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The Role of Protein Convertases in Bigdynorphin and Dynorphin A Metabolic Pathway

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Résumé

Les dynorphines sont des neuropeptides importants avec un rôle central dans la nociception et l'atténuation de la douleur. De nombreux mécanismes régulent les concentrations de dynorphine endogènes, y compris la protéolyse. Les Proprotéines convertases (PC) sont largement exprimées dans le système nerveux central et clivent spécifiquement le C-terminale de couple acides aminés basiques, ou un résidu basique unique. Le contrôle protéolytique des concentrations endogènes de Big Dynorphine (BDyn) et dynorphine A (Dyn A) a un effet important sur la perception de la douleur et le rôle de PC reste à être déterminée. L'objectif de cette étude était de décrypter le rôle de PC1 et PC2 dans le contrôle protéolytique de BDyn et Dyn A avec l'aide de fractions cellulaires de la moelle épinière de type sauvage (WT), PC1 $^{-/+}$ et PC2 $^{-/+}$ de souris et par la spectrométrie de masse. Nos résultats démontrent clairement que PC1 et PC2 sont impliquées dans la protéolyse de BDyn et Dyn A avec un rôle plus significatif pour PC1. Le traitement en C-terminal de BDyn génère des fragments peptidiques spécifiques incluant dynorphine 1-19, dynorphine 1-13, dynorphine 1-11 et dynorphine 1-7 et Dyn A génère les fragments dynorphine 1-13, dynorphine 1-11 et dynorphine 1-7. Ils sont tous des fragments de peptides associés à PC1 ou PC2. En plus, la protéolyse de BDyn conduit à la formation de Dyn A et Leu-Enk, deux peptides opioïdes importants. La vitesse de formation des deux est réduite de manière significative dans les fractions cellulaires de la moelle épinière de souris mutantes. En conséquence, l'inhibition même partielle de PC1 ou PC2 peut altérer le système opioïde endogène.

Mots-clés: Dynorphines, Dynorphine A, Proprotéines convertases, Protéolyse, Peptides opioïdes, Moelle épinière, Spectrométrie de masse, Douleur, Synapse.

Abstract

Dynorphins are important neuropeptides with a central role in nociception and pain alleviation. Many mechanisms regulate endogenous dynorphin concentrations, including proteolysis. Proprotein convertases (PCs) are widely expressed in the central nervous system and specifically cleave at C-terminal of either a pair of basic amino acids, or a single basic residue. The proteolysis control of endogenous Big Dynorphin (BDyn) and Dynorphin A (Dyn A) levels has a profound impact on pain perception and the role of PCs remain unclear. The objective of this study was to decipher the role of PC1 and PC2 in the proteolysis control of BDyn and Dyn A levels using cellular fractions of spinal cords from wild type (WT), $PC1^{-/+}$ and $PC2^{-/+}$ animals and mass spectrometry. Our results clearly demonstrate that both PC1 and PC2 are involved in the proteolysis regulation of BDyn and Dyn A with a more important role for PC1. C-terminal processing of BDyn generates specific peptide fragments Dynorphin 1-19, Dynorphin 1-13, Dynorphin 1-11 and Dynorphin 1-7 and C-terminal processing of Dyn A generates Dynorphin 1-13, Dynorphin 1-11 and Dynorphin 1-7, all these peptide fragments are associated with PC1 or PC2 processing. Moreover, proteolysis of BDyn leads to the formation of Dyn A and Leu-Enk, two important opioid peptides. The rate of formation of both is significantly reduced in cellular fractions of spinal cord mutant mice. As a consequence, even partial inhibition of PC1 or PC2 may impair the endogenous opioid system.

Keywords: Dynorphins, Dynorphin A, Proprotein convertases, Proteolysis, Opioid peptides, Spinal cords, Mass spectrometry, Pain, Synapse.

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Abbreviations list

(-/+)	Knockdown
(-/-)	Knockout
A	Aspartic acid
ACTH	Adrenocorticotropic hormone
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APCI	Atmospheric pressure chemical ionization
ASIC	Acid-sensing ion channel
ATP	Adenosine triphosphate
BDyn	Big dynorphin
C	Cysteine
Ca_v	calcium voltage-gated channels
cDNA	Complementary deoxyribonucleic acid
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
D	Aspartic acid
DEG/ENaC	Degenerin/epithelial Na^+ channel
DOR	δ-opioid receptor
DRG	Dorsal root ganglion
Dyn	Dynorphin
E	Glutamic acid
EM	Endomorphin
End	Endorphin
ER	Endoplasmic reticulum
ESI	Electrospray ionization
ESI-MS	Electrospray ionization coupled mass spectrometer
F	Phenylalanine
g	Gravitational constant
G	Glycine
GABA	γ-Aminobutyric acid
H	Histidine

HPLC	High performance liquid chromatography
HRAM	High resolution accurate mass
I	Histidine
K	Lysine
KCNK	K^+ channel subfamily K
KOR	κ-opioid receptor
L	Leucine
Leu-Enk	Leu-Enkephalin
M	Metionine
m/z	Mass to charge ratio
Met-Enk	Met-Enkephalin
min	Minute
MOR	μ-opioid receptor
MRM	Multiple reaction monitoring
MS	Mass spectrometer
MSH	Melanocyte-stimulating hormone
N	Asparagine
Na_v	Voltage-gated sodium channels
NMDA	N-methyl-D-aspartate
NK1/2	Neurokinin-1/2 receptor
NKA	Neurokinin A
NKB	Neurokinin B
NPLC	Normal-phase liquid chromatography
p	Significance level (in statistics)
P	Proline
PAG	Periaqueductal
PC	Proprotein convertase
PDyn	Prodynorphin
PEnk	Proenkephalin
pH	power of hydrogen (acidity measurement)
PNS	Peripheral nervous system
POMC	Proopiomelanocortin

Q	Glutamine
Q-orbitrap	Quadrupole coupled orbitrap mass spectrometer
QqQ	Triple quadrupole mass spectrometer
Q-TOF	Quadrupole coupled TOF mass spectrometer
R	Arginine
RPLC	Reverse phase liquid chromatography
RVM	Rostral ventromedial medulla
S	Serine
SP	Substance P
SRM	Selected reaction monitoring
T	Threonine
TGN	*trans*-Golgi network
TIC	Total ion current chromatogram
TOF	Time of flight mass analyzer
TRPA	Transient receptor potential ankyrin
TRPM	Transient receptor potential melastatin
TRPV	Transient receptor potential vanilloid
V	Valine
W	Tryptophan
WT	Wild type
XIC	Extract ion chromatogram
Y	Tyrosine

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Introduction

Patients suffering from chronic or neuropathic pain have a highly compromised quality of life and account for approximately 20-25% of the population worldwide. Given the importance of managing pain in medicine, the complex mechanisms that encompass pain perception, transduction and modulations are currently subject of intense research.

Pain is usually caused by a noxious stimulation of the peripheral nervous system (PNS). The PNS is responsible for perceiving those stimuli and to project that information through afferent neurons to the spinal cord. The synaptic transmission takes place at the external laminae of the dorsal root between afferent neurons and secondary order neurons. This process is called first synapse and regulates the intracellular signaling between the PNS and the central nervous system (CNS), relaying nociceptive information to the brain where it is perceived as pain. The communication between neurons is mediated by the release of excitatory neurotransmitters from the terminal of the afferent neurons. The neurotransmitters, like glutamate and substance P (SP), activate secondary neurons by their interaction with post-synaptic receptors such as N-methyl-D-aspartate (NMDA) and neurokinin-1 (NK1) receptors. When a noxious stimulus is perceived, the release of those excitatory neurotransmitters is enhanced. As a response to that perception, the brain is able to modulate the activity at first synapses through different modulatory pathways. The release of endogenous opioid peptides into the first synapse is the principal endogenous mechanism for the alleviation of pain.

Four families of endogenous opioid peptides have been described to date. They include endorphins, endomorphins, enkephalins and dynorphins. These peptides are synthetized as large and inactive proneuropeptides and requires endoproteolytic processing to generate the bioactive peptides, which play an essential role in the endogenous modulation of pain. Several studies have shown that protein convertases (PCs), specifically PC1 and PC2, are involved into C-terminal endoproteolytic processing of proneuropeptides through their cleavage at basic residues of proteins and peptides. In neuronal cells, proneuropeptides and PCs are synthetized and packed into dense-core vesicles. During the axonal transport of these vesicles, proneuropeptides are processed by PCs prior their release by exocytosis at the first

synapse. As a consequence of a noxious stimulation, the production and the release of vesicles containing endogenous opioid peptides are significantly enhanced.

Dynorphins have been identified as an important family of endogenous opioid peptides with potent analgesic effects. Prodynorphin is the proneuropeptide precursor of dynorphins. Early studies have partially described the endoproteolytic processing of prodynorphin, including a fundamental role for PC1 and PC2. Those preliminary studies established that the action of PC1 and PC2 is needed for the formation of different high molecular weight dynorphin-peptides, Bigdynorphin (BDyn), Dynorphin A (Dyn A) and Dynorphin B (Dyn B). However, the different contribution of each endoprotease, including PC1 and PC2, in the regulation of endogenous BDyn and Dyn A levels still remains unclear.

The presence of paired and single basic residues on the primary sequence of BDyn and Dyn A suggest further C-terminal processing catalyzed by PC1 and PC2 leading to the formation of several important N-terminal metabolites. Further processing of BDyn and Dyn A can lead to the formation of bioactive peptides including Dynorphin 1-19 (Dyn 1-19), Dynorphin A (Dyn A), Dynorphin 1-13 (Dyn 1-3), Dynorphin 1-11 (Dyn 1-11), Dynorphin 1-10 (Dyn 1-10), Dynorphin 1-7 (Dyn 1-7) and Dynorphin 1-6 (Dyn 1-6). Interestingly all these prodynorphin-derived peptides encode a copy of Leu-Enkephalin (Leu-Enk), another important opioid peptide, at their N-terminal. Thus, Leu-Enk might be an important metabolic product of BDyn and Dyn A. The objective of this project is to study the metabolism of BDyn and Dyn A, identify and quantify the rate of formation of the metabolites, as well as clarify the role of PC1 and PC2 in regulation of the concentration of both neuropeptides.

This study was designed to develop an *in vitro* experimental procedure to show the enzymatic degradation of BDyn and Dyn A and to elucidate the roles of PC1 and PC2 in the proteolytic control of endogenous dynorphins levels. Mice lumbar spinal cord S9 fractions were isolated from 3 different mice genotype, wild type (WT), PC1-knockdown ($PC1^{-/+}$) and PC2-knockdown ($PC2^{-/+}$), and the cellular homogenates containing among other enzymes PC1 and PC2, were used as a source of endogenous enzymes for the *in vitro* digestion. High performance liquid chromatography (HPLC) separation coupled with an electrospray

ionization mass spectrometer (ESI-MS) was used for the identification and quantification of BDyn and Dyn A metabolites. Moreover, an isotopic dilution method was employed for peptide quantification.

The study was designed to provide a better understanding of the mechanisms involved in the endogenous control of peptide levels and their impact on pain modulation pathways. Since opioid drugs are widely used in pain treatment with serious side-effects, a better mechanistic understanding of endogenous opioid metabolic pathways could lead to the development of innovative strategies in the treatment of pain.

LITERATURE REVIEW

CHAPTER I-ENDOGENOUS MECHANISMS OF PAIN

I.1-Pain generalities

Pain has been defined by the International Association for the Study of Pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. This experience is essential for human survival since it protects body tissues from damage and alerts of stimulation in any part of the body by activating the surveillance mechanism and evoking the so-called muscular reflex arc.

Pain can be caused by diverse methods including physical injuries, infections or other forms of noxious stimulation. Understanding the nature of pain as well as its complex molecular mechanisms associated with pain perception and transduction is a challenge and many of these mechanisms are still not well understood.

Depending on its nature, pain can be subdivided into neuropathic, nociceptive and inflammatory pain. They are each generated for different reasons and need to be treated accordingly.

I.2-Pain pathologies

I.2.1-Neuropathic pain

The central nervous system (CNS) is responsible for pain perception. Any alteration on the CNS can lead to an inadequate perception making pain persist long after the initiating cause has ceased (Gold and Gebhart, 2010). Neuropathic pain is provoked from disorders, damage or injuries on peripheral nerves, dorsal root ganglion (DRG) or the CNS. Patients suffering neuropathic pain exhibit persistent pain independent of a stimulus (Devor, 2006). Depending its influence in the sympathetic nervous system, pain perception ranges from a persistent burning sensation evoked for any alteration on C-sensory neurons, to acute pain perception resulting by large myelinated A fibers. Alterations on the CNS also evoke two

characteristic pain sensitizations; hyperalgesia and allodynia (Ossipov and Porreca, 2005). Hyperalgesia is characterized as an enhanced and more prolonged pain response to noxious stimuli as a result of abnormal processing of nociceptor input. This is a pain hypersensitivity that usually accompanies inflammation (Schaible, 2006; Woolf and Salter, 2000). On the other hand, allodynia is a state of pain characterized by a pain sensation caused by a normally innocuous stimulus. It can be evoked by the action of low threshold myelinated Aβ fibers on an altered CNS and by the reduction of the activation threshold of nociceptors present in the PNS (Woolf and Mannion, 1999).

I.2.2-Nociceptive pain

Nociceptive pain, also described as physiological pain, is caused by injuries like cuts, burns and other excessive stimulation of nociceptors. Its purpose is to protect tissues from further damage activating withdraw reflexes (Schaible, 2006). It is characterized by aching, sharp, or throbbing sensations. Well localized, constant and time limited, this kind of pain typically resolves once the tissue damage heals (Woolf and Salter, 2000). Treatments with opioids-like drugs such as morphine or codeine tends to respond well to nociceptive pain (Matthes, et al., 1996; Mogil et al., 1996).

I.2.3-Inflammatory pain

After injury, primary sensory neurons and other non-neural cells respond producing and releasing chemical mediators at the site of tissue injury. This ensemble of chemicals, known as inflammatory soup, includes peptides (bradykinins, prostaglandins, interleukins), lipids and neurotransmitters (serotonin, ATP) among others chemical compounds. The interaction of those compounds with nociceptors leads to an increase of nociceptors sensitivity altering neuronal excitability (Julius and Basbaum, 2001). That results in the generation of pain in the absence of any stimulation. Moreover, the release of neurotransmitters, such as Substance P (SP) and Calcitonin Gene Related Peptide (CGRP), from the terminal fibers induces vasodilation and activation of many non-neuronal cells, including mast cells and neutrophils, which will contribute to the release of additional molecules into the inflammatory soup (Chiu et al., 2012). Inflammatory pain is treated using

anti-inflammatory drugs which reduce the release of essential molecules involved in neurogenic inflammation (Piomelli et al., 2014; Murata et al., 1997).

I.3-Pain physiology

I.3.1-Nociceptive process

The nociception process includes the detection of noxious stimuli at cutaneous and deep somatic tissues innervated by primary afferent neurons, and the subsequent transmission of that information to the brain. The afferent neurons present nociceptors on their terminals that are responsible for noxious or damaging stimuli transduction being activated when the stimuli are sufficient to initiate an action potential (Kidd and Urban, 2001). The information encoded as an action potential is then transmitted through afferent neurons located in the dorsal horn of the spinal cord where, within specific laminae, a synaptic transmission will occur with second order neurons. Sensory information is then carried through these secondary order neurons to supraspinal structures where it is transduced and finally perceived as pain. (Woolf and Salter, 2000).

I.3.1.1-Primary sensory neurons

Nociceptors are commonly associated with primary afferent neurons (Aδ and C fibers) relaying information about noxious perceptions from the periphery to the CNS, making up the so-called nociceptive system. Primary sensory neurons are activated when a harmful or damaging stimuli are perceived by the nociceptors. C-fibers and Aδ-fibers are considered as the afferent fibers signaling nociceptive perceptions whereas Aβ-fibers are not involved on pain transmission and they predominantly carry information concerning innocuous perceptions such light touch or pressure (Figure 2).

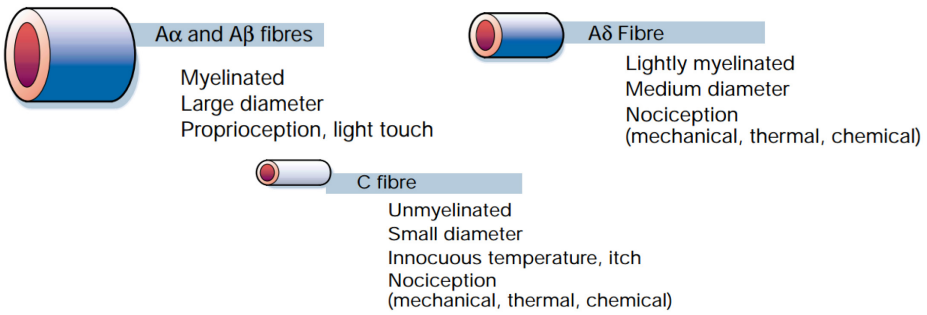

Figure 1: *Primary afferent neurons include large-diameter (Aβ), medium-diameter (Aδ) and small-diameter unmyelinated (C) afferent fibers (Adapted from Julius and Basbaum, 2001)*

Aβ fibers have the largest diameter and are myelinated. The layer of myelin, a dielectric compound which prevents the electric current from leaving the axon, confers a high speed of propagation of impulses along these fibers (Russell, 1992). Aβ fibers are not involved in pain perception but allow the integration of innocuous stimuli such as cold or warm feeling, sense of touch, vibration and light pressure (Julius and Basbaum, 2001).

Aδ fibers are medium-diameter fibers and are also myelinated. They are polymodal and more importantly, play a central role in the transmission of intense heat, mechanical and chemical noxious perception. The high speed of propagation of noxious stimuli though these fibers, conferred by their myelination and diameter, link Aδ fibers on the perception of acute and fast pain called first pain (Adriaensen et al., 1983).

C-fibers, are the smallest in diameter and are unmyelinated. Their small diameter and the lack of myelination results in a slow conduction velocity (Bouhassira, 2009). These fibers mediate the so-called second or slow pain. C-fibers are also polymodal responding to thermal, mechanical and chemical stimuli (Basbaum et al., 2009). Both Aδ and C-fibers have elevated activation threshold and are involved in noxious stimulus perceptions (Schaible, 2006).

I.3.1.2-Ion channels

Harmful or damaging stimuli perception is mediated in the first instance by voltage-gated ion channels composed of complex transmembrane proteins. The activation of ion channels leads the efflux or influx of specific ions through the channel resulting in a polarization or depolarization of the cell membrane modulating the electrical excitability of

neurons. A vast majority of nociceptors are ion channels or are associated with them allowing rapid membrane depolarization (Takayama et al., 2015). Ion channels permeable to different ions have been identified along the CNS. They play a fundamental role in the generation of action potentials and their propagation through afferent fibers.

I.3.1.2.1-Sodium channels

Voltage-gated sodium channels (Na_v) are present in all sensory neurons. They initiate action potentials in neuronal cells though the influx of Na^+, producing a fast depolarization of the membrane. Many Na_v have been identified and they have similar functions (Goldin et al., 2000). Nav1.7, Nav1.8 and Nav1.9 have been demonstrated to be critical for pain perception. Genetically engineered mice lacking those channels shown a clear insensibility to pain (Cummins et al., 2007).

I.3.1.2.2-Calcium channels

In neuronal cells, calcium voltage-gated channels (Ca_v) are the principal ionic channels involved in regulating the release of neurotransmitters during synaptic transmission. $Ca_v2.2$, located on nerve terminals, has been described to be essential for initiating presynaptic neurotransmitter release by neuronal cells as a response to the influx of Ca^{2+} (Catterall, 2000; Catterall and Few, 2008). Moreover, the influx of Ca^{2+} through Ca_v into cell cytosol is also crucial to regulate the activity of cytosolic enzymes and other biochemical processes (Flavell and Greenberg, 2008). In addition, as shown below, several nociceptors, such as TRPV receptors are in fact Ca_v, the activation of which leads to membrane depolarization and the generation of action potentials (Fernández-Carvajal et al., 2011).

I.3.1.2.3-Potassium channels

Potassium channels are primarily involved in repolarizing the membrane. The influx of K^+ though these channels modulates the formation of action potentials on sensory neurons. The activity of potassium channels is essential to control the length and frequency of the action potential (Brady et al., 2005). In addition, potassium channels have been demonstrated to be associated with various opioid receptors, which open specific potassium channels and prevent the excitation and propagation of action potentials (Ocaña et al., 2004).

I.3.1.2.4-Chloride channels

The concentration of Cl^- ions in neurons is low, therefore an influx or efflux of Cl^- through chloride channels drastically leads to a rapid membrane polarization or depolarization respectively, making these channels crucial for the control of neuronal excitability (De Koninck, 2007). Chloride channels such as anoctamin 1 have been found associated with nociceptors providing a rapid depolarization mediated by the efflux of Cl^- (Takayama et al., 2015). In contrast, the activation of the chloride channels γ-aminobutyric acid (GABA) receptors, one of the most important synaptic receptors, leads to a hyperpolarization of the membrane by the influx of Cl^-, resulting in a reduction in the probability of action potential initiation and causing neuronal inhibition (Brady et al., 2005; Duran et al., 2010).

I.3.1.3-Pain receptors, nociceptors

Pain usually starts with the activation of sensory receptors known as nociceptors (Woolf, 2011; Scholz and Woolf, 2002). Nociceptors are located at terminal axons of peripheral sensory neurons that innervate skin, organs, joints and viscera. Nociceptors are able to respond selectively to different tissue stimulations (Gold and Gebhart, 2010). They respond to specific noxious threshold stimuli but do not respond to innocuous stimuli. Their activation can result from noxious heat and cold perception (heat/cold sensitive receptors), from chemical compounds (sensitive to chemical compounds receptors) or from mechanical stimuli (mechano-transducers) (Figure 1) (Schaible, 2006). The activation threshold of those different nociceptors depends on the tissue or organ innervated. As an example, activation threshold in tissues such as the cornea is low compared to other tissues such as the skin (Gold and Gebhart, 2010). Moreover, the sensitivity of nociceptors can be altered after tissue injury owing to the release of inflammatory chemical compounds which reduce nociceptor activation threshold (Chiu et al., 2012). The excitation of nociceptors by mechanical, thermal and chemical stimuli evokes membrane depolarization, leading to the generation of an action potential. (Gold and Gebhart, 2010)

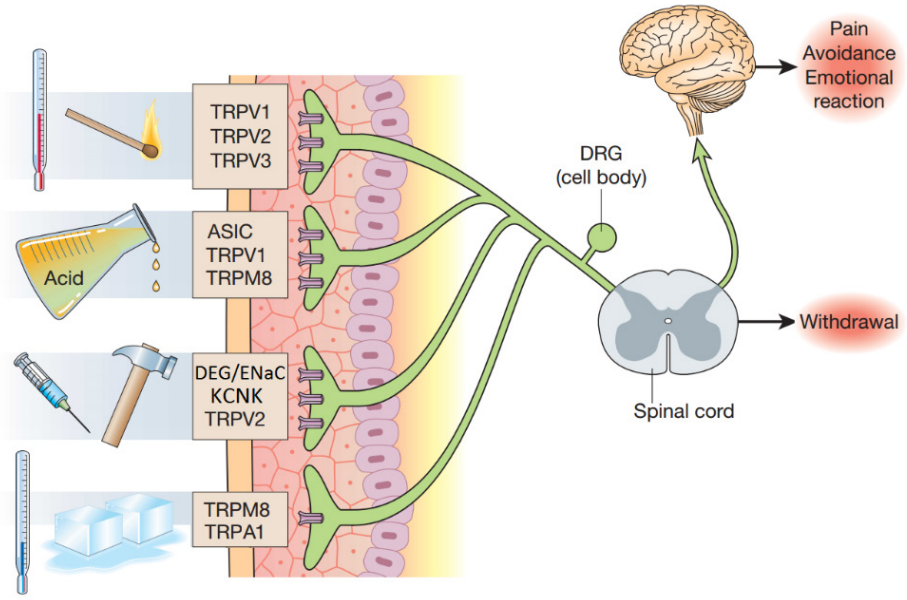

Figure 2: *Thermal, mechanical and chemical noxious stimuli are perceived by nociceptors located at the terminal of afferent neurons. The activation of nociceptors leads to the generation of action potentials which rely noxious perceptions to the brain (Adapted from Scholz and Woolf, 2002).*

I.3.1.3.1-Heat sensitive receptors

Transient receptor potential vanilloids (TRPV) are responsible for heat stimuli perception. Their thermal stimulation open the channels and allow the influx of Ca^{2+}, resulting in cell depolarization (Schaible, 2006). Heat-sensitive TRPV receptor family comprises 3 essential receptors TRPV1, TRPV2 and TRPV3. TRPV1 and TRPV2 are activated by noxious heat (>42° C) (Fernández-Carvajal et al., 2011), whereas TRPV3 is activated by innocuous warmth (30°C–40°C), maintaining its activation at noxious temperatures. Those receptors and expressed at high levels along the PNS and CNS in small diameter C and Aδ fibers. Its activity is enhanced under inflammatory pain conditions (Julius and Basbaum, 2001).

I.3.1.3.2-Chemical sensitive receptors

Acid-sensing ion channel receptors (ASICs) are the most important and studied chemical sensitive receptor. ASICs are Na^{+} channels opening at acidic pH (<5) (Sazanavets and Warwicker, 2015; Babinski et al., 1999). They serve among other things as a receptor

for extracellular proton release following a tissue injury (Sutherland et al., 2001). In addition, some thermo-sensitive nociceptors such as TRPV1 and TRPM8 are also considered chemically sensitive receptors. TRPM8 can be activated, in addition to thermal stimulus, by menthol and TRPV1 by capsaicin and acids (Karashima et al., 2007; Bandell et al. 2004). The importance of the polyvalence of some nociceptors is reflected, for example, by the involvement of TRPV1 receptors on the burning pain perceived during inflammation, which is a result of the interaction of protons contained in the inflammatory soup with TRPV1 heat-sensitive receptors (Reid and Flonta, 2001). Chemical sensitive receptors have been reported to be present along PNS and CNS in C and Aδ fibers (Iida et al., 2003).

I.3.1.3.3-Mechano-transducers

Although the molecular mechanisms of mechano-transducers are not fully understood, recent studies suggested a major role for degenerin/epithelial Na^+ channel (DEG/ENaC) and K^+ channel subfamily K (KCNK) for the transduction of mechanical stimulus (Mano and Driscoll, 1999; Bautista et al., 2008). Likewise, the TRPV2 channels can respond to osmotic stretch in addition to noxious heat, denoting their role in mechano-transduction (Basbaum, et al., 2009). A variety mechano-transducer ranging from high threshold activation mechano-receptors are found on C and Aδ fibers and low threshold mechano-transducers which are found on Aβ fibers capable of detecting light pressure, vibration or texture.

I.3.1.3.4-Cold sensitive receptors

Transient receptor potential melastatin 8 (TRPM8) and transient receptor potential ankyrin 1 (TRPA1) ion channels have been proposed as the most essential cold sensitive receptors, whose activation allows the entry of Na^+ and Ca^{2+} ions to the cell. TRPM8 is the best known cold sensitive receptor. This receptor is responsible for cold perceptions within the range of innocuous temperatures below 30°C. (Fernández-Carvajal et al., 2010). On the other hand, TRPA1 receptor is responsible for the perception of cold stimuli within noxious range (<15ºC) (Basbaum, et al., 2009). Both TRPM8 and TRPA1 are also sensitive cooling agents such as menthol and eucalyptol (Karashima et al., 2007; Bandell et al. 2004). These

nociceptors were identified on C and Aδ fibers, into the CNS and in a variety of tissues (Dhaka et al. 2007; Simone and Kajander 1996).

I.3.2-Ascending pathway

Sensory information perceived by the nociceptors is carried through the primary afferent neurons and reach the spinal cord. The ascending pathway comprises the process of signaling between primary afferent neurons and secondary order neurons at the spinal cord, as well as the ascension of the information through the secondary order neurons to supraspinal structures (Figure 3). In the brain noxious information is translated and then perceived as a noxious stimuli. The signaling process between primary afferent neurons and secondary order neurons is known as the first synapse. This process takes place at the spinal cord and allows the communication between neurons via the release of neurotransmitters.

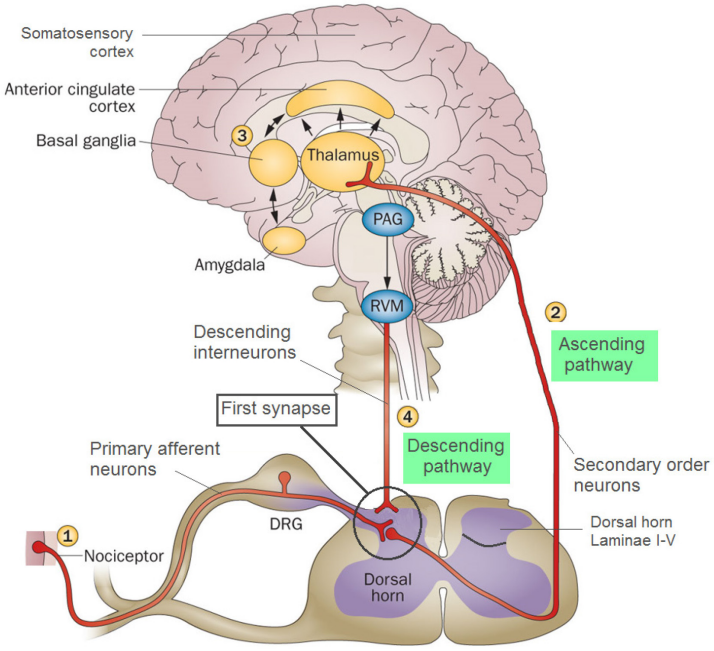

Figure 3: *Schematic of the pain pathway. (1) The PNS is responsible of noxious stimuli perception and the transmission of noxious information to the spinal cord though primary afferent neurons. (2) Signaling between afferent and secondary neurons take place at the first synapse. (3) These secondary order neurons transmit the information to the thalamus which engage the somatosensory cortex, providing information about the location and intensity of the painful stimulus. Other projections engage the thalamus with cingulate cortex and amygdala, contributing to the emotive component of the pain experience. (4) Noxious stimulus inputs activates the endogenous modulating system, stimulating the synthesis of endogenous opioids and their release into the first synapse, resulting in a modulation of the synaptic activity (Adapted from de Lalouvière et al. 2014).*

I-3.2.1-First Synapse

In the late 19th century the first neuronal connections were discovered. The connections were found to be discontinuous and the communication between neurons was observed to be carried out by the so-called synaptic transmission (López-Muñoz et al., 2006). Nowadays the communication between neurons is known to be mediated by the release of excitatory neurotransmitters from the terminal of primary neurons. Through the interaction of these released neurotransmitters with their receptors located on secondary neurons, the secondary neurons are activated and the information is transferred. The first synapse is the process through which the PNS and the CNS are connected. It takes place at the dorsal horn of the spinal cord within laminae I to V and allows the signaling between afferent and secondary order neurons which relay information to supra-spinal structures (Basbaum et al., 2009).

The synaptic process is a dynamic mechanism of neurotransmitters release which keeps a basal activity of sensory neurons. However, noxious stimulations of the PNS and CNS enhances the activity at the first synapse. Action potentials generated as a consequence of a harmful perception by the nociceptors, are propagated through primary sensory neurons stimulating the synthesis of specific peptidergic neurotransmitters at the DRG along with the opening the Ca^{2+} channels at the terminal of the neuron. These peptides involved in the signaling of noxious information between neurons are packed into secretory vesicles and are carried through the axons to the presynaptic terminal (Zhang et al., 1995). The high concentrations of Ca^{2+} at the terminal, caused by the Ca^{2+} influx through the Ca^{2+} channels activated by the action potential, facilitate the release of the vesicle content from the presynaptic terminal into the synaptic gap by exocytosis (Margeta et al., 2008; Südhof, 2004). The interaction between the released neurotransmitters with specific post-synaptic receptors stimulates the postsynaptic terminal and generates a new action potential at the secondary order neuron (Brady et al., 2005).

I-3.2.1.1-Principal synaptic neurotransmitters and receptors

Several neurotransmitters are involved in the neuronal synaptic communication. Glutamate and ϒ-aminobutyric acid (GABA) are the major chemical neurotransmitters involved, and it is believed that approximately 80-90% of the synapses in the CNS are glutamatergic (Brady et al, 2005). Glutamine is synthesized in neurons and metabolized to glutamate by the mitochondrial enzyme glutaminase (Olsen and DeLorey, 1999). Glutamate binds to N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) postsynaptic receptors producing excitatory postsynaptic response (Purves et al., 2001). The NMDA receptor is an ionotropic receptor which, following activation, produces an influx of cations mainly Na^+ and K^+ but also Ca^{2+} into the cell. On the other hand, AMPA receptor is also an ionotropic transmembrane receptor, and which gates mainly Na^+ and K^+, but not Ca^{+2} ions. Their activation and the consequent influx of cations produces a membrane depolarization at the postsynaptic terminal, resulting in the generation of an action potential (Purves et al., 2001). Glutamate is the precursor for GABA, a major inhibitory neurotransmitter. GABA activates GABA receptors, which are ionotropic channels permeable to Cl^-. The transmembrane influx of Cl^- through GABA receptors leads to membrane hyperpolarization reducing the presynaptic release of neurotransmitters as well as the excitability of postsynaptic receptors (Petroff et al., 2002; Schousboe et al., 2007).

The perception of intense stimulus is directly associated with the release of peptidergic excitatory neurotransmitters including tachykinins and calcitonin gene-related peptide (CGRP). Thus, perceptions on the noxious range promote the production of tachykinins and CGRP at the DRG and their subsequent release into the synaptic gap. Tachykinins are a family of neurotransmitters that includes peptides such as Substance P (SP), Neurokinin A (NKA) and Neurokinin B (NKB). These tachykinin-related peptides act on neurokinin receptors (i.e. NK1, NK2 and NK3). The activation of neurokinin receptors, particularly the activation of NK1 by SP, generates a greater post-synaptic response and enhances NMDA receptors activity (Gao and Peet, 1999; Teodoro et al., 2013). CGRP also plays an important role in nociception, it is a potent vasodilator and the release of CGRP potentiates the action of SP by inhibiting its enzymatic degradation and by enhancing its release (Gangula et al., 2000; Bennett et al., 2000; Biella et al., 1991).

I.3.3-Descending pathway

The perception of well-being is the result of the equilibrium between an incessant release of excitatory neurotransmitters at spinal levels, which allow the brain to perceive noxious information, and the suppressive influences of the same importance that descends from the brain. When the ascending signal is more intense than the suppressing action, pain appears (Beaulieu 2005).

By the time the brain receives noxious stimuli inputs this perception is projected to the periaqueductal gray (PAG) and the rostral ventromedial medulla (RVM) activating the descending pathway (see Figure 3) and leading to a nociceptive modulation (Lovick, 1991; Helmstetter et al., 1998). This modulating process is principally mediated by the release of endogenous opioid peptides from descending interneurons into the first synapse. Through the interaction of these opioid peptides with their respective pre- and post-synaptic opioid receptors the synaptic activity is modulated, reducing the intensity of noxious perceptions.

I.3.3.1-Opioid receptors and endogenous opioid peptides

Since their discovery in 1970s, research related to opioid peptides revealed fundamental CNS functions (Brownstein, 1993). Endogenous opioid peptides participate in pain modulation producing analgesia and a sense of well-being (Froehlich, 1997). Synthetized as large and biologically inactive precursors at the DRG, pituitary and adrenal gland, they require enzymatic processing to generate active opioid peptides. Similar to other neuropeptides, opioid peptides are synthetized and packed into dense core vesicles (Hook, 2006). Following noxious stimuli, the synthesis and release of these vesicles are enhanced, leading to measurable modulatory effects on pain perception (Alberts et al., 2002).

Opioid peptides are classified into four major families; endorphins, endomorphins, enkephalins and dynorphins. Each family is derived from a distinct precursor (Figure 4) and has a characteristic anatomical distribution and physiological activities (McDonald and Lambert, 2014). Moreover, each family of endogenous opioid peptides has specific affinity for the opioid receptors present into the CNS. These receptors include μ-opioid receptors (MOR), κ-opioid receptors (KOR) and δ-opioid receptors (DOR) (Table 1).

Precursor	Endogenous peptide	Amino acid sequence	Affinity for Opioid receptors		
			MOR	DOR	KOR
Pro-Opiomelanocortin	β-endorphin	YGGFTMTSEKSQTPLVYLFKNAIIKNAYKKGE	+++	++	-
Unknown	Endomorphin-1	YPWF-NH2	+++	-	-
	Endomorphin-2	YPFF-NH2	+++	-	-
Pro-Enkephalin	Met-Enkephalin	YGGFM	++	+++	-
	Leu-Enkephalin	YGGFL	+	+++	-
Pro-Dynorphin	Dynorphin A	YGGFLRRIRPKLK	+	-	+++
	Dynorphin B	YGGFLRRQFKVVT	+	+	+++

Table 1: *Most representative opioid peptide derived from the processing of their respective precursors. Affinity observed for opioid receptors (Stein et al. 2009; Merg et al., 2006; Beaulieu, 2005; Mansour et al., 1995; Raynor et al., 1994).*

I.3.3.1.1-Opioid receptors

Opioid receptors are G protein-coupled receptors characterized by 7 transmembrane domains. Pharmacological, behavioral, and receptor binding studies have suggested the existence of at least three types of opioid receptors including MOR, KOR and DOR (Snyder and Pasternak, 2003). These receptors are highly abundant in the brain and the spinal cord. Opioid receptors are mainly located in the superficial dorsal horn (Zöllner and Stein, 2007). These receptors induce specific pharmacological response and they differ in their binding characteristics, even though a specific opioid peptide can interact with more than one type of opioid receptor (Lutz and Pfister, 1992; Ji et al., 1995; Mcnally and Akil, 2002).

The binding of opioid peptides to opioid receptors initiates a series of biochemical events that usually culminates in the stimulation of potassium efflux though the potassium ion channels, leading to a repolarization that results in various effects, including analgesia and euphoria (Ikeda et al., 2002; Maldonado et al., 2001; North et al., 1987). KORs are located presynaptically on primary afferent neurons in the dorsal horn of the spinal cord where they participate in the inhibition of the release of excitatory neurotransmitters such as SP, CGRP and glutamate. DORs are present on postsynaptic terminals of secondary order

neurons, and they decrease the excitability provoked by the activation of other postsynaptic receptors such as NK1 and NMDA. MORs are located either at presynaptic or postsynaptic terminals, therefore, MORs can either modulate the release of excitatory neurotransmitters or decrease the excitability of postsynaptic receptors (McDonald and Lambert, 2014).

I.3.3.1.2-Endorphins

Endorphins are endogenous opioid peptides produced during arduous exercise, excitement or pain perception, specifically inducing analgesia and well-being feeling (Sprouse-Blum et al., 2010). They are found widely distributed in the PNS and CNS (Marvizón et al., 2009). Endorphins are derived from the precursor proopiomelanocortin (POMC) a 241 amino acids protein (Smith et al., 1988). Its endoproteolytic processing by protein convertases (PCs) generate various bioactive peptides, such as β-endorphin (β-End), in addition to several non-opioid neuropeptides including adrenocorticotropic hormone (ACTH) and α-, β- and γ- melanocyte-stimulating hormone (α-MSH, β-MSH and γ-MSH) (Mousa et al., 2004). Binding affinity experiments of β-End for opioid receptor have shown a primary affinity for MOR, even though a high affinity for DOR was also reported (Mansour et al., 1995).

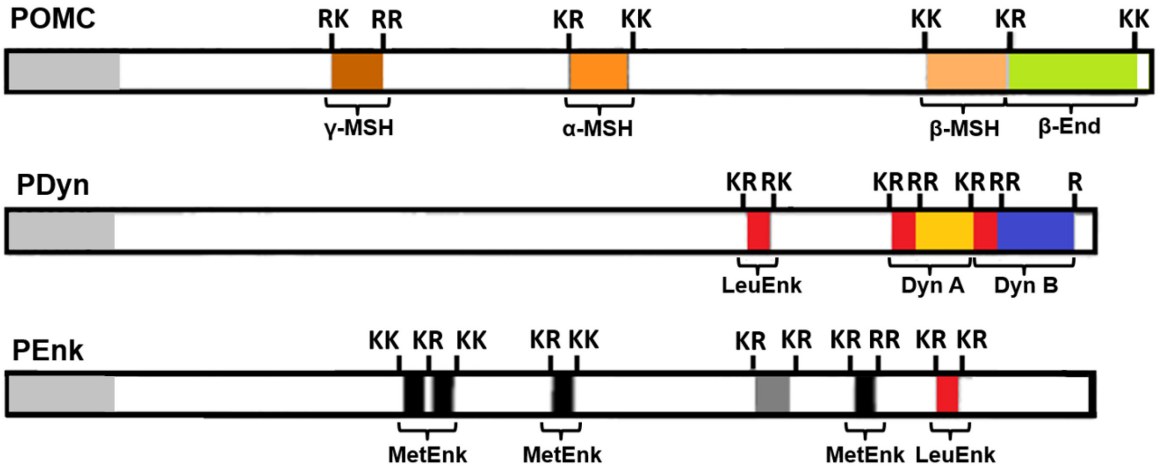

Figure 4: *Opioid-derived peptides are generated from the endoproteolytic processing of proopioid precursors POMC, PDyn and PEnk. With the exception of dynorphins, the enzymatic formation of opioids peptide are regulated by cleavages at dibasic amino acid positions.*

I.3.3.1.3-Enkephalins

Enkephalins are pentapeptides found in many different regions in the CNS, suggesting that these peptides are involved in many physiological functions. Among other functions enkephalins are involved in pain perception, mood and behavior by altering emotional responses as well as by acting on cardiovascular and respiratory functions (Przewłocki and Przewłocka, 2001; Mediavilla, 1977). In pain modulation, enkephalins have a potent action but with a short duration due to their rapid degradation by metallopeptidases (Mosnaim et al., 2008). Proenkephalin (PEnk), a protein constituted of 267 amino acids, is proteolytically cleaved into enkephalin peptides. PEnk processing by PCs results in the generation of 4 copies of Met-enkephalin (Met-Enk) and one copy of Leu-Enkephalin (Leu-Enk) (Loh et al., 2002). Both enkephalins have high affinity for DOR and moderate affinity, approximately tenfold lower, for MOR (Roques et al., 2012; Zöllner and Stein, 2007).

I.3.3.1.4-Endomorphins

The most recent family of endogenous opioid peptides discovered is endomorphins. They have a key role in pain perception, responses related to stress, and complex functions such as reward, arousal, and vigilance (Fichna et al., 2007). Although the endomorphin precursor still remains unidentified, two endomorphin peptides have been identified; endomorphin-1 (EM-1) and endomorphin-2 (EM-2). Both endomorphins differ just in one amino acid and are quite distinct from the other opioid peptides (endorphins, enkephalins and dynorphins), which all share the YGGF amino acid sequence at the N-terminus. EM-1 and EM-2 are widely distributed throughout the CNS and bind selectively to the MOR. Both endomorphin peptides have high affinity and similar potency for MOR (Zadina et al., 1997; Hackler et al., 1997; Martin-Schild et al., 1999).

I.3.3.1.5-Dynorphins

Endogenous dynorphin peptides are generated from the endoproteolytic processing of their precursor prodynorphin by PCs. This family of dynorphin peptides includes big dynorphin, dynorphin A and dynorphin B. They have primary affinity for KOR and their biological

functions comprise, among several others, a key role in pain modulation. The functions of the principal dynorphin peptides will be thoroughly described in the next chapter.

CHAPTER II-DYNORPHINS

II.1-Dynorphins generalities

The modulation of sensory information has been shown to take place in the dorsal horn of the spinal cord, more specifically at the first synapse (Honore et al., 2000; Levine, 1993). Dynorphins are a family of endogenous opioid peptides that have potent analgesic effects and have been identified as neuropeptides involved in endogenous pain inhibition (Kuner, 2010; Mika et al., 2011). Dynorphin peptides are widely distributed in the mammalian CNS and have a primary affinity for KOR (Stein et al. 2009; Schaible, 2006). This class of opioid peptides is known to be involved in a wide range of functions, including mood, motor activity and homeostatic response to injury and contributes to perceptual distortion in schizophrenia, dementia and bipolar disorders. An altered expression of dynorphins is also observed in drug abusers and psychiatric patients (Solbrig and Koob, 2004; Hurd, 2001; Hurd et al., 1996). Like other endogenous opioid peptides, bioactive dynorphins result from the processing of its large and inactive precursor, Prodynorphin (PDyn). PCs, specifically PC1 and PC2, found in neuronal and endocrine cells, were reported to be involved into PDyn proteolytic processing by cleaving its basic amino acid residues (Berman et al., 2000; Dupuy et al., 1994; Day et al., 1998).

II.2- Prodynorphin

Dynorphin peptides are derived from PDyn, a 254 amino acid biologically inactive protein which undergoes enzymatic degradation by PCs generating dynorphin peptides. PDyn was first characterized in 1982 from porcine neuronal tissues by using cDNA hybridization (Kakidani et al., 1982). As shown in figure 5, mouse, rat and human PDyn present a high homology at the amino acid level, sharing an identical sequence for the region encoding dynorphin A (Dyn A) and dynorphin B (Dyn B) (Civelli et al., 1985).

PDyn, similarly to other proneuropeptides, is synthesized in the endoplasmic reticulum (ER), of neuronal cells, and is transferred to the Golgi apparatus where it is packed in dense core vesicles together with the endoproteases PC1 and PC2 (Hook et al., 2008).

Dense core vesicles are then transported along the axons to the nerve terminal. Classical models postulated that PDyn processing by endoproteases starts at the *trans*-Golgi network (TGN) and continues during the axonal transport of the secretory vesicles (Hökfelt et al., 2000; Alberts et al., 2002). Potassium-induced depolarization produced by neuronal activity, stimulates the production, the migration and the exocytotic release of dynorphin-containing vesicles into the first synapse (Seidah and Chrétien, 1999; Zhou et al., 1999; Arvan and Castle, 1998). Others have proposed that vesicles containing unprocessed PDyn are stored at the nerve terminal waiting for depolarization to induce precursor processing and its release into the synaptic space (Yakovleva et al., 2006).

The highest concentrations of unprocessed PDyn are found in the hypothalamus, striatum, and hippocampus. Less important amounts are also found in the midbrain, nucleus tractus, brainstem, and cerebral cortex. In non-brain tissues, PDyn is found in the adrenal gland, spinal cord, testis, and anterior pituitary (Civelli et al., 1985).

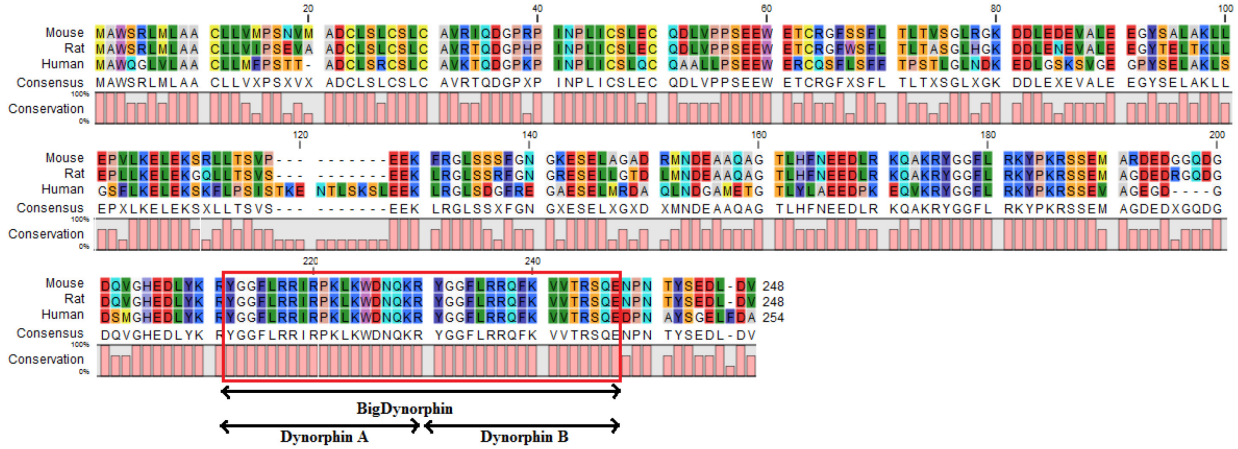

Figure 5: *Mouse, rat and human Prodynorphin proteomic alignment. High homology is observed in multiple regions between species. The region encoding BDyn is completely homologue for the three species. This homology shows high conservation of this gene through evolution.*

II.3-Endogenous Dynorphin peptides

Several active dynorphin peptides have been identified in mammalian brain and spinal cord as potential products from PDyn proteolytic processing. These PDyn-derived peptides include BDyn, Dyn 1-19, Dyn A, Dyn B, Dyn 1-13, Dyn 1-11, Dyn 1-10, Dyn 1-9, Dyn 1-8, Dyn 1-7 and Dyn 1-6 (Lu et al., 2001; Mansour et al., 1995; Reed et al., 2003; Prokai et al., 1998; Chou et al., 1994) (Table 2). So far, BDyn, Dyn A and Dyn B have been the dynorphin peptides which were extensively studied with a strong emphasis on Dyn A physiological activities.

Peptide	Amino acids	Sequence
Bigdynorphin	1-32	*YGGFLRRIRPKLKWDNQKRYGGFLRRDFKVVT*
Dynorphin 1-19	1-19	*YGGFLRRIRPKLKWDNQKR*
Dynorphin A	1-17	*YGGFLRRIRPKLKWDNQ*
Dynorphin B	20-32	*YGGFLRRDFKVVT*
Dynorphin 1-13	1-13	*YGGFLRRIRPKLK*
Dynorphin 1-11	1-11	*YGGFLRRIRPK*
Dynorphin 1-10	1-10	*YGGFLRRIRP*
Dynorphin 1-9	1-9	*YGGFLRRIR*
Dynorphin 1-8	1-8	*YGGFLRRI*
Dynorphin 1-7	1-7	*YGGFLRR*
Dynorphin 1-6	1-6	*YGGFLR*

Table 2: *Prodynorphin-derived peptides amino acid sequences*

Dyn 1-19, in spite of being the major product from Bigdyn endoproteolytic processing (Berman et al., 2000), owing to its rapid conversion into Dyn A by carboxipeptidases is not a highly abundant peptide in the CNS and its role is still quite unclear (Berman et al. 2001).

Dyn 1-13 has been presented as an extraordinarily potent opioid peptide with a long duration of action (Goldstein et al., 1979), acting on KOR (Oka et al., 1982). Its administration was reported to induce catalepsy and analgesia in rats (Herman et al., 1980). Studies also shown that the expression of opiate withdrawal symptoms after the administration of Dyn 1-13 were suppressed in mice (Takemori et al., 1992 and 1993; Khazan

et al., 1983; Aceto et al., 1982; Hooke et al., 1995), suggesting that, in vivo, Dyn 1-13 does not behave as a typical KOR agonist, but its role still needs to be clarified.

On the other hand, the effects of Dyn 1-11, Dyn 1-10, Dyn 1-9, Dyn 1-8, Dyn 1-7 and Dyn 1-6 remains relatively unexplored. Even though they have been identified as proteolytic products of PDyn, BDyn or Dyn A, just few studies have been published corroborating their antinociceptive effects, while their specific physiological role still remains to be determined (Lu et al., 2001; Mansour et al., 1995; Reed et al., 2003; Prokai et al., 1998; Chou et al., 1994; Herman et al., 1980).

II.3.1-BigDynorphin

BDyn is the largest and bioactive PDyn-derived peptide, consisting on Dyn A and Dyn B bound by arginine-lysine (KR) paired basic amino acids. In addition to serving as a precursor for Dyn A and Dyn B, this peptide may also have its own function. Found at substantial levels in the pituitary gland, brain and spinal cord (Xie and Goldstein, 1987; Day and Akil, 1989), the effects of BDyn differs from those of the other dynorphin peptides. The intrathecal and intracerebral administration of BDyn to mice were reported to produce a nociceptive behavioral response of the animal (Tan-No et al. 2002). These nociceptive responses were associated with the interaction of BDyn with NMDA receptors (Merg et al., 2006; Chen et al., 1995). In addition, the binding affinities and potency of BDyn for opioid receptors were also studied, showing that BDyn also has a strong affinity for KOR. Although BDyn affinity for KOR was comparable to Dyn A, its potency was significantly greater compared to other dynorphins such as Dyn A or Dyn B (Merg et al., 2006; Kuzmin et al., 2006).

The selectivity and potency of BDyn in activating KOR along with the behavioral effects observed in mice, mediated by the activation of NMDA receptors, suggest that a deficient processing of PDyn, resulting in high levels of BDyn, could lead to an enhancement on nociceptive perception, while a normal processing of PDyn leads generally to a predominant activation of KOR by BDyn and other dynorphin peptides, resulting in antinociceptive effects.

II.3.2-Dynorphin A

Dyn A was the first dynorphin peptide to be identified (Cox et al., 1975). The observation of potent analgesic effects when administered intrathecally to mice raised the interest in this opioid peptide for further studies in pain research (Hayes et al., 1983). Dyn A is widely found throughout the CNS, being more abundant especially in the brain, in areas like hypothalamus, substantia nigra and periaqueductal gray. High concentrations are also found in the dorsal horn of the spinal cord and in the pituitary gland. (Tan-No et al., 1997).

Studies revealed that Dyn A participates in pain modulation pathways, mediated in part by its release in the brain and spinal cord (Mizoguchi et al., 2006). Its participation in the first synapse was corroborated *a posteriori* when high levels of Dyn A were found in intrinsic neurons projecting into laminae I, II and V of the spinal cord dorsal horn (Draisci et al., 1991; Przewlocki et al., 1983), where neurons responding to noxious input are contained (Mika et al., 2011). Subsequent studies based on the injection of Dyn A into the subarachnoid space of the spinal cord of rats showed a strong and long-lasting analgesic effect, with a greater potency than morphine (Merg et al., 2006). That effect was completely reversed by the administration of naxolone, a potent opioid receptor antagonist, demonstrating that Dyn A acts on KOR (Han and Xie, 1982; Nakazawa et al., 1985). The affinity and potency of Dyn A for KOR has been reported to be substantially higher than for other dynorphin peptides (Merg et al. 2006). However, intrathecal administration of Dyn A at high doses was observed to induce long-lasting mechanical and thermal allodynia in rats, a response that was not blocked by naloxone, suggesting that when Dyn A is administered at high doses, it is able to interact with other receptors such as NMDA in addition to KOR (Laughlin et al. 1997; Vandera et al. 1996; Shukla et al. 1994). Those studies suggested that Dyn A has inhibitory or excitatory effects, depending on its concentration (Caudle et al. 1994).

II.3.2.1-Physiological role of Dynorphin A

EM-2 has been proposed to have an important involvement in antinociception mediated by Dyn A at spinal cord levels (Draisci et al., 1991). The mechanism proposed by Mizoguchi and co-workers (Mizoguchi et al. 2006) and later supported by Fichna and co-workers (Fichna et al. 2007) establish that the release of Dyn A from descending interneurons

and its subsequent interaction with presynaptic KOR, promote the production and release of EM-2 from the terminal of primary afferent neurons. The released EM-2 preferentially stimulates MOR, a presynaptic and postsynaptic receptor, leading to the inhibition of the release of excitatory neurotransmitters such as SP, glutamate and CRGP, as well as causing a decrease of the excitability of postsynaptic receptors such as NMDA or NK1 receptors (Figure 5). Moreover the presence of MOR in the descending dynorphin-containing neurons also make EM-2 having an important regulator of the release of Dyn A (Ohsawa et al., 2001; Sakurada et al., 2001; Mizoguchi et al., 2006; Fichna et al., 2007).

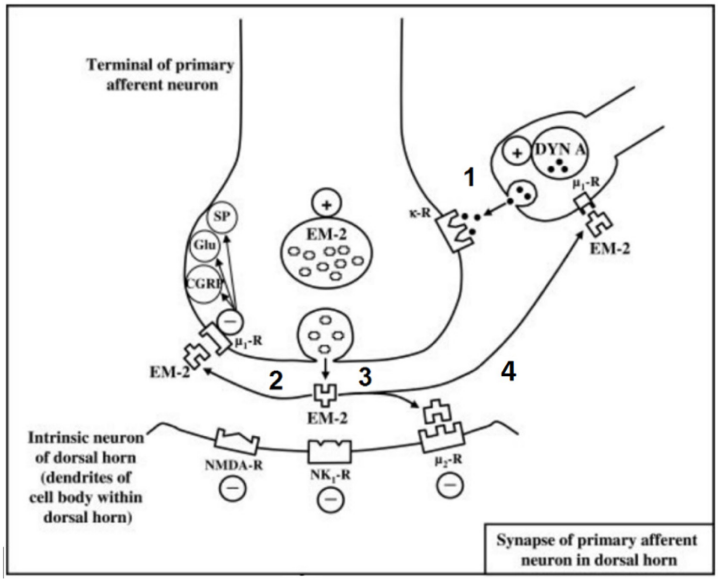

Figure 6: *Involvement of Dyn A in pain modulation. (1) The release of Dyn A enhances the production and release of EM-2. Thus through its interaction with MOR, EM-2 (2) inhibits the release of excitatory neurotransmitters and (3) decreases the excitability of postsynaptic receptors in addition to (4) regulate the release of Dyn A. (Fichna et al., 2007)*

II.3.3-Dynorphin B

As a primary product from Bigdyn, Dyn B is present in the same neuronal fibers and tissues that contains Dyn A (Zamir, 1984). Dyn B has a primary affinity for KOR, however its affinity for the receptor and its potency is lower compared to Dyn A (Merg et al., 2006). Intrathecal injections of Dyn B in rats produced potent and long-lasting analgesic effects (Han and Xie, 1982). Nevertheless, the physiological role of Dyn B is still far from clear.

Mizoguchi and colleges reported that the physiological role of Dyn B completely differs from Dyn A. They observed that Dyn B activated KOR induced the release of another potent antinociceptive peptide, analogue to dermorphin (Mizoguchi et al. 2006a). This mechanism of action was similar to the role observed for Dyn A involving the release of EM-2. These interesting findings strongly suggest that the dynorphin peptides act through separate pathway to bring about pain relief.

II.4-Proprotein convertases

Several neuropeptides and hormones as well as a variety of other endogenous peptides are derived from large and inactive proteins which require endoproteolytic processing for the biosynthesis of the active peptides. Protein convertases (PCs) are a family of enzymes catalyzing protein cleavage at monobasic amino acid residues such as single lysine (K) or arginine (R) as well as at paired basic residues such as RR, KR, RK and KK (Hook and Brennand, 2014; Rouillé et al., 1995). As an exception, when a basic position is flanked by a proline at the C-terminal, the conformational restrictions imposed by this amino acid on the peptide avoids any possible cleavage by PCs (Vanhoof et al. 1995).

Seven PCs were identified in mammalian tissues including PC1, PC2, PC4, PC5 , PACE4, PC7 and furin, officially named as proprotein convertase subtilisin/kexin type 1, 2, 4, 5, 6,7 and furin respectively (Seidah et al. 1998). Like most secreted proteins, PCs are synthesized in the ER as immature PCs which require post-translational modifications to become fully active (Seidah et al. 2008). Following their synthesis, PCs are transported to downstream compartments of the secretory pathway where they are N-glycosylated at various sites and folded into an active conformation within the ER (Benjannet et al. 1993; Steiner, 1998). As an exception, PC2 does not follow this process, instead, immature PC2 is transported with a binding protein (7B2) to acidic immature secretory granules, where it is autocatalytically activated (Mousa et al., 2004; Mbikay et al., 2001).

PCs and other secreted proproteins are then packed in secretory vesicles as they leave the ER. During the transport of those vesicles, active PCs process proproteins within the vesicles (Alberts et al., 2002). PCs require Ca^{2+} as a cofactor to cleave their substrates (Linard et al., 1995). In addition, PC1 and PC2 are maximally active in the acidic pH

environment (5.5-6.5) of immature secretory granules (Shennan, 1995), being less effective outside the vesicles (Seidah and Prat, 2012). Since all PCs possess overlapping functions, substrate specificity is dictated by different tissue and cellular distribution (Steiner et al., 1992).

II.4.1-Tissue and cellular expression of PCs

The tissue distribution and intracellular localization of PCs is varied as presented in Table 3. Furin and PC7 are widely distributed in the lymphatic system, in the liver and in the kidney, and are localized predominantly in endosomes, on the cell surface and in the trans-Golgi Network (TGN) (Hatsuzawa et al., 1990; Schalken et al., 1987). PC5 and PACE4 are expressed in both endocrine and non-endocrine tissues, primarily in the brain, the digestive system and the adrenal cortex. They can be found intracellularly in the cell surface and in the extracellular matrix (ECM) (Lusson et al., 1993; Nakagawa et al., 1993). The expression of PC4 differs from other PCs since this enzyme is predominantly synthesized in testicular germ cells, the placenta and the ovary. PC4 is important in fertility, but its intracellular location is still not well characterized (Seidah and Prat, 2012; Torii et al., 1993). Neuronal and endocrine cells are rich in PC1 and PC2. They are found inside the cells in the TGN and are stored within the acidic regulated secretory vesicles (Seidah et al., 2008 and 1999). PC2 has been described to be the major protein convertase within the CNS (Seidah et al., 1998).

Tissue and cellular location of PCs are fundamental in the determination of their role and target substrate. The rich expression of PC1 and PC2 in neuroendocrine cells confers a key role of these enzymes in the processing of several proneuropeptides and prohormones (Hook et al., 2008).

Protein convertase	Tissue distribution	Subcellular localization
PC1 and PC2	Neuroendocrine	Acidic regulated secretory granules
Furin	Ubiquitous	TGN, cell surface, endosomes
PC4	Germinal	??
PC5	Widespread: adrenal cortex, intestine, kidney,ovary	Cell surface ECM
PACE4	Widespread: muscle, heart, pituitary, intestine, cerebellum, kidney	Cell surface ECM
PC7	Ubiquitous	TGN, cell surface, endosomes

Table 3: *PCs tissue distribution and subcellular location. Addapted from (Seidah and Prat, 2012).*

II.4.2-Proneuropeptide processing

Bioactive neuropeptides usually result from the removal of N- or C-terminal residues, being commonly flanked by dibasic residues within the proneuropeptide sequence. C-terminal and N-terminal proneuropeptide processing has been reported to be mediated by different proteases. PCs cleave at the C-terminal side and Cathepsin L at the N-terminal (Hook et al., 2008; Hook, 2006). The removal of basic residues from the intermediates differs between C- and N- terminal processing. Carboxypeptidases (CP), specifically carboxypeptidase E (CPE), removes basic residues from the C-terminal (Seidah and Prat, 2002; Scamuffa, 2006). On the other hand, aminopeptidase is required for the removal of basic residues from the far N-terminal as shown in Figure 7 (Hook et al., 2008; Hwang et al., 2007). The contribution of both pathways is controversial, and it has been suggested that the biosynthesis of most neuropeptides occurs by endoproteolytic cleavage on the C-terminal side (Zhang et al. 2010; Minokadeh et al. 2010).

The main C-terminal peptide-processing endopeptidases identified in neuroendocrine tissues are PC1 and PC2. Their enzymatic activities have been extensively studied using a variety of approaches (Zhang et al., 2010; Miller, 2003; Breslin et al., 1993; Day et al., 1998; Johanning et al., 1998). PC1 and PC2 are often jointly involved in the production of a variety of neuropeptides due to their colocalization in neuroendocrine tissue. The role of PC2 was

extensively described and it was shown to be essential for the processing of a vast variety of proneuropeptides (Berman et al., 2000; Brakch et al., 1997; Paquet, 1996).

PC1 and PC2 have been shown to efficiently cleave at dibasic sites, showing a primary affinity for dibasic KR residues, although cleavage at KK, RR, and RK dibasic sites have also been described (Hook et al., 2008; Berman et al., 2000; Day et al., 1998). Moreover, cleavage at monobasic R and K residues has been experimentally evidenced specially by PC2 (Zhang et al., 2010; Day et al., 1998). Additionally, it has been suggested that paired basic amino acid residues can be cleaved in between basic amino acids (Hook and Bandeira, 2015). Due to their similar specificity and localization, PC1 has been reported to compensate for PC2 activity when the latter is absent (Miller et al., 2003), however the presence of other processing enzymes such as Cathepsin L makes this compensation difficult to be estimated. Factors including the substrate structure and localization of paired basic residues were reported to be important in the determination of PC specificity (i.e. enzyme-substrate complex thermodynamic stability) as occurs when a basic position is flanked by a proline at the C-terminal (Vanhoof et al., 1995; Breslin et al., 1993).

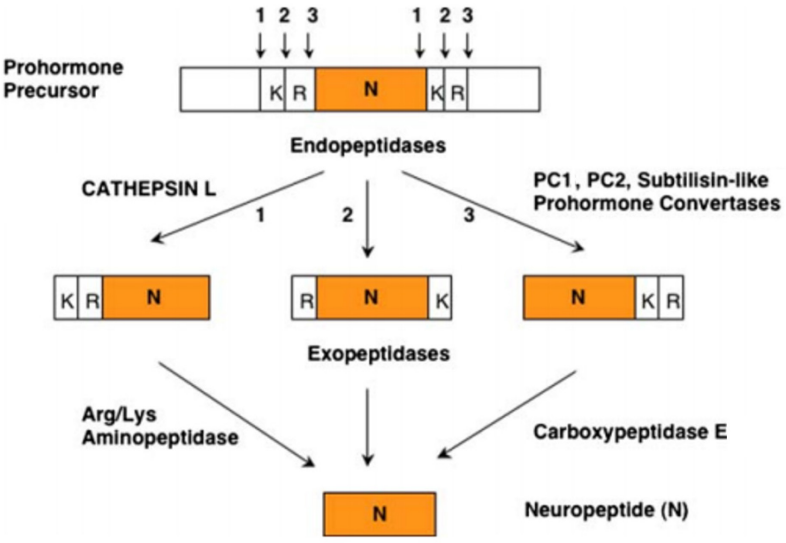

Figure 7: *Pathways for proneuropeptides processing (Hook, 2006).*

Cathepsin L proteolytic processing occurs in parallel with reactions catalyzed by PC1 and PC2. Cathepsin L is also present in secretory vesicles and contributes to the biosynthesis of neuropeptides. In this case, cleavage occurs at the N-terminal side of the dibasic residues, or between the dibasic residues (Hook, 2006; Hook et al., 2008; Yasothornsrikul et al., 2003). Overall, the synthesis of neuropeptides is dependent on a joint contribution of Cathepsin L, PC1 and PC2 to generate specific N- and C-terminal bioactive neuropeptides (Minokadeh et al., 2010). In contrast, the reduced concentrations of N-terminal derived neuropeptides in PC2 knockout mice suggest that PCs are also involved in N-terminal processing and reveal an alternative pathway to Cathepsin L (Zhang et al., 2010).

II.4.3-Prodynorphin processing by PC1 and PC2

Contrary to other opioid precursors such as PEnk and POMC (Loh et al., 2002; Johanning et al., 1998; Zhou et al., 1993), the study of PDyn processing presents an extra challenge because the formation of dynorphin peptides requires processing at dibasic and monobasic residues as shown in Figure 4.

Just a few studies have examined the involvement of PC1 and PC2 in PDyn processing and mainly by *in vitro* techniques, using either purified recombinant enzymes or gene overexpression systems (Dupuy et al., 1994; Day et al., 1998). These studies have shown that both PC1 and PC2 are involved in PDyn processing and suggest a significant role of PC1 in the formation of a 10 kDa fragment resulting from PDyn dibasic processing. Trace amounts of an 8 kDa fragment were identified and shorter fragments from single basic processing were also observed. These data revealed an important contribution of PC1 to dibasic processing and a lesser role in monobasic processing of PDyn (Dupuy et al., 1994). In contrast, PC2 processing of PDyn was observed to generate notable amounts of shorter fragments including BDyn, Dyn 1-19, Dyn A, Dyn B and Dyn 1-8, in addition to the formation of the 10 kDa Dyn-containing fragment (Figure 8). These results demonstrated the ability of PC2 to cleave either at monobasic or dibasic residues. However, studies using recombinant enzymes and protein precursors are probably not representative of physiological conditions. The use of certain concentrations of enzymes and substrate may force thermodynamically unfavorable reactions due to entropic effects.

Interestingly, Berman and co-workers (Berman et al., 2000) studied PDyn processing using mice tissues lacking active PC2. By comparing with WT mice, the authors corroborated earlier findings, observing that under physiological conditions the concentration of dynorphin peptides, such as BDyn, Dyn A, Dyn B and Dyn 1-8 were considerably decreased whereas there was a significant increase in either unprocessed PDyn or high-molecular-weight Dyn-containing fragments, such as the 10 or the 8 kDa fragments.

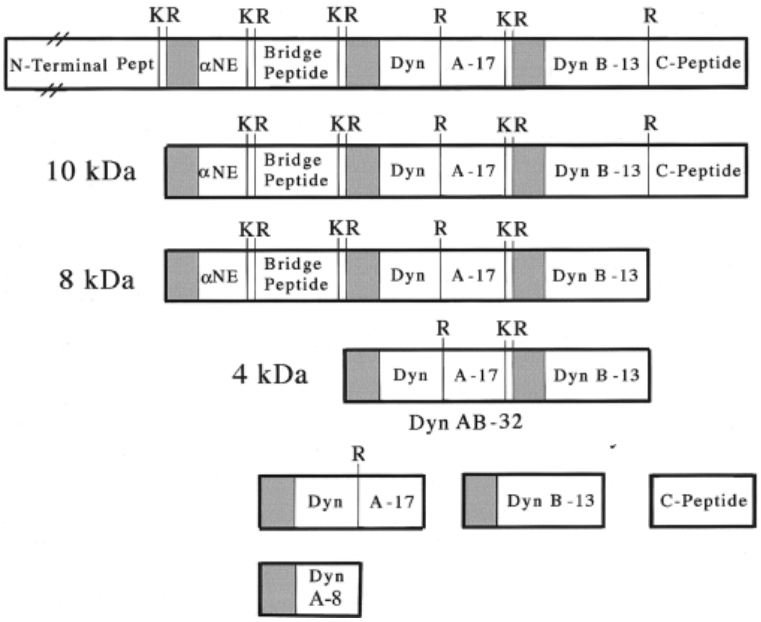

Figure 8: *Schematic representation of PDyn precursor shows possible paired and single basic cleavage sites (KR and R). Various processing intermediates, such as 10-, 8- and 4-(BDyn) kDa peptides were identified, as well as some final opioid peptide products such as Dyn A, Dyn B and Dyn 1-8. The shaded portions represent Leu-Enk (Berman et al., 2000).*

Later studies identified other dynorphin fragments, such as Dyn 1-13, Dyn 1-11, Dyn 1-7 and Dyn 1-6, as Dyn A metabolic products by analyzing *in vivo* cerebral microdialysates (Reed et al., 2003; Prokai et al., 1998).

Considering the results outlined by these few studies, PC1 and PC2 appear to play a central role in the endoproteolytic processing of PDyn leading to the formation of several dynorphin metabolites including 10 and 8 kDa high-molecular-weight fragments, BDyn, Dyn 1-19, Dyn A and Dyn B. However, the different contribution of PC1 and PC2 to PDyn processing showed in previous studies is still far from being clarified, and more importantly

the involvement of PC1 or PC2 in the metabolic turnover of bioactive dynorphins such as BDyn and Dyn A has never been studied.

As shown in Figure 9, the presence of mono and dibasic residues on BDyn and Dyn A amino acid sequences suggest that, PC1 and PC2 could be involved in the regulation of BDyn and Dyn A endogenous levels and in the formation of several BDyn and Dyn A derived peptides.

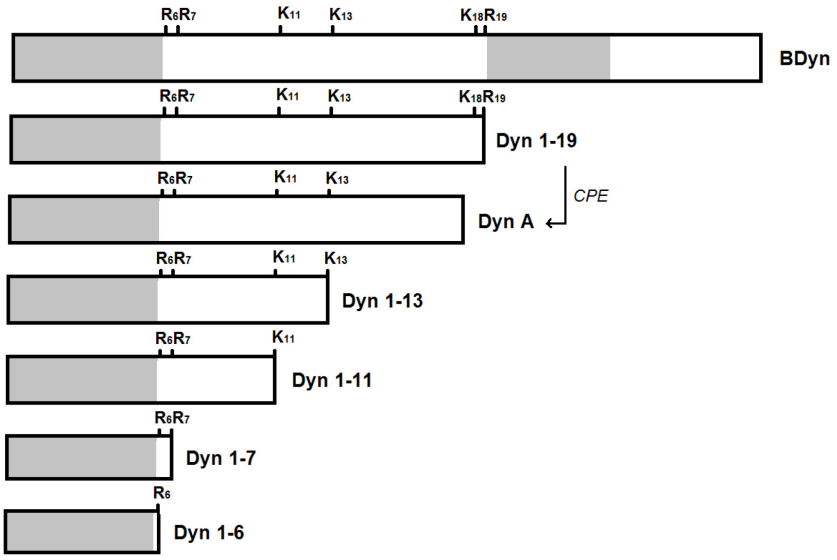

Figure 9: *Potential BDyn and Dyn A-derived peptides from PC1 and PC2 cleavage*

II.4.4-PC1$^{-/-}$ and PC2$^{-/-}$ mice phenotypes

The essential role of PC1 and PC2 in the synthesis of neuropeptides and hormones was also corroborated by several studies showing that PC1-knockout (PC1$^{-/-}$) and PC2 knockout (PC2$^{-/-}$) mice presented several abnormalities associated with the deficient processing of hormones and neuropeptides. PC2$^{-/-}$ mutant mice were reported to exhibit elevated levels of unprocessed prohormones such as proinsulin (Furuta et al., 1997), proglucagon and prosomatostatin (Furuta et al., 2001; Laurent et al., 2002), as well as lesser amounts of several neuropeptides such as endorphins (Allen et al., 2001), enkephalins (Miller et al., 2003), and dynorphins (Berman et al., 2000). Even though these mice do not show significant differences on their survival rate compared with wild type (WT) animals, this

strain showed important reproductive problems (Furuta et al., 1997). On the other hand, $PC1^{-/-}$ mice show several deficiencies of proneuropeptides and prohormones processing and a low survival rate, just a third of $PC1^{-/-}$ animals survive beyond seven days (Zhu et al., 2002). In particular, $PC1^{-/-}$ mice show grievous growth defects, presenting about 60% reduction of normal size at 10 weeks due to their lack of mature growth hormone (Lloyd et al., 2006; Zhu et al., 2002; Scamuffa et al., 2006). Furthermore, behavioral studies were performed on $PC2^{-/-}$ animals to examine the consequences of PC2 deficiency on nociception and analgesia; interestingly, the $PC2^{-/-}$ mice were significantly less sensitive to pain (Croissandeau et al., 2006).

The behavioral responses observed in PC2 mutant mice to noxious stimulation were very similar to the behaviors presented by tachykinin-1-knockout ($Tac1^{-/-}$) mice (Zimmer et al., 1998). Tac1 mutant mice present a deficiency of active SP, resulting in insensitivity to noxious perceptions. These observation, together with the reported involvement of PC2 on the formation of SP (Saidi et al., 2015), suggest that the behavioral responses observed in $PC2^{-/-}$ mice could be due to either the accumulation of intermediate opioid peptides with potent analgesic effects, or to a deficient formation of active nociceptive peptides such as SP (Croissandeau et al., 2006).

CHAPTER III-MASS SPECTROMETRY

III.1-Introduction to mass spectrometry

Mass spectrometry is a powerful analytical technique with high sensitivity and specificity for the identification and quantification of large and small molecules including peptides, proteins, lipids, and organometallic among other inorganic compounds (Yates et al., 2009; Becker, 2008; Hopfgartner et al., 2004; Colton et al., 1995). Mass spectrometers (MS) operate by converting analyte molecules into charged ions. Ionized analytes and any fragment ion produced are subsequently measured on the basis of their mass to charge ratio (m/z) (El-Aneed et al., 2009; Glish et al., 2003).

A general schematic presentation of the main components of a MS instrument is presented in figure 10. At the ion source, analytes are converted into gas phase ions and transported through the interface by a high density electric field to the mass analyzer, where ions will be separated and identified according to their m/z. Ions are then ejected from the mass analyzer to the detector where the signal produced by the incident ions is transduced into output data represented as a mass spectrum (Ruse and Yates, 2006).

Several different technologies are available for both the ionization and ion analysis depending on sample complexity and the goals of analysis, resulting in various types of mass spectrometers with specific capabilities (Glish et al., 2003). For the analysis of proteins and peptides, the combination of high performance liquid chromatography (HPLC) with an electrospray ionization-mass spectrometer (ESI-MS) has become the main tool in proteomic analysis owing to its high specificity, selectivity and its ability to handle complex mixtures.

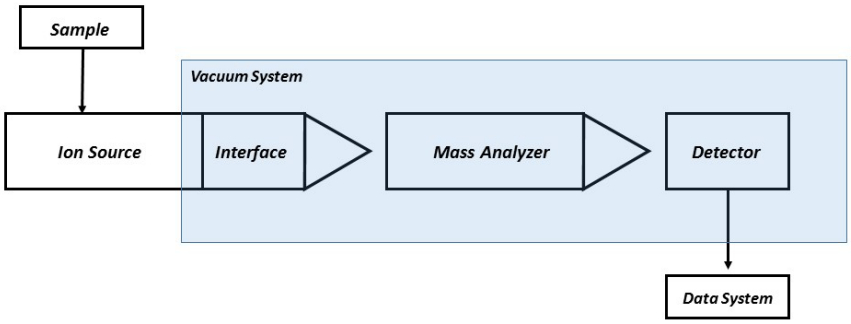

Figure 10: *Schematic representation of the different sections of a MS system.*

III.1.1-Sample separation

The analysis of complex matrices such as biological fluids or tissues requires separation of analytes prior the analysis by mass spectrometry. A suitable separation provides a better efficiency of the ionization process, significantly improving the sensitivity (Cravatt, 2007; Jemal and Xia, 1999). The most widely used technique for an efficient separation of peptides is HPLC. The HPLC system is interfaced to the MS via the ion source such as electrospray (ESI), which combines the separating power of HPLC, with the detection power of the MS.

III.1.1.1-HPLC system

HPLC was developed in the mid-1970's and rapidly gained popularity amongst analytical chemists due to the simplicity and the convenience of the technique. HPLC is a chromatographic technique used to separate components in a mixture based on their polarity and hydrophobicity (Bakes, 2000). This technique is based on the passage of the molecules contained in a sample carried by a mobile phase through a functionalized chromatographic column. The mobile phase is composed of a solvent or a mixture of solvents of a specific polarity and the column is packed with a stationary phase consisting of sorbent particles which are chemically functionalized (e.g. C18, C8, C4, Phenyl, CN) bound to a support, typically consisting of silica or polymers (Dorsey et al., 1998).

According to the polarities of the analytes subject to chromatographic separation, both the mobile phase and the chromatographic column must be chosen to ensure that the analytes of interest are retained, but not so strongly that they cannot be eluted. For the chromatographic separation of nonpolar compounds or compounds with limited solubility in water, normal-phase liquid chromatography (NPLC) is used. This technique uses columns packed with polar stationary phases (CN, silica) combined with nonpolar or moderately-polar mobile phases (hexane, tetrahydrofuran) (William 2006). On the other hand, compounds containing functional groups which provide some degree of hydrophilic character to the molecule, are usually separated by reverse phase liquid chromatography (RPLC). Since a vast majority of compounds present a certain hydrophilic character, RPLC is the most commonly used separation technique in HPLC. This technique utilizes nonpolar stationary

phases (C18, C8), and aqueous-based polar mobile phases (water/methanol, water/acetonitrile).

RPLC generally uses gradient elution when compounds with similar polarities need to be separated. A gradient elution refers to a variation on the polarity of the mobile phase during the chromatography. Thus, gradient-RPLC starts under aqueous conditions allowing the analytes to be strongly adsorbed to the surface of the stationary phase. By increasing the percentage of organic solvent on the mobile phase, each analyte is desorbed from the stationary phase within a very narrow variation on the concentration of organic solvent (Figure 11B) (Hodges and Mant, 1991; Kumar and Kumar, 2012). The elution order of the analytes is related to their hydrophobicity; more polar solutes move the fastest and appear first, followed by solutes of decreasing polarity (Figure 11B).

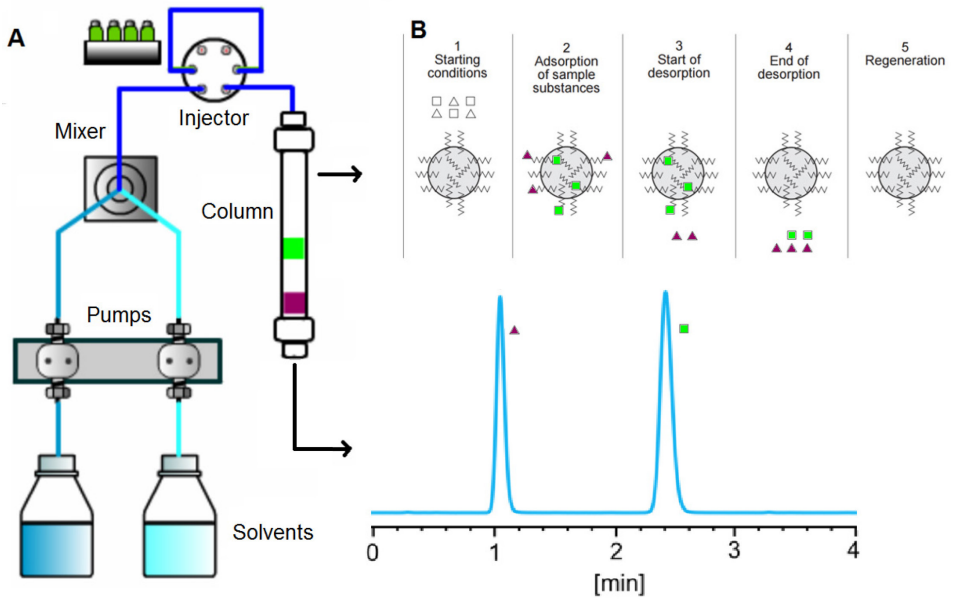

Figure 11: *(A) HPLC operating mode. The sample is injected into the system, the mobile phase is composed by a mixture of organic and aqueous solvents in a specific proportion and carry the sample into the column. The type of interaction between the molecule with the stationary and mobile phases makes each analyte to leave the column at different retention times (chromacademy.com). (B) Principle of RPLC with gradient elution (Kumar and Kumar, 2012).*

By the late 1970's, HPLC methods started to be developed and became popular especially in pharmaceutical laboratories to support the analysis and the isolation of molecules containing

similar functional groups using RPLC separation. HPLC was a new technique with significant improved separation, identification, purification and quantification features over previous chromatographic methods such as column chromatography or thin layer chromatography (Synder and Kirkland, 1979). Since then, continuous improvements in column packing material (Gritti and Guiochon, 2012), design of new instruments (Snyder et al., 2011) and development of computational methods and automation (Hanai, 2005) have improved the reproducibility, efficiency and robustness of this technique and simplified HPLC methods (Snyder, 2000).

III.1.2-Sample Ionization

Following HPLC separation, molecules need to be converted into gas phase ions prior to their introduction to the MS entrance. An atmospheric pressure ion source is necessary when any instrument that separates molecules in a liquid phase is coupled to a detector that identifies the ions by manipulations in the gas phase such as a MS. The ion source is needed to evaporate the liquid, produce ions and generate an electric field to transport the ions into the vacuum system of the mass spectrometer (Ashcroft, 1997). Due to its high ionization efficiency and its compatibility with HPLC fluidics, ESI has become the most popular technique capable of ionizing both small and large molecules of different polarities. Additionally, neutral or thermally stable molecules may require ionization by atmospheric pressure chemical ionization (APCI) or other related techniques (Carvalho et al., 2008; Nagy et al., 2007; Nelson et al., 2004).

III.1.2.1-Electrospray Ionization

ESI-MS was introduced by Yamashita and Fenn (1984) and has made a significant commercial impact since the 1990s (Balogh, 1998). In an ESI source, the liquid from the HPLC is directed through the free end of a capillary (electrode) set at 3 to 5 kV. In the case of pure ESI, the high electric field at the tip of the capillary pulls the liquid emanating from the electrode into a fine jet that breaks up, typically a millimeter from the tip of the electrode, into a fine spray of electrified droplets. Figure 12 illustrates the ESI process.

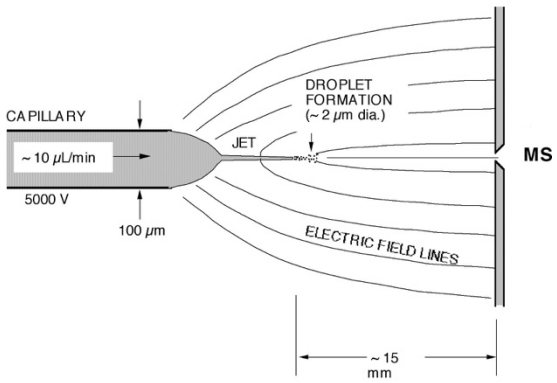

Figure 12: *Schematic of a typical electrospray source*

The fine droplets in the spray, evaporate in about one millisecond to liberate charged molecules from the droplets as ions, which the electric field of the electrode tip then transports toward the entrance of the MS. Figure 13 illustrates the ionization and the evaporation mechanism involved during the ESI process.

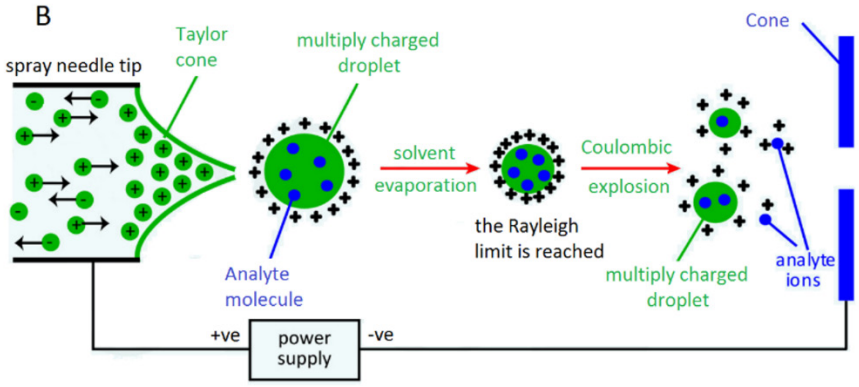

Figure 13: *(A) HPLC eluent containing the sample analytes is sprayed into small droplets and converted into gas phase ions. The ions carried by the electric field and the high vacuum are then introduced through a transfer capillary into the MS. (B) The potential applied into the needle tip makes that the droplets of the same polarity are repelled from the needle towards the cone generating the so-called Taylor cone. As the droplets traverse the space between the needle tip and the cone, solvent evaporates until it reaches the point that the surface tension can no longer sustain the charge (the Rayleigh limit) at which point a "Coulombic explosion" occurs and the droplet is dissociated producing charged analyte molecules (Adapted from Gates, 2014).*

Currently, the ESI process is one of the softest ionization techniques available and has the advantage of generating molecular ions such as $[M+nH]^{n+}$ or $[M-nH]^{n-}$. The fact that the ions

observed in a given mass spectrum are produced in solution, and therefore no extra internal energy is imparted to the ions, makes ESI the most suitable ionization technique to convert analytes into gas phase ions without extensive degradation (Yates et al., 2009; Bruins et al., 1987).

Several other techniques were derived from this general concept such as ionspray or pneumatic assisted (Bruins et al., 1987), microspray (Covey, 1995) and nanospray (Wilm and Mann, 1996). Ionspray is the most often used version of ESI currently in bioanalysis, however the different techniques available encompass a wide range of nebulization capacities and flow rates.

III.1.3-Mass analyzer

Once the ions are generated at the ion source and introduced into the MS, ions are carried through the interface to the mass analyzer. The interface is made up of a series of optics, including skimmers, lenses and quadrupoles/octopoles that accelerate and focalize the ions from the atmospheric pressure zone, the ion source, to the mass analyzer which remains at high vacuum (Domon and Aebersold, 2006).

The mass analyzer is the central component of a MS. It differentiates and separates the ions based on their relative m/z and progressively ejects the ions into the detector (Jansen et al., 2005). Several types of analyzers are available, the more frequently encountered ones for the analysis of proteins and peptides are quadrupole-based analyzers, time of flight (TOF) analyzers, ion traps and orbitraps (Mann et al., 2001). Moreover several hybrid instruments are also available. These instruments combine two or more mass analyzers providing a better sensitivity and ion separation than instruments equipped just with one analyzer. Hybrid instruments include, among others, the triple quadrupole (QqQ), the quadrupole coupled TOF (Q-TOF) and the quadrupole coupled orbitrap (Q-orbitrap) (Domon and Aebersold, 2006). Each instrument has its own features and depending on the desired application, the selection of the analyzer is an important step.

III.1.3.1-Quadrupole

Quadrupole mass spectrometers have been widely used for many years. Generally, these devices are constructed from four parallel metallic rods. Radio frequency and direct current voltages are applied to the rods. The potential applied to adjacent rods are of the same magnitude but opposed, establishing a two-dimensional and dynamic electric field within the quadrupole. Mass selection is achieved by choosing a combination of radio frequency and direct current voltages such that ions within a narrow m/z are stable over the length of the quadrupole and reach the detector. Contrary, the ions which m/z differs from the range established are wasted (De Hoffmann et al., 2003) (Figure 14).

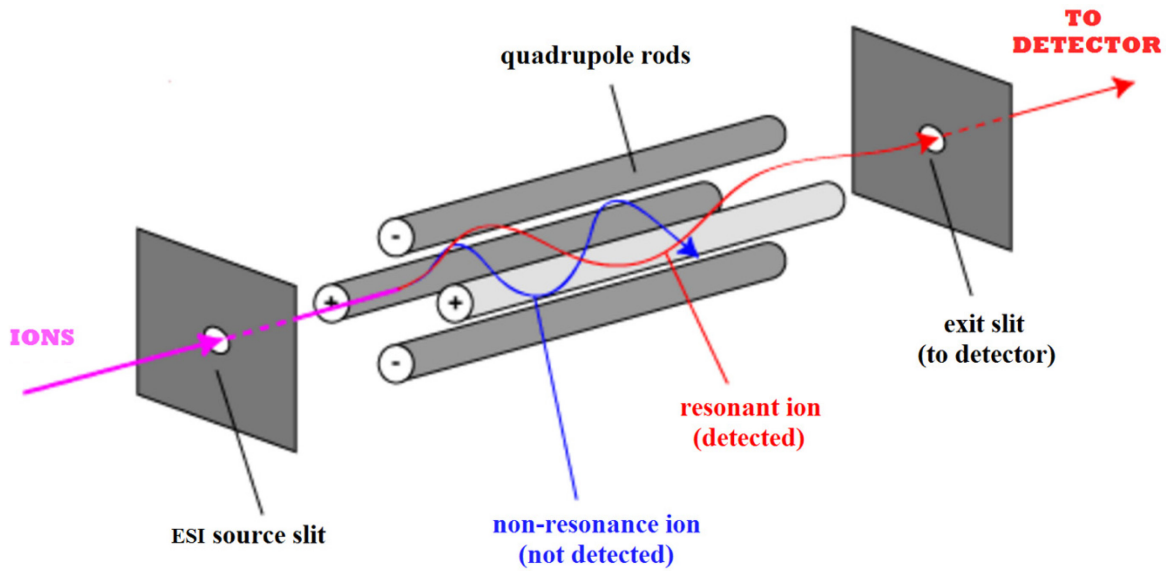

Figure 14: *Schematic illustration of a quadrupole mass analyzer (www.bris.ac.uk/nerclsmsf/techniques/gcms.html)*

The QqQ have been the traditional MS used on most analytical laboratories. The main feature of this instrument is its high detection sensitivity. This sensitivity is achieved though the combination of three quadrupoles arranged in series. The first and the third quadrupoles work as a mass filter allowing the passage of the ions within the selected m/z range. The second quadrupole consists of a collision cell where ions can be fragmented though their collision with an inert gas (N_2, He), allowing the acquisition of MS^2 spectra (Glish et al.,

1982). MS^2 (or MS/MS), consisting of a single fragmentation of a selected ion (precursor ion) and the subsequent analysis of its ion fragments, is the most used technique when structural information is required (Tozuka et al., 2003).

III.1.3.2-Time of flight

First time of flight (TOF) mass analyzers emerged in the 1960s but were soon replaced by quadrupole instruments owing to the lack of technologies to facilitate the recording and processing of the mass spectrum in a microsecond time-frame. Afterwards these facilitating technologies started to emerge and currently TOF analyzers are widely used for qualitative and quantitative high resolution analysis of small and large molecules (Guilhaus, 1995; Szájli et al., 2008).

TOF mass analyzers work on the basis of measuring the time it takes for an ion to go over a distance when it is accelerated by an electric field of known strength. This acceleration results in an ion having the same kinetic energy as any other ion that has the same charge. However, for a given kinetic energy the velocity of the ion depends on its mass; the heavier the ions the lower the velocity. Thus, the time that the ion takes to cross a known distance of the flight path and reach the detector is measured (Figure 15). This time will depend on the m/z of the particle (Guilhaus et al., 2000).

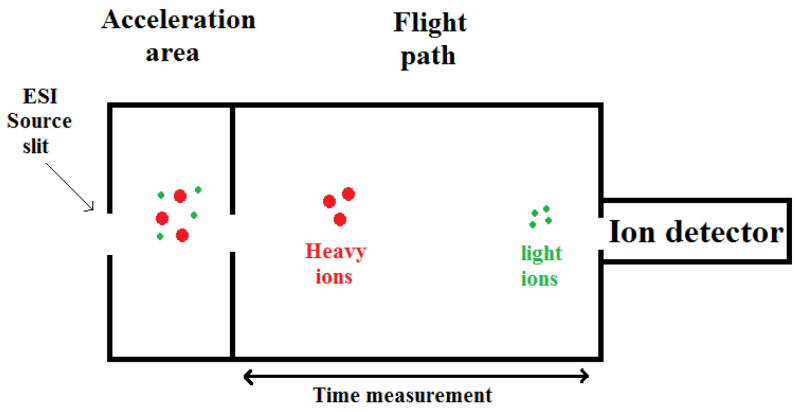

Figure 15: *Schematic of a TOF mass analyzer*

The Q-TOF is the most widely used TOF-hybrid instrument which can be coupled with an ESI source. It consists of the combination of a triple quadrupole coupled with a TOF analyzer.

This instrument has the highest selectivity, resolution and mass precision features among the different mass spectrometers, however, its detection limit is lower (Williamson and Bartlett, 2007; Bristow, 2006).

III.1.3.3-Ion traps

One of the first linear traps was constructed by Church (1969). Nowadays, ion trap mass analyzers are widely used for the analysis of small and large molecules, and its main feature is its high sensitivity particularly for untargeted analysis (Douglas et al., 2005).

Ion trap analyzers work on the basis of storing ions in a "trap" and manipulating the ions by using static and radio frequency voltages. The amplitude of the applied voltages enables the analyzer to trap ions of specified m/z within the analyzer, and non-selected ions are given a trajectory by the electrostatic field that causes them to exit the trap (Payne and Glish, 2005). Ions contained inside the trap are then sequentially ejected based on their m/z values to the detector to create a mass spectrum (Figure 16). Alternatively, a specific ion can be isolated into the trap by the application of a specific voltage while other ions are ejected. By filling the trap with an inert gas such as N_2 or He, fragmentation of those selected ions is possible. This isolation and fragmentation can be performed several times in succession before the final mass spectrum is obtained, resulting in a so called MS^n spectrum.

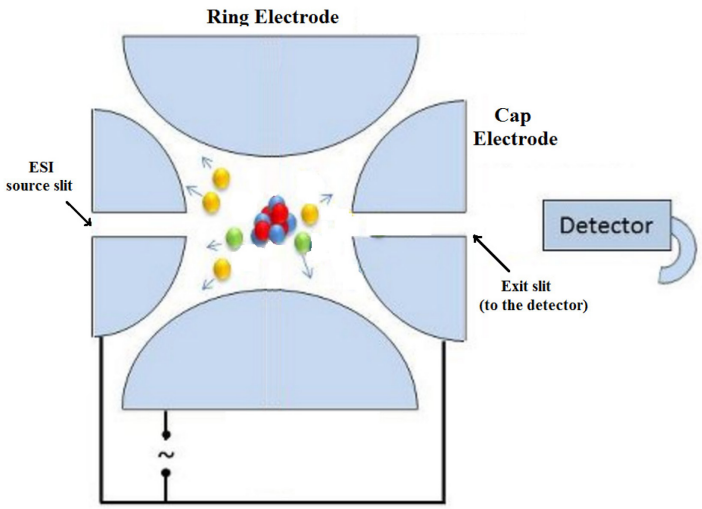

Figure 16: *Schematic representation of an ion trap mass analyzer. Ions of a specified m/z are trap in the analyzer. Non-selected ions are ejected from the trap (adapted from www.york.ac.uk/chemistry/staff/resstaff/yoshikawan)*

Two different ion trap mass analyzers are available: 3D-ion trap, and linear ion trap. They differ in their design and geometry as well as their features, but their working principle is complementary. In most of cases, linear ion traps provide higher sensitivity, resolution and mass precision compared to 3D ion traps (Hager, 2002; Schwartz, 2002). Ion traps can be combined with other mass analyzers in hybrid instruments. The LTQ-XL from Thermo Scientific, combines a linear ion trap with a quadrupole which allows the isolation of a desired m/z range ions to reach the ion trap, thus improving the sensitivity of the analysis (Hager, 2002).

III.1.3.4-Orbitrap

The Orbitrap was first presented to the general MS public at a conference of the American Society for Mass Spectrometry in 1999. It quickly made its debut in mainstream MS in 2005 as an accurate and compact mass analyzer (Makarov et al., 2006).

Orbitrap mass analyzers operate by making the ions oscillate around a central barrel-like electrode sustained at high voltage. The strong electrical field inside the trap generated by the central electrode initiates the axial oscillations of the ions, whose harmonic frequency oscillation along the electric field axis is then measured. Since the ion oscillation frequency is proportional to the ion m/z, fast Fourier transform calculations allows the conversion of the detected oscillating signal is into a mass spectrum (Figure 17).

Orbitrap mass analyzers provide high performance and high resolution (i.e. 140 000 FWHM and < 3 ppm mass precision) analysis. The high resolution powers of this type of analyzer provides high resolution MS spectra, from where the exact mass and the isotopic distribution of the ions can be accurately determined (Van der Heeft et al, 2009). The wide range of applications of this analyzer and its performance characteristics, including its high resolution, high mass accuracy, dynamic range and tandem MS capabilities, makes this analyzer one of the most powerful and commercially claimed MS instrumentation of the last decade (Hardman and Makarov, 2003). Hybrid Orbitrap instruments, such as the Q-Exactive from Thermo Science, combine the Orbitrap with a quadrupole allowing an early m/z range selection, a collision cell where ions can be fragmented and an ion trap (C-trap) that

progressively injects the ions fragmented or selected into the Orbitrap analyzer (Michalski et al., 2011; Hu et al., 2005).

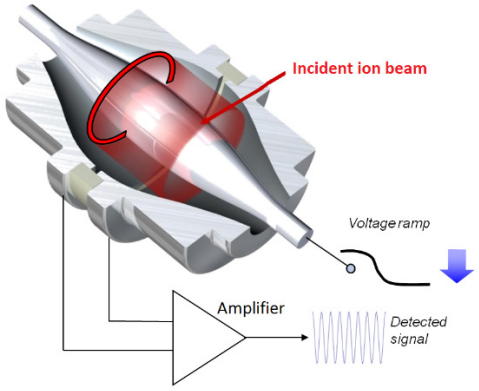

Figure 17: *Orbitrap operating basis. Ions are injected into the Orbitrap, where, as a consequence of the high voltage applied on the central electrode, ions oscillate around the electrode driven by the strong electric field inside the Orbitrap. Ions oscillation frequency is then measured and converted into a useful signal.*

III.1.4-Ion Detector

The final element of the mass spectrometer is the detector, except for instruments based on Orbitrap mass analyzer where the detector is embedded in the analyzer. Once the ions have passed the mass analyzer, they have to be detected and transformed into a usable signal. The detector is an important element of the mass spectrometer that generates a signal from incident ions. Electron multipliers are the most commonly used detectors. These types of detector are made up of a series of dynodes which generates an amount of secondary electrons that are proportional to the number of incident ions. Those secondary electrons are then focalized to further dynodes that will amplify the signal. Electrons will finally fall upon an electron collector anode, transducing the signal into data (Gaire et al., 2007; Funsten et al., 2005; Céolin et al., 2005).

III.1.5-Nomenclature for peptide fragmentation

MS^2 (also MS^3) analysis is the most frequent operating mode for qualitative and quantitative MS analysis of proteins and peptides. It is especially useful when structural

information is required. The ion fragments generated during the fragmentation process are named in accordance to the position where the peptide or protein was fragmented. As shown in Figure 18, the fragments produced from fragmentation at the peptide bound between two amino acids are called “y”, for C-terminal fragments, and “b” fragments, for N-terminal fragments. When fragmentation occurs between the chiral carbon and the carbonyl group within a specific amino acid, “x” and “a” fragments are obtained. Lastly, if the fragmentation takes place between the amide group and the chiral carbon of amino acids “z” C-terminal fragments and “c” N-terminal fragments are obtained. In addition, fragments are numbered in accordance to the position of the amino acid subject to fragmentation within the amino acid sequence of the peptide.

Thus, as an example, the b_4 fragment observed in the MS/MS spectra obtained for Leu-Enk (YGGFL) singly charged ion (pg. 75), corresponds to the fragment GGFL generated by the brake down of the peptide bound between Y and G amino acids.

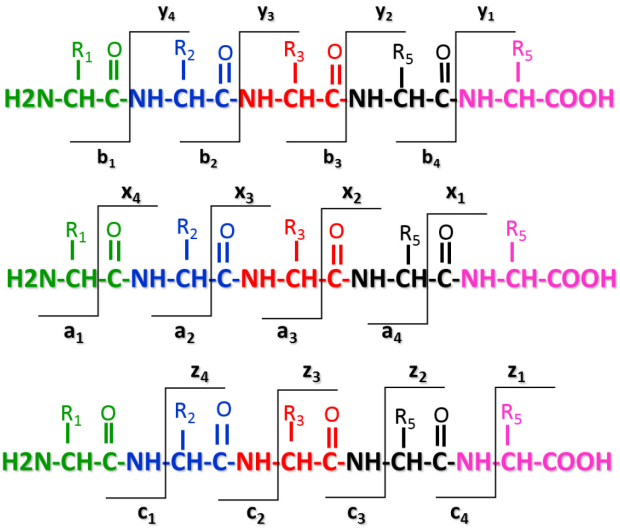

Figure 18: *Peptide fragmentation nomenclature*

III.1.6-MS data acquisition modes

III.1.6.1-Full scan MS mode

Full scan mode allows the monitoring of ions comprised on a set m/z range (Figure 19A). A typical mass scan range will cover from 200-2000 m/z (5 to 10 Hz) and will detect ions within that range over a set time period typically fixed according to HPLC conditions. Several molecule libraries are available, including extensive protein and peptide databases containing experimental and *in silico* generated mass spectra used for analyte identification and characterization (Mueller et al., 2008; Perkins et al., 1999). The Full scan mode is very useful when identifying unknown compounds in a sample or indispensable for untargeted assays (Rossi and Sinz, 2001). Moreover, working on Full scan mode with high resolution mass analyzers such as Orbitraps, allows the identification of peaks by observing the exact mass (< 3 ppm) and the isotopic distribution of the specific ion, tools that can be used to reduce false positive identification (Van der Heeft et al, 2009).

III.1.6.2-SRM and MRM modes

The basic concept of single reaction monitoring (SRM) and multiple reaction monitoring (MRM) modes is to monitor the presence and intensity of a specific transition of a selected precursor ion, which is isolated in the mass analyzer, into a single or multiple fragment ions (Figure 19B). The double selection criteria (precursor/fragment ions) provide high specificity for peptide detection since any undesired transitions can be avoided. Furthermore, dependent MS^n scans can be used to provide further sequence information for the selected peptides, thus increasing the specificity of the technique. The use of SRM or MRM has a compromise in that SRM analysis provides lower detection limits whereas MRM provide higher selectivity and specificity (Addona et al., 2009; Keshishian et al., 2007). Its selectivity and specificity features make this operation mode ideal for quantitative analysis.

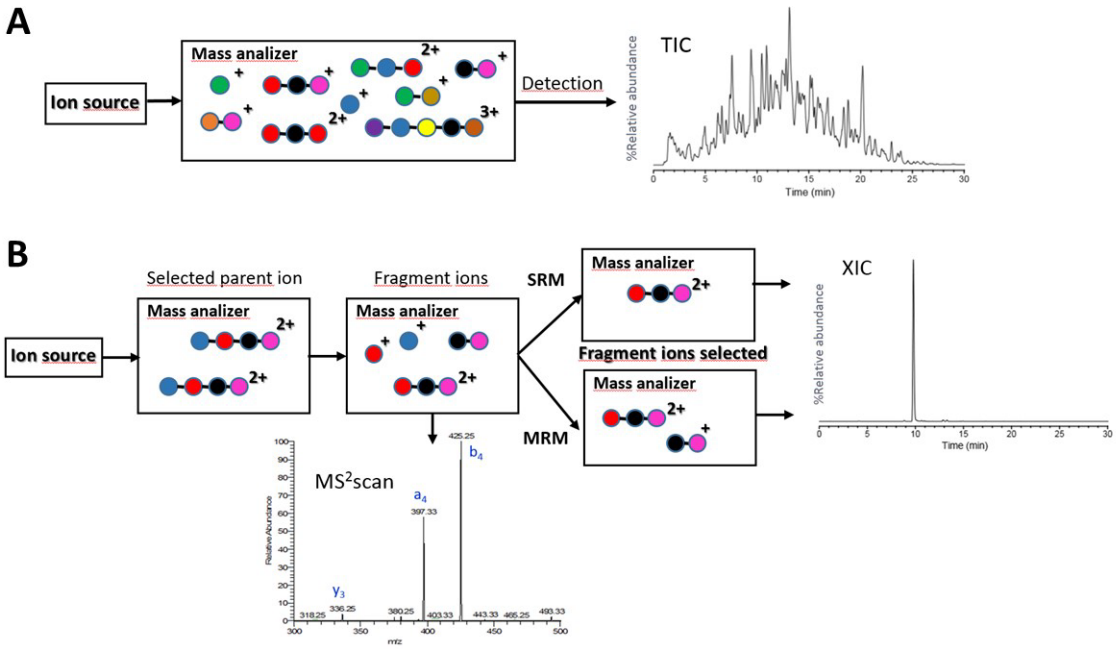

Figure 19: *(A) Full scan acquisition mode. All ions generated at the ions source are injected into the detector. The total ion chromatogram (TIC) shows all the ions detected on the basis of their retention time. (B) SRM and MRM mode. Specific m/z ions are selected and fragmented. Extract ion chromatogram (XIC) is extracted by monitoring a specific transition. A specific ion (precursor ion) is selected and fragmented. Then, one (SRM) or more (MRM) of its fragment ions generated, are isolated and injected into the detector. This operating mode also allows to obtain structural information from the MS^2 spectra by studying the fragmentation pattern.*

III.1.7-Absolute quantification by isotope dilution

Isotope dilution has been used for several decades for the quantitative analysis of proteins and peptides by HPLC-MS (De Leenheer and Thienpont, 1992) and has been recognized as the reference method for internal standardization (Brun et al., 2009). The absolute quantification by isotope dilution is based on the addition of defined quantities of isotopically label standards which are created by the substitution of specific atoms for one of its isotopes. The most common labeling comprises the substitution of ^{1}H by ^{2}H (d) or ^{12}C by ^{13}C. These labeled standards exhibit the same chromatographic and ionization behavior as the unlabeled peptide, but they can be distinguished by their mass difference (Figure 20) and isotopic signature (Ong and Mann, 2005). Thus, independent extract ion chromatograms (XICs) can be extracted from selecting the same parent ion or the same MS^2 transition for the unlabeled and labeled peptides. The concentration of the targeted peptides in the sample can

be determined by using the peak area ratio in a biological sample (Keshishian, 2007). The most important advantage of this quantitative technique is the alleviation of the matrix effect that might take place during the ionization process since both peptides are ionized with the same efficiency (Pailleux and Beaudry, 2012).

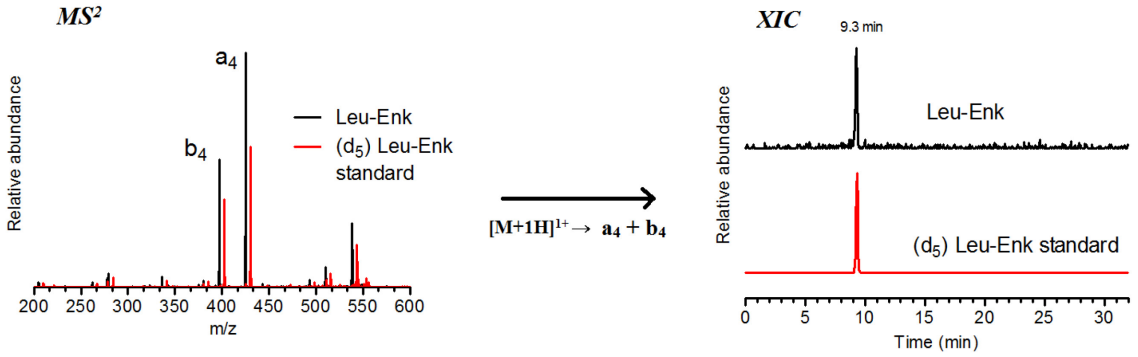

Figure 20: *Leu-Enk MS^2 spectra shows how the peaks corresponding to the fragment ions from the unlabeled standard and the labeled peptide can be differentiated owing to the differences between their masses. XICs are extracted for each peptide by monitoring the same specific MRM transition for both peptides.*

HYPOTHESIS AND OBJECTIVES

The endoproteolytic regulation of BDyn and Dyn A levels has a profound impact on pain perception. Nevertheless, the role of PCs in the regulation of BDyn and Dyn A levels remains unclear. Both BDyn and Dyn A contain paired or single basic residues where PCs could be involved in their C-terminal processing, leading to several important N-terminal metabolites. It is hereby hypothesized that PC1 and PC2 are crucial for BDyn and Dyn A processing through cleavage at these basic positions. In addition, the presence of one copy of Leu-Enk at the N-terminal of dynorphin peptides, suggest that Leu-Enk could be an important metabolic product of BDyn and Dyn A processing.

The objectives of this study were to elucidate the role of PC1 and PC2 in the proteolytic control of BDyn and Dyn A levels. For this purpose, S9 cellular fractions of mice spinal cords were used to perform the *in vitro* digestion of BDyn and Dyn A. Differences in the activity of PC1 and PC2 were evaluated by comparing the metabolism in spinal cord S9 fractions obtained from WT versus $PC1^{(-/+)}$/$PC2^{(-/+)}$ animals. Thereafter, an HPLC-MS/MS method was developed to characterize BDyn and Dyn A metabolites and an isotope dilution method was used for their quantitation.

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Characterization of Endoproteolytic Processing of Dynorphins by Proprotein Convertases using Mouse Spinal Cord S9 Fractions and Mass Spectrometry

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Abstract

Dynorphins are important neuropeptides with a central role in nociception and pain alleviation. Many mechanisms regulate endogenous dynorphin concentrations, including proteolysis. Proprotein convertases (PCs) are widely expressed in the central nervous system and specifically cleave at C-terminal of either a pair of basic amino acids, or a single basic residue. The proteolysis control of endogenous Big Dynorphin (BDyn) and Dynorphin A (Dyn A) levels has a profound impact on pain perception and the role of PCs remain unclear. The objective of this study was to decipher the role of PC1 and PC2 in the proteolysis control of BDyn and Dyn A levels using cellular fractions of spinal cords from wild type (WT), $PC1^{-/+}$ and $PC2^{-/+}$ animals and mass spectrometry. Our results clearly demonstrate that both PC1 and PC2 are involved in the proteolysis regulation of BDyn and Dyn A with a more important role for PC1. C-terminal processing of BDyn generates specific peptide fragments Dynorphin 1-19, Dynorphin 1-13, Dynorphin 1-11 and Dynorphin 1-7 and C-terminal processing of Dyn A generates Dynorphin 1-13, Dynorphin 1-11 and Dynorphin 1-7, all these peptide fragments are associated with PC1 or PC2 processing. Moreover, proteolysis of BDyn leads to the formation of Dyn A and Leu-Enk, two important opioid peptides. The rate of formation of both is significantly reduced in cellular fractions of spinal cord mutant mice. As a consequence, even partial inhibition of PC1 or PC2 may impair the endogenous opioid system.

Keywords: Dynorphins, Dynorphin A, Proprotein convertases, Proteolysis, Opioid Peptides, Spinal cords, Mass spectrometry

1. Introduction

In mammals, the presences of tissue-damaging stimuli are sensed by primary afferent nociceptors. The sensation of pain produced by a noxious stimulus is not always consistent and depends on numerous factors influencing the neurophysiology of pain transmission (Julius and Basbaum, 2001; Gold, M.S. and Gebhart 2010). The nervous system has developed very complex mechanisms that control how noxious sensory input is perceived by the organism (Basbaum et al., 2009). It has been shown that extensive modulation of sensory information happens in the dorsal horn of the spinal cord, more specifically at the first synapse (Honore et al., 2000; Levine et al., 1993). There are various molecular events contributing to the transmission of the sensory information at the first synapse and several major neuropeptides were identified including dynorphin, enkephalin and tachykinin peptides (Kuner, 2010; Mika et al., 2011; Felippotti et al., 2012; Pailleux et al., 2013; Ferland et al., 2011).

Neuropeptides are either neurotransmitters or neuromodulators at various levels in the central nervous system and play a critical role in pain transmission (Levine et al., 1993; Seybold, 2009). Many members of the tachykinin family (e.g. Substance P) are mostly pro-nociceptive neuropeptides and have been known to play a fundamental role in central sensitization leading to hyperalgesia and allodynia (Lecci et al., 2000; Teodoro et al., 2013). Opioid peptides (i.e. endomorphins, enkephalins and dynorphins) have potent analgesic effects in the central nervous system (CNS) and play a fundamental role in endogenous pain inhibition (Machelska, 2007; Wahlert et al., 2013; Bali et al., 2014). They are interacting with μ, κ and δ-opioid receptors expressed widely in the brain and in the spinal cord (Carr and Lovering, 2000; Stanojevic et al., 2008; Mulder et al., 1989).

Dynorphin A (Dyn A), one of the major proteolytic fragments of prodynorphin (Civelli et al., 1985; Dores et al., 1985), is an endogenous ligand of the κ and μ-opioid receptors (Beaulieu et al., 2005; Mika et al., 2011). The agonist activity of Dyn A significantly reduces neuronal activity (Chavkin et al., 1982; Mizoguchi et al., 2006). However, the concentration of Dyn A in the spinal cord can be regulated rapidly by endoproteolysis (Cho and Basbaum, 1989). Neuropeptides are derived from larger protein precursors (i.e. proneuropeptides) and their primary structures include the sequence of the active form of at least one neuropeptide

within its full-length (Funkelstein et al., 2010). It has been revealed that many neuropeptides are synthesized by the actions of protein convertases (PCs) and endopeptidases within dense core vesicle (Harrison and Geppetti, 2001). Particularly, recent studies outlined the significant contribution of PC1 and PC2 into the proteolytic processing of proneuropeptides (Zheng et al., 1994; Cui et al., 1998; Jin et al., 2005; Miller et al., 2003). PCs, particularly PC1 and PC2 are widely expressed in the CNS and specifically cleave at C-terminal of either a pair of basic amino acids (KR-, RR-, RK- and KK-), or a single basic residue (R or K). Early prodynorphin (PDYN) endoproteolytic studies suggest that PCs play a significant role in PDYN processing (Berman et al., 2000; Day et al., 1998). These preliminary results generated using indirect methods suggest that the action of PC2 is needed for the formation of Big dynorphin (BDyn), Dyn A and dynorphin B (Dyn B) (Day et al., 1998). The proteolytic control of endogenous BDyn and Dyn A levels has a profound impact on pain perception and the role of PCs remain unclear. As shown in Figure 1, both peptides contain paired or single basic residues and the action of PCs could be involved in C-terminal processing of BDyn and Dyn A leading to several important N-terminal metabolites. Interestingly, BDyn, Dyn A and all N-terminal metabolites encode one copy of Leu-enkephalin (Leu-Enk), an important opioid peptide. The objective of this study was to decipher the role of PC1 and PC2 in the proteolysis control of BDyn and Dyn A levels using cellular fractions of spinal cords from wild type (WT), $PC1^{-/+}$ and $PC2^{-/+}$ animals.

2. Materials and Methods

2.1 Chemicals and reagents

Big Dynorphin (BDyn), Dynorphin A (Dyn A), Dynorphin 1-13 (Dyn 1-13), Dynorphin 1-11 (Dyn 1-11), Dynorphin 1-10 (Dyn 1-10), Dynorphin 1-9 (Dyn 1-9), Dynorphin 1-7 (Dyn 1-7), Dynorphin 1-6 (Dyn 1-6) and Leu-Enkephalin (Leu-Enk) were purchased from Phoenix Pharmaceuticals (Belmont, CA, USA). Dynorphin 1-19 (Dyn 1-19) and deuterium labeled analogue peptides were synthesized *de novo* (CanPeptide, Inc., Pointe-Claire, QC, Canada). Other chemicals, including acetonitrile, hexane, formic acid, Trifluoroacetic acid (TFA), sodium phosphate dibasic and sodium phosphate monobasic were purchased from Fisher Scientific (Ottawa, ON, Canada).

2.2 Sample Preparation

Spinal cord tissues (n=6 per genotypes) from male WT (C57BL/6J), male $PC1^{-/+}$ (product #006327) and male $PC2^{-/+}$ mice (product #002963) were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA) and kept frozen at -80 °C until analysis. Heterozygote animals only were used since $PC1^{-/-}$ and $PC2^{-/-}$ exhibit many abnormalities and the survival rate after one week is extremely low. Animal genotyping was performed for each animal by Jackson Laboratory using a standard PCR assay. All mice were 8 weeks old at time of tissues collection. The animals from all groups (WT, $PC1^{-/+}$ and $PC2^{-/+}$) were euthanized with an overdose of isoflurane followed by a transection of the cervical spine. A flush of saline was performed within the spinal canal to collect the spinal cord lumbar enlargement. Tissue samples were snap-frozen in cold hexane (-60 °C) and stored immediately at -80 °C pending analyses. The study protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine of the Université de Montréal and it was performed in accordance with the guidelines of the Canadian Council on Animal Care.

For each animal group, three spinal cords were pooled and homogenized in a 0.1 M phosphate buffer, pH 7.4 at a ratio of 1:5 (w:v). Samples were sonicated 20 minutes and the homogenates were centrifuged at 9,000 *g* for 10 minutes. The total amount of protein in each supernatant was determined using the standard Coomassie protein assay (Bradford). This procedure was necessary in order to assure the same amount of protein was used for each experiments. Supernatant aliquots, designated as S9 fractions, were kept at -80 °C until usage.

2.3 Peptide metabolism study

The incubations were performed minimally in triplicate. The incubations were performed in a microcentrifuge tubes and contained 2.5 nmol/mL of BDyn or Dyn A, 0.25 mg/mL of S9 fraction proteins diluted in 0.1 M phosphate buffer, pH 7.4. Spinal cord S9 enzyme suspensions (total volume of 1 mL) were preincubated with 1mM $CaCl_2$ in 0.1 M phosphate buffer (pH 7.4) at 37°C for 5 min prior fortification with BDyn or Dyn A. Immediately after fortification of the full-length peptide into the spinal cord S9 fraction suspension containing 1mM $CaCl_2$, the sampling point for t=0 was taken, and the reaction was quenched after 30 minutes incubation. Two hundred microliters of samples were taken and mixed with 200 µL

of an aqueous solution containing 1% TFA to stop the reaction. Samples were centrifuged at 12,000 g for 10 min and 150 µL of the supernatant was transferred into an injection vial and spiked with 150 µL of the deuterated internal standard solution (50 pmol/mL labeled peptides in 0.1% TFA) for MS analysis. The rate of formation (v_i) was calculated based on the concentration of each metabolite measured after 30 minutes incubation of the full-length peptides using Equation 1.

$$v_i = \frac{d[P]}{dt} = \frac{[peptide\ fragments]}{30\ min} \quad (1)$$

2.4 Instrumentation

The HPLC-MS/MS system included a Thermo Accela autosampler, a Thermo Accela pump and a Thermo LTQ-XL Linear Ion Trap Mass Spectrometer (San Jose, CA, USA). Linear ion trap instruments typically have unit mass resolution throughout the mass range. The instrument was calibrated and the resolution was set at 0.5-0.7 Da at full width at half maximum (FWHM). Data were acquired and analyzed with Xcalibur 2.2 (San Jose, CA, USA), and regression analyses were performed with PRISM (version 6.0d) GraphPad software (La Jolla, CA, USA) using nonlinear curve-fitting module with an estimation of the goodness of fit. The calibration lines were constructed from the peak-area ratios of targeted neuropeptides and corresponding deuterated labeled peptides used as internal standards. Further analyses were performed using a Thermo Scientific Q-Exactive Orbitrap Mass Spectrometer (San Jose, CA, USA) interfaced with a Thermo Scientific UltiMate 3000 Rapid Separation UHPLC system using a pneumatic assisted heated electrospray ion source.

2.5 Bioanalytical methods

The chromatography was achieved using a gradient mobile phase along with a microbore column Thermo Biobasic C8 100 × 1 mm, with a particle size of 5 µm. The initial mobile phase condition consisted of acetonitrile and water (both fortified with 0.1% of formic acid) at a ratio of 5:95. From 0 to 1 min, the ratio was maintained at 5:95. From 1 to 12 min, a linear gradient was applied up to a ratio of 45:55 and maintained for 3 min. The mobile phase composition ratio was reverted at the initial conditions and the column was allowed to re-equilibrate for 15 min for a total run time of 32 min. The flow rate was fixed at 75 µL/min and 2 µL of sample were injected using full loop mode. All targeted neuropeptides and

deuterium labeled peptides eluted between 6.2 to 9.3 min. The mass spectrometer was coupled with the HPLC system using a pneumatically assisted electrospray ion source (ESI). The sheath gas was set to 25 units and the ESI electrode was set to 4000 V in positive mode. The capillary temperature was set at 300°C and the ion transfer tube voltage to 46 V. All scan events were acquired with a 100 ms maximum injection time. An activation q = 0.25 and activation time of 30 ms were used for all targeted peptides. The mass spectrometer operated for quantitative analyses in full scan MS/MS and the quantification was based on specific post-processing multiple reaction monitoring (MRM) extracted ion chromatograms. Specific analysis details are presented in Table 1. Two specific production ions were used to generate post acquisition MRM extracted ion chromatograms for quantification purposes. The method used an isotope dilution mass spectrometry (IDMS) strategy for the quantification of the targeted peptides. Due to synthesis yield issues related to generate labeled BDyn and Dyn 1-19, deuterated Dyn A was used as an internal standard for BDyn and Dyn 1-19 quantification along with the BDyn and Dyn 1-19 reference standards. The labeled peptides were used at a constant concentration of 50 pmol/mL. The peptide concentrations were determined using the peak area ratio of the light and heavy analog peptide. Additional analyses were performed using a hybrid Quadrupole-Orbitrap mass spectrometer (i.e. Thermo Q-Exactive). The chromatographic condition used was identical. The MS detection was performed in positive ion mode and it was operating in full-scan mode at high-resolution, and accurate-mass (HRAM). Nitrogen was used for sheath and auxiliary gases and they were set at 10 and 5 arbitrary units. The ESI probe was set to 4000 V and the ion transfer tube temperature was set to 300°C. The scan range was set to *m/z* 300-1500. Data was acquired at a resolving power of 140,000 (FWHM), resulting to a scanning rate of ≈ 700 msec/scan when using automatic gain control target of 3.0×10^6 and maximum ion injection time of 200 msec.

2.6 Statistical analysis

All data were analyzed using a one-way ANOVA followed by Dunnett multiple comparison test. Significance was set a priori to $p < 0.05$. The statistical analyses were performed using PRISM (version 6.0f).

3. Results and Discussion

3.1 Mass Spectrometry and Isotopic Dilution Method

Full-scan and product ion mass spectra for all peptides and internal standards were obtained in positive ion mode. The full-scan electrospray mass spectrum of targeted peptides displayed the formation of characteristic pseudo molecular ions $[M+nH]^{n+}$ and the fragment ions observed in MS/MS spectra were annotated based on the Roepstorff and Fohlman nomenclature (Roepstorff and Fohlman, 1984). Details on MS parameters and MRM transitions are reported in Table 1. Full-scan and product ion mass spectra are necessary to identify and characterize each neuropeptide. The full-scan electrospray mass spectra of targeted neuropeptides showed a base peak pseudo molecular ions at m/z 569.9 (7+) for BDyn, 487.1 (+5) for Dyn 1-19, 716.4 (+3) for Dyn A, 535.3 (+3) for Dyn 1-13, 682.0 (+2) for Dyn 1-11, 617.9 (+2) for Dyn 1-10, 434.7 (+2) for Dyn 1-7, 356.7 (+2) for Dyn 1-6 and 556.3 (+1) for Leu-Enk. Corresponding labeled peptides shown compatible pseudo molecular ion profiles. All precursor ion masses are listed for each unlabeled and labeled peptide in Table 1. Figure 2 presents product ion spectra (MS/MS) for targeted neuropeptides obtained and typical a, b, c, y and z positive ion fragments were observed. The observed collision-induced dissociation spectra were all compatible with the neuropeptide sequences. Additionally, we selected and optimized two of the most abundant and specific product ions for each neuropeptide to generate post acquisition MRM extracted ion chromatograms in order to achieve the best sensitivity, selectivity and reproducibility. Furthermore, selected MRM transitions were monitored for spinal cord S9 fraction supernatants and compared with reference materials. The overlay MRM extracted ion chromatograms display in Figure 3 demonstrate a good concordance between peptide reference standards and peptides observed in spinal cord S9 fraction supernatants. Due to the important dilution factor of spinal cord S9 fraction used during this study, the endogenous levels were below the limit of quantification.

The heavy-label versions of Dyn A, Dyn 1-11, Dyn 1-10, Dyn 1-9, Dyn 1-7, Dyn 1-6 and Leu-Enk were spiked in spinal cord S9 fraction supernatants to quantify BDyn and Dyn A metabolites. After optimization, the concentration of spiked labeled peptides, it was determined that a final concentration of 50 pmol/mL would lead to adequate measurements of the peptides selected MRM transitions. All labeled peptides are clearly distinguishable

from unlabeled version by mass spectrometry, and the ratio of the unlabeled peptides to stable isotope-labeled peptides found in mouse spinal cord S9 fraction supernatants can be used to calculate the absolute concentration of each peptide monitored. The linearity response was tested at concentration ranging from 1 to 200 pmol/mL for each peptide. Correlation assessments between measured peak area ratios and nominal dilution ratios were performed and results show excellent correlations (R^2 = 0.9906 to 0.9999). The precision (%CV) was comprised between 1.3% and 9.1% and the accuracy (%NOM) was contained between 88.2% and 110.9% for all targeted neuropeptides. Accordingly, the analytical method provided adequate figures of merit for targeted peptide analysis performed during this study.

3.2 *Metabolic stability of BDyn and Dyn A in* mouse spinal cord S9 fractions

Big dynorphin and Dyn A concentration in mouse spinal cord is regulated by proteolysis generating a series of peptide metabolites. Tissue S9 fractions are widely used to study metabolism since this fraction contains the cytosol and microsomes (Duffus et al., 2007). To determine the pathways for BDyn and Dyn A degradation in mouse spinal cord S9 fractions, spinal cord S9 fractions were incubated with full-length BDyn and Dyn A for 30 min and then analyzed the quenched reactions by HPLC-MS/MS (i.e. Thermo LTQ-XL) to determine BDyn and Dyn A metabolic stability and identify fragments that had been produced. The Figure 4 revealed that proteolysis of BDyn and Dyn A is occurring in mouse spinal cord S9 fractions. The results shown that > 86% of the full-length peptides degraded in 30 minutes. Furthermore, negative control (i.e. no S9 proteins) shows no degradation after a 30 minutes incubation period for both full-length peptides. These results revealed significant BDyn and Dyn A degrading activity in mouse spinal cord S9 fractions.

3.3 Metabolite profiling using HRAM MS analysis

Neuropeptides are generally synthesized as larger precursors that undergo endoproteolysis at specific sites. Several neuropeptide-processing enzymes were identified in mammalian cells (Fricker and Devi, 1995). Endoprotease of the subtilisin family of serine proteases, including proprotein convertases (PCs), cleave peptide precursors at paired or single basic residue (Conn and Smith, 1995; Minokadeh et al., 2010). Following the precursor cleavage at paired or single basic residue, carboxypeptidases remove basic amino acids from the C-terminal

(Zheng et al., 1994; Fricker, 1988) of the resulting metabolites (Hook et al. 2008). As shown in Figure 1, schematic representation of BDyn and Dyn A indicates possible paired and single basic cleavage sites. Full-length peptides, BDyn and Dyn A, were incubated for 30 minutes in mouse spinal cord S9 fractions and following the reaction termination, supernatant were analyzed using a Quadrupole-Orbitrap high-resolution mass spectrometer. The HRAM MS analysis reveals specifically the presence of several expected BDyn and Dyn A metabolites with mass accuracy within 2 ppm as shown in Table 2 and 3. Many expected and targeted PC1 and PC2 primary and secondary metabolites were detected. Interestingly, following the degradation of BDyn, we can detect the presence of Dynorphin B but also Dyn 12-32 and Dyn 14-32, two complementary metabolites of Dyn 1-11 and Dyn 1-13. This result suggests that Dyn 1-11 may not be sequentially produced following the formation of Dyn 1-13. Interestingly, C-terminal processing of Dyn A leads to the formation of N-terminal fragments Dyn 1-13, Dyn 1-11 and Dyn 1-7, all potential metabolites resulting from PC1 or PC2 cleavage. Additionally, we were able to detect complementary C-terminal fragments including Dyn 14-17, Dyn 12-17 and Dyn 8-17 denoting they are most likely primary cleavage products. Our results clearly revealed the presence of BDyn and Dyn A metabolites compatible with PC1 or PC2 processing. N-terminal processing at dibasic residue by cathepsin L was reported to be involved for the production of enkephalin peptides (Hook *et al.*, 2008; Funkelstein et al., 2010). HRAM MS analysis reveals the presence of Leu-Enk following BDyn and Dyn A incubation in mouse spinal cord S9 fractions. No other significant peaks associated with N-terminal processing at dibasic residue were observed. Leu-Enk can be directly produce by cathepsin L N-terminal processing of BDyn and Dyn A or by C-terminal processing of Dyn 1-7, an important C-terminal processing metabolite observed.

3.4 Analysis of PC1 and PC2 in mouse spinal cord S9 fractions

We have previously performed the quantification of PC1 and PC2 in WT, $PC1^{-/+}$ and $PC2^{-/+}$ mouse spinal cord S9 fractions (Saidi et al., 2015). Briefly, we quantified PC1 and PC2 in WT, $PC1^{-/+}$ and $PC2^{-/+}$ mouse spinal cord S9 fractions using high-resolution MS. The analytical strategy was based on a targeted peptide mass fingerprinting (PMF) method that specifically relies on upstream identification of specific PC1 and PC2 proteolytic peptides

using *in silico* digestion to generate a mass list. The protein analyses were performed using a hybrid Quadrupole-Orbitrap mass spectrometer operating in MS at a resolution of 140,000 (FWHM) and in MS/MS at a resolution of 17,500 (FWHM). Two specific proteolytic peptides for PC1 and PC2 were observed within 1 ppm of the exact mass. Specific XIC's (exact mass ± 5 ppm) of each targeted proteotypic peptides were used to perform label-free relative quantification based on observed ion abundance. Our results reveal that PC1 and PC2 are significantly down-regulated (i.e. 38 to 49%) in $PC1^{-/+}$ and $PC2^{-/+}$ mouse spinal cord S9 fractions respectively (Saidi et al., 2015). Additionally, all collected MS/MS spectra were coherent with the amino acid sequence of each tryptic peptide.

3.5 Contribution of PC1 and PC2 in BDyn C-terminal processing

Some studies have examined the involvement of PC1 and PC2 in PDYN processing but none have shown the involvement of PC1 or PC2 in the metabolic turnover of BDyn and Dyn A (Berman et al. 2000). BDyn is a proteolytic product of PDYN containing one copy of Dyn A, Dyn B and two copies of Leu-Enk. Full-length BDyn was incubated for 30 minutes in WT, $PC1^{-/+}$ and $PC2^{-/+}$ mouse spinal cord S9 fractions and metabolites quantified by HPLC-MS/MS. The rate of formation (v_i) was determined using Equation 1. As shown in Figure 5, cleavage at paired and single basic sites were observed forming Dyn 1-19, Dyn 1-13, Dyn 1-11 and Dyn 1-7. As expected, no trace of Dyn 1-9 was detected since PCs do not cleave when single or paired basic amino acids are followed by a Proline (P). The contribution of PC1 and PC2 in the degradation of BDyn is noteworthy. As shown in Figure 5, PC1 and PC2 mediate processing of BDyn to Dyn 1-19, but only PC1 appears to be significant for the formation of Dyn 1-11. No statistically significant differences were noted for Dyn 1-13 and Dyn 1-7. However, this does not mean that PC1 and PC2 are not involved in the formation of these metabolites but only suggest that they are not principal metabolites of BDyn since the enzymes active sites are largely saturated with BDyn. Rate of formation changes will be noticed only when substrate concentration saturates or the enzymes active sites nearly saturates. Therefore, rate of formation differences for minor metabolites of BDyn produced by the same metabolic pathway can't be clearly observed. The stability of the enzyme-peptide complex is closely related with the enzyme structure. It plays a central role in defining the energetically favored binding cluster of the peptide into the active enzyme site (Sun and

Scott, 2010). As shown *in silico*, the structure of the binding cluster may lead to different metabolites or affect the rate of formation (Sun and Scott, 2010). Our results suggest that the formation of Dyn 1-19 and Dyn 1-11 is favored following the formation of the enzyme-peptide complex. Interestingly, metabolites generated following the removal of basic amino acids from the C-terminal by carboxypeptidases were also impacted. Figure 6 reveals that rate of formation of Dyn 1-10, Dyn 1-6 and Leu-Enk in $PC1^{-/+}$ mouse spinal cord S9 fractions is significantly hampered with some noticeable reduction in the formation of Dyn A (not statistically significant). Furthermore, Dyn A formation is reduced in $PC2^{-/+}$ mouse spinal cord S9 fractions. These results clearly demonstrate that PCs regulate BDyn concentrations through C-terminal processing. Turnover kinetics of neuropeptides is important to describe the formation of all bioactive peptides that play an important role in synaptic transmission. As shown in Figure 5 and 6, proteolysis of BDyn leads to the formation of important opioid peptides, including specifically, Dyn A and Leu-Enk. BDyn is a precursor of Dyn A and has two copies of Leu-Enk encoded in its primary sequence. More specifically, the interaction of Dyn A with κ-opioid receptors located at the presynaptic terminals inhibits the release of SP and therefore plays a central role in alleviating pain. Proteolytic processing of BDyn leads to the formation of Leu-Enk, an agonist of the δ-opioid receptors (Chen et al., 2007; Fichna et al., 2007). The activation of δ-opioid receptors by endogenous enkephalins mediates analgesic effects. Both peptides have a fundamental function in the endogenous opioid system. As a consequence, even partial inhibition of PC1 or PC2 may impair the endogenous opioid system.

3.6 Contribution of PC1 and PC2 in Dyn A C-terminal processing

Spinal concentration of Dyn A was reported to increase in patients suffering of acute and chronic pain (Wang et al., 2001). As mentioned before, Dyn A inhibits pain transmission since it impedes SP release in the spinal cord (Zachariou and Goldstein, 1997). Also, it can affect nociceptive synaptic transmission by blocking the calcium channels (Werz and Macdonald, 1984; Werz and Macdonald, 1985). Others have indicated that dynorphins act as pronociceptive peptides by activating non-opioid receptors (Lai et al., 2006; Tang et al., 2000) and participate in the generation of chronic pain (Wang et al., 2001). Thus, understanding proteolytic regulation of Dyn A can be insightful on the pathophysiology of

nociceptive pain and potential new strategies for treatment. Full-length Dyn A was incubated for 30 minutes in WT, $PC1^{-/+}$ and $PC2^{-/+}$ mouse spinal cord S9 fractions and metabolites quantified by HPLC-MS/MS. The rate of formation (v_i) was determined using Equation 1. Figure 7A distinctly demonstrates that PC1 is involved in the proteolysis control of Dyn A in the spinal cord. PC1 clearly mediates the processing of Dyn A to Dyn 1-13, Dyn 1-11 and Dyn 1-7, metabolites that resulted from a cleavage at paired or single basic residues. Similarly to BDyn results, Dyn A metabolites generated following the removal of basic amino acids from the C-terminal by carboxypeptidases were also impeded. As illustrate in Figure 7B, the rate of formation (v_1) of Dyn 1-10, Dyn 1-6 and Leu-Enk was significantly hampered in $PC1^{-/+}$ mouse spinal cord S9 fractions. To a much lesser extent, the rate of formation (v_1) of Dyn 1-10 and Dyn 1-6 was significantly reduced in $PC2^{-/+}$ mouse spinal cord S9 fractions. Again, interestingly, the proteolysis controls of Dyn A generated Leu-Enk, another important opioid peptide mediating potent painkilling effects (Holden et al., 2005; Akil et al., 1997). Thus, proteolysis regulation of BDyn and Dyn A play an important role in the control of endogenous concentrations but also play a significant role in the production of peptide fragments with opiate-like properties with strong antinociceptive effects. Pharmacological manipulation of PC1 and PC2 may therefore have a profound impact on the endogenous pain-relieving mechanisms.

4. Conclusion

In neuronal cells, almost all neuropeptides are produced through endoproteolysis specifically at C-terminal pairs of basic residues during their transport along the secretory pathway. Interestingly, it is recognized that proneuropeptides are cleaved using a cell-specific mechanism by members of the family of calcium-dependent subtilisin-like endoproteases (Steiner et al., 1998; Seidah et al., 1999; Hook et al., 2008). PC1 and PC2 are predominantly expressed in neural cells and our results suggest they play a central role in the proteolysis controls of endogenous BDyn and Dyn A levels generating important peptide metabolites. It is important to recognize that physiologically, the differential expression of PC1 and PC2 in

different neuroendocrine cells and neurons may generate varied mixtures of BDyn and Dyn A metabolites since observed metabolic products are intimately related to the thermodynamic stability of the enzyme-peptide complexes and the turnover number (k_{cat}) is associated with the enzyme concentration. Several dynorphins and enkephalins play a central role in pain modulation and inhibition and the proteolytic regulation of endogenous dynorphins has a profound impact on the pathophysiology of pain. The pronociceptive tachykinin system (SP-NK1) and the analgesic opioid systems are important for normal pain sensation and these systems strongly interact together (Saidi and Beaudry, 2015). Our results clearly demonstrate that both PC1 and PC2 are involved in the proteolysis regulation of BDyn and Dyn A with a more important role for PC1. Interestingly, PCs may also play a role in the proteolytic processing of the protachykinin-1 protein (Saidi et al., 2015) and therefore, be directly or indirectly involved in the formation of SP, a neuropeptide playing a critical role in nociceptive transmission. These observations may have insightful impact on our basic knowledge on the proteolytic regulation of neuropeptides in the central nervous system and for future analgesic drug developments.

5. Acknowledgements

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Table 1. Summary of peptide quantification parameters used for HPLC-MS/MS analysis and post processing MRM

Peptides	Sequences	Precursor ions	Product ions	Collision energy (%)
BigDynorphin	YGGFLRRIRPKLKWDNQKR YGGFLRRDFKVVT	570 (z = 7)	645.3 (b_{31}^{6+})	45
			561.4 (a_{27}^{6+})	
Dynorphin 1-19	YGGFLRRIRPKLKWDNQKR	487 (z = 5)	629.9 (**b**$_{15}^{3+}$)	45
			564.0 (**z**$_{18}^{4+}$)	
Dyn A	YGGFLRRIRPKLKWDNQ	716 (z = 3)	944.2 (b_{15}^{2+})	45
			630.1 (b_{15}^{3+})	
(d_5)Dyn A	YGG**F**(d_5) LRRIRPKLKWDNQ	719 (z = 3)	947.2 (b_{15}^{2+})	45
			631.6 (b_{15}^{3+})	
Dyn 1-13	YGGFLRRIRPKLK	535 (z = 3)	493.0 (c_{12}^{3+})	35
			449.0 (b_{11}^{3+})	
(d_5)Dyn 1-13	YGG**F**(d_5) LRRIRPKLK	537 (z = 3)	494.4 (c_{12}^{3+})	35
			451.0 (b_{11}^{3+})	
Dyn 1-11	YGGFLRRIRPK	682 (z = 2)	618.0 (c_{10}^{2+})	35
			560.3 (b_9^{2+})	
(d_5)Dyn 1-11	YGG**F**(d_5) LRRIRPK	684 (z = 2)	620.5 (c_{10}^{2+})	35
			563.0 (b_9^{2+})	
Dyn 1-10	YGGFLRRIRP	618 (z = 2)	560.4 (b_9^{2+})	35
			539.4 (b_5)	
(d_5)Dyn 1-10	YGG**F**(d_5) LRRIRP	621 (z = 2)	563.0 (b_9^{2+})	35
			541.9 (b_5)	
Dyn 1-9	YGGFLRRIR	570 (z = 2)	491.42 (c_8^{2+})	45
Dyn 1-7	YGGFLRR	435 (z = 2)	694.5 (b_6)	45
			631.6 (z_5)	
(d_5)Dyn 1-7	YGG**F**(d_5) LRR	437 (z = 2)	699.5 (b_6)	45
			636.5 (z_5)	
Dyn 1-6	YGGFLR	357 (z = 2)	549.5 (y_5)	35
			492.4 (y_4)	
(d_5)Dyn 1-6	YGG**F(**d_5**)**LR	359 (z = 2)	554.4 (y_5)	35
			497.5 (y_4)	
Leu-Enk	YGGFL	556 (z = 1)	425.3 (**b**$_4$)	35
			397.3 (**a**$_4$)	
(d_5)Leu-Enk	YGG**F(**d_5**)**L	561 (z = 1)	430.3 (**b**$_4$)	35
			402.3 (**a**$_4$)	

Table 2. Summary of the most significant C-terminal processing BDyn peptide metabolites detected using high-resolution accurate mass spectrometry

Peptides	Sequences	Charge states z	Theoretical mass	Observed mass	Δm (ppm)
BigDynorphin	YGGFLRRIRPKLKWDNQKRYG GFLRRDFKVVT	+7	569.9008	569.9009	0.18
Dynorphin 1-19	YGGFLRRIRPKLKWDNQKR	+5	487.0847	487.0893	1.64
Dyn A	YGGFLRRIRPKLKWDNQ	+3	716.4043	716.4033	- 1.40
Dyn 1-13	YGGFLRRIRPKLK	+3	535.3351	535.3349	- 0.37
Dyn 1-11	YGGFLRRIRPK	+2	681.9095	681.9094	- 0.15
Dyn 1-10	YGGFLRRIRP	+2	617.8620	617.8618	- 0.32
Dyn 1-7	YGGFLRR	+2	434.7430	434.7433	0.69
Dyn 1-6	YGGFLR	+2	356.6925	356.6923	- 0.56
Leu-Enk	YGGFL	+1	556.2766	556.2761	0.90
Dyn B	YGGFLRRDFKVVT	+3	524.2999	524.2994	- 0.95
Dyn 12-32	LKWDNQKRYGGFLRRDFKVVT	+5	528.6994	528.6998	0.76
Dyn 14-32	WDNQKRYGGFLRRDFKVVT	+5	480.4636	480.4636	0.00

Table 3. Summary of the most significant C-terminal processing Dyn A peptide metabolites detected using high-resolution accurate mass spectrometry

Peptides	Sequences	Charge states z	Theoretical mass	Observed mass	Δm (ppm)
Dyn A	YGGFLRRIRPKLKWDNQ	+3	716.4043	716.4037	- 0.84
Dyn 1-13	YGGFLRRIRPKLK	+3	535.3351	535.3350	- 0.19
Dyn 1-11	YGGFLRRIRPK	+2	681.9095	681.9092	- 0.44
Dyn 1-10	YGGFLRRIRP	+2	617.8620	617.8616	- 0.65
Dyn 1-7	YGGFLRR	+2	434.7430	434.7429	- 0.23
Dyn 1-6	YGGFLR	+2	356.6925	356.6922	- 0.84
Leu-Enk	YGGFL	+1	556.2766	556.2762	- 0.72
Dyn 14-17	WDNQ	+1	562.2256	562.2255	-0.18
Dyn 12-17	LKWDNQ	+2	402.2060	402.2058	-0.50
Dyn 8-17	IRPKLKWDNQ	+3	433.2507	433.2505	-0.46

Figure Legends

Figure 1. Prodynorphin (PDYN) processing by proprotein convertases (PC1 and PC2). Dynorphins have several paired and single basic amino acid cleavage sites and based on the sequence, various processing intermediates can be derived leading to dynorphin 1-19, dynorphin 1-13, dynorphin 1-11, dynorphin 1-7 and other active neuropeptides. Moreover, Dynorphin A encode one copy of Leu-Enk (YGGFL) suggesting, it could be an important proteolytic product.

Figure 2. . Product ion spectra (MS^2) of dynorphins. MS/MS spectra were annotated based on the Roepstorff and Fohlman nomenclature. Product ions observed were compatible with the primary sequence of each peptide analyzed.

Figure 3. Reconstructed ion chromatograms for targeted dynorphins. Reference standards (black) and incubated spinal cord (SC) S9 fractions were compared. Please note that at time 0, no metabolites were observed.

Figure 4. Evaluation of the metabolic stability of BDyn and Dyn A in mouse spinal cord S9 fractions. The degradation of full-length peptides occurred only with the presence of S9 proteins and demonstrates that BDyn and Dyn A undergoes proteolytic processing in mouse spinal cord S9 fractions.

Figure 5. Assessments of PC1 and PC2 involvement in the proteolysis of big dynorphin. Results suggest that PC1 and PC2 are involved in the proteolytic processing of big dynorphin leading to the formation of dynorphin 1-19 and dynorphin 1-11.

Figure 6. Impact of PC1 and PC2 on the formation of other big dynorphin metabolites. The formation of dynorphin A, dynorphin 1-10, dynorphin 1-6 and Leu-Enk is severally impaired particularly in $PC1^{-/+}$ spinal cord S9 fractions.

Figure 7. Assessments of PC1 and PC2 involvement in the proteolysis of dynorphin A. (A) Illustrates the important role of PC1 in the formation of dynorphin 1-13, dynorphine 1-11 and dynorphin 1-7. (B) The formation of dynorphin 1-10, dynorphin 1-6 and Leu-Enk is significantly reduced in $PC1^{-/+}$ and $PC2^{-/+}$ spinal cord S9 fractions.

Figure 1.

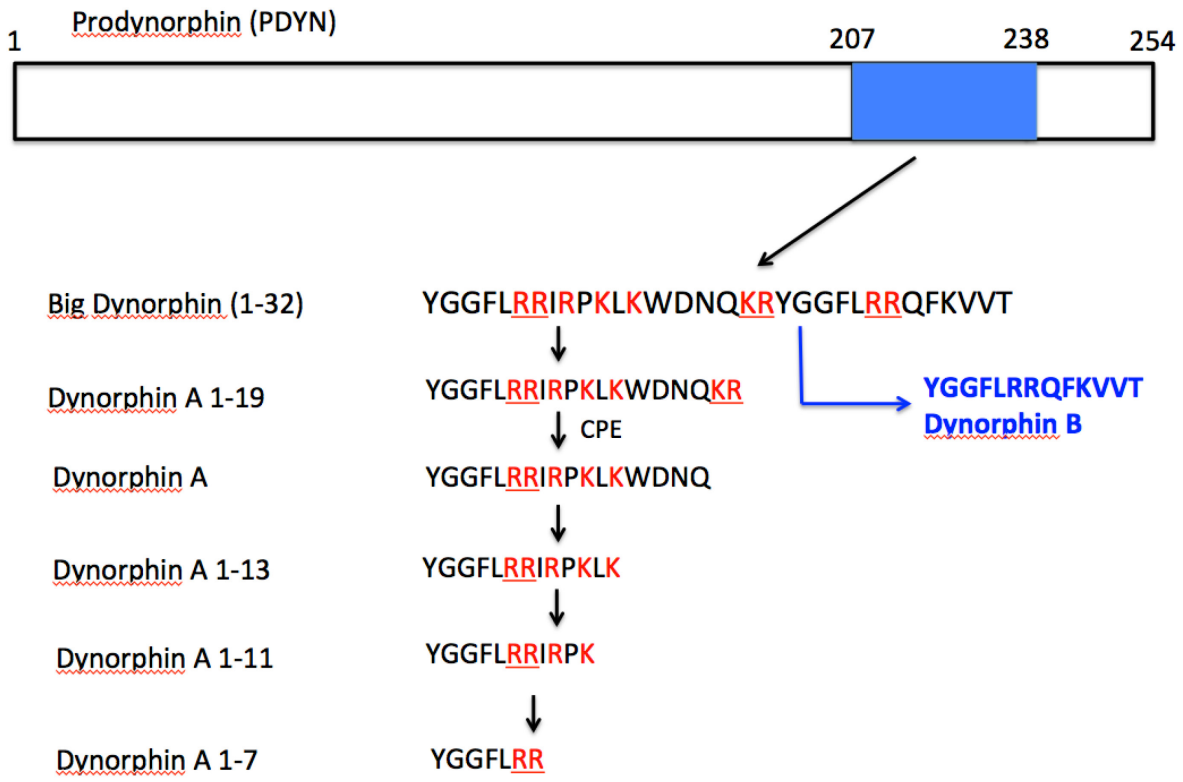

Prodynorphin (PDYN)
1
207
238
254
Big Dynorphin (1-32)
YGGFLRRIRPKLKWDNQKRYGGFLRRQFKVVT
Dynorphin A 1-19
YGGFLRRIRPKLKWDNQKR
YGGFLRRQFKVVT
Dynorphin B
CPE
Dynorphin A
YGGFLRRIRPKLKWDNQ
Dynorphin A 1-13
YGGFLRRIRPKLK
Dynorphin A 1-11
YGGFLRRIRPK
Dynorphin A 1-7
YGGFLRR

Figure 2.

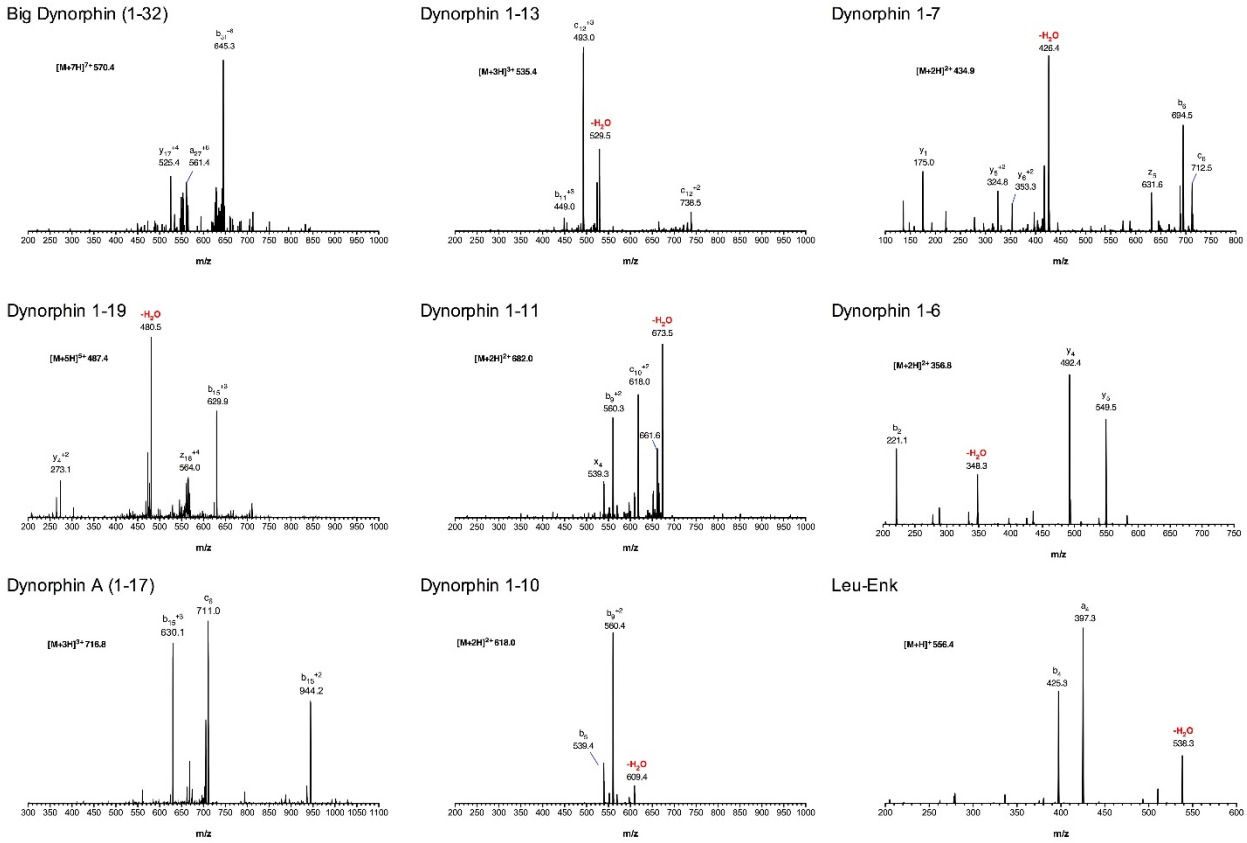

Big Dynorphin (1-32)
[M+7H]7+ 570.4
645.3
525.4
561.4
m/z
Dynorphin 1-13
[M+3H]3+ 535.4
493.0
529.5
449.0
736.5
m/z
Dynorphin 1-7
[M+2H]2+ 434.9
426.4
694.5
175.0
324.8
353.3
631.6
712.5
m/z
Dynorphin 1-19
[M+5H]5+ 487.4
480.5
629.9
273.1
564.0
m/z
Dynorphin 1-11
[M+2H]2+ 682.0
673.5
618.0
580.3
661.6
539.3
m/z
Dynorphin 1-6
[M+2H]2+ 356.8
492.4
549.5
221.1
348.3
m/z
Dynorphin A (1-17)
[M+3H]3+ 716.8
711.0
630.1
944.2
m/z
Dynorphin 1-10
[M+2H]2+ 618.0
560.4
539.4
609.4
m/z
Leu-Enk
[M+H]+ 556.4
397.3
425.3
538.3
m/z

Figure 3.

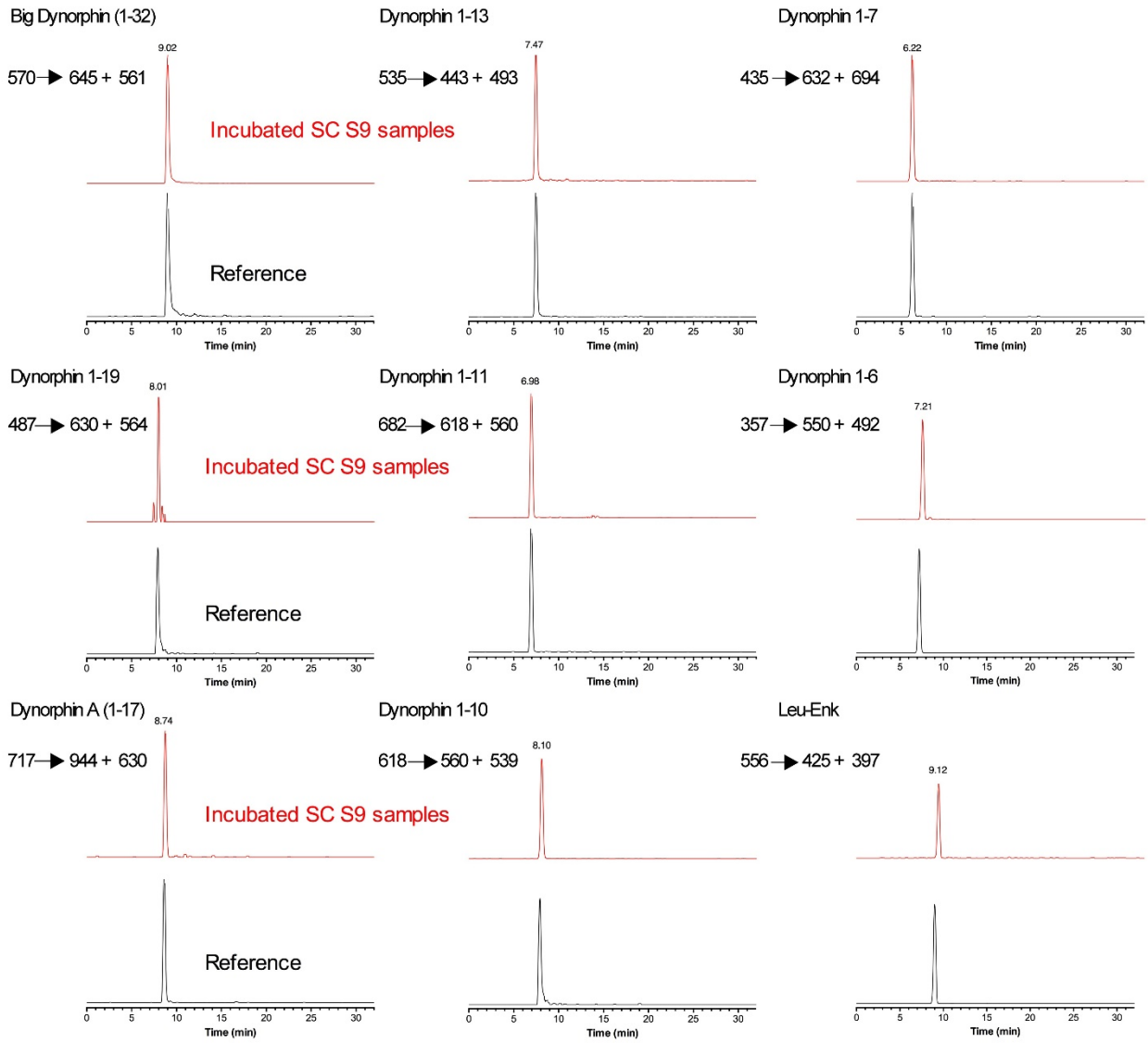

Big Dynorphin (1-32)
570 → 645 + 561
9.02
Incubated SC S9 samples
Reference
Dynorphin 1-13
535 → 443 + 493
7.47
Dynorphin 1-7
435 → 632 + 694
6.22
Dynorphin 1-19
487 → 630 + 564
8.01
Dynorphin 1-11
682 → 618 + 560
6.98
Dynorphin 1-6
357 → 550 + 492
7.21
Dynorphin A (1-17)
717 → 944 + 630
8.74
Dynorphin 1-10
618 → 560 + 539
8.10
Leu-Enk
556 → 425 + 397
9.12
Time (min)

Figure 4.

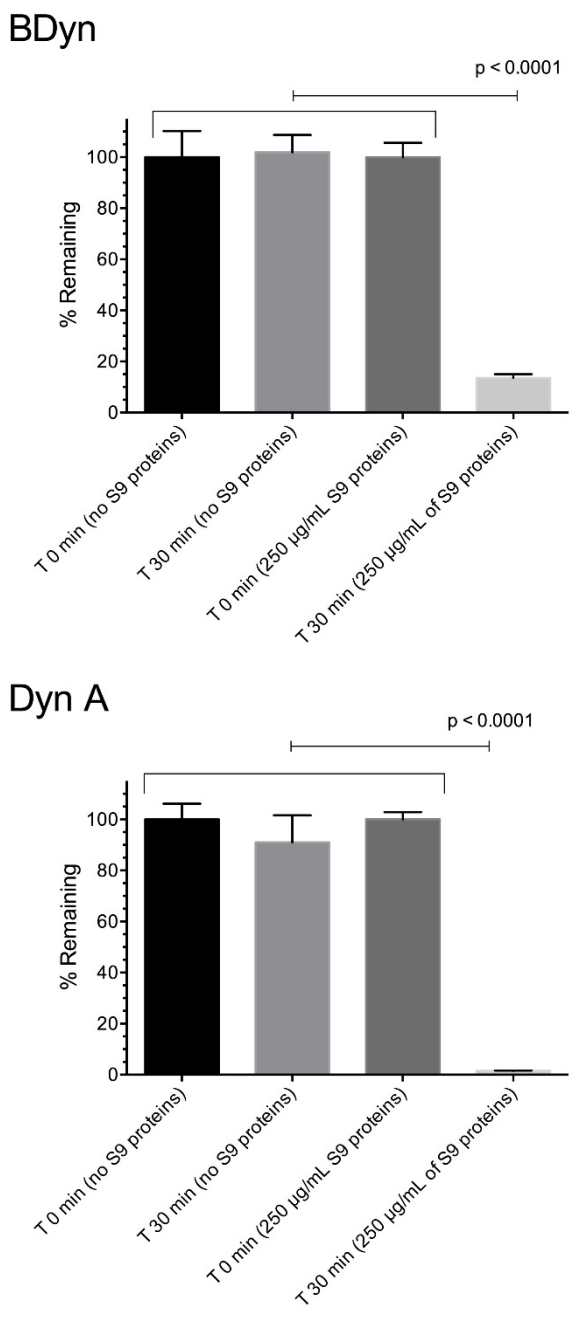

BDyn
p < 0.0001
% Remaining
0
20
40
60
80
100
T 0 min (no S9 proteins)
T 30 min (no S9 proteins)
T 0 min (250 µg/mL S9 proteins)
T 30 min (250 µg/mL of S9 proteins)
Dyn A
p < 0.0001
% Remaining
0
20
40
60
80
100
T 0 min (no S9 proteins)
T 30 min (no S9 proteins)
T 0 min (250 µg/mL S9 proteins)
T 30 min (250 µg/mL of S9 proteins)

Figure 5.

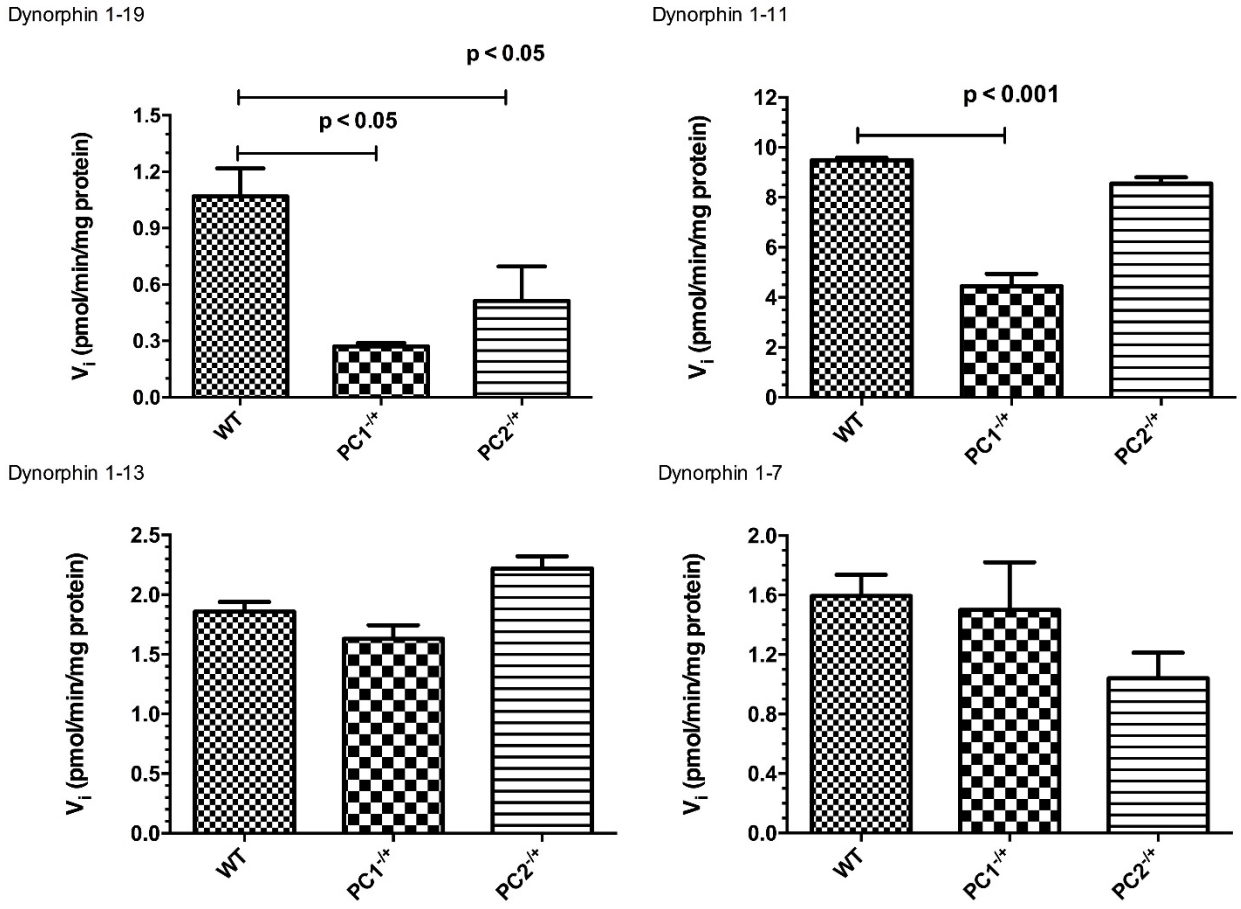

Dynorphin 1-19
p < 0.05
p < 0.05
V_i (pmol/min/mg protein)
1.5
1.2
0.9
0.6
0.3
0.0
WT
PC1-/+
PC2-/+
Dynorphin 1-11
p < 0.001
V_i (pmol/min/mg protein)
12
10
8
6
4
2
0
WT
PC1-/+
PC2-/+
Dynorphin 1-13
V_i (pmol/min/mg protein)
2.5
2.0
1.5
1.0
0.5
0.0
WT
PC1-/+
PC2-/+
Dynorphin 1-7
V_i (pmol/min/mg protein)
2.0
1.6
1.2
0.8
0.4
0.0
WT
PC1-/+
PC2-/+

Figure 6.

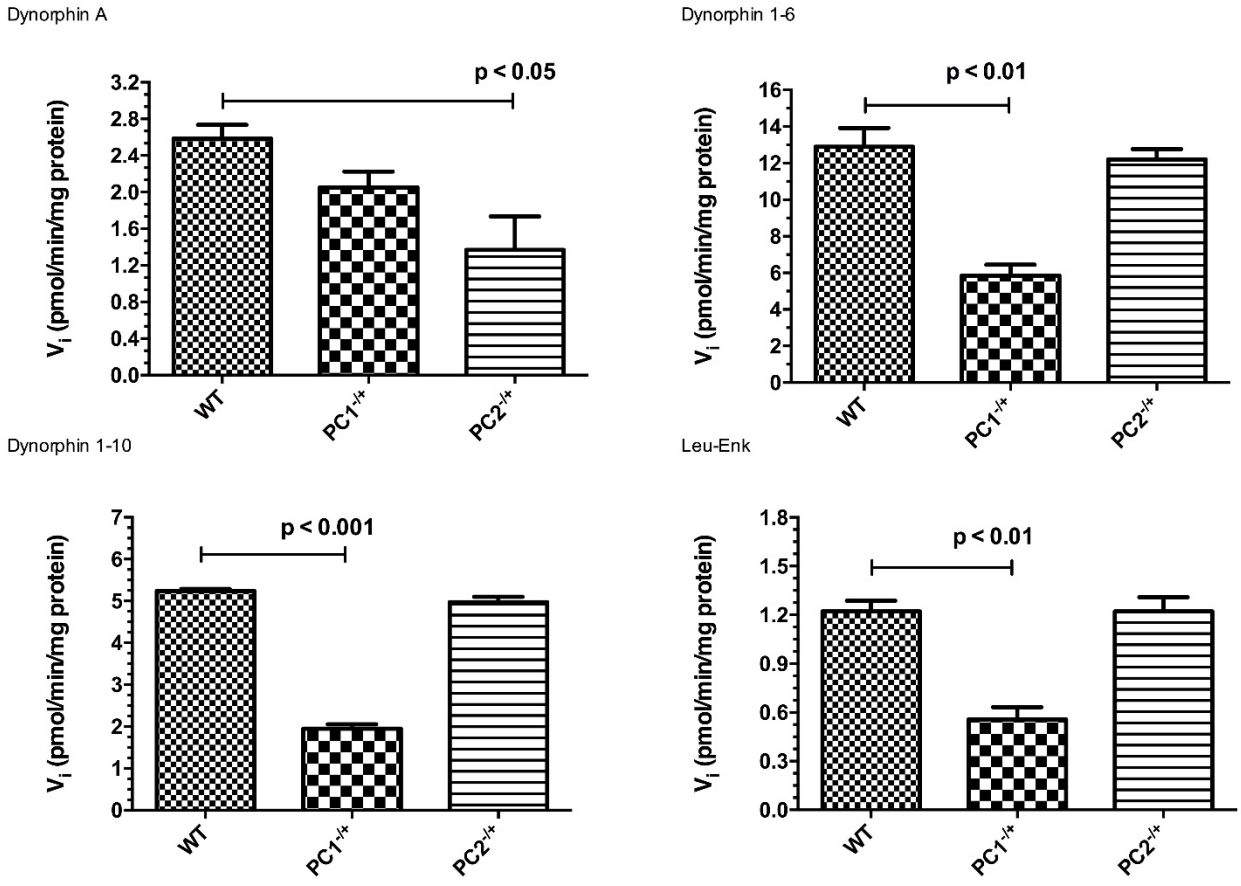
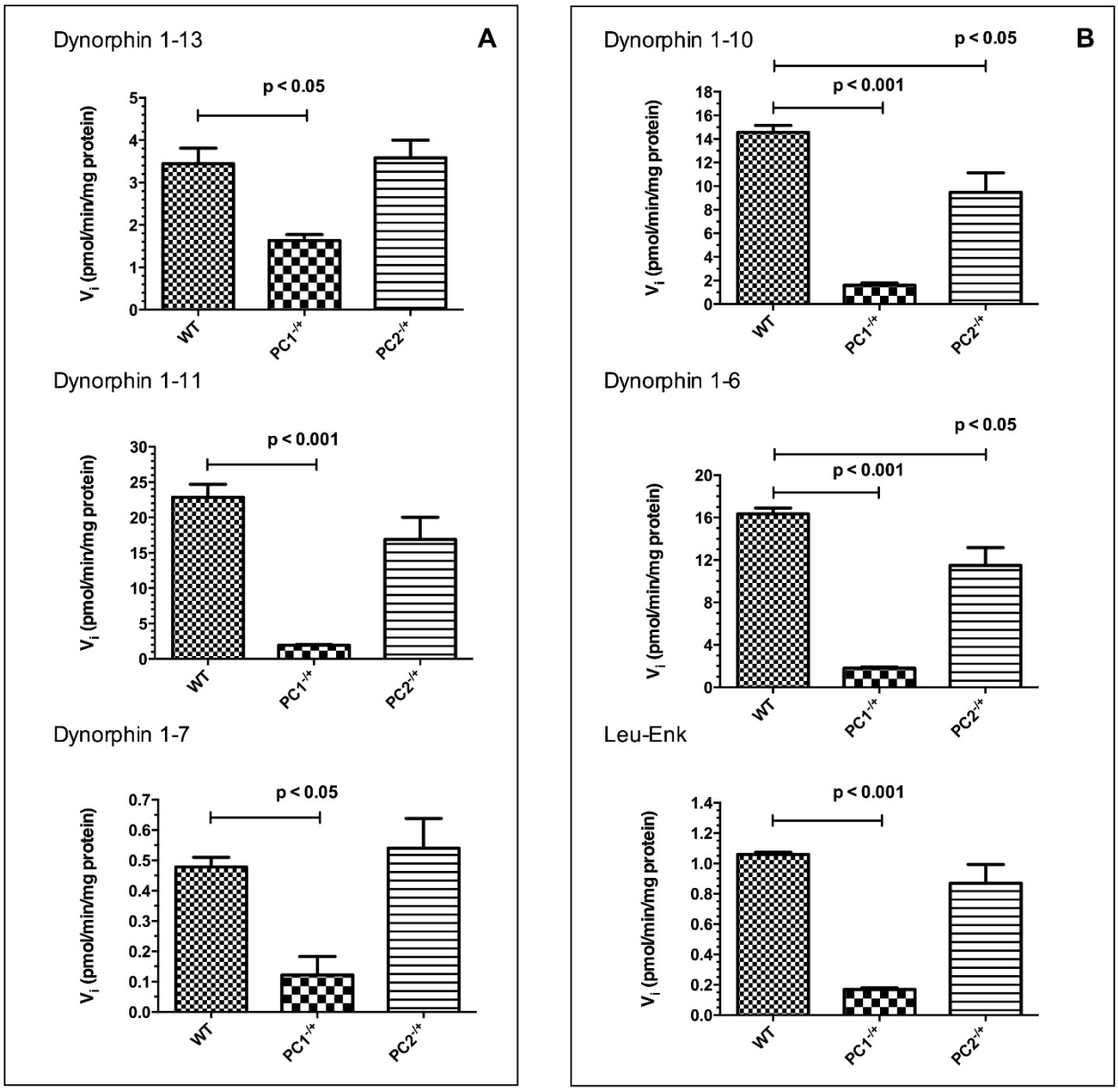

Dynorphin A
p < 0.05
V_i (pmol/min/mg protein)
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
WT
PC1-/+
PC2-/+
Dynorphin 1-6
p < 0.01
V_i (pmol/min/mg protein)
16
14
12
10
8
6
4
2
0
WT
PC1-/+
PC2-/+
Dynorphin 1-10
p < 0.001
V_i (pmol/min/mg protein)
7
6
5
4
3
2
1
0
WT
PC1-/+
PC2-/+
Leu-Enk
p < 0.01
V_i (pmol/min/mg protein)
1.8
1.5
1.2
0.9
0.6
0.3
0.0
WT
PC1-/+
PC2-/+

Figure 7.


A
Dynorphin 1-13
p < 0.05
Vi (pmol/min/mg protein)
WT
PC1-/+
PC2-/+
Dynorphin 1-11
p < 0.001
Dynorphin 1-7
p < 0.05
B
Dynorphin 1-10
p < 0.05
p < 0.001
Dynorphin 1-6
p < 0.05
p < 0.001
Leu-Enk
p < 0.001

GENERAL DISCUSS

Determining complex molecular mechanisms involved in pain modulation is necessary for the understanding of the causes and the evolution of neuropathic and chronic pains. The endogenous opioid system might be a target through which new pain treatments could be designed. Current analgesic treatments induce analgesia by just masking the causes that generate pain. These treatments are principally based on the administration of opioid-like drugs which act on opioid receptors leading to a rapid relief of pain. Even though their effects are potent and effective, lengthy treatments have shown to cause several side-effects (Gallagher and Rosenthal, 2008; Sindrup and Jensen, 1999). These side-effects include the formation of undesirable metabolic products and CNS plasticity problems that might change the normal stimulus-response characteristics (Woolf and Salter, 2000). Targeting events upstream of the endogenous opioid system and its regulation, could lead to a novel strategy through which other antinociceptive strategies could be designed.

Dynorphins are an important family of endogenous opioid peptides, which play an essential role in pain modulation as well as in the progression of various diseases such as inflammation due to pathogenic infections (Seidah and Prat, 2012). Nevertheless, the metabolic pathway and the physiological role of dynorphins are still unclear. Dyn A is known to be one of the most abundant dynorphin found in the mammalian CNS, and it induces potent analgesic effects at spinal levels (Kuner, 2010; Mika et al., 2011). The metabolic stability of endogenous dynorphin peptides is an important factor to take into consideration since it regulates the duration of dynorphins-induced analgesia. Thus, if high levels of endogenous Dyn A are maintained by prolonging its metabolic stability, it could lead to long-lasting and effective antinociception.

Endogenous levels of dynorphins are regulated by the action of PCs, which are involved in the endoproteolytic processing of the dynorphins precursor, PDyn (Berman et al., 2000; Day et al., 1998). Early studies suggest that the action of PC2 is needed for the formation of BDyn, Dyn A and Dyn B (Day et al., 1998), even though no studies on the role of PCs on BDyn and Dyn A metabolism have been reported. As a consequence, the metabolic stability and the potential metabolic products of BDyn and Dyn A still remains unclear.

1-Summary of results

Experiments reported here were focused on the evaluation of the metabolic stability of BDyn and Dyn A in mouse spinal cord S9 fractions. The results show that the degradation of full-length peptides occurred only with the presence of S9 proteins, demonstrating that BDyn and Dyn A undergoes proteolytic processing when incubated in mouse spinal cord S9 fractions. The designed HPLC-MS method allowed for the selective identification of several metabolites resulting from the enzymatic degradation of both precursors. In addition, by using isotope dilution as a quantification method, it was possible to quantify the metabolites and evaluate their relative rate of formation. Moreover, HRAM MS analyses were used to provide a second verification and to assess peptides identity with a mass accuracy within 2 ppm.

From the *in vitro* digestion of BDyn and Dyn A precursors, several dynorphin peptides were identified as products from C-terminal processing at basic positions of both precursors. The enzymatic degradation of BDyn lead to the generation of dynorphin peptides such as Dyn 1-19, Dyn 1-13, Dyn 1-11. The identification of their complementary fragment peptides, including Dyn B, Dyn 12-32, Dyn 14-32 supported those results and suggested that they are most likely primary cleavage products from BDyn. On the other hand, Dyn A processing was observed to generate Dyn 1-13, Dyn 1-11 and Dyn 1-7. Their respective complement fragments Dyn 14-17, Dyn 12-17 and Dyn 8-17 were also observed. BDyn and Dyn A processing pattern were compatible with PC1 and PC2 processing, showing a crucial processing role of these enzymes. Other dynorphin peptides, including Dyn A, Dyn 1-10, Dyn 1-6 and Leu-Enk were also identified. These peptides are considered as secondary metabolites and their formation is associated with the removal of C-terminal basic residues by CPE.

To elucidate the role of PC1 and PC2 on BDyn and Dyn A processing, cellular S9 fractions were isolated from WT, $PC1^{(-/+)}$ and $PC2^{(-/+)}$ mice and used to perform the *in vitro* digestion of the full-length precursor peptides. In order to assess differences on the expression of PC1 and PC2 between the 3 genotypes, the relative abundance of PC1 and PC2 were quantified in the three cellular S9 fractions. The quantification was performed following

a bottom-up proteomic strategy and using high-resolution MS. The concentration of both enzymes on the S9 fractions isolated from the mutant mice were expressed as a relative concentration of the enzyme compared with the WT mice. The results revealed that the expression of PC1 and PC2 were significantly down-regulated, around 40% in the $PC1^{-/+}$ and around 50% in the $PC2^{-/+}$ S9 fractions respectively.

The *In vitro* digestion of both precursors were then performed and the data obtained was statistically compared between the three genotypes. The presence of different PCs target cleavage points on BDyn sequence, including dibasic KR and RR and monobasic K and R positions, presents an opportunity to study differences on cleavage affinity of both enzymes for basic positions. Results generated from BDyn digestion clearly revealed an important contribution of both, PC1 and PC2, in precursors processing at KR dibasic residues. Interestingly, both enzymes show a similar affinity for this cleavage point but we can't exclude that PC1 could compensate for the reduction of PC2 activity in cleaving dibasic residues when the former is absent and *vice versa*. On the contrary, cleavage at the dibasic RR position was not observed for PC2, and PC1 as shown by a non-significant affinity for this position. However processing at monobasic positions, K and R were shown to be completely regulated by PC1. On the other hand, those results were corroborated by the data obtained from the incubation of Dyn A with the S9 fractions. Dyn A presents one dibasic position of cleavage, RR, as well as K and R monobasic amino acids, all of them possible targets for PC1 and PC2 cleavage. Surprisingly, the data obtained from Dyn A in *in vitro* digestion show that Dyn A degradation was completely governed by PC1 and that PC2 did not play a role in its processing. PC1 was observed to efficiently cleave at RR, K and R positions, being the enzyme responsible of the regulation of Dyn A. Moreover, the data suggest that PC2 did not compensate for the reduction of PC1 activity in cleaving these specific sites.

2-Relevance of the observations

PC1 and PC2 have been proposed to be involved in PDyn processing, leading to the generation of high molecular weight dynorphin peptides including BDyn. The generation of the peptides is the result of the proteolytic processing of dynorphins by PC1 and PC2,

cleaving mainly at dibasic KR amino acids, but also at R monobasic position. These early studies suggested that the action of PC2 is needed for the formation of most of the high molecular weight dynorphin peptides. PC2 is thus involved in long peptide processing at the monobasic and dibasic positions (Day et al., 1998; Berman et al., 2000). On the other hand, PC1 has been reported to be implicated in the processing of PDyn into high molecular weight peptides, but its contribution is significantly lower than PC2. The main cleavage sites for PC1 is at the KR paired amino acids (Berman et al., 2000). These results lead to the hypothesis that PC1 and PC2 might have a similar role in the processing of low molecular weight peptides such as BDyn and Dyn A.

According to the proposed hypothesis in the present study, the results clearly show that PC1 and PC2 are also involved in the processing of BDyn and Dyn A. Furthermore, the KR paired amino acids found on BDyn sequence, were observed to be a targeted point of cleavage either by PC1 or PC2. Contrary to the data presented in previous studies, the affinity of both enzymes on KR processing sites were observed to be similar for both enzymes. Moreover, BDyn and Dyn A monobasic processing was observed to differ from previous results reported. Results shown here indicate that monobasic processing of BDyn and Dyn A was completely regulated by PC1 both at K and R positions.

Taking earlier observations and the present results together, PC2 seems to be more active in cleaving large peptides, while PC1 appears to be more involved at processing smaller peptides. Affinity of enzymes for their respective substrates are mostly regulated by binding affinity. The formation of the enzyme-substrate complex is governed by the thermodynamic stability of the complex. Thus, steric hindrance is an important factor in the impairment of the cleavage process. PC1 is a protein consisting of 753 amino acids, whereas PC2 consist of 638 amino acids (Saidi et al., 2015). This suggests that the steric hindrance of PC1 cleaving large proteins might be greater than for PC2. This factor could be one reason that explains the different role and affinity of PC1 and PC2 depending on the size of the protein subject to cleavage.

To confirm one of the previous hypotheses, it is important to point out the formation of Leu-Enk, a metabolic product resulting from the *in vitro* digestion of BDyn or Dyn A.

This observation demonstrates that Leu-Enk might be a metabolic product from PDyn in addition to PEnk.

3-Implication of the results

Endogenous antinociceptive system is governed by the levels of active opioid peptides. Dynorphins, enkephalins and endomorphins are some of those endogenous peptides which participate in the endogenous modulation of pain. Specifically, Dyn A is one of the major dynorphins found in mammalian CNS (Civelli et al., 1985; Dores et al., 1985; Tan-No et al., 1997). Dyn A is an endogenous ligand of KOR and MOR producing analgesic effects and significantly reducing neuronal activity (Mizoguchi et al., 2006). Other dynorphins such as Dyn 1-13, Dyn 1-11 or Dyn 1-6 have also been reported to mediate analgesia when they are directly injected into rats, even though the duration of their activity seems to be short-lasting (Goldstein et al., 1979; Takemori et al., 1992; Reed et al., 2003). This observation might be caused, in addition to the differences in their affinities and kinetics for binding KOR, by a lower stability of short peptides compared to larger ones. The smaller the peptide, the shorter is its stability. The presence of degrading enzymes in neuronal cells make active opioid peptides to tend to degrade rapidly (Mosnaim et al., 2008).

The high abundance of Dyn A might be due to its high metabolic stability. That means that even though the potency of other dynorphins might be higher than Dyn A, their absolute effect on producing analgesia could be more significant. The above observation shows that Dyn A is an important dynorphin peptide regarding its activity and stability.

The observations extracted from the present experiments clearly show that PC1 regulates the metabolism of Dyn A. Therefore, controlling the activity of PC1 can result in prolonged stability of Dyn A resulting in a more active modulation of pain through endogenous mechanisms. Also, PC1 is also involved in the endoproteolysis of protackykinins (Saidi et al., 2015) and partial inhibition can lead to a significant reduction of SP levels in the synaptic gap leading to a reduction of SP-NK1 interactions impeding the pronociceptive effect of SP. However, it will be useful to perform an exhaustive phenotyping study on $PC1^{-/+}$ mice using validated pain models in order to elucidate if a reduction of PC1 activity is

sufficient to significantly reduce pain behavior. It is important to note that completely alleviate pain is undesired since it may further aggravate injuries.

4-Limitations

PC1 and PC2 participate in the synthesis of several endogenous opioid peptides from its respective precursors such as Enkephalins (Breslin et al., 1993; Johanning et al., 1996 and 1998) and endorphins (Zhu et al., 2002; Miller et al., 2003). Those studies also converge on the fact that PC2 appears to be more capable of generating active opioid units from PEnk and POMC than is PC1. This premise supports the concept that even if the activity of PC1 is partially inhibited, the action of PC2 might lead to an efficient formation of active opioid peptides such as enkephalins and endorphins, in addition to favoring the stability of certain opioids such as Dyn A.

Even if PC1 were to be inhibited in order to prolong the activity of Dyn A, its inhibition could result in the lack of processing of protachykinin-1, the neuroprecursor that encodes SP. SP is a neurotransmitter that plays a central role in nociceptive transmission in the CNS. PC1 and PC2 have been observed to be equally involved in protachykinin-1 processing (Saidi et al., 2015). This fact suggests that a partial inhibition of PC1 or PC2 would lead to a reduction in the amount of active SP. As a consequence of the reduction in the release of SP, the endogenous opioid system could not be activated.

CONCLUSIONS

The *in vitro* digestion experiments performed on the present study using cellular S9 fractions, demonstrate that PC1 and PC2 are crucial for the endoproteolytic processing of BDyn and Dyn A. Moreover, the results generated from WT, $PC1^{(-/+)}$ and $PC2^{(-/+)}$ mice have shown that PC2 is involved in the processing of BDyn through the cleavage at dibasic KR positions leading to the generation of Dyn 1-19. However, PC2 cleavage at monobasic positions was not observed. On the other hand, PC1 was able to cleave at KR dibasic positions with a similar efficiency as PC2. Interestingly, PC1 was also observed to cleave at RR dibasic positions and at monobasic R and K amino acids. Thus, PC1 can lead to the formation of Dyn 1-19, Dyn 1-13, Dyn 1-11, Dyn 1-7 and Dyn 1-6. In addition, other dynorphin peptides were also identified from the processing of BDyn and Dyn A, including Dyn A from BDyn, and Dyn 1-10 and Leu-Enk from either BDyn or Dyn A. These peptides are most probably metabolic products from the removal of terminal basic amino acids by CPE from Dyn 1-19, Dyn 1-11 and Dyn 1-6 respectively.

Our results establish that PC1 is involved in the proteolytic control of Dyn A, since its processing is exclusively regulated by cleavage at mono basic residues (K or R). Contrary, PC2 was not observed to participate on the metabolism of Dyn A, but its activity is important for the formation of Dyn 1-19 from BDyn, which leads to the generation of Dyn A through the subsequent action of CPE.

In addition, the present study also establish, for the first time, that Leu-Enk is a significant metabolic product of BDyn and Dyn A, showing another metabolic route of formation in addition to PEnk.

Although further studies representing physiological conditions will be needed to substantiate the role of PC1 and PC2 on the processing of further proneuropeptides and prohormones, these findings suggest that a partial inhibition of the activity of PC1 might favor the maintenance of high endogenous levels of active opioid peptides such as Dyn A. Thus, the results presented could represent the first step towards an important advancement on the design of new analgesic drugs.

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