young female Sprague-Dawley rats weighing 225-250 g (3 months of age) were purchased to Charles River Laboratories (Barcelona, Spain). They were housed together in groups of 3-4 per cage with unrestricted access to water and food, and under controlled temperature and light conditions (22 ± 2°C; lights on between 7 a.m. and 7 p.m.). Surgical procedures and all behavioral experiments were conducted during the light period of the 12:12 h cycle.

### **Surgery: two-vessel Carotid artery occlusion procedure**

One week after animal's arrival to the Rodent Facility, permanent global ischemia and sham surgery was performed as described elsewhere [28,29]. Briefly, animals were anaesthetized for surgery with a mixture of ketamine (75 mg/kg BW, i.p.) plus medetomidine (4 mg/kg BW, i.p.). A neck ventral midline incision was made and both common carotid arteries were exposed and carefully separated from carotid sheath, cervical sympathetic and vagus nerve. For occlusion, each carotid artery was double-ligated with 5-0 silk sutures just below the carotid bifurcation. Sham-operated animals were subjected to the same surgical procedures without carotid artery ligation.

During the surgery, the animals were breathing spontaneously and body temperature maintained around 37°C by a self-regulating heating pad. After the procedure, rats were i.p. injected with the medetomidine reversing agent mixture (Antisedan®, dose of 1 mg/kg BW) and kept on heating pad until they recovered from the anesthesia. Afterwards, all animals were returned to their home cages with free access to food and water. During the first 24h post-surgery, Budale® was administrated for pain relief purposes (dose volume of 0.05mL/150-300g BW q 8-12h). Animals were closely monitored during the post-operative recovery for appearance, activity, feeding behavior and body weight. Like in larger surgical interventions, 2VO is typically followed by an initial decrease in body weight [3] but all animals involved in this study regained their weight before behavioral testing. Body weight was measured before the surgery and controlled whenever the animals were handled for i.p. injection and/or experimentation.

### **Rat treatment regimen**

KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub> were dissolved in physiological saline solution (0.9 % NaCl containing 5 % of dimethyl sulfoxide, DMSO) prior to intraperitoneal (i.p.) injection (in a dosing volume of 1 mL/kg BW). KTP-derivatives were administrated as a chronic treatment regimen during 7 consecutive days (single i.p. dose/day) in two different timings: (A) one week and (B) four weeks after the onset of 2VO surgery.

Experimental groups were as follows: (1) sham-operated group; (2) 2VO control group; (3) 2VO animals receiving KTP-NH<sub>2</sub> i.p. (32.3 mg/kg = 96 µmol/kg); and (4) 2VO animals receiving IbKTP-NH<sub>2</sub> i.p. (24.2 mg/kg = 46 µmol/kg). The selected doses of KTP-derivatives were based on our previous results concerning their analgesic action profile [18-20]. Moreover, the control 2VO- and sham-operated groups were i.p. injected with the vehicle solution. A time line of experimental events is depicted in **Figure 2**.

### **Behavioral test procedures**

All behavioral studies were carried out between 9 a.m. and 6 p.m. in animals accustomed to the testing room and to researchers performing the trials. During the fifth week post-2VO surgery (**Fig. 2**), rats were tested in standard behavioral paradigms to examine spontaneous locomotor activity (open-field test) and spatial recognition memory (Y-maze). All behavioral

apparatus were thoroughly cleaned after each rat to mitigate olfactory stimuli. At the day of experiments, animals were brought into the testing room for at least 2 h prior to the start of the behavioral session.

### **Motor function testing: Open field**

Open field test (OFT) is a widely used test of locomotion activity, exploratory and anxiety behaviors [30]. The open field apparatus consisted of an empty square box (67 x 67 x 51 cm height), “virtually” divided in three concentric squares: borders (near the walls), periphery and center. Without prior habituation, locomotor behavior was measured in a quiet room with dimmed light. Testing protocol has been described in detail previously [20]. Briefly, fifteen minutes after i.p. injection (with one of KTP derivatives or with the vehicle) rats were placed individually in the center of the arena box and allowed to explore the apparatus for 5 min. Their behavior was video-recorded during the testing period. Animal tracking along the different areas on the open field arena was analyzed using specific software (Smart version 2.5.10 program; Panlab, S.L.U, Barcelona, Spain). The experimenter was out of sight from the rat during the OFT. To evaluate the number of crossings, the open field arena was also virtually divided into 16 equal rectangles, and the number of times each animal crossed between two areas was measured. Results are shown as average velocity, % of time resting, number of crossings and % of time spent in the center of arena. Each animal was considered to be resting if the mean velocity was < 3 cm/s. Average velocity is the mean velocity with the resting time excluded. All animals were tested only once.

### **Memory testing: Y-Maze**

Y-maze is a simple two-trial recognition test for measuring spatial memory skills [31]. The experimental protocol was similar as the one described in [32]. Briefly, Y-shaped apparatus comprises three identical arms at a 120° angle from each other (arm dimensions, 35cm long x 10cm wide x 20cm high). Those three arms were designated as “Start arm”, in which the rat starts to explore (always open), “Novel arm”, which is blocked at the 1st trial, but open at the 2nd trial, and “Other arm” (always open). Animal bedding was used to cover the floor of the maze and was mixed after each individual trial to prevent rats from using odor cues in maze

navigation. The experimenter was never present in the room while the animals explored the maze. Two separated trials were performed: the first one had 10 min duration and allowed the animal to explore freely only two arms (Start and Other) since the third arm (Novel) was blocked with black Plexiglas. Then, after an interval of 1 h, the rat was placed in same starting arm, with free access to all three arms for 5 min (2nd trial). Animal behavior was video-monitored, enabling the number of total entries (sum of entries in all three arms, i.e. Novel+Other+Start arms) and time spent in Novel and Other arms to be analyzed. Entry was considered to be complete when all four limbs were within an arm. No animal had jumped out of the maze arena during the testing period.

### **Histopathology and Immunofluorescence**

After the last behavioral test, rats were anesthetized using ketamine/medetomidine mixture and perfused transcardially with 0.9% saline solution, followed by 4% paraformaldehyde in phosphate buffer (pH 7.4). Following decapitation, their brains were carefully removed, maintained for post-fixation in the same fixative solution at 4°C for 24 h and then cryoprotected with a 30% sucrose solution for at least two days.

Brains were gelatin-embedded and then sectioned at a thickness of 15 µm on a cryostat (LEICA CM 3050S, Nussloch, Germany). Only the coronal sections located at the level of dorsal hippocampus (around - 3.6 mm posterior from bregma [33]) were collected, mounted on SuperFrost® Plus slides (Menzel-Glaser, Braunschweig, Germany) and further processed for Hematoxylin-Eosin (H&E) staining and immunofluorescence.

H&E staining was performed in the first set of sections, in order to detect the location of areas of ischemic brain damage, as well as to examine changes in laminar structures of the hippocampus namely pyramidal cell layers in the cornu ammonis (i.e., CA1, CA2 and CA3) subfields. The H&E-stained sections were observed under a brightfield microscope (Leica DM2500, 12.5x and 50x magnifications).

For the immunofluorescence studies, the second set of hippocampal sections was double-stained for the astrocytic marker mouse anti-gial fibrillary acidic protein (GFAP, 1:200; catalog #MAB3402, Milipore) and for the neuronal marker rabbit anti-neurofilament-L protein (NFL, 1:50; catalog #AB9568, Milipore). Briefly, slides were placed in PBS for 10 min at 37°C to remove gelatin from brain tissue. Sections were subsequently treated with 0.1% Triton X-100

in PBS (PBS-Tx) for membrane permeabilization, blocked for 1 h with 2 % bovine serum albumin (BSA) in PBS at room temperature (RT), and then incubated at 4 °C overnight with the mixture of primary antibodies prepared in blocking solution (2% BSA in PBS). In the following day, sections were washed in PBS-Tx and incubated with the secondary antibodies goat anti-mouse IgG Alexa 488 (1:200; catalog #A11017, Molecular Probes) and goat anti-rabbit IgG Alexa 594 (1:200; catalog #A11012, Molecular Probes) for 1 h at RT in a humidified dark chamber. For nuclei staining, sections were incubated with Hoechst 33342 (Life Technologies) for 10 min at RT protected from light. At the end, sections were cover slipped with ProLong® Gold antifade reagent (Life Technologies). Negative controls in which the primary antibodies were omitted were performed simultaneously.

All samples were analyzed on a confocal point-scanning microscope (Zeiss LSM 510 META) using excitation wavelengths of 405, 488 and 594 nm. Immunofluorescence images were capture (400× magnification) in two randomized areas of dorsal CA1 subfield in both hemispheres, i.e. CA1 images were taken bilaterally in each rat. All acquisition conditions were kept constant between samples during the capture process.

Tissue background was determined and since its autofluorescence was negligible, background subtraction was not required for immunofluorescence quantification. Image J 1.48C Software was used to measure the intensities of the fluorescence signals for GFAP and NFL, after gray-scale threshold determination. One measurement was taken from each hippocampus of each animal (2 measurements per rat, 3 to 4 rats per group) rendering a total of 6 to 8 data points per group.

### **Statistical analysis**

Data are represented as the groups' mean  $\pm$  SEM (standard error of the mean).

All statistical analyses were calculated with Prism Software (GraphPad Software, version 6, La Jolla, CA, USA). The significance of differences between groups was analyzed with one way ANOVA followed by Tukey's or Bonferroni's multiple comparison test when indicated. Differences in immunofluorescence measurements between two groups were analysed with Student's t-test (unpaired). Statistical significance was defined as  $p < 0.05$ .

## RESULTS

### Histopathological and immunofluorescence evaluation

Representative photomicrographs of hippocampal sections stained with H&E are shown in **Figure 3**. In some animals subjected to 2VO surgery, brain tissue loss was observed (**Fig. 3 A**): ischemic regions were colored white, while the non-ischemic regions were colored pink.

As can be seen in **Figure 3 B**, 2VO-control animals showed significant unilateral changes (right or left hemisphere) in the histoarchitecture of the cornu ammonis (i.e., CA1, CA2 and CA3) subfields reflected by the loss of pyramidal cells layers. These degenerative changes were not observed in the two KTP-treated 2VO groups (i.e., KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub>).

In order to evaluate the effects of KTP derivatives on astrocytic responses and against neuronal damage in CA1 subfield, immunofluorescence studies were performed. One of the major components of the axonal cytoskeleton, the neurofilament-L protein (NFL) was used as neuronal marker. Glial fibrillary acidic protein (GFAP) was used as astrocytic marker.

Representative images of hippocampal CA1 region double-stained for GFAP and NFL are presented in **Figure 4**. As can be seen, the NFL signal intensity for the 2VO-control group was particularly lower when compared with the sham - control group and both KTP-treated 2VO groups. Apparently, no major changes in GFAP signal were detected between all animal groups.

To confirm this qualitative analysis, the fluorescence signals for GFAP and NFL were quantified (**Table 1**). In fact, no significant effects on GFAP immunofluorescence were observed in the three 2VO-groups when compared to the sham-control group. As expected, there was a significant decrease in NFL content in 2VO-control group ( $P=0.0004$  vs. sham group;  $P< 0.0001$  vs. KTP-NH<sub>2</sub> group;  $P=0.009$  vs. IbKTP-NH<sub>2</sub> group). In contrast, the NFL immunofluorescence results for KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub> groups were similar to those in the sham - control group.

### Open Field test

Locomotor activity in a new environment was measured in an open field apparatus. A 5 min session was chosen as this time period is known to emphasize rodent exploratory behavior and

response to novelty [30] as well as to detect anxiety-like tendencies, significant hyperactivity or behavioral sedation [30].

As shown in **Figure 5**, there were no differences between the vehicle-treated animals (i.e., sham-operated and 2VO controls) for the velocity parameter, % of time resting and number crossings. In fact, the only significant difference between the sham-operated rats and the three 2VO-groups was the % of time spent in the center of the arena (**Fig. 5 C**), i.e., all the 2VO-animals exhibit a more pronounced anxious behavior.

Moreover, the pattern of locomotor response is clearly different in IbKTP-NH<sub>2</sub>-treated 2VO group as those animals moved slower (**Fig.5 B**,  $p < 0.01$  vs. both controls), spent more time resting (**Fig.5 A**,  $p < 0.001$  vs. both controls) and crossed less frequently between areas (**Fig.5 D**,  $p < 0.001$  vs. both controls). These results show that the locomotor performance was affected to a certain extent. No significant effects of KTP-NH<sub>2</sub> are detected relative to 2VO-controls.

## Y Maze

**Figure 6** shows the results for the performance of KTP-treated 2VO animals in Y-maze task, in which short-term spatial recognition memory was evaluated.

To determine if the motivation to explore the maze apparatus was the same among the groups during the testing period, the number of total arm entries was analyzed. There was no difference found between all animal groups in terms of total number of arm entries (**Fig.6 A**). Analysis of which arm was chosen to be explored first revealed similar outcomes between sham-operated and IbKTP-NH<sub>2</sub>-treated groups: i.e., all those animals entered the Novel arm as the first choice (100%), whereas the 2VO-control group chose unanimously the Other arm to go first. The percentage of animals that entered Novel arm as first choice in KTP-NH<sub>2</sub> group was 60%.

As can be seen in **Figure 6**, there was no difference between the sham-operated group and the KTP-treated 2VO groups for the % of time spent in the Other arm and in the Novel arm (**Fig.6 C**). On the other hand, the 2VO-control group spent less time in the Novel arm when compared with sham-operated animals. Moreover, the analysis within each experimental group showed that the ischemic 2VO-control group explored the Other arm significantly more than Novel arm

( $p < 0.0028$ , **Fig.6 C**), whereas all the other groups (sham and KTP-treated 2VO animals) spent similar amount of time both in the Novel and Other arms (**Fig.6 C**).

## DISCUSSION

It is now consensual that ischemic episodes of brain, which is critically dependent of a continuous blood supply to maintain its activity, trigger a cascade of degenerative events similar to those that ultimately culminate in irreversible dementia of Alzheimer's phenotype (for review see [34]). Besides  $\beta$ -amyloid peptide and hyperphosphorylated tau protein accumulation in postischemic brains, human epidemiological studies indicated a higher incidence of dementia (i.e., up to 9-fold) following a few months of ischemic injury [34].

Permanent bilateral ligation of the common carotid arteries (2VO) in rats is a suitable experimental model to investigate the neurodegeneration and cognitive consequences of chronic brain hypoperfusion. Like in human aging and dementia states, the 2VO-animals show a progressive loss of hippocampal neurons, which leads to cognitive decline and behavioral changes [3]. The usefulness of the 2VO-rat model for the development and testing of potentially neuroprotective drugs, against ischemic damage and/or dementia, has been emphasized [3].

In this work, two analgesic kyotorphin derivatives, KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub>, were studied regarding their ability to improve cognitive and behavioral functions in 2VO-rat model.

The "time window of opportunity" for an effective neuroprotective treatment, in relation to the timing of the ischemic episode, is an important factor to be considered. Herein, KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub> were administrated in two periods during the chronic phase of brain hypoperfusion: female rats were injected for 7 days in the first week and also in the fourth week after the onset of 2VO. This drug administration schedule was chosen taking into account the neuropathologic changes in the hippocampal CA1/CA3 subfields over time reported in 2VO model, i.e., comprising the periods of negligible neuronal death and when hippocampal neuronal injury is obvious [3].

From our histopathological data, both KTP derivatives were effective in preventing extensive neuronal death at the hippocampus. In contrast, in 2VO-animals treated with saline solution (2VO-control group) there was a notorious damage at CA1, CA2 and CA3 subfields reflected by the disappearance of the well-defined layer of pyramidal neurons (**Fig. 3**). Moreover, our immunofluorescence studies clearly demonstrate the beneficial effects of KTP derivatives on CA1 neurons. Loss of neurofilament-L (NFL) proteins may be closely related with the selective

vulnerability of CA1 neurons in cerebral ischemia [35]. Chronic hypoperfusion caused a significant reduction in the NFL signal in the CA1 subfield (2VO-control group, **Fig. 4, Table 1**). On the other hand, no changes were seen in NFL immunofluorescence for KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub> groups when compared with sham-control group indicating that KTP derivatives treatment afforded neuronal protection.

Cerebral ischemia triggers reactive astrogliosis, a condition characterized by an increase of GFAP levels in astrocytes [3]. In our study there was no evidence of major astrogliosis in hippocampal CA1 region after 6 weeks of 2VO surgery. Although astrocytic activation and proliferation can be detected in the cortex [3] and hippocampus [2] after 1 week of the occlusion, GFAP increase may be not evident until 6 months later [3].

Rats subjected to global cerebral ischemia may exhibit hypoactivity or hyperactivity or normal activity when tested in the open field (for references see [36]). Although ischemic injury may affect brain areas related to motor function (i.e, cortex and neocortex regions), there are no obvious signs of motor deficits in 2VO-rats [3]. Moreover, variations in environmental conditions, namely the level of illumination, may explain in part the different locomotor results found in literature [37].

In our study, the comparable results of the control groups (sham-operated and 2VO) in terms of velocity, % of resting and number crossings, suggested that the permanent two-vessel occlusion did not impair locomotion performance. This was also observed for the ischemic animals treated with KTP-NH<sub>2</sub>. In fact, when tested in the Y-maze arena all the 2VO-animals showed a similar behavior as the sham-operated ones in the total number of arm visits (another locomotor activity index, [32]), reinforcing that locomotor activity remained intact in 2VO-animals and which is also corroborated by other authors [2, 3]. We should emphasize that the mild motor impairment observed in the IbKTP-NH<sub>2</sub>-treated group was not induced by the chronic administration of the compound but rather a consequence of the single bolus dose administrated immediately before the open field test. This behavior was already reported by us in normal rats (without any type of surgery, and also after 15 min of i.p. injection) and seems to be due a synergistic effect of ibuprofen and kyotorphin [20].

Although the motor function was not affected by 2VO surgery, all the female rats subjected to bilateral occlusion were more anxious than the sham-controls in the open field, as measured by the decreased time spent in the center of the arena. These results are in agreement with previous studies of global ischemia, in which tested animals developed an anxious behavior [38]. On the other hand, hippocampus is important in many species-typical behaviors (like anxiety), potentially influencing performance in a range of behavioral tests [39]. Previous studies indicate that dorsal CA1 lesioned mice are hyperactive upon exposure to a novel environment and have spatial working memory impairments in the Y-maze [40].

Clinical studies have also shown that neurodegenerative conditions are also associated with mood alterations and increased anxiety levels in AD patients [41]. In our study the anxious behavior of the operated animals was not affected by the administration of kyotorphin derived compounds (**Fig. 1**), showing that these peptides probably do not possess intrinsic anxiolytic properties, and/or did not act on key areas responsible for this behavior.

Spatial recognition memory of female rats was measured by the two-trial Y-maze test [31, 32]. The Y-maze paradigm is based on an innate tendency of rodents to explore a novel environment but not on learning a new behavior or rule, allowing to measure behavior parameters such as recognition vs. discrimination memory and spatial exploration [31, 32]. Rodents typically prefer to investigate a new arm of the maze (unfamiliar) rather than returning to one that was previously visited (familiar). Therefore, if memory and novelty-seeking behavior are not affected, rat females are expected to enter the Novel arm more than the Other arm. In our study, although all animals shown to be equally motivated to explore the Y-maze (total entries into all arms were similar), their response to novelty inside the maze apparatus was not the same. A difference was seen between groups related to the first arm choice and percent of time spent in Novel vs Other arms with the 2VO-control group spending more time and choosing the Other arm to go first. This indicates that the 2VO surgery induced short-term memory deficits, making those females unable to discriminate novelty and familiarity. On the other hand, the two KTP-treated groups and the sham-animals chose more often first the Novel arm and spent similar time in the Novel vs Other arm. In fact, the percentage of animals that entered the Novel arm as the first choice was 100% in both sham- and IbKTP-NH<sub>2</sub> groups and 60% in KTP-NH<sub>2</sub> group. Since the first choice for Novel arm reflects recognition of the unfamiliar arm (discrimination memory), is obvious that the administration

over time of IbKTP-NH<sub>2</sub> and KTP-NH<sub>2</sub> improved the ability of 2VO-animals to distinguish the Novel arm from the familiar ones. Therefore, IbKTP-NH<sub>2</sub> and KTP-NH<sub>2</sub> treatment enhanced spatial recognition memory in 2VO animals, with a greater effect of IbKTP-NH<sub>2</sub>.

Several studies have found a direct correlation between cerebral hypoperfusion-induced memory deficits and hippocampal CA1 neuronal damage [42,43]. Therefore, improved memory abilities seen in KTP-treated 2VO animals may be attributed to the neuroprotective effects of those peptides on CA1 neurons.

Moreover, IbKTP-NH<sub>2</sub> showed to be more effective in restoring normal cognitive function than KTP-NH<sub>2</sub>. The mechanism underlying this observation seems to involve, at least in part, the presence of the NSAID ibuprofen in IbKTP-NH<sub>2</sub> derivative, which may attenuate some of the neuroinflammatory processes in 2VO-ischemic brain. In rodent models of AD dementia, chronic administration of ibuprofen prevents oxidative damage, significantly inhibits amyloid formation and deposition, and improves cognitive functions [44,45]. Further studies are needed to unveil which are the molecular targets of IbKTP-NH<sub>2</sub>.

In conclusion, our experimental findings show that KTP-derivatives improved spatial working memory and prevented neuronal damage in hippocampal CA1 subfield induced by chronic cerebral hypoperfusion.

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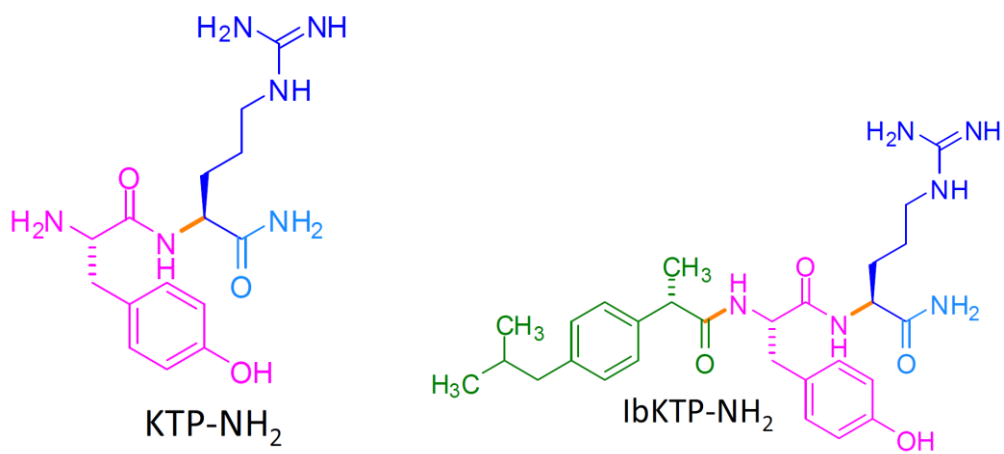
## FIGURES

### Figure Legend

**Figure 1-** Chemical structures of KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub>

*Tyrosyl residue in pink, Arginyl residue in dark blue, ibuprofenyl residue in green*

**Figure 1**

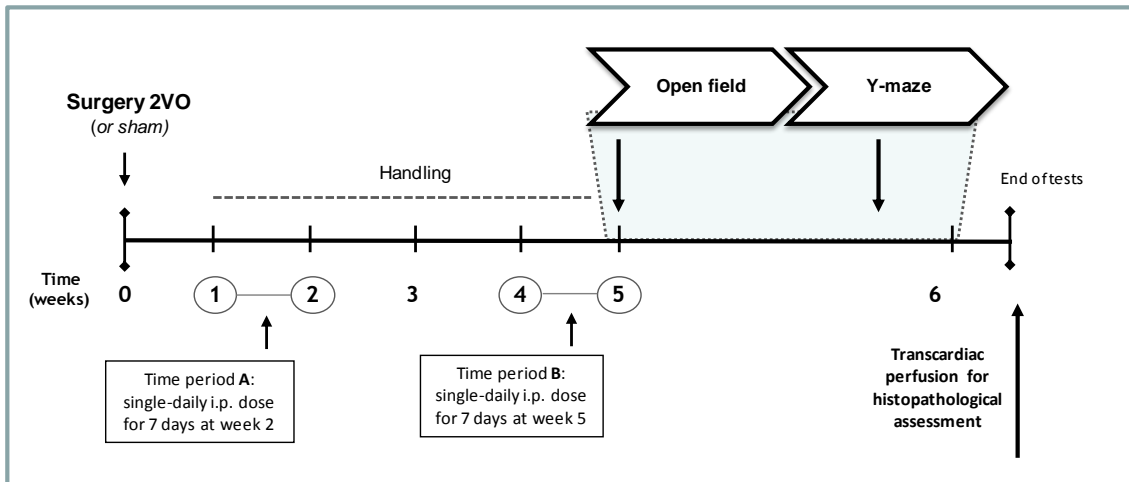


## Figure Legend

**Figure 2-** Time course of the experiments planned. Animals were divided into groups according to the type of surgery and to the injected compound during the two time periods **A** and **B**: i.e., KTP-NH<sub>2</sub> (32.3 mg/kg = 96 μmol/kg), IbKTP-NH<sub>2</sub> (24.2 mg/kg = 46 μmol/kg) or saline solution (vehicle).

Abbreviations: i.p., intra-peritoneal; PFA, paraformaldehyde; 2VO, two-vessel occlusion.

**Figure 2**

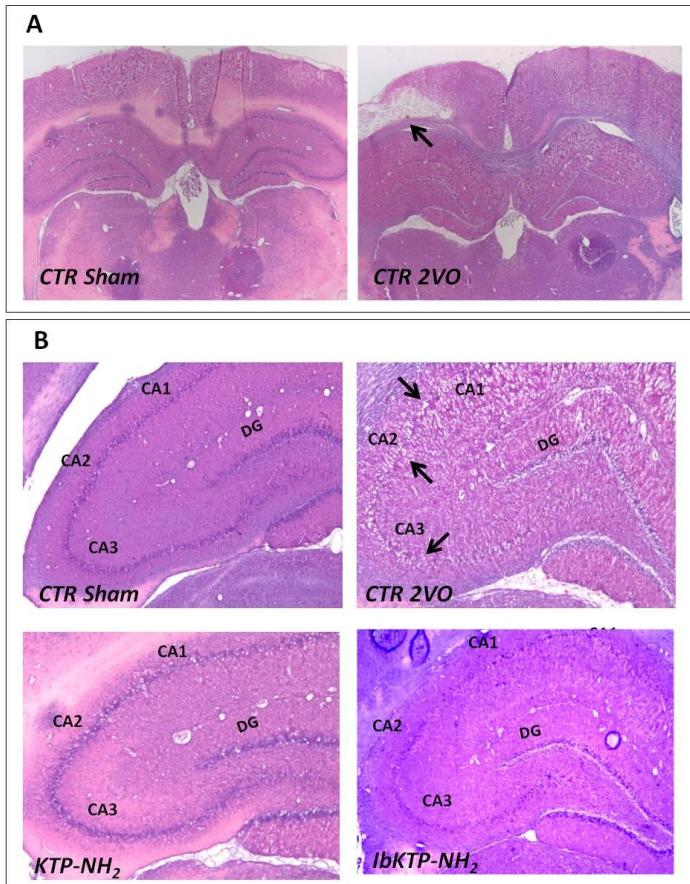


## Figure Legend

**Figure 3** - Representative H&E staining photomicrographs of hippocampal sections from sham-operated and 2VO female rats. Histological evaluation was performed in the sixth week after the onset of 2VO surgery. **(A)**: images showing ischemic lesion (white area, black arrow) in 2VO rat (12.5x magnification); **(B)**: images showing histological changes of the CA1, CA2 and CA3 pyramidal cell layers in 2VO-control group (black arrows are pointing at damaged layers) (50x magnification). 2VO-control animals showed significant unilateral changes (right or left hemisphere) in the histoarchitecture of the cornu ammonis (i.e., CA1, CA2 and CA3) subfields reflected by the loss of pyramidal cells layers. These degenerative changes were not observed in the two KTP-treated 2VO groups (i.e., KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub>).

*Abbreviations:* CTR, control; 2VO, two-vessel occlusion; CTR sham, control sham-operated animals; DG, dentate gyrus; H&E, hematoxylin and eosin.

**Figure 3**



### Figure Legend

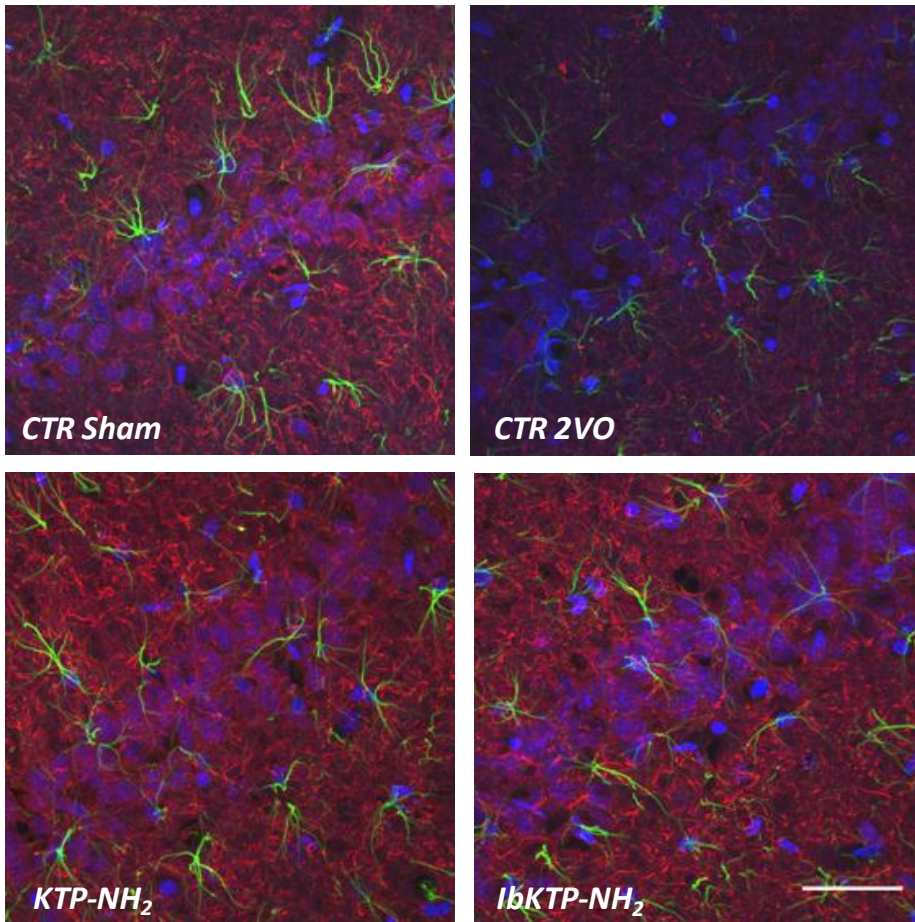
**Figure 4-** Representative images of double immunofluorescence staining of GFAP and NFL in the hippocampal CA1 subfield from sham-operated and 2VO female rats sacrificed six weeks after surgery. Neuronal marker NFL is shown in red whereas the astrocytic marker GFAP is shown in green. Nuclei are stained blue (Hoechst).

Upper panels: control groups, sham and 2VO (left and right panel, respectively). Lower panels: KTP derivatives treated groups, KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub> (left and right panel, respectively) (400x magnification).

Note a significant decrease in the NFL signal in the 2VO-control group.

Abbreviations: CTR, control; 2VO, two-vessel occlusion; CTR Sham, control sham-operated animals; GFAP, glial fibrillary acidic protein; NFL, neurofilament-L protein. Scale bar= 50 μm

**Figure 4**

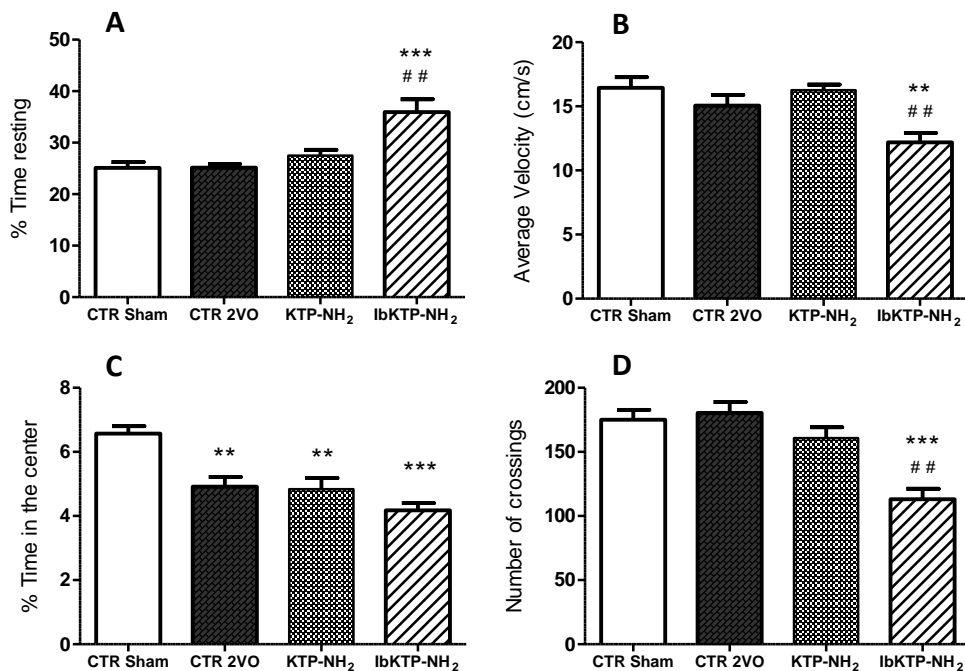


## Figure Legend

**Figure 5** - Locomotion performance in the open field of KTP-treated rat females, five weeks after the onset of 2VO surgery. Animals were subjected to two chronic treatment regimens for 7 days (single i.p. dose/day of peptides or vehicle) at first week and fourth week after 2VO surgery. In the OFT, 2VO-animals were individually placed in the center of the test apparatus 30 min after being i.p. injected with KTP-NH<sub>2</sub> (32.3 mg/kg), IbKTP-NH<sub>2</sub> (24.2 mg/kg), or vehicle (saline with 5 % DMSO used as a control). Sham-operated animals were injected with the vehicle. Behavior was video-recorded for a 5 min time period and data are shown as % time spent resting (**A**), average velocity (**B**), % time spent in the center of the arena (**C**) and the number of crossings (**D**). In all experiments, n=5 per group. In (**A**), (**B**) and (**C**): \*\*P< 0.01, \*\*\*P< 0.001 versus control 2VO group and sham-operated group; ##P< 0.01, ###P< 0.001 versus KTP-NH<sub>2</sub>, one way ANOVA [P = 0.0003 in (**A**), P = 0.0027 in (**B**), P = 0.0001 in (**C**) and in (**D**)] followed by Tukey's post test.

Abbreviations: CTR, control; 2VO, two-vessel occlusion; CTR sham, control sham-operated animals. Mean ± SEM for all groups.

**Figure 5**



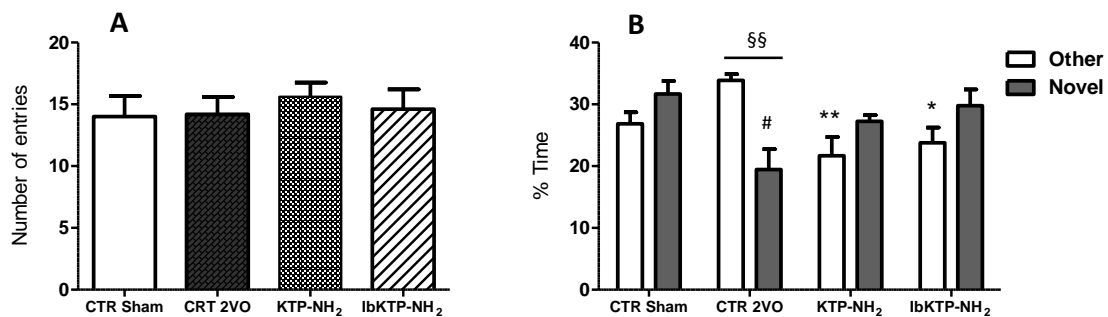
## Figure Legend

**Figure 6** - Cognitive performance in the Y-maze of KTP-treated rat females, five weeks after the onset of 2VO surgery. Animals were subjected to two chronic treatment regimens at first week and at fourth week after 2VO surgery (single i.p. dose/day during 7 consecutive days): KTP-NH<sub>2</sub> (32.3 mg/kg), IbKTP-NH<sub>2</sub> (24.2 mg/kg), or vehicle (saline with 5 % DMSO used as a control). Sham-operated animals were injected with the vehicle. In the Y-maze, animals were individually placed in the Start arm of the apparatus and allowed to explore freely the entire maze. Behavior was video-monitored for a 5 min time period and data are shown as number of total entries (**A**) and % time spent in the Other and Novel arms (**B**). In all experiments, n≥4 per group.

In (**B**): #P< 0.05 versus sham-operated group; \*P< 0.05, \*\*P< 0.01 versus control 2VO group, one way ANOVA [P =0.0083 for % time in Other arm, P=0.0283 for % time in Novel arm] followed by Tukey's post test. Comparison between % time in Other arm and % time in Novel arm: <sup>§§</sup>P=0.0028, unpaired two-tailed Student's test.

Abbreviations: CTR, control; 2VO, two-vessel occlusion; CTR sham, control sham-operated animals. Mean ± SEM for all groups.

**Figure 6**



**Table 1**

Quantification of the immunofluorescence signals of GFAP (astrocytic marker) and NFL (neuronal marker) in the hippocampal CA1 subfield from sham-operated and 2VO female rats sacrificed six weeks after surgery. Measurements were performed bilaterally from 3 to 4 rats per group, rendering at least 6 data points per group.

Data expressed as percentage of the sham-control group (mean  $\pm$  SEM for all groups).

\*\*\*P=0.0004 versus sham-operated group; ##P=0.009, #####P<0.0001 versus control 2VO group, unpaired two-tailed Student's t-test.

<b>Group</b>	<b>GFAP</b>	<b>NFL</b>
<b>CTR 2VO</b>	114 $\pm$ 29	45,1 $\pm$ 8,8***
<b>KTP-NH<sub>2</sub></b>	92,7 $\pm$ 3,7	119,8 $\pm$ 9,3#####
<b>IbKTP-NH<sub>2</sub></b>	97,4 $\pm$ 4,2	109,6 $\pm$ 20,2##



## **ARTICLE 5**

# **Side-effects of Analgesic Kyotorphin derivatives: advantages over clinical opioid drugs**

***Amino Acids, 2013***

*I, Sara Matos Santos, declare that the experimental design, the laboratory work, the data analysis and discussion were carried out by Marta Ribeiro, Sónia Sá Santos et al. under supervision of Prof. Miguel A.R.B. Castanho. Sara Matos Santos participated in the experimental design, data collection, analysis and discussion and preparation of the manuscript. The manuscript was written under the guidance of my supervisor, Prof. Miguel A.R.B. Castanho.*

## **Outline and main findings of the experimental work**

Previous laboratory work (Ribeiro, 2011a, Ribeiro 2011b) enabled us to obtain results with the KTP derivatives that were promising enough to consider a pharmaceutical development. After designing and achieving insight over the mechanism of action of these new peptides, a characterization of their potential side-effects was missing. This characterization is fundamental since most of the drugs, namely opioids, do not thrive in the clinical practice due to their side effects (Buenaventura, 2008).

In our study we had promising kyotorphin amidated derivatives that had demonstrated very interesting results: KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub>. These drugs were selected for their side-effects evaluation in vivo and opioid pathways are involved in their mechanism of action, they were at the same time compared with two reference opioids commonly used in the clinics: morphine and tramadol. Both KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub> showed a strong analgesic effect in the absence of the major side-effects associated with classical opioids.

## Side-effects of analgesic kyotorphin derivatives: advantages over clinical opioid drugs

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**Abstract** The adverse side-effects associated with opioid administration restrain their use as analgesic drugs and call for new solutions to treat pain. Two kyotorphin derivatives, kyotorphin-amide (KTP-NH<sub>2</sub>) and ibuprofen-KTP-NH<sub>2</sub> (IbKTP-NH<sub>2</sub>) are promising alternatives to opioids: they trigger analgesia via an indirect opioid mechanism and are highly effective in several pain models following systemic delivery. In vivo side-effects of KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub> are, however, unknown and were evaluated in the present study using male adult *Wistar* rats. For comparison purposes, morphine and tramadol, two clinically relevant opioids, were also studied. Results showed that KTP-derivatives do not cause constipation after systemic administration, in contrast to morphine. Also, no alterations were observed in blood

pressure or in food and water intake, which were only affected by tramadol. A reduction in micturition was detected after KTP-NH<sub>2</sub> or tramadol administrations. A moderate locomotion decline was detected after IbKTP-NH<sub>2</sub>-treatment. The side-effect profile of KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub> support the existence of opioid-based mechanisms in their analgesic actions. The conjugation of a strong analgesic activity with the absence of the major side-effects associated to opioids highlights the potential of both KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub> as advantageous alternatives over current opioids.

**Keywords** Kyotorphin · Analgesic peptides · Opioids · Side-effects · Morphine · Constipation

Marta M. B. Ribeiro, Sónia Sá Santos contributed equally to this work.

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### Abbreviations

BBB	Blood-brain barrier
BP	Blood pressure
CNS	Central nervous system
DMSO	Dimethyl sulfoxide
i.p.	Intraperitoneal
KTP	Kyotorphin (Tyr-Arg)
KTP-NH <sub>2</sub>	Kyotorphin-amide
IbKTP-NH <sub>2</sub>	KTP-NH <sub>2</sub> linked to ibuprofen
MCs	Metabolic cages
SBP	Systolic blood pressure
SEM	Standard error of the mean

### Introduction

Opium and its alkaloids are powerful centrally acting compounds in the treatment of chronic pain. In parallel, they trigger several side-effects, such as constipation, nausea,

separately (funnelled into underside cylinders) as well as food and water intake. Animals were i.p. administered with each of the compounds or vehicle and immediately placed in the MC (1 rat/cage). Ad libitum access to a known amount of water (150 mL) and food (40 g) was available via a graduated drinking tube and a feeder chamber, respectively. The testing period was 6 h. This time period was chosen so as to cover the lifetimes of the compounds' analgesic activity and of their metabolization. At the end of the testing period, the remaining amount of water and food as well as the faecal pellets and urine expelled were quantified and registered. The urine pH was also measured using a pH universal indicator (Merck).

#### Evaluation of locomotion

Evaluation of locomotor function was performed with an open field apparatus and carried out in a quiet and diffusely lit room with each group equally represented at the time of testing. The open field arena was an empty square box (67 × 67 × 51 cm high). Animals were i.p. administered with one of the compounds or with the vehicle. Fifteen minutes later, rats were placed individually in the centre of the arena, and their behaviour was video-recorded for 5 min using a camera mounted on the ceiling above the open field box. This 5-min time period is known to emphasize mice/rats exploratory behaviour (Gould et al. 2009), as well as to be appropriate to detect significant hyperactivity or behavioural sedation (Crawley 2007). Animal tracking along the different areas on the open field arena was analysed using the software Smart, version 2.5.10 (Panlab, S.L.U., Barcelona, Spain). For motor performance analysis, the arena was virtually divided into three concentric squares: borders (near the walls), periphery and centre. For the evaluation of the number of crossings, the open field arena was also virtually divided into 16 equal rectangles, and the number of times each animal crossed between two areas was measured. This analysis is independent of the squares being at the centre or at the periphery of the arena (Tuon et al. 2008). Results are shown as average velocity, % time spent resting, number of crossings and % time spent in the centre of the arena. Each animal was considered to be resting if the mean velocity was <3 cm/s. Average velocity is the mean velocity with the resting time excluded.

#### Blood pressure evaluation

The effects of KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub> in blood pressure (BP) were evaluated according to previous protocols using the non-invasive tail-cuff method (Tavares et al. 1997). Briefly, after a training period of 7 days, the rats were inserted into a restrainer (LE 5610, Panlab S.I.,

Barcelona, Spain) and placed on a heating pad at 38 °C for 10–15 min. A tail-cuff detector (LE 5008 05PL Panlab S.I., Barcelona, Spain) was positioned on the animals' tail. Five BP measurements were collected as baseline values for each animal. Animals were unrestrained and allowed to recover for 10 min, after which they were injected i.p. with 32.3 mg/kg of KTP-NH<sub>2</sub> or 24.2 mg/kg of IbKTP-NH<sub>2</sub>. Five BP measurements were obtained 15, 30 and 60 min after administration.

#### Statistical analysis

Data are represented as the groups' mean ± SEM (standard error of the mean). The significance of differences between groups was analysed with one way ANOVA followed by Tukey's multiple comparison test. The significance of differences in each group was analysed with Friedman's test followed by Dunn's multiple comparison test. All statistical analyses were calculated with Prism Software (GraphPad Software, version 5).

## Results

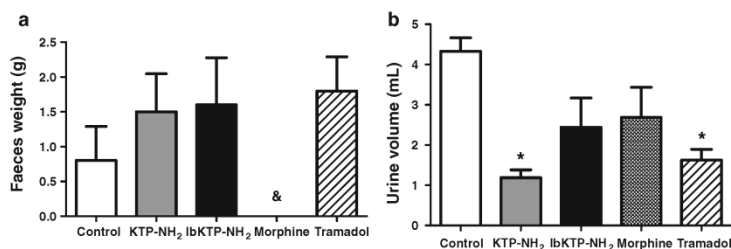
#### Metabolic study

The metabolic response of *Wistar* male rats after KTP-derivatives or opioids administration was assessed using metabolic cages, which allowed accurate measurement of metabolic intake and output—the ingestion of food and water, and the production of faeces and urine. Morphine administration led to a blockade of bowel function with a total absence of faecal pellets for the whole 6-h period (Fig. 1a). On the other hand, KTP-NH<sub>2</sub>, IbKTP-NH<sub>2</sub>- and tramadol-treated groups did not exhibit significant alterations of this behaviour.

All tested compounds appear to cause a reduction in urine output when compared to the control (Fig. 1b). Statistical significance was, however, only obtained for KTP-NH<sub>2</sub> and tramadol-treated animals. Urine pH measurement detected no statistical difference between the groups, with values ranging from 6.5 to 8.0 randomly. In addition to reducing voiding, tramadol was the only drug that changed eating and thirst patterns by causing a significant increase in the quantities of food and water intake (Fig. 2).

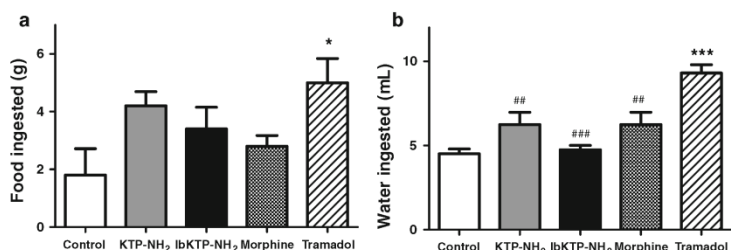
#### Evaluation of locomotion

The locomotion behaviour of animals after administration of KTP-derivatives, morphine and tramadol was recorded in an open field apparatus. Although no differences between groups were observed regarding the average velocity (Fig. 3a), IbKTP-NH<sub>2</sub> treated animals spent more



**Fig. 1** Effect of KTP derivatives and opioids on faecal (a) and urine (b) output in rats, observed in animals i.p. administered with KTP-NH<sub>2</sub> (32.3 mg/kg), IbKTP-NH<sub>2</sub> (24.2 mg/kg), morphine (5 mg/kg), tramadol (10 mg/kg) or vehicle (saline with 5 % DMSO used as control) and individually placed in metabolic cages for 6 h.

The quantity of faeces and urine was measured following this time period. In all experiments,  $n = 5$  per group. \* $P < 0.05$  versus control, one way ANOVA ( $P = 0.0124$ ) followed by Tukey's post test. &No faecal pellets were collected in the morphine group. Mean + SEM for all groups



**Fig. 2** Effect of KTP derivatives and opioids on food (a) and water intake in rats (b) observed in animals i.p. administered with KTP-NH<sub>2</sub> (32.3 mg/kg), IbKTP-NH<sub>2</sub> (24.2 mg/kg), morphine (5 mg/kg), tramadol (10 mg/kg) or vehicle (saline with 5 % DMSO used as control) and individually placed in metabolic cages for 6 h.

The amount of food and water consumed was measured after this time period. In all experiments,  $n \geq 4$  per group. \* $P < 0.05$ , \*\*\* $P < 0.001$  versus control; ## $P < 0.01$ , ### $P < 0.001$  versus tramadol, one way ANOVA [ $P = 0.0396$  in (a),  $P < 0.0001$  in (b)] followed by Tukey's post test. Mean + SEM for all groups

time resting (Fig. 3b) and displayed a lower number of crossings between areas (Fig. 3c), which suggests the occurrence of motor impairments. Also, these animals were the only ones with a reduced time spent in the centre of the arena (Fig. 3d). KTP-NH<sub>2</sub> and opioids morphine and tramadol did not affect the evaluated locomotion parameters.

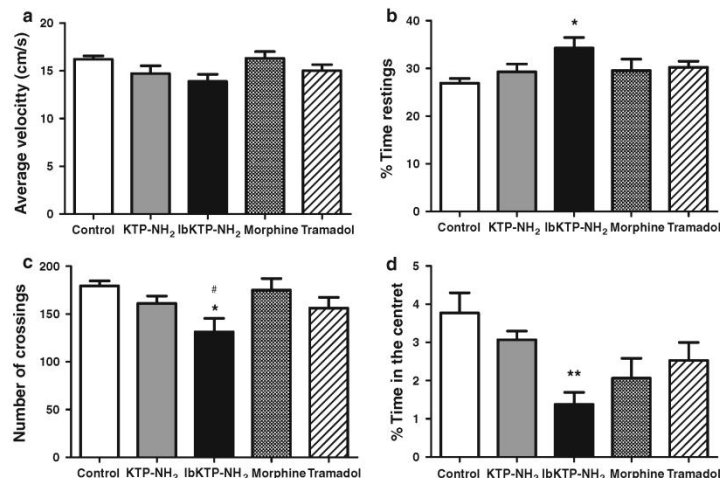
#### Blood pressure evaluation

The effects of KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub> in systolic BP are shown in Fig. 4. KTP-NH<sub>2</sub> induced a transient and non-statistically significant increase in BP 15 min after injection ( $83.33 \pm 26.84$  mmHg), after which BP values decreased to baseline values. IbKTP-NH<sub>2</sub> induced a transient and non-significant decrease in BP values 15 min following injection ( $35.00 \pm 13.05$  mmHg) after which BP values returned to baseline values. No differences from baseline values were detected 60 min after the injection of KTP-NH<sub>2</sub> or IbKTP-NH<sub>2</sub>.

#### Discussion

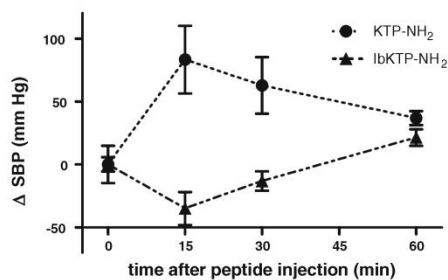
Improving the safety of opioid-like drugs, currently of limited applicability due to major side-effects, is crucial for further pharmaceutical development. In this work, two new kyotorphin derivatives, KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub>, were studied regarding their side-effect profiles and compared with the widely used opioids morphine and tramadol.

The absence of constipation after KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub> injection is an important advantage of these peptides over many opioids, namely morphine. The constipation observed as a result of morphine administration is mainly related to morphine's high affinity to  $\mu$ -opioid receptors (Reimer et al. 2009). Tramadol, on the other hand and in spite of its  $\mu$ -agonist activity, has about 6,000-fold lower affinity for  $\mu$  receptors than morphine. This may explain the maintenance of gastrointestinal motility in tramadol-treated animals (Benyamin et al. 2008; Grond and Sablotzki 2004).



**Fig. 3** Locomotion functions evaluation after administration of KTP derivatives and opioids. Animals were individually placed in an open field apparatus 15 min after being i.p. injected with KTP-NH<sub>2</sub> (31.3 mg/kg), IbKTP-NH<sub>2</sub> (24.2 mg/kg), morphine (5 mg/kg), tramadol (10 mg/kg), or vehicle (saline with 5 % DMSO used as a control). Behaviour was recorded for a 5-min time period and data are

shown as average velocity (a), % time spent resting (b), number of crossings (c) and % time spent in the arena centre (d). In all experiments,  $n \geq 6$  per group. \* $P < 0.05$ , \*\* $P < 0.01$  versus control; # $P < 0.05$  versus morphine, one way ANOVA [ $P = 0.0811$  in (b),  $P = 0.0225$  in (c),  $P = 0.0065$  in (d)] followed by Tukey's post test. Mean + SEM for all groups



**Fig. 4** Effect of Kyotorphin derivatives on animals' systolic blood pressure (SBP) after i.p. administration of KTP-NH<sub>2</sub> (31.3 mg/kg) and IbKTP-NH<sub>2</sub> (24.2 mg/kg). Results expressed as the variation of the SBP at each evaluation time point in relation to baseline values (time 0, obtained immediately before injection). In all experiments,  $n \geq 4$  per group. Data expressed as mean ± SEM

Both KTP-NH<sub>2</sub> and tramadol lowered the micturition volume. Opioid-receptor mediated inhibition of micturition can occur mainly at the CNS, caused by stimulation of  $\mu$ -opioid receptors but also  $\delta$  receptors (Pandita et al. 2003). Therefore, KTP-NH<sub>2</sub> results agree with previous data regarding the central activity of this peptide through opioid pathways (Ribeiro et al. 2011a). It should be stressed that KTP-NH<sub>2</sub> has probably a minor physiological

effect since urine output reduction did not result in major urinary retention because increases in blood pressure were not triggered. On the other hand, IbKTP-NH<sub>2</sub> did not cause a similar effect. However, the molar concentration dose of IbKTP-NH<sub>2</sub> used is about half of the KTP-NH<sub>2</sub>. Also, non-steroidal anti-inflammatory drugs, like ibuprofen, do not induce urinary retention (Cole et al. 1988; Malinovsky et al. 1998). This may provide at least a partial explanation for the differences between the effect of KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub> in urinary retention.

As to tramadol, doses up to 10 mg/kg were previously reported to increase bladder storage capacity without impairing bladder emptying (Pandita et al. 2003), which may explain the reduction in urine output observed. This effect is possibly due to supraspinal  $\mu$ -opioid receptor activation and 5-hydroxytryptamine reuptake inhibition (Pandita et al. 2003). Such activity of tramadol is potentially interesting in the treatment of detrusor overactivity and nocturia. Similarly, the KTP-NH<sub>2</sub> effect may suggest interesting properties for the treatment of micturition disturbances.

Our study revealed that only tramadol changed eating and thirst patterns, causing an increase in both. Increase in water intake might be related to dry mouth, a frequent side-effect of tramadol (Grond and Sablotzki 2004). Further interpretation of data is hampered because the role of

opioids in ingesting and drinking behaviour is still unclear (Yeomans and Gray 2002). Nevertheless, the fact that none of the KTP-derivatives affected food and water intake is a clear benefit especially because humans subjected to opioid treatment are frequently bedridden or have reduced motility.

Locomotion studies revealed that IbKTP-NH<sub>2</sub> impairs motor capacities and may eventually induce an anxiogenic effect because IbKTP-NH<sub>2</sub> treated animals spent less time in the centre of arena (Belzung 1999; Gould et al. 2009; Stohr et al. 1998). KTP-NH<sub>2</sub>, on the other hand, did not change the parameters evaluated, which supports previously published locomotion data using the Rotarod-test (Ribeiro et al. 2011a). Therefore, one can conclude that KTP amidation brings no adverse effects regarding locomotor capacities. Original kyotorphin itself was shown not to induce ataxia, catalepsy or hypermobility in rats and goldfish (Kolaeva et al. 2000). Structurally, the only difference between KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub> resides on the ibuprofen residue. Simultaneous administration of ibuprofen and opioids, mainly hydrocodone, is commonly used in clinical settings as this improves analgesic performance (Kolesnikov et al. 2003). However, some adverse effects are also associated with this treatment: anxiety, nausea and sleepiness, which are probably a synergistic combination of the side-effects of the two drugs. The results reported here for IbKTP-NH<sub>2</sub> treated animals in the open field test may be due to a similar synergistic effect of an opioid-like analgesic, kyotorphin, and ibuprofen. Nevertheless, these motor alterations do not interfere with the conclusion that IbKTP-NH<sub>2</sub> is an analgesic substance, since the nociceptive activity of neurons at the spinal cord is lower in IbKTP-NH<sub>2</sub> treated animals (Ribeiro et al. 2011b).

As to possible cardiovascular effects of KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub>, the present data did not show any alterations in blood pressure. This agrees with our preliminary data for KTP-NH<sub>2</sub> obtained 30 min after administration (Ribeiro et al. 2011a) and with results obtained with unmodified KTP: a transient and dose-dependent BP increase was detected after intracerebroventricular injection, but systemic administration did not cause any effects on BP (Summy-Long et al. 1998). These data show that amidation and conjugation of KTP with ibuprofen do not lead to cardiovascular effects of the peptide. Due to ethical constraints on the use of laboratory animals, the effects of morphine and tramadol in cardiovascular parameters were not evaluated since the literature provides extensive and clear data on their effects on BP. As to morphine, all studies performed with systemic administration reported effects on BP with the main difference depending on whether the animal is anaesthetized or not. For example, i.v. administration of morphine (0.1–2.5 mg/kg) in rats lightly anesthetized with pentobarbital sodium (Randich et al. 1991) induced dose-dependent hypotension. Gomes

and colleagues stated that i.v. morphine (10 mg/kg) transiently increased BP in conscious rats whereas in anaesthetized animals hypotensive responses were obtained even with low doses (1 mg/kg) (Gomes et al. 1976). In the dose used in the present study (5 mg/kg), morphine was shown to produce a short lasting hypotension in non-anaesthetized animals (Gomes et al. 1976). As to tramadol, it is considered haemodynamically stable (Mildh et al. 1999) but at high doses (5 and 10 mg/kg) cardiac depression and hypotension are induced (Raimundo et al. 2006). The present experiments allow us, therefore, to conclude that KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub> do not appear to induce effects in BP, suggesting that they do not stimulate cardiovascular effects.

In this work, we characterized the side-effect profile of new KTP-derivatives and showed their advantages over opioids morphine and tramadol. Data reported here clearly demonstrate that KTP-derivatives display a safer side-effect profile and give further evidence on the mechanisms of action of these peptides, in agreement with previously obtained results (Ribeiro et al. 2011a, b): (1) opioid receptors are involved in KTP-derivatives mechanism of action, (2) their activity is mainly at the CNS-level, (3) KTP-NH<sub>2</sub> does not cause motor impairment or blood pressure changes, and (4) KTP-derivatives mechanisms of action are distinct from those of classic opioids.

The only side-effect of KTP-NH<sub>2</sub> is a reduction in micturition, which may be exploited as a positive effect in cases of detrusor overactivity. IbKTP-NH<sub>2</sub> causes a mild motor impairment that is, however, less harmful than the collection of severe side-effects resulting from the administration of morphine and tramadol. In conclusion, KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub> associate strong analgesic activities with very important side-effect reduction when compared to morphine and tramadol (Table 1), which make them promising drugs for the future treatment of pain.

**Table 1** Comparative display of the side-effects following administration of either KTP-NH<sub>2</sub> (32.3 mg/kg), IbKTP-NH<sub>2</sub> (24.2 mg/kg), morphine (5 mg/kg), or tramadol (10 mg/kg)

Side-effect	Tested compounds			
	KTP-NH <sub>2</sub>	IbKTP-NH <sub>2</sub>	Morphine	Tramadol
Obstipation	–	–	+	–
Micturition reduction	+	–	–	+
Food intake increase	–	–	–	+
Water intake increase	–	–	–	+
Motor impairment	–	+	–	–
Blood pressure alterations	–	–	+ <sup>a</sup>	+ <sup>b</sup>

<sup>a</sup> From Gomes et al. (1976)

<sup>b</sup> From Raimundo et al. (2006)

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Section 4

# Final Conclusions

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Pain and neurodegeneration are huge clinical problems and even if, through the last decades, significant progresses have been made towards designing improved therapeutic interventions, considerable efforts are still needed in the search of the most appropriate, safe and effective strategies in the management of these conditions. Interventions over the vicious cycle between chronic pain and Alzheimer's disease is of extreme importance in order to slow down the whole process and lower the associated suffering. The present study was an approach towards that goal, following a multi-target combination of clinical and basic science.

The findings shown in the previous chapters gather epidemiologic information and molecular analytic results using mass spectrometry, demonstrating the need of a biomarker for pain in patients with AD, and that kyotorphin may be one of the links between the molecular mechanism of pain and neuroprotection.

In article 2 we show that pain is underestimated in Alzheimer's disease patients and their caregivers, reinforcing the need for a proper evaluation in the clinical context, through the use of validated scales instead of solely relying on the caregiver perception. This is an important finding because we are probably undertreating pain in this group of patients and untreated pain has several consequences, with symptoms such as sadness, anxiety, depression, irritability, sexual dysfunction, disruptions in sleeping, eating, mobility, impaired immune function, poor appetite and weight loss that are sometimes regarded as symptoms of other conditions and treated accordingly. The conclusion of this study is a confirmation that AD patients do not valorize their pain complaints, even in moderate stages of the disease and that a marker for their pain, would be of the utmost importance.

The clinical component of this work evolved into the determination of kyotorphin levels in the cerebro-spinal fluid of AD patients (article 3), and its correlation with a biomarker of the disease (p-Tau protein). We collected and processed samples of CSF patients with AD and of healthy ones (individuals who did not have any known

neurodegenerative diseases or other serious diseases related to the central nervous system). The collaboration with the University Hospital of Santiago de Compostela was critical in order to create a mass spectrometry method capable of determining the low levels of such a small peptide in a complex matrix such as CSF. A clear negative correlation between KTP levels and p-Tau was found. The hypothesis of KTP being a molecular link between pain and neurodegeneration is thus valid and new avenues are open in clinical research in the largely unexplored domain of pain in patients with cognitive impairment. It is equally important to pursue the stimulating hypothesis that KTP derivatives of pharmacological interest to fight pain may also have in itself a neuroprotective effect, unifying in a single strategy the amelioration of pain and neurodegeneration. More important, from the clinical point of view, KTP seems to follow the progression of AD. Since KTP may be an important molecule with neuroprotective properties (Bocheva, 2004; Nazarenko, 1999), we can hope that KTP may be a key-molecule in the link between pain and neurodegeneration. Moreover, it could also act as a molecular marker of pain, at least in this group of patients, unable to correctly express their suffering.

With these results in hand, we moved to the next step: evaluation of the effect of kyotorphin derivatives in the behavior of an animal model of AD. It must be remembered that the compounds KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub> had been previously tested (Ribeiro 2011a, Ribeiro 2011b) and were all able to elicit antinociceptive effects in animals, following systemic administration (i.p. or oral) at a relatively low dose and with no toxicity. KTP-NH<sub>2</sub>, in particular, showed to be active in several models of acute and chronic pain, including neuropathic pain. At the same time, derivatization with Ibuprofen granted KTP the ability to cross the BBB. More than being just a lipophilic sequence or an anti-inflammatory drug, Ibuprofen appears to work as a delivering agent, transporting the KTP across the BBB until its target site. These studies gave new insights regarding KTP derivatives mechanism of action: the administration of an opioid receptor antagonist (naloxone) previously to KTP peptides injection abolished

the nociceptive action of KTP-NH<sub>2</sub>, while for IbKTP-NH<sub>2</sub> the effect was reduced but still significant, what suggests that KTP-NH<sub>2</sub> is totally dependent on opioid pathways to elicit its activity, whereas IbKTP-NH<sub>2</sub> has a bivalent mechanism of action: opioid dependent and independent. This duality may explain IbKTP-NH<sub>2</sub> increased analgesic effect when compared to KTP-NH<sub>2</sub>. Anyway, both act at the CNS, as evidenced in the lower nociceptive activity of neurons in the spinal cord.

In accordance with our previous work, the AD animal model chosen was obtained through the bilateral occlusion of the carotid arteries (2 VO model) of female rats. In this model, the chronic hypoperfusion to key brain areas leads to neuronal death, mainly in the hippocampus and forebrain, therefore mimicking the pattern of AD. Moreover, in recent years, investigators have increasingly tended to accept that AD may be primarily a vascular disease with neurodegenerative consequences, rather than a neurodegenerative disorder with vascular consequences. Two key factors for the development of AD have thus been proposed: aging and decreased cerebral perfusion. The convergence of them will result in an entity called "critically attained threshold of cerebral hypoperfusion" (CATCH). It was proposed that the CATCH leads to a distortion of the architecture of the brain capillary, involving the release of nitric oxide (NO), which will affect the signaling between the immune, nervous and cardiovascular systems. NO is the product of the enzyme nitric oxide synthase (NOS) which exists in three isoforms: eNOS (located in the endothelium), nNOS (located in neurons), iNOS (inducible in neurons and glial cells after activation by the cytokines) (Zhu *et al*, 2007). Tissues that do not maintain the basal level of NO are more predisposed to an amendment of their regulation, extending the excitatory state. Thus, the concomitance of advanced age and a risk factor may contribute to vascular lesions involving a deficit of NO to such a degree that this could initiate the neurodegenerative changes characteristic of AD (De la Torre, 2000). The combination of this information found in the literature with our personal experience with kyotorphin led us to consider that, given the coincidence of cellular pathways involved,

KTP presents important properties that may influence the course of AD: potent analgesic, changing currents of calcium, substrate for nNOS, and neuromodulator/neuroprotector in key brain areas in Alzheimer's disease. Using behavioral tests such as the open field and Y-maze we found that KTP-NH<sub>2</sub> and Ibuprofen-KTP NH<sub>2</sub> were neuroprotective in the hippocampus and improved spatial working memory of rats subjected to the 2-VO procedure (article 4).

The evaluation of possible side effects of the derivatives and the comparison with current opioid drugs is shown in the article 5. First of all, we know that the original KTP does not bind to opioid receptors (Rackham, 1982) and that morphine and KTP derivatives seem to have distinct modes of action. The assay of opioid receptor binding clearly established this: KTP-NH<sub>2</sub> and IbKTP-NH<sub>2</sub> bind weakly to opioid receptors and without specificity (Ribeiro 2011a). We showed that common side effects intrinsically correlated with binding to opioid receptors as constipation, blood pressure alterations and variations in eating pattern were absent from the amidated derivatives. Opioid pathways are indirectly involved in KTP peptides mode of action, although in the case of IbKTP-NH<sub>2</sub>, blocking opioid receptors does not suffice to abolish its analgesic activity. There are studies showing that the existence of a specific receptor for KTP is unlikely (Lopes, 2006) and data support that KTP analgesic activity is probably through NO formation leading to the release of met-enkephalin (Arima, 1996; Arima, 1997).

Overall this project helped to build a bridge between two entities with a strong epidemiological burden and consequences at personal and social level: pain and Alzheimer's disease. It has been shown that kyotorphin and its derivatives are drugs with analgesic potential together with diminished side effects.

On the other hand, as we showed in our animal models, kyotorphin derivatives have neuromodulatory and/or neuroprotective properties and KTP itself can serve as a biomarker of Alzheimer's disease. In fact our study showed a clear negative correlation between KTP levels and p-Tau with KTP seeming to follow the progression of AD. In this study, Alzheimer's disease patients have demonstrated a degree of pain

underestimated even by their close caregivers; thus, a biomarker for pain in this group of patients is very important.

On the other hand, the usual age of AD onset coincides with that of the highest incidence of pathologies associated with chronic pain, whereby a drug combining analgesic and neuromodulatory properties is extremely promising.

In brief, this thesis revealed kyotorphin and its derivatives as potential biomarkers of pain in neurodegenerative conditions such as Alzheimer's disease, as well as drugs capable of neuromodulation, opening a door for more extensive studies in this domain and raising the hope of fighting pain even in patients incapable of expressing their suffering.

## Section 5

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