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OPIOID AND NON-OPIOID ACTIVITIES OF THE DYNORPHINS

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ABSTRACT

Endogenous opioid peptides α-neoendorphin, dynorphin A (Dyn A), dynorphin B (Dyn B) and big dynorphin (Big Dyn) consisting of Dyn A and Dyn B, collectively known as dynorphins are derived from the precursor protein prodynorphin (PDYN). Dynorphins regulate pain processing and memory acquisition and modulate reward induced by intake of addictive substances. Such actions are generally mediated through the κ-opioid receptors. However, excitotoxic effects of Dyn A and Big Dyn relevant for neuropathic pain are non-opioid; the mechanisms of these effects are unknown but glutamate receptors are apparently involved. *PDYN* mRNA and dynorphin peptides have been extensively characterized in the rat and human brain, whereas little is known about PDYN, its biochemical properties, and localization and processing in the brain.

The general aim of the present study was (1) to characterize mechanisms of non-opioid interactions of dynorphins with cells, (2) to identify non-opioid biochemical targets for these peptides, (3) to characterize biochemical properties, intracellular localization and distribution in the brain of the precursor protein PDYN, and (4) to assess role of dynorphins in human neurodegenerative disorders including Alzheimer disease.

Our analysis revealed similarity of dynorphins with cell-penetrating peptides (CPPs) capable of translocating into cells. The ability of dynorphins to translocate across the plasma membrane was tested using immunofluorescence, confocal fluorescence microscopy, and fluorescence correlation spectroscopy on fixed and live cells. Big Dyn and Dyn A but not Dyn B were found to be capable to penetrate into cells. Big Dyn showed higher translocation potential compared with that of Dyn A, while Dyn A and transportan-10, a prototypical CPP translocated into cells with similar efficacy. The translocated dynorphins were predominantly located in the cytoplasm where they were associated with the endoplasmic reticulum. Fluorescence and circular dichroism spectroscopy imply that two structural features, the ability to form α-helix and high positive charge, may in conjunction determine the membrane translocation potential of Big Dyn and Dyn A. Translocation of dynorphins into cells followed by interactions with intracellular targets might represent an evolutionary ancient mechanism of intercellular communications and signal transduction (Paper I).

For identification of intracellular dynorphin targets - non-opioid binding sites in neuronal cells and brain, radioligand-binding assay was used. A novel soluble factor that binds Dyn A and Big Dyn with high specificity and affinity (IC_{50} 5-10 nM) was found. Binding of Dyn A to the factor was virtually irreversible and resulted in conversion of Dyn A into Leu-enkephalin, suggesting that the Dyn A-binding factor (DABF) functions as an oligopeptidase. This enzyme may potentially degrade other neuropeptides. Dynorphins may regulate this degradation acting as competitors when they bind to the enzyme (Paper II).

Dynorphins generation may be regulated at the levels of gene transcription, translation, protein trafficking and processing. As the first step in the analysis of these processes, the biochemical properties of PDYN, its intracellular localization and distribution in the rat and human brain were characterized. The focus was on structures where PDYN is synthesized and where mature dynorphins are located. PDYN distribution pattern in rat brain determined by immunohistochemistry and western blot was similar to that of dynorphin peptides with highest levels in the amygdala, hippocampus and striatum and lower levels in the cerebral cortex. PDYN in unprocessed form was also present in the ventral tegmental area (VTA) and the hippocampal CA3 region that do not have cell bodies of neurons producing PDYN but contain axons and axon terminals originating from PDYN-ergic neurons. Thus PDYN is transported to and stored in axon terminals. PDYN in axon terminals may be processed to mature peptides prior to their release from cells. This notion is supported by the observation that K^+-evoked depolarization of PDYN producing cells increases the total amounts of dynorphins in cells and medium demonstrating that PDYN processing is activated. Stimulation of PDYN processing in axon terminals and dendrites by neuronal activity and extracellular signals may represent a mechanism for the local regulation of synaptic transmission (Paper III).

Dyn A through non-opioid glutamate receptor-mediated mechanism may induce excitotoxicity resulting in neuronal death (related paper 1). Neuronal death in Alzheimer disease (AD) and other neurodegenerative diseases may be associated with upregulation of Dyn A, which may potentially contribute to neurodegeneration. To assess the relationship between neurodegeneration and dynorphins, the status of the dynorphin system was evaluated in AD. The levels of Dyn A and Dyn B and the related neuropeptide nociceptin in the inferior parietal lobule (Brodmann area 7) of control, AD, Parkinson disease (PD) and cerebro-vascular disease (CVD) groups were determined. AD subjects displayed robustly elevated levels of Dyn A, whereas the levels of Dyn B and nociceptin were unaltered. Subjects with PD and CVD showed no changes in all three peptides. The levels of PDYN and the activity of DABF did not change in AD, whereas the levels of the processing enzyme pro-hormone convertase 2 (PC2), which processes PDYN, were elevated. The levels of Dyn A correlated with the density of neuritic plaques. These results suggest a non-opioid role of Dyn A in the pathogenesis of AD (Paper IV).

LIST OF PUBLICATIONS

1.1 PAPERS INCLUDED IN THE THESIS

I. Marinova Z*, Vukojevic V*, Surcheva S, Yakovleva T, Cebers G, Pasikova N, Usynin I, Hugonin L, Fang W, Hallberg M, Hirschberg D, Bergman T, Langel U, Hauser KF, Pramanik A, Aldrich JV, Graslund A, Terenius L, Bakalkin G[#]. Translocation of dynorphin neuropeptides across the plasma membrane: A putative mechanism of signal transmission.
The Journal of Biological Chemistry, 280:26360-26370, 2005

II. Marinova Z*, Yakovleva T*, Melzig MF, Hallberg M, Nylander I, Ray K, Rodgers DW, Hauser KF, Ekstrom TJ, Bakalkin G[#]. A novel soluble protein factor with non-opioid dynorphin A-binding activity.
Biochemical and Biophysical Research Communications, 321:202-209, 2004

III. Yakovleva T, Cebers G, Marinova Z, Vlaskovska M, Pasikova N, Usynin I, Nikoshkov A, Colago EE, Gileva I, Matskevitch A, Johansson B, Hochgeschender U, Singh IN, Hauser KF, Ekström TJ, Terenius L, Hurd YL, Pickel VM, Bakalkin G[#]. Prodynorphin is located in axon terminals and dendrites where processing to dynorphins is regulated by neuronal activity.
Manuscript

IV. Yakovleva T, Marinova Z[#], Kuzmin A, Seidah NG, Haroutunian V, Terenius L, Bakalkin G. Dysregulation of dynorphins in Alzheimer disease.
Submitted

The papers will be referred to by their Roman numbers throughout the thesis.

[#] corresponding author

* the first two authors contributed equally to this study

1.2 RELATED PAPERS

1. Singh IN, Goody RJ, Goebel SM, Martin KM, Knapp PE, Marinova Z, Hirschberg D, Yakovleva T, Bergman T, Bakalkin G, Hauser KF. Dynorphin A (1-17) induces apoptosis in striatal neurons in vitro through alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate/kainate receptor-mediated cytochrome c release and caspase-3 activation.
   Neuroscience 122:1013-1023, 2003

2. Tan-No K, Takahashi H, Nakagawasai O, Niijima F, Sato T, Satoh S, Sakurada S, Marinova Z, Yakovleva T, Bakalkin G, Terenius L, Tadano T. Pronociceptive role of dynorphins in uninjured animals: N-ethylmaleimide-induced nociceptive behavior mediated through inhibition of dynorphin degradation.
   Pain 113:301-309, 2005

3. Nikoshkov A, Hurd YL, Yakovleva T, Bazov I, Marinova Z, Cebers G, Pasikova N, Gharibyan A, Terenius L, Bakalkin G. Prodynorphin transcripts and proteins differentially expressed and regulated in the adult human brain.
   The FASEB Journal, 19:1543-1545, 2005

LIST OF ABBREVIATIONS

Aβ	beta-amyloid
AD	Alzheimer disease
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APP	amyloid precursor protein
Big Dyn	big dynorphin
cAMP	cyclic adenosine monophosphate
CPP	cell penetrating peptide
CREB	cAMP response element binding protein
CSF	cerebrospinal fluid
CTF	C-terminal fragment
CVD	cerebro-vascular disease
DABF	dynorphin A - binding factor
DCE	dynorphin-converting enzymes
DTT	dithiothreitol
Dyn A	dynorphin A
Dyn B	dynorphin B
FCS	fluorescence correlation spectroscopy
kDa	kilodalton
NMDA	N-methyl-D-aspartate
NP-40	Nonidet P-40
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PC	pro-hormone convertase
PD	Parkinson disease
PDYN	prodynorphin
POMC	proopiomelanocortin
Rh	rhodamine
RIA	radioimmunoassay
SDS	sodium dodecyl-sulfate
TAT	transactivator of transcription
TOP	thimet oligopeptidase
VTA	ventral tegmental area

CONTENTS

1 INTRODUCTION

1.1 NEUROPEPTIDES

Neurotransmitters are a group of substances released upon excitation from the axon terminal of a presynaptic neuron of the central or peripheral nervous system and travel across the synaptic cleft to either excite or inhibit the target cell. Several chemically distinct families of neurotransmitters have been described, including amino acids and their metabolites (such as excitatory glutamate and aspartate, and inhibitory gamma-aminobutyric acid and glycine), biogenic monoamines (including, acetylcholine, dopamine, norepinephrine and serotonin), neuropeptides, and "gaseous" molecules.

The neuropeptides are 3-50 amino-acid residues long and up to 50 times larger in molecular weight than classic neurotransmitters. They are widely expressed in neurons of the central and peripheral nervous system and are also present in various types of glial cells. Neuropeptides coexist with one or more classic neurotransmitters suggesting that they are involved in modulatory processes. Besides being involved in normal physiological processes, some neuropeptides may play a role when the nervous system is stressed, challenged or afflicted by disease. Peptidergic systems may, therefore, be important targets for novel therapeutic strategies (Hökfelt et al., 2003).

Some neuropeptides are neurohormones that cause release of other hormones, such as corticotropin-releasing factor and growth hormone-releasing hormone. There are pituitary peptides such as adrenocorticotropic hormone, prolactin and growth hormone. A number of peptides were originally discovered in the gut, but also have a variety of actions in the brain, such as cholecystokinin and vasoactive intestinal peptide. The endogenous opioids are also an important class of peptide neurotransmitters.

A major difference between classical transmitters and peptides is the mode of synthesis and replacement after release. It has been assumed that peptides can only be produced ribosomally, synthesized de novo in the cell bodies, while classic transmitters can also be synthesized locally in the nerve endings. Bioactive neuropeptides are parts of larger molecule precursors, which, after synthesis in the endoplasmic reticulum, are transported to the Golgi apparatus for packaging, followed by centrifugal transport and exocytotic release. In neurons, processing of neuropeptides precursors is thought to take place during transport from the perikaryon to nerve terminals. The precursor

proteins are transported and stored in the so called large dense core vesicles or secretory granules together with processing enzymes, also called convertases, which cut out the bioactive peptides. The majority of endoproteolytic cleavages occur in the secretory granules, mainly at pairs of basic amino acid residues. In addition, important post-translational enzymatic modifications of the final peptide may occur, such as glycosylation, C-terminal amidation, acetylation, phosphorylation and sulfation. Classic transmitters often have a membrane reuptake mechanism through specific transporter molecules, which allows reutilization of the transmitter. The neuropeptides are apparently degraded in the extracellular space by peptidases and replacement after release has to occur via axonal transport from the cell bodies to the nerve endings (Hökfelt et al., 2000).

1.2 OPIOID PEPTIDES

Opioid peptides are endogenous ligands for the opioid receptors. They are defined as such, because their activity can be blocked by the opioid antagonist naloxone. However, several opioid peptides produce also non-opioid actions.

The opioid receptors were identified simultaneously by three groups in 1973 as the binding sites of opiates (Terenius, 1973; Pert and Snyder, 1973; Simon et al., 1973). The prototypical opiate is morphine, an alkaloid derived from the poppy plant. Historically, opiates have been among the most important agents in medicine due to their ability to relieve pain and reduce suffering. No one has succeeded in finding other pain-relieving agents nearly as powerful. At the same time they have high addictive potential bringing great toll on society.

After the opioid receptors were discovered, the question appeared: “Man was not born with morphine in him: might there be endogenous opiate-like neurotransmitters?” In 1975 Hughes et al. reported the isolation and amino-acid sequence of the two endogenous enkephalin opioid pentapeptides, Met-enkephalin and Leu-enkephalin (Hughes et al., 1975). This was followed by the isolation and characterization of β-endorphin (Bradbury et al., 1976) and dynorphin A 1-13 (Dyn A 1-13) (Goldstein et al, 1979). The generic term “endorphin” was used to refer to any opioid peptide.

There are three major classes (Table 1) of endogenous opioid peptides, rising from three different precursors. They bind preferentially to different classes of opioid receptors and all contain at least one copy of the opioid core peptide sequence Tyr-Gly-Gly-Phe (Met, Leu) (Dores et al., 1984; Khachaturian et al., 1985).

Table1. Endogenous opioid peptides

Precursor	Typical peptide	Preferred receptor
Proopiomelanocortin	β-endorphin	μ + δ
Proenkephalin	Leu-enkephalin	δ + μ
	Met-enkephalin	
Prodynorphin	Dynorphin A	κ
	Dynorphin B	
	α-neoendorphin	

β-endorphin, ligand for the μ-opioid receptor, is derived from the precursor protein proopiomelanocortin (POMC). POMC was found to be pluripotent and besides β-endorphin/β-lipoprotein contains also the non-opioid adrenocortictropic hormone and melanocyte-stimulating hormone. End-products of the POMC system are synthesized in the pituitary, the arcuate nucleus and the nucleus tractus solitarius.

Enkephalins, ligands for the δ-opioid receptors, are derived from the precursor protein proenkephalin. Proenkephalin contains seven enkephalin sequences. These include one copy of Leu-enkephalin, four copies of Met-enkephalin, and one copy each of Met-enkephalin-Arg-Phe and Met-enkephalin-Arg-Gly-Leu. End products of the proenkephalin precursor are found in the adrenal medulla and are distributed widely in the central nervous system.

The precursor protein prodynorphin (PDYN) gives rise to Dyn A 1-17, dynorphin B (Dyn B), and α-neoendorphin. The mature peptides obtained from PDYN are ligands for the κ-opioid receptors. End products of the PDYN system have also wide distribution in the brain.

Enkephalins and β-endorphin through actions on the μ-opioid receptors induce potent analgesia and euphorigenic activity similar to the actions of opiates. Dynorphins seem to act as neuromodulators at different levels of the central nervous system (Terenius, 2000).

In addition to the three classical groups of endogenous opioid peptides, several related peptides were described later. Endomorphins are a group of peptides with a characteristic atypical structure and high affinity for the μ-opioid receptor (Zadina et al., 1997). Endomorphin-1 is present mainly in the brain and endomorphin-2 is present mainly in the spinal cord. Little is known about their synthesis.

Nociceptin/orphanin FQ is a related peptide that is derived from the pronociceptin protein and acts on separate NOP-receptors. The NOP-receptor was initially identified by homology screening and named ORL1 (orphan receptor L-1) since no natural ligand was known at that time. A few years later, however, a ligand was found and named nociceptin/orphanin FQ (Meunier et al., 1995; Reinscheid et al., 1995). Nociceptin shares a partial sequence homology with dynorphins and particularly with Dyn A. At the behavioral level, systematic application of nociceptin elicits a wide range of effects on pain processing such as hyperalgesia, analgesia, and allodynia, as well as anxiolytic actions, modulation of opioid-mediated processes, and influence on learning and memory (Meis, 2003).

1.3 DYNORPHIN PEPTIDES – DISTRIBUTION IN THE BRAIN, SYNTHESIS AND PROCESSING

Dyn A 1-17, Dyn B and α-neoendorphin, collectively known as dynorphins, are cleaved from the precursor protein PDYN. These peptides may be metabolized into shorter forms: Leu-enkephalin-Arg^6, Leu-enkephalin, Dyn A 1-13, Dyn A 1-8, Dyn A 2-17 that demonstrate different receptor profile with preferential binding to δ-opioid receptor (Leu-enkephalin-Arg^6, Leu-enkephalin), κ-opioid receptor (Dyn A 1-13, Dyn A 1-8), or non-opioid activity (Dyn A 2-17). Besides mature dynorphins, higher molecular weight intermediates of PDYN processing are present in the pituitary and the brain. One of them is "big dynorphin" (Big Dyn) – 32 amino acid dynorphin peptide consisting of Dyn A and Dyn B sequences bridged with KR amino acids (Fischli et al., 1982). Big Dyn may serve as an intermediate in the processing to mature dynorphins and/or may have its own function.

As mentioned earlier, dynorphin opioid peptides and their cognate receptors, the κ-opioid receptors, exhibit widespread distribution throughout the mammalian brain. Immunoreactive dynorphin perikarya are found in several cerebral cortical areas, striatum, amygdala, hippocampus, several hypothalamic nuclei, midbrain periaqueductal gray, brainstem areas, in the spinal cord dorsal horn, and in the pituitary anterior lobe. Additionally, fiber immunoreactivity is seen in many other areas of the brain. The anatomical distribution of dynorphins provides some insight into their functional role. The peptides are involved in pain regulation, modulation of reward induced by addictive substances, motor control, memory acquisition and learning and stress-induced analgesia.

The dynorphin system (Figure 1) has been studied extensively at the levels of *PDYN* mRNA and the mature dynorphin peptides but little is known about the precursor protein PDYN. Altered *PDYN* mRNA levels have been observed in the brain of animals treated with cocaine (Turchan et al., 1998), of drug abusers and psychiatric patients (Hurd and Herkenham, 1993; Hurd, 2002) and allelic variations in the *PDYN* promoter have been associated with cocaine abuse and epilepsy (Chen et al., 2002; Stogmann et al., 2002). Altered levels of dynorphins have also been observed in several psychopathological situations (Heikkila et al., 1990; Risser et al., 1996). Upregulation of *PDYN* mRNA and mature dynorphins has been detected in experimental animals in conditions associated with neuropathic pain (Faden et al., 1985; Przewlocki et al., 1988).

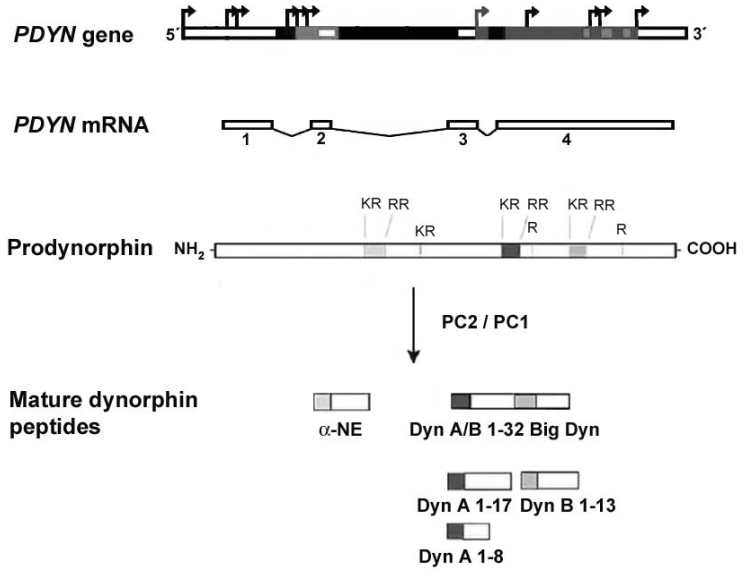

Figure 1. *Synthesis and processing of PDYN*

It is important to study the dynorphin system at the level of the precursor protein; changes in post-translational modifications and post-translational processing of the precursor protein as well as the timing of the enzymatic cleavage of PDYN (before or after packaging in vesicles) would influence the production and effects of mature dynorphin peptides both under normal circumstances and in pathological conditions.

Therefore, one of the tasks of this thesis was to develop the tools and study the precursor protein PDYN in terms of its distribution in the brain, the form in which it is present in it, and the dependence of its processing and secretion on neuronal activity.

Two major enzymes involved in the processing of PDYN to mature dynorphin peptides are the pro-hormone convertases PC2 and PC1/3. PC1/3 cleaves PDYN at both single and paired basic residues to yield processing intermediates of 8 or 10 kilodaltons (kDa) (Dupuy et al., 1994), while PC2 cleaves PDYN to produce Dyn A 1-17, Dyn B 1-13 and α-neoendorphin, without a previous requirement for PC1/PC3 (Day et al, 1998).

1.4 DYNORPHIN ACTIONS MEDIATED THROUGH THE κ-OPIOID RECEPTORS

Dynorphins are endogenous ligands for the kappa opioid receptors. At the same time several dynorphins have also actions that cannot be blocked by the opiate receptor antagonist naloxone and are therefore non-opioid (Figure 2).

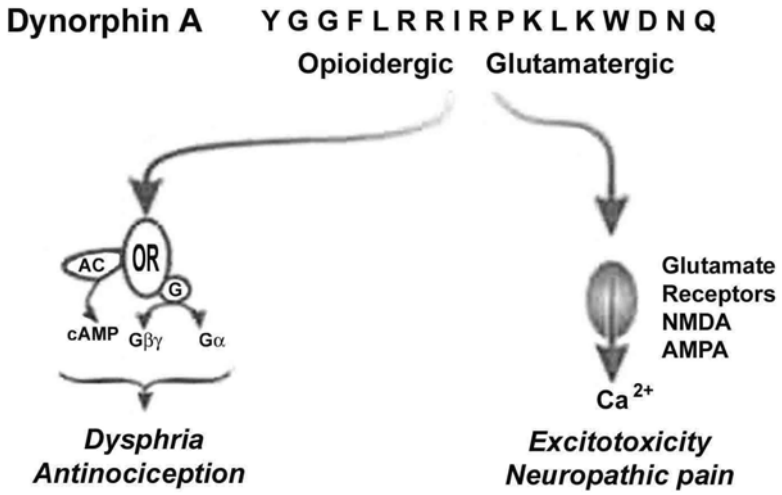

Figure 2. *Opioid and non-opioid activities of Dyn A*

The kappa opioid receptors belong to the superfamily of G-protein-coupled receptors. The kappa opioid receptor has been cloned and found to be coupled to inhibition of adenylate cyclase and to subsequent decrease in cyclic adenosine monophosphate (cAMP) levels (Zhu et al., 1995; Mansson et al., 1994; Attali et al., 1989). Furthermore, activation of kappa opioid receptors leads to an increase in inward rectifying K^+ conductance and decrease in Ca^{2+} conductance through N-type channels.

Activation of the kappa opioid receptors produces many effects including dysphoria and psychotomimetic effects, antinociception, inhibition of locomotion,

modulation of memory formation and neuroendocrine responses, and water diuresis (see Costigan and Woolf, 2002; de Vries and Shippenberg, 2002; Kreek, 2001; Solbrig and Koob, 2004).

1.4.1 Modulation of reward induced by addictive substances

Drug addiction is a chronic, relapsing disorder, in which compulsive drug-seeking and drug-taking behavior persists despite serious negative consequences. Generally, addictive substances can act as positive reinforcers (producing euphoria) or as negative reinforcers (alleviating symptoms of withdrawal or dysphoria). Continued use induces adaptive changes in the central nervous system that lead to tolerance, physical dependence, sensitization, craving, and relapse (Cami and Farre, 2003).

Different drugs of abuse bind to separate molecular targets (for example morphine to opioid receptors, cocaine to dopamine and norepinephrine transporters). Despite such mechanistic differences, drug actions converge in producing some common effects. Prominent among them is the activation of the mesolimbic dopamine system, critical for mediating motivational effects of drugs of abuse and for facilitating the encoding of learned associations necessary for the development of addiction. This activation involves increased firing of dopamine neurons in the ventral tegmental area (VTA) of the midbrain and a subsequent increase of dopamine released in the nucleus accumbens and the prefrontal cortex. Once a person is addicted, a pathological form of plasticity in excitatory transmission is apparently important for the uncontrollable urge to obtain drugs and for relapse (Kalivas and Volkow, 2005). There are many mechanisms by which repeated exposure to a drug of abuse could alter gene expression in the brain and best studied so far is the regulation of gene transcription. One of the transcription factors altered by drugs of abuse is the cAMP response element binding protein (CREB) (Nestler, 2004).

Administration of kappa opioid agonists to humans induces dysphoric and psychotomimetic effects blocked by kappa opioid antagonists (Pfeiffer et al, 1986). Furthermore, kappa opioid receptor agonists modulate the effects of rewarding substances in animal studies. For example, kappa opioid agonists attenuate the self-administration, rewarding effect, drug discriminative effect and locomotor enhancement induced by morphine (Glick et al., 1995; Narita et al., 1993; Spanagel et al., 1994), and antagonize cocaine-induced conditioned place preference (Suzuki et al., 1992).

Dynorphin expression is induced in the nucleus accumbens after exposure to addictive drugs and this effect seems to be mediated by CREB. Dynorphin decreases dopamine release within the nucleus accumbens through an action on kappa opioid receptors that are located on presynaptic dopamine-containing nerve terminals in the region (Figure 3). This effect of dynorphin contributes to the negative emotions (dysphoria and anhedonia) present during the early phases of abstinence (Nestler, 2001).

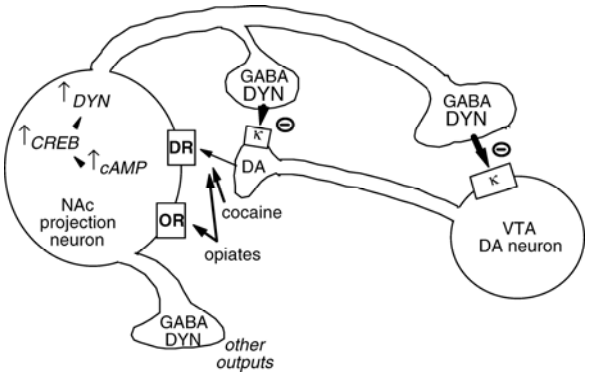

Figure 3. *Interactions between dopamine and dynorphins* (reproduced with permission from the publisher from Nestler, 2004)

1.4.2 Antinociceptive actions

Kappa opioid agonists demonstrate some antinociceptive activity after intrathecal administration. Slight antinociceptive activity after intrathecal injection of dynorphins, most probably mediated through kappa opioid receptors, has also been reported. However, antinociceptive effects of dynorphins are hindered by their neurotoxic actions, mediated through non-opioidergic mechanism (Przewlocki et al., 1983). Generally, spinal action of opioids acting at kappa opioid receptors apparently depends on the nature of the noxious stimulus (Przewlocki and Przewlocka, 2001).

1.4.3 Effects on memory and learning

A number of reports demonstrate that dynorphins are involved in modulation of memory and learning under conditions of stress, pain or tissue injury. Dynorphins are upregulated in rodents with age and this upregulation apparently contributes to the age-associated cognitive impairment (Jiang et al., 1989; Nguyen et al., 2005).

1.5 NON-OPIOID ACTIONS OF DYNORPHINS

Several effects of Dyn A and Big Dyn are not blocked by the opioid antagonist naltrexone and are also exerted by non-opioid derivatives of Dyn A, strongly suggesting that they are non-opioid. Non-opioid effects of Dyn A appear to be relevant for several pathophysiological processes including chronic neuropathic pain and spinal cord injury. Clinically, neuropathic pain is a chronic syndrome that may arise from injury to peripheral nerves and is associated with hyperalgesia, spontaneous pain, allodynia and repetitive discharge of nociceptors (Lai et al., 2001).

Intrathecally administered Dyn A and its non-opioid derivatives produce hindlimb paralysis, loss of neuronal cell bodies and long-lasting hyperresponsiveness to innocuous mechanical and noxious stimuli in rats (Faden and Jacobs, 1984; Skilling et al., 1992; Vanderah et al, 1996). *PDYN* mRNA expression and dynorphin immunoreactivity are elevated in peripheral nerve injury and spinal cord trauma (Przewlocki et al., 1988; Cox et al., 1985). The elevation of dynorphins is consistent with the duration of the experimental pain state and may contribute to secondary neurodegeneration. The importance of dynorphins in the maintenance of neuropathic pain states has been confirmed with studies on mice in which the *PDYN* gene is deleted (Wang et al., 2001). These mice generally have a normal phenotype (Sharifi et al., 2001), show normal responses to innocuous and noxious stimuli and develop neuropathic pain within two to three days after injury. However, by ten days after injury, the knockout mice are no more sensitive than they were prior to injury, while the wild-type animals in contrast display neuropathic pain past day fourteen (Wang et al, 2001). These data suggest that spinal dynorphins do not contribute to initial pain onset, but rather are critical for the maintenance of neuropathic pain.

The mechanisms of Dyn A induced non-opioid activity are not fully elucidated but NMDA and AMPA receptors are apparently involved. Thus, Dyn A-induced paralysis (Faden and Jacobs, 1984; Caudle and Isaac, 1987), mechanical and thermal allodynia and mechanical hyperalgesia (Vanderah et al., 1996, Laughlin et al., 1997) can be prevented by pretreatment with the NMDA receptor antagonist MK-801. Furthermore, Dyn A induces toxicity in striatal neurons through a mechanism that involves the activation of AMPA/kainate receptors and the caspase-3 apoptotic cascade (Singh et al., 2003 – related paper 1).

The interaction of endogenous Dyn A and NMDA receptors may be either direct or indirect. Dyn A may directly bind to the closed/desensitized state of the NMDA receptor, although the physiological relevance of such interaction remains to be established (Massardier and Hunt, 1989; Tang et al., 1999). Alternately, NMDA-mediated effects of Dyn A can represent an indirect interaction mediated by the initial release of excitatory amino acids that in turn stimulate NMDA receptors. Indeed, the application of Dyn A to the rat hippocampus results in a localized, dose-dependent release of glutamate and aspartate (Faden, 1992). Thus, Dyn A may indirectly potentiate NMDA receptor activity by enhancing the release of excitatory neurotransmitters, thereby resulting, for example, in hindlimb paralysis and neuronal cell loss. However, several non-opioid actions of dynorphins cannot be accounted for by an NMDA-dependent mechanism. Thus, the non-opioid peptide Dyn A 2-17 appears to stimulate an acute time and dose-dependent increase in $[Ca^{2+}]_i$ in cortical neurons in a way that is independent of NMDA receptors (Tang et al., 2000).

Since non-opioid activities of dynorphins have important implications in pathological situations, we set out to search for alternative mechanisms of interactions of dynorphins with cells and for non-opioid targets of these peptides.

1.6 CELL PENETRATING PEPTIDES

Cell penetrating peptides (CPPs) are cationic peptides that have the unconventional ability to translocate across the plasma membrane into cells, seemingly without any chiral receptors. Members of the CPPs group include the human immunodeficiency virus transactivator of transcription (TAT) protein, the Antennapedia homeodomain (penetratin), or represent oligoarginine polymers or chimeric peptides (transportan) (Frankel and Pabo, 1988; Joliot et al., 1991; Pooga et al., 1998; Mitchell et al., 2000). The basic nature due to the high content of arginine or lysine, the amphipathic character, and/or the presence of a hydrophobic core sequences have been suggested as important structural features of CPPs (Wender et al., 2000; Christiaens et al., 2004). Dynorphins have structural similarity to CPPs in possessing a high content of basic amino acids (they are probably the most basic neuropeptides known) and especially a large number of arginine residues (Table 2).

Table 2. Amino acid sequences and properties of dynorphins compared with CPPs

Peptide	Sequence
Dyn A	YGGFLRRIRPKLKWDNQ
Dyn B	YGGFLRRQFKVVT
Big Dyn	YGGFLRRIRPKLKWDNQKRYGGFLRRQFKVVT
Big Dyn 6-26	RRIRPKLKWDNQKR YGGFLRR
TAT	RKKRRQRRR
Penetratin	RQIKIWFQNRRMKWKK
R7	RRRRRRR

CPPs have mostly been studied with the hope of using them as a tool to target therapeutic substances into cells or for gene regulation (Joliot and Prochiantz, 2004; Järver and Langel, 2004). Little is known about the physiological functions of the translocation process.

The mechanism of translocation of CPPs is not well understood. Initial studies suggested binding of CPPs to the plasma membrane through an electrostatic interaction between the CPP (positive charge) and the membrane phospholipids and polysaccharides (negative charge), with subsequent uptake via an unknown receptor- and endosomal-independent pathway. More recent data have challenged this view and have shown that internalization of CPPs occurs at least partially via endocytosis and that internalized peptides are localized almost exclusively in the cytoplasm. However, a large number of studies demonstrate CPP-mediated delivery of biologically active cargos, including proteins, peptides and oligonucleotides, which suggests that a small fraction of CPPs enters cells by an endocytosis-independent pathway or that at least partial endosomal release of CPPs occurs (Magzoub and Gräslund, 2004; Oehlke et al., 2005).

Since there is a structural similarity between CPPs and dynorphins, we decided to test whether dynorphins are able to translocate across the plasma membrane into cells similarly to CPPs, and whether this property could potentially contribute to their non-opioid activities.

1.7 OLIGOPEPTIDASES

Following interactions with kappa opioid receptors or non-opioid targets, dynorphins are inactivated by enzymatic degradation. Enzymatic degradation is the general way for inactivation of neuropeptides in extracellular space. Active peptides are

frequently converted to a fragment or to several fragments that retain or in some cases have different biological actions. The peptidases responsible for the conversion of the parent neuropeptides can be anchored to the extracellular surface of the plasma membrane, or can be present in the extracellular fluid or in the cytoplasm. Most of these enzymes may cleave a variety of peptide targets, although they exhibit a high specificity for the nature of the amino acid residues in proximity to the cleaved bond. Other proteases are reported to be rather specific for certain neuropeptides (Hallberg and Nyberg, 2003).

The dynorphin-converting enzymes (DCE) constitute a group of proteinases that are capable of transforming PDYN-related opioid peptides to enkephalins (Nyberg et al., 1985). Although the conserved sequences of the NH_2-terminal pentapeptide (Leu-enkephalin), flanked from the C-terminus by dibasic amino acid residues, is present in all dynorphins, they are processed differentially by the DCE (Silberring et al., 1992; Silberring and Nyberg, 1989). The release of Leu-enkephalin from dynorphins results in a conversion of a κ-agonist to a product acting on μ/δ-receptors. This type of conversion may be of relevance in brain areas where kappa signals oppose those of μ/δ receptors as in the brain reward system (Figure 4).

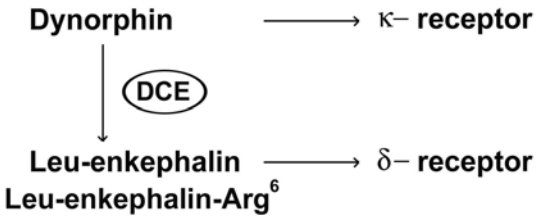

Figure 4. *Conversion of dynorphins to enkephalins by dynorphin-converting enzymes* (modified from Hallberg and Nyberg, 2003)

After identifying the ability of dynorphins to translocate across the plasma membrane, we searched for factors that they can interact with in the cell interior. We found a novel soluble Dyn A-binding factor with oligopeptidase activity that we refer to as Dyn A-binding factor (DABF).

1.8 ALZHEIMER DISEASE AND DYNORPHINS

Alzheimer disease (AD) is the most common cause of dementia worldwide that that accounts for up to 65% of dementia cases in older people. It is characterized by severe neurodegeneration and a progressive loss of cognitive function. In brains of individuals with AD neurons of the hippocampus and cerebral cortex are selectively lost, and extracellular amyloid (or senile) plaques and intracellular neuro-fibrillary tangles of hyperphosphorylated tau protein are present. Amyloid plaques contain small, toxic cleavage products, denoted as beta-amyloid(Aβ)40 and Aβ42, of the amyloid precursor protein (APP). The apolipoprotein E4 (apoE4) genotype and advanced age are major risk factors for the development of AD. Most cases of AD are idiopathic but some are familial AD.

According to the amyloid hypothesis the accumulation and deposition of fibrillar β-amyloid is the primary driver of neurodegeneration and cognitive decline leading to dementia (Glenner and Wong, 1984; Selkoe, 1991). Over the past 20 years, the amyloid hypothesis has been strongly supported by a wealth of evidence, including data from genetic studies of AD (Tanzi and Bertram, 2005). All four of the established AD genes, the β–amyloid precursor protein (APP), presenilin 1 and 2 (PSEN1, PSEN2) and apolipoprotein E (APOE) harbor mutations or variants that influence the accumulation of the Aβ peptide. In the last years a shift to the synaptic Aβ hypothesis that places a greater emphasis on the pathogenic role of non-fibrillar soluble Aβ oligomers, promoting neurodegeneration and disrupting cognitive function, has been observed (Tanzi, 2005). The mechanisms by which Aβ impairs synaptic function are complex but dysregulation of intracellular calcium signaling and NMDA receptors is apparently involved (LaFerla, 2002; Louzada et al., 2004).

Abnormalities in the expression of several neuropeptides have been observed in AD. Thus, somatostatin, corticotropin-releasing factor and substance P are downregulated in AD (Davies et al., 1980; Bissette et al., 1985; Beal and Mazurek, 1987). Since somatostatin activates the metabolism of Aβ, which is catalyzed by neprilysin, somatostatin downregulation may trigger accumulation of Aβ, leading to late-onset sporadic AD (Saito et al., 2005). As mentioned previously, dynorphins are upregulated in rodents with age and this upregulation contributes to the age-associated cognitive impairment. Furthermore, Dyn A induced non-opioid effects result in

neurodegeneration and neuronal death and may be relevant for the AD neuropathology (Singh et al., 2003 – related paper1; Skilling et al., 1992; Hauser et al., 2001).

Therefore, we hypothesized that the dynorphin system may be altered in AD and may contribute to the pathogenesis of the disorder. To test this hypothesis, we evaluated the status of the dynorphin system in AD and control subjects, and, for comparison, in subjects with Parkinson disease (PD) and cerebro-vascular disease (CVD).

2 AIMS

The main objectives of the work presented in this thesis were as follows:

1. To characterize mechanisms of non-opioid interactions of dynorphins with cells and to identify non-opioid biochemical targets for these peptides:

    a. To test whether dynorphins, which are similar to cell penetrating peptides (CPP) in high content of basic and hydrophobic amino acids, may also translocate across the plasma membrane.

    b. To identify non-opioid dynorphin binding sites in neuronal cells and brain tissues.

2. To characterize biochemical properties, intracellular localization and distribution in the rat brain of the precursor protein PDYN.
3. To assess a role of dynorphins in human neurodegenerative disorders including Alzheimer disease.

3 METHODS

Detailed description of the utilized methods is provided in the individual papers. More general characterization of the methods is presented here.

3.1 CELL CULTURING

Several immortalized cell lines and primary cells cultures were used in the present thesis. Human cervical carcinoma cell line (HeLa), African green monkey kidney cell line (COS-1) and cerebellum granule cell line (from 8-day-old Sprague-Dawley rats) were used to study the ability of dynorphins to translocate across the plasma membrane into cells (Paper I).

Human neuroblastoma cell line (SK-N-MC), neuroblastoma × glioma hybrid cell line (NG 108-15), choriocarcinoma cell line (JEG-3), small cell lung carcinoma cell lines (U-2020, U-1690, and U-1906), and lymphoblast-like cell line (IM-9) were used to identify and characterize dynorphin binding sites in neuronal cells and brain tissues (Paper II).

Mouse insulinoma cell line (MIN 6), cerebral cortical cell line (from gestation day 17-19 Sprague-Dawley rats) and striatal neuronal culture (from embryonic day 15 ICR mice) were used to study the stimulated secretion of the precursor protein PDYN (Paper III). HeLa, COS-1 and rat pheochromocytoma (PC12) cells were transfected with plasmid PDYN DNA-lipofectamine mixture to study PDYN (Paper III).

3.2 SECRETION ASSAY

It has been suggested that both peptide synthesis and release are related to neuronal activity. In vitro studies, in which neuropeptides secretion is stimulated and the amount of released neuropeptides is measured by radioimmunoassay (RIA), have been used to study this phenomenon. Such studies have limitations deriving from the fact that there is a gap between the time scale used for estimating release and that used for exocytosis. However, they are valuable in providing evidence for peptide release related to neuronal firing. A common strategy to induce neurotransmitter release involves the inclusion of high concentrations of potassium ions in the medium. Diffusion of these ions across the plasma membrane generates depolarisation, leading to neurotransmitter release. Glucose stimulation has been

demonstrated to induce expression of PDYN and an increase in both synthesis and secretion of the endoproteolytic cleavage product, Dyn A.

To study whether secretion of dynorphins and PDYN processing are related to membrane depolarization, in the present study MIN6 cells, cerebral cortical cultures and striatal neuronal cells were stimulated by K^+ and glucose.

MIN6 cells were incubated in a modified Krebs-Ringer solution including 5.9 mM KCl and 3 mM glucose (basal conditions) or in the same buffer with 25 mM KCl or 20 mM glucose (stimulated conditions) for 20 min. Acetic acid was added to cell extracts or culture medium to a 1M final concentration and peptides were analyzed by RIA. A protein fraction of the medium, precipitated by 10% trichloroacetic acid, was washed in acetone, resuspended in 1 × sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and analyzed by western blot.

Cerebral cortical cultures prepared from Spague-Dawley rats (17-19 days gestation) cultivated for 14-20 days were incubated for 0.5 and 2h with Locke 5 mM K^+, or Locke 90 mM K^+. Striatal neuronal cultures prepared from embryonic day 15 ICR mice, were grown for 7 days and incubated in low- (including 5 mM KCl and 118 mM NaCl) or high- (including 40 mM KCl and 83 mM NaCl) potassium buffer for 1 h. Culture medium mixed with acetic acid (1M final) and cells, extracted with 1M acetic acid, were analyzed by RIA.

3.3 CELL AND TISSUE EXTRACTS FOR WESTERN BLOT ANALYSIS

For protein extraction, tissue samples/powder or cell suspension were homogenized in solubilization buffer containing detergent, buffer salt and protease inhibitors. The presence of high salt concentrations contributed to disruption of protein-protein interactions and DNA precipitation. Extraction buffers were supplemented with protease inhibitors and all procedures were carried out on ice to prevent proteolysis. Ionic detergents are usually better solubilizing agents than non-ionic and amphoteric detergents, but they tend to denature proteins by destroying native three dimensional structures. This denaturing ability can be useful for SDS-PAGE, but is detrimental where native structure is important, as when functional activities must be retained. In the present study, for analysis of PDYN, PC2, and DABF (Papers II, III, IV) tissue samples/powder were homogenized in 4 volumes of nondenaturing buffer C called also Dignam's buffer that includes the non-ionic detergent 0.2 % Nonidet P-40 (NP-40). For analysis of PDYN (Paper III), in some cases cell/tissue samples were homogenized in 4

volumes of nondenaturing Triton X-100 lysis buffer that includes the non-ionic detergent 1 % Triton X-100. After incubation, the mixture was centrifuged for 10-15 min at 20 000 × g. Soluble proteins, and membrane proteins solubilized in the detergent were contained in the supernatant. About 50 % of PDYN was soluble in Triton X-100 lysis buffer while almost full extraction of PDYN was achieved in buffer C. To achieve full extraction of PDYN after Triton X-100 extraction, the insoluble material was solubilized in SDS buffer including 4 % SDS, boiled and DNA was sheared by sonication.

Several assays were used to quantify total protein: the Bio-Rad protein assay kit based on coomassie dye-binding, the bicinchinonic Micro BCA™ assay or DC Protein assay, which are copper-based assays.

3.4 WESTERN BLOT ANALYSIS AND DENSITOMETRIC SEMI-QUANTIFICATION

Western blot analysis (referred to also as immunoblotting) is used to identify specific antigens recognized by polyclonal or monoclonal antibodies. In the most commonly used one dimensional gel methods, the detergent SDS is used to solubilize, denature and impart a strong negative charge to proteins. Following electrophoretic separation of proteins and transfer from the gel to an appropriate membrane, the immobilized proteins are probed with specific antibodies to identify and quantitate any antigens present.

In papers III and IV, aliquots of tissue extracts were resolved by SDS-PAGE on tricine or tris-glycine gels. Before loading on the gels, samples were heated for 5 min at 95 °C. In some cases, to be able to study PDYN in the form of monomers, heating was carried out in the presence of the reducing agent dithiothreitol (DTT) that reduces disulfide bonds, and was followed by alkylation at room temperature with iodoacetamide that keeps –SH groups blocked. 20-80 µg protein were loaded per well in the different experiments. Proteins were transferred at 4 °C onto nitrocellulose membranes and stained by Memcode Reversible Protein Stain Kit to assure the same amount of protein was loaded on each lane. After destaining non-specific binding sites on the membranes were blocked for 30 min in 5% non-fat dry milk and membranes were incubated with primary antibodies. They included antibody directed against human PDYN C-terminal fragment (CTF 241-254), anti-PC2 antibody, anti-BiP antibody, antibody directed against rat PDYN CTF, anti-recombinant PDYN antibody.

After washing, membranes were incubated with secondary HRP-conjugated anti-mouse or anti-rabbit antibodies (BioRad). The immunocomplex was detected with an Amersham ECL kit. HRP catalyses the oxidation of a substrate in the ECL solution whereby the product reacts with peroxide under emission of light that is captured on autoradiography film.

In paper IV, the amount of protein detected by western blot was quantified by densitometric scanning of the film that measured the density of the bands captured on it, using an Image Analysis System (Image Gauge V3.12, Fujifilm). An aliquot of pooled "standard" human brain was assayed on three lanes of each gel and respect densitometry values were used for intra- and extrablot comparison. Densitometry values for total protein determined with Memcode membrane staining were used for normalization of the data for each individual subject.

3.5 CELL AND TISSUE EXTRACTS FOR RADIOIMMUNOASSAY

Small and relatively hydrophilic peptides are efficiently extracted by dilute acids at high temperatures. For RIA determinations of dynorphins, tissue samples were heated in 1 ml hot (95 °C) 1 M acetic acid to inactivate enzymes, cooled on ice, sonicated to break cells and DNA, and reheated. After centrifugations, the supernatant that contained dynorphins was subjected to SP-Sephadex chromatography. For the same purpose, cells were collected, acetic acid was added to 1 M, and they were heated, cooled on ice, sonicated, reheated, centrifuged and the supernatant was subjected to SP-Sephadex chromatography. The medium was mixed with 100% acetic acid to 1 M, heated, and then processed by SP-Sephadex chromatography.

3.6 CHROMATOGRAPHY

Irrespective of the method of extraction, it is often necessary to concentrate and partially purify neuropeptides-containing fractions. Several chromatographic methods are routinely used for this purpose, including reverse-phase chromatography, in which polar mobile-phase and a non-polar stationary phase are used (C18) and ion-exchange chromatography, in which a charged solute molecule in the mobile phase is electrostatically attracted to the oppositely charged stationary phase (Sephadex chromatography). Prior to analysis by RIA, chromatography-concentrated samples may be dried in a speed-vac concentrator.

In paper III, to concentrate cerebrospinal fluid (CSF) samples for determination of proteins, 200 ml of human CSF collected from nearly 40 subjects was mixed with trifluoroacetic acid (1% final) and loaded on SEP-PAC columns (500 mg of C18; Waters Inc., UK) equilibrated with 1% trifluoroacetic acid. After washing with 1% trifluoroacetic acid, peptides and proteins were eluted with 97% acetonitrile/3% trifluoroacetic acid, dried and analyzed by SDS-PAGE.

In paper III and IV, to prepare cell/tissue extracts for RIA measurements, cells/tissue extracts in 1 M acetic acid and cell culture medium mixed with acetic acid, were run through SP-Sephadex ion exchange C-25 column. Peptides were eluted stepwise with 0.018 M pyridine and 0.1 M formic acid, pH 3.0 (fraction 1), 0.1 M pyridine and 0.1 M formic acid, pH 4.3 (elutes Leu-enkephalin; fraction 2), 0.35 M pyridine and 0.35 M formic acid, pH 4.3 (elutes Leu-enkephalin-Arg^6; fraction 3), and 1.6 M pyridine and 1.6 M formic acid, pH 4.3 (elutes Dyn A, Dyn B, nociceptin, fraction 5). For detection of PDYN and its fragments, acidified extracts and medium were boiled, lyophilized and digested with trypsin to liberate Leu-enkephalin-Arg^6, PDYN marker.

In paper II, SK-N-MC extract was incubated with labeled Dyn A, mixed with dextran/charcoal suspension, and centrifuged. The supernatant containing labeled peptide–protein complexes was mixed with an equal volume of 99% methanol/0.1% HCl at 4 °C, incubated for 30 min at 4 °C, centrifuged and the resulting supernatant diluted with buffer for faction I, was loaded and stepwise eluted as described above onto SP-Sephadex C-25 column.

3.7 RADIOIMMUNOASSAY MEASUREMENTS

RIA depends on the competition between radioactively-labeled and unlabeled antigen for the specific binding site of an antibody. The amounts of antibody and labeled antigen are fixed, the only variable being the unlabeled antigen concentration. After separation of the bound from the free antigen, the amount of labeled antigen bound to antibody can be compared. Some of the procedures for the separation of bound from free peptide fraction in the RIA include secondary antibody precipitation and adsorption on dextran-coated charcoal. Fractions containing Dyn A, Dyn B, nociceptin and Leu-enkephalin-Arg^6 from the cation-exchange chromatography were lyophilized and redissolved in methanol/0.1 M HCl (1:1).

RIAs for Dyn A, Dyn B and nociceptin followed the so-called non-immunoglobulin pre-precipitating method (Papers III, IV). An aliquot of the sample or standard, antiserum, and radioactively labeled peptide were incubated overnight at 4 °C. The antisera and labeled peptides were diluted in assay buffer. To separate antibody-bound and free dynorphin, immunoprecipitate was added. The samples were incubated for 1 h at 4 °C. After centrifugation in a Beckman Microfuge, the supernatant was discarded and the pellet was counted in a gamma counter.

For Leu-enkephalin-Arg^6 aliquot of sample or standard, antiserum, and labeled peptide were incubated overnight at 4 °C (Paper III). Antisera and labeled peptide were diluted in gelatin containing phosphate buffer. The samples were incubated 10 min with dextran-coated charcoal to separate antibody-bound and free peptide. After centrifugation in a Beckman Microfuge, 300 µl of the supernatant was counted in a gamma counter.

3.8 RADIOACTIVITY BINDING ASSAY

Binding reaction was used to study the activity of DABF (Papers II, IV). Cell/tissue extract (from 2 to 20 µg of protein, 10 µg in most experiments) and radioactively labeled Dyn A (30,000 cpm) in assay buffer were incubated for 30 min or longer under reducing conditions in the majority of experiments. An equal volume of charcoal/dextran suspension was added and after 5 min incubation mixture was centrifuged in a Beckman microfuge. In this procedure, unbound peptide was precipitated with charcoal, whereas labeled peptide that had formed complexes with extract components was retained in the supernatant.

3.9 INDIRECT IMMUNOFLUORESCENCE

Indirect immunofluorescence relies on fixation of cells to retain cellular distribution of antigen and to preserve cellular morphology. After fixation, the cells are exposed to the primary antibody directed against the protein of interest, in the presence of permeabilizing reagents to ensure antibody access to the antigen epitope. The unbound antibody is removed and the bound primary antibody is then labeled by incubation with a fluorescently labeled secondary antibody, directed against the primary antibody host species. After removal of the secondary antibody, the specimen is ready for viewing on the fluorescence microscope.

Indirect immunofluorescence was used in the present project for identification and characterization of dynorphins translocation across the plasma membrane (Paper I). HeLa, PC12, COS-1 or cerebellar granule cells were incubated with Big Dyn, Dyn A or Dyn B at 37 ºC in the presence of fetal calf serum or in its absence. They were washed with phosphate-buffered saline (PBS), fixed in paraformaldehyde solution in PBS under varying conditions, washed again with PBS, blocked in 100 mM glycine, and permeabilized with 0.1 % Triton X-100. After washing with PBS, the cells were incubated in a blocking buffer and then overnight at 4 °C with rabbit anti-dynorphin B-antiserum, IgG fraction, or rabbit anti-dynorphin A-antiserum, IgG fraction. After washing cells were incubated with goat anti-rabbit IgG $F(ab')_2$ fragments conjugated with Alexa Fluor 488, washed and mounted in DAKO fluorescence media.

In the assay with biotinylated peptides, HeLa cells were incubated with biotinylated Big Dyn, Dyn A, Dyn B, and Big Dyn 6-26 central fragment at 37 ºC. Thereafter, the cells were washed with PBS, fixed in paraformaldehyde solution in PBS, again washed with PBS, blocked, permeabilized, and after washing incubated in blocking buffer. For peptide visualization, cells were incubated with streptavidin Alexa Fluor 633 conjugate for 1 h.

For colocalization studies with transferrin, HeLa cells were incubated with Big Dyn and transferrin conjugated with Alexa Fluor 546, fixed and labeled with rabbit anti-dynorphin B antibody and goat anti-rabbit IgG $F(ab')_2$ fragments conjugated with Alexa Fluor 488. For colocalization with the markers of the endoplasmic reticulum ERp29 and the Golgi complex GM 130, HeLa cells were incubated with Big Dyn, fixed and stained with rabbit anti-dynorphin B antibody and monoclonal antibodies against ERp29 or GM 130 as primary antibodies and goat anti-rabbit IgG F $(ab')_2$ fragments conjugated with Alexa Fluor 488 and anti-mouse IgG antibodies conjugated with Alexa Fluor 546 as secondary antibodies.

Confocal images were recorded using a Leica TCS SP confocal laser scanning microscope (Leica Microsystem, Heidelberg, Germany). Images were prepared using Photoshop 6.0 (Adobe; San Jose, California, USA).

3.10 LIVE CELL VISUALIZATION

In detecting cell penetrating peptides, one should carry in mind that fixation may lead to redistribution of peptide in the cell. Therefore, we wanted to detect the translocation of dynorphins fluorescently labeled with rhodamine (Rh) in live cells.

HeLa cells were incubated with 10 μM Rh-Dyn A or Rh-Dyn B for 15 min and after three additional washings with the medium viewed under a confocal microscope.

3.11 FLUORESCENCE CORRELATION SPECTROSCOPY

In fluorescence correlation spectroscopy (FCS), a highly sensitive confocal technique with single molecule detection sensitivity, statistical analysis of the time course and amplitudes of spontaneous fluctuations in fluorescence intensity in a small volume element was performed to evaluate dynamic processes. FCS was performed on a ConfoCor instrument (Carl Zeiss) with confocal illumination of a laser volume element of 0.2 fl. Prior to FCS measurements, HeLa cells were washed and incubated with fluorescently labeled peptides Rh-Dyn A, Rh-Dyn B, Rh-TP10, or free Rh in phenol red-free Iscove's medium with 10% calf serum at room temperature. Fluorescence intensity was measured at different intervals after incubation in the medium and at different subcellular locations. The average fluorescence intensity was estimated from 10 s measurements.

3.12 STATISTICAL ANALYSIS

In paper IV for comparison of biochemical parameters between four diagnostic groups ANOVA was used, followed, when appropriate, by Newman-Keuls test. For comparison of biochemical markers between two groups Student’s t-test was used. ANCOVA analyses with pH and gender as covariates were also performed. Pearson Product-Moment correlation procedures were used to assess the correlation between the various continuously distributed variables. Statistical analysis was performed using Statistica for Windows (release 6.0, Statsoft Inc. Tulsa, OK). P level was set at 0.05 as statistically significant.

3.13 OTHER METHODS

Detailed description of mass spectrometry (Paper I), biophysical methods for studying model vesicles (Paper I), fast protein liquid chromatography (Paper II), immunocytochemistry (Paper III), electron microscopy (Paper III) and *in situ* hybridization (paper III) can be found in the individual papers.

4 RESULTS AND DISCUSSION

4.1 TRANSLOCATION OF DYNORPHINS ACROSS THE PLASMA MEMBRANE (PAPER I)

Dynorphins are rich in arginine and lysine residues and are probably the most basic neuropeptides. Because hydrophobicity and/or basic nature are general properties of CPPs, we hypothesized that the basic dynorphins may also translocate across the plasma membrane into cells, which may be relevant for interneuronal communication in the central nervous system. To test this hypothesis in Paper I we studied the ability of dynorphins to translocate into cells. We also investigated the kinetics of translocation of the dynorphin peptides, their intracellular distribution, and the interactions with phospholipids membranes relevant for the translocation into cells.

Four dynorphin peptides were tested for their translocation properties: Dyn A, Dyn B, Big Dyn and a central fragment of Big Dyn - Big Dyn 6-26, which contains the most basic part of its sequence.

To determine whether dynorphins translocate into cells initially two strategies were used. Cells were incubated with Dyn A, Dyn B or Big Dyn, fixed, probed with anti-Dyn A or anti-Dyn B antibody and secondary fluorescently labeled antibody or alternatively cells were incubated with biotinylated Dyn A, Dyn B, Big Dyn or Big Dyn 6-26, fixed and probed with streptavidin conjugated to fluorescent dye. The biotin-streptavidin assay allowed detection of biotinylated peptides with equal sensitivity, allowed the inclusion of Big Dyn 6-26 in the analysis for which no antibody was available and ensured that the results obtained are not dependent on the detection assay. With both assays translocation of Big Dyn into cells was detected at 1 μM concentration, translocation of Dyn A was detected at 10 μM concentration, while no translocation of Dyn B or Big Dyn 6-26 was detected at either 1 or 10 μM concentrations. Both translocated Big Dyn and Dyn A demonstrated punctate cytoplasmic labeling, with negligible signal in the cell nucleus and on the plasma membrane (Fig. 5 A, B). Opioid receptors were not involved in the translocation process, since HeLa cells do not express opioid receptors at detectable levels and since pretreatment with opioid antagonists to block opioid receptors that might be expressed at low levels did not have any effect on the translocation process.

Studies of other cell penetrating peptides have shown that under certain circumstances fixation may cause artificial redistribution of peptides (Richard et al., 2003). Therefore, we studied translocation of both fluorescently labeled with rhodamine (Rh) Dyn A and Dyn B into live HeLa cells by confocal microscopy. Rh-Dyn A at 10 μM translocated in 30-50% of the cells it was incubated with, while Rh-Dyn B did not penetrate into cells. Fluorescence of Rh-Dyn A was intense in the cytoplasm, although some bright fluorescent domains were visible in the cell nucleus. The signal was generally much weaker at the plasma membrane and in the cell nucleus. Thus, Dyn A exhibits a higher potential to translocate into live cells compared with Dyn B, and the process of fixation did not lead to redistribution of these peptides from plasma membrane into cell interior (Fig. 5 C, D).

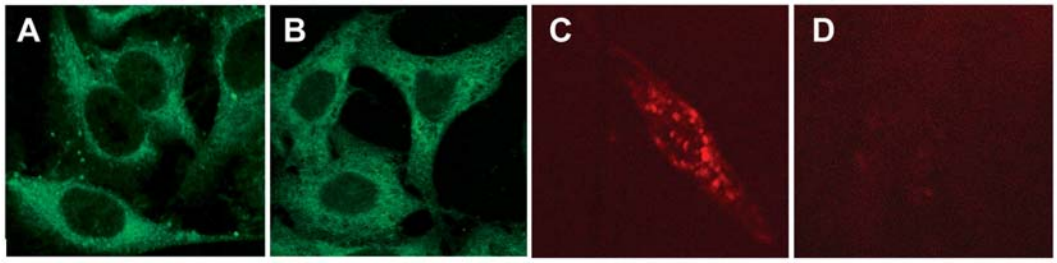

Figure 5. *Translocation of dynorphins into cells* **A.** Big Dyn (1 μM) immunofluorescence; **B.** Dyn A (10 μM) immunofluorescence; **C.** Fluorescence of Rh-Dyn A (10 μM) in live cells; **D.** Fluorescence of Rh-Dyn B (10 μM) in live cells.

Using fluorescently labeled peptides is the current standard for studying translocation of peptides into live cells. However, in order to discriminate the effect of the fluorescent label, to study translocation of dynorphins at lower concentrations and to analyze the kinetics of translocation, FCS, a confocal technique for examining molecular events in real time and with single molecule detection sensitivity, was used. Interactions of Rh-Dyn A with live cells were compared with those of Rh-Dyn B, Rh-transportan-10, a prototypical CPP, and the fluorescent label alone.

FCS measurements demonstrated that Rh-Dyn A translocates efficiently into live HeLa cells at concentrations as low as 10 nM. The Rh-Dyn A accumulation in the cytoplasm exhibited a hyperbolic type kinetic profile with a rapid initial uptake phase lasting 1-5 min followed by a steady-state phase. Rh-Dyn B did not show similar

propensity to translocate into cells. Rh-transportan-10, a prototypical CPP, showed a membrane/cytoplasm distribution and kinetic profile of translocation into HeLa cells similar to Rh-Dyn A. The penetration pattern of these two peptides markedly contrasted with that of free Rh used for comparison; Rh showed an initial accumulation predominantly on the plasma membrane with slow onset of penetration into the cytoplasm.

The pattern of distribution of dynorphins in the cells suggests that these peptides are associated with subcellular membrane structures. This was investigated in colocalization experiments with markers of the endoplasmic reticulum (ERp29), Golgi complex (GM 130), and clathrin-mediated endocytosis (transferrin). Colocalization was evident for Big Dyn and ERp29. Colocalization of Big Dyn with GM 130 and transferrin was not observed. The latter observation suggests that clathrin-mediated endocytosis was not involved.

The first event in the CPPs translocation into cells is their interaction with the negatively charged plasma membrane. Peptide-membrane interactions are generally modeled with phospholipid vesicles. To characterize the dynorphin-membrane interactions, the binding of dynorphins to small, unilamellar, and partially negatively charged phospholipids vesicles was studied by fluorescence and circular dichroism spectroscopy. All four peptides were found to bind strongly to lipid vesicles, suggesting their efficient binding to the lipid phase of the plasma membrane. Upon interaction with lipid vesicles about 30% α-helical structure was induced in Big Dyn, Dyn A and Dyn B, while there was virtually no induction of α-helical structure in Big Dyn 6-26. Among these peptides, Big Dyn and Dyn A, which translocate into cells, have higher positive charge compared with Dyn B. Thus, two structural features, the ability to form α-helical structure and high positive charge, may in conjunction determine the membrane translocation potential of Big Dyn and Dyn A.

Big Dyn is more effective than Dyn A in inducing non-opioid nociceptive effects, whereas Dyn B is not active (potency order is Big Dyn > Dyn A >> Dyn B) (Tan-No et al., 2002). The rank of potency correlates well with the ability of these peptides to translocate across the plasma membrane, suggesting that peptide translocation may be relevant for pathophysiological processes such as the development of chronic pain or for neurotoxic effects of Dyn A.

Potential targets for the action of dynorphins after their translocation into cells include subunits of the NMDA-receptors, G-proteins or intracellularly located opioid receptors. A conserved negatively charged segment of the C terminus of the NR1 subunit of the NMDA receptor that is localized intracellularly has been identified as a target for Dyn A (Woods et al., submitted). Synthetic peptides containing this epitope form stable complexes with Dyn A, functionally antagonizing the potentiation of NMDA receptor-activated responses produced by Dyn A, preventing Dyn A-induced cell death and reducing dynorphin-evoked allodynia in the spinal cord. Another neuropeptide, substance P has been demonstrated to be able to translocate across the plasma membrane, bind to the G protein subunit, and induce mast cell degranulation (Lorenz et al., 1998). By analogy, G proteins may be targeted also by basic dynorphins penetrated into the cells. This assumption is supported by the observation that Dyn A stimulates histamine release from mast cells, an effect that is not mediated though opioid receptors (Casale et al., 1984; Shanahan et al., 1984). Dyn A interacts with all three subtypes of opioid receptors with reasonably high affinity (Zhang et al., 1998) and a large proportion of δ-opioid receptors are located in the neuronal cytoplasm (Cahill et al., 2001). Dynorphins translocated into cells may potentially interact with intracellular δ-opioid receptors and also with μ- and κ-opioid receptors undergoing transport and recycling.

4.2 INTERACTIONS OF DYNORPHINS WITH DYN A-BINDING FACTOR (PAPER II)

In paper II we aimed to identify non-opioid intracellular targets/binding sites for Dyn A and Big Dyn, with which these peptides may interact after translocation into cells. We also set out to characterize these binding sites/factors biochemically in cell lines, brain and spinal cord.

To test whether there are cellular components that interact specifically with Dyn A, radioactively labeled Dyn A was incubated with extracts of neuroblastoma SK-N-MC or neuroblastoma × glioma NG 108-15 cell line (Figure 6). Dextran-coated charcoal was used to separate free and bound labeled peptide and after centrigugation unbound peptide was present in the charcoal pellet, while bound form of Dyn A was present in the supernatant. Dyn A radioactivity in the supernatant was 2- to 10-fold

higher in the presence of cell extract than in its absence, suggesting the formation of complex between Dyn A and a factor from the cellular extract that we refer to as Dyn A-binding factor (DABF). Preincubation of extracts with 1 μM unlabeled Dyn A reduced the level of bound peptide to levels observed in the absence of extract. The degree of observed binding depended on the extract concentration and on the concentration of labeled peptide. Importantly, radioactively labeled Dyn B and Leu-enkephalin-Arg^6 did not form complexes with SK-N-MC and NG 108-15 cell extracts.

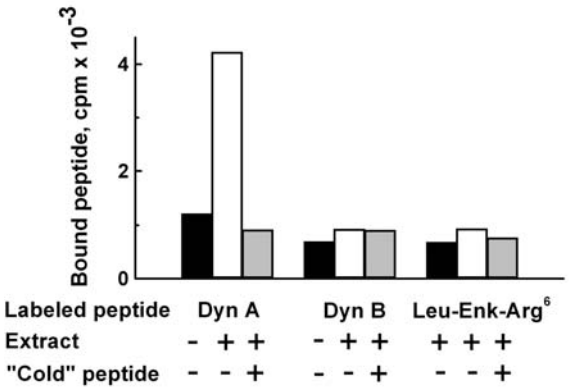

Figure 6. *Binding of labeled peptides to SK-N-MC cell extract*

Next we wanted to examine the affinity and specificity of Dyn A interactions with DABF and map the binding epitope of Dyn A to DABF. For this reason competition experiments were used, in which cellular extract was preincubated with different concentrations of dynorphins, longer PDYN fragments, other opioid and non-opioid peptides, and non-peptide ligands of opioid receptors and the effect of the preincubation on the formation of radioactive Dyn A-DABF complex was analyzed. Preincubation of cell extracts with Dyn A 1–17, Dyn A 2–17, Dyn A 1–13, Big Dyn, and Big Dyn 6–26 concentration-dependently inhibited the specific binding of labeled Dyn A and 50 % of inhibition was observed in the 5–10 nM range. For both Arg^{11,13}-Dyn A 1–13-Gly-NH-$(CH_2)_5$-NH_2 and Dyn A 3–13 50 % of inhibition was observed at 50–100 nM concentrations. Dyn A fragments 1–10 and 6–12 displaced 50% of labeled peptide at 10^{-5} M concentration, whereas Leu-enkephalin, Leu-enkephalin-Arg^6, Dyn A 1–8, Dyn A 9–17 and Dyn B at this and lower concentrations did not interfere with binding. DABF did not form complexes with the other studied opioid and non-opioid peptides, opiates, and benzomorphans. Thus, the binding of DABF to Dyn A is highly specific. A binding epitope was mapped to Dyn A 6-13. However, extension of the

binding core (6–13) either from the N-terminus or from the C-terminus increased the binding affinity.

The complex Dyn A – DABF has low dissociation rate, since 1 μM unlabeled Dyn A added to the labeled Dyn A preincubated with cell extract for 20 min did not displace labeled peptide from the preformed complex. To analyze in what form Dyn A bound to DABF is present, the complex Dyn A-DABF was dissociated with methanol-HCl and analyzed by SP-Sephadex C-25 chromatography or by PAGE after lyophilization. Labeled peptide extracted from the complex was eluted in the same fraction and demonstrated the same mobility on polyacrylamide gels as labeled Leu-enkephalin. Thus, Dyn A apparently undergoes conversion to its fragment Leu-enkephalin upon binding to or dissociation from DABF, suggesting that DABF has Dyn A-specific oligopeptidase activity. Size-exclusion chromatography of SK-N-MC cell and spinal cord extracts revealed a single peak of dynorphin-binding activity with a molecular weight of about 70 kDa, suggesting that this activity is associated with a single protein in the analyzed protein extracts.

DABF levels were compared in adult rat and murine tissues and several tumor cell lines. In adult rat brain, DABF activity was high in the periaqueductal gray, cortex, pituitary gland, and striatum, intermediate in dental gyrus, hypothalamus, and cerebellum, and low in the VTA. DABF activity was present in all adult mouse tissues examined including the cerebrum, cerebellum, liver, and kidney. Thus, there is no correlation between the levels of DABF and Dyn A.

Similarity in molecular size, thiol dependence, and ability to hydrolyze oligopeptides suggested a DABF identity with thimet oligopeptidase (TOP), known to interact with Dyn A with high specificity (Rioli et al., 2003; Oliveira et al., 2001). Experiments with recombinant TOP, however, did not support this suggestion but demonstrated that TOP may form complexes with Dyn A corroborating the idea that DABF belongs to the same class of enzymes as TOP.

DABF is thiol-sensitive, demonstrated by the fact that the reducing agent DTT improved binding of DABF and Dyn A, while SH-group blockers *p*-hydroxy-mercuribenzoic acid and *N*-ethylmaleimide prevented formation of the complex. Cysteine oligopeptidases appear to play a critical role in the degradation of Dyn A and its conversion into shorter enkephalins (Nyberg and Silberring, 1990; Silberring et al.,

1992). In our studies, i.t.-administered *N*-ethylmaleimide produced nociceptive behavior in wild type but not in PDYN knockout mice. Similar behavior was also induced by synthetic Dyn A but not Dyn B i.t.-administered into mice. *N*-ethylmaleimide inhibited degradation of Dyn A in spinal cord extracts, suggesting that induction of nociceptive behavior by this inhibitor is due to the inhibition of degradation/conversion of endogenous Dyn A by cysteine proteases (Tan-No et al., 2005 – related paper 2). In this regard, it is important to evaluate whether DABF is involved in degradation of Dyn A, a major pathogenic factor in chronic pain, or in its conversion into shorter enkephalins in future studies. Another function of DABF may be to regulate the levels of Dyn A available for interactions with the opioid or NMDA receptors by the sequestration of this peptide through formation of stable complexes. The stable Dyn A–DABF complexes may represent a transport form of this peptide from the sites of Dyn A release to the peptide cellular targets. DABF, as an oligopeptidase, may be involved in degradation of other neuropeptides, and thereby Dyn A may inhibit this process by competition for binding to the DABF catalytic site.

4.3 CHARACTERIZATION OF PRODYNORPHIN IN THE RAT BRAIN (PAPER III)

The dynorphin system has been thoroughly characterized at the level of mRNA and mature peptides but not at the protein precursor level. With aim to develop tools for analysis of the precursor protein PDYN, we raised and characterized a panel of rabbit polyclonal antibodies against rat and human PDYN. Antibodies against rat PDYN C-terminal fragment (anti-r-CTF 251-254), recombinant rat PDYN (rec-PDYN) and human PDYN CTF (anti-h-CTF) showed the highest sensitivity and specificity.

PDYN distribution in the rat brain was determined by light microscopic immunocytochemistry. Anti-r-CTF and anti-rec PDYN antibodies produced identical distribution pattern of PDYN-immunoreactivity that was generally consistent with previous analysis of dynorphins with the highest levels observed in the amygdala, hippocampus and striatum and in lower amounts in the cerebral cortex (Fallon and Leslie, 1986; Khachaturian et al., 1982; Pierce et al. 1999; van Bockstaele et al., 1994). PDYN-immunoreactivity was also present in brain structures such as the VTA and CA3 region of the hippocampus that do not have cell bodies of neurons producing

PDYN but contain axons and axon terminals originating from PDYN-ergic neurons in the ventral striatum and dentate gyrus, respectively.

In immunoblotting PDYN was detected as a dominant band with an apparent molecular size of 28 kDa, identical to the calculated PDYN molecular size, in rat pituitary gland and brain structures in which PDYN-immunoreactivity was present. The specificity of the PDYN identification was confirmed using blocking procedure and PDYN knockout mice.

The VTA and the CA3 region of the hippocampus lack PDYN-ergic neuronal cell bodies, but have axon terminal of PDYN-ergic neurons projecting from the nucleus accumbens and the dentate gyrus, respectively. Therefore, the levels of PDYN in dentate gyrus, CA3 region of the hippocampus, nucleus accumbens and VTA were determined by western blot to clarify whether PDYN is transported to axon terminals in unprocessed form (Figure 7). PDYN was found in all four regions and its levels in VTA and CA3 region of the hippocampus were slightly lower than those in the ventral striatum and dentate gyrus, respectively. In situ hybridization histochemistry detected negligible levels of *PDYN* mRNA in the CA3 subfield of the hippocampus and VTA, excluding the possibility of a local synthesis of the precursor at these sites. RIA demonstrated that Dyn A is present in the rat brain in the VTA and CA3 area of the hippocampus at levels similar or exceeding those in the dentate gyrus and striatum. Together, these results suggest that PDYN is at least partially transported to axon terminals in unprocessed form.

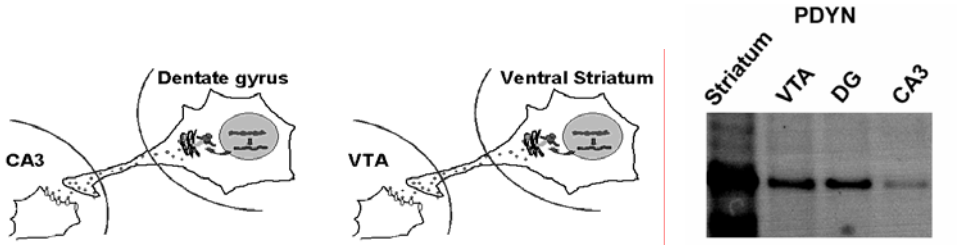

Figure 7. *PDYN in neuronal cell bodies and axon terminals*

PDYN was normally extracted from tissues with buffer C, which contained 0.42 M NaCl, detergent NP-40, and the reducing agent DTT. The extraction procedure was developed after recognizing that PDYN is not a soluble protein. PDYN was partially extracted (30 to 70%) with 1% TritonX-100 lysis buffer and even after four subsequent extractions with Triton X-100 lysis buffer a substantial amount of PDYN was found in

an insoluble form in striatal and hippocampal CA3 area tissues. The insoluble fraction of PDYN may consist of its aggregates or oligomers, formed with the participation of Cys residues at the N-terminus of PDYN. To address this question, soluble and insoluble fractions of mouse pancreatic β-cell line MIN6 cells, producing PDYN at high levels, were analyzed by PAGE. High molecular weight PDYN complexes were identified in the insoluble fraction analyzed under non-reducing conditions and they dissociated to PDYN monomers in the presence of the reducing agent DTT. The level at which insoluble high molecular weight PDYN complexes were observed was strongly increased when brefeldin A, which blocks protein transport from the endoplasmic reticulum to the Golgi compartment, was added. These results imply that insoluble PDYN consists of oligomers, stabilized by disulphide bonds, and these oligomers are formed in the endoplasmic reticulum. The disulfide bonds generated in the lumen of the endoplasmic reticulum are essential for the assembly and oligomerization of secretory proteins that determines their intracellular transport (Arvan and Castle, 1998; Cawley et al., 2000; Dannies, 1999; Tooze, 1998). The N-terminus of POMC, which also forms the disulfide-bonded loop, could direct the protein to the regulated secretory pathway (Cool et al., 1995). Identical arrangement of Cys residues at N-termini of POMC and PDYN, both opioid precursors, implies that the homologous PDYN segment also functions as a sorting signal. The presence of insoluble PDYN in the CA3 region suggests its oligomeric nature in axon terminals. PDYN oligomers may represent the storage form of the protein in secretory granules in axon terminals and dendrites. Considerable amplification of the signal is possible if PDYN, stored in a concentrated form, is processed immediately before it is released. Storage as oligomers may be a specific property of PDYN and other precursor proteins that need to be cleaved into functional peptides. Oligomers may readily dissociate into monomers that are potentially better substrates for processing than oligomers themselves, and this dissociation may be regulated within secretory vesicles. A similar mechanism of oligomerization-based sorting and storage has been proposed for POMC (Cawley et al, 2000).

PDYN in axon terminals may be processed to mature peptides prior to their release from secretory granules, or secreted as intact precursor. To test this suggestion MIN6 cells and rat cortical and striatal neuronal cultures were used as model systems.

In MIN6 cells secretion of Dyn A was stimulated and the total levels of the mature peptide in cells plus medium were elevated (indicative of activated PDYN processing)

in the presence of high concentrations of K^+ or glucose. The total levels of the precursor protein PDYN secreted by MIN6 cells in the presence of high concentrations of K^+ or glucose were also elevated 2.5- and 1.8-fold, respectively, compared to the ones under basal conditions. In addition, long PDYN fragment of 21 kDa molecular size was identified with anti-r-CTF antibodies in the medium upon K^+ depolarization that was absent under the basal conditions. Thus, K^+ depolarization activates processing of PDYN into its long fragment and mature opioid peptides in MIN6 cells.

PDYN processing and the secretion of mature peptide products were activated by K^+ depolarization in neuronal cultures, too. Upon depolarization, release of Dyn B and Leu-enkephalin-Arg^6 was stimulated 2-6-fold. Furthermore, the levels of trypsin-liberated Leu-enkephalin-Arg^6 in the medium were much higher than those of mature Leu-enkephalin-Arg^6, suggesting that a substantial fraction of PDYN is secreted by neurons in unprocessed or partially processed forms.

To test whether PDYN and its long fragments are secreted from neurons in the brain, a protein fraction of human cerebrospinal fluid was concentrated on a SEP-PAC column and analyzed by western blot with anti-h-CTF-antibodies. 18 kDa PDYN fragment was detected in the CSF, whereas no 28 kDa PDYN band was evident.

With respect to the association of processing and secretion, PDYN is similar to pro-atrial natriuretic factor that is stored in the myocytes and cleaved to atrial natriuretic factor when cells receive a signal that triggers release of the mature peptide (Sei et al., 1992). The stimulation of PDYN processing in axon terminals and dendrites by neuronal activity and extracellular signals may represent a novel mechanism for the regulation of synaptic transmission.

4.4 STATUS OF THE DYNORPHIN SYSTEM IN ALZHEIMER DISEASE (PAPER IV)

Dynorphins can modulate memory and learning and Dyn A can induce excitotoxicity resulting in neuronal loss. These actions may contribute to cognitive impairments and neurodegeneration in several neuropathological conditions including Alzheimer disease (AD). To test this hypothesis, the status of the dynorphin system and the related nociceptin system for comparison was evaluated in parietal cortex (Brodmann area 7) postmortem samples from AD subjects (n=21) and compared with

that in control (n=18), Parkinson disease (PD) (n=12) and cerebro-vascular disease (CVD) (n=21) subjects. In the course of experiments it became apparent that Dyn A is elevated in AD; to address the mechanism of this upregulation we determined the levels of the precursor protein PDYN, the processing convertase PC2, and the neuroendocrine protein 7B2, required for processing of PC2 precursor to catalytically active PC2, as well as DABF activity.

The levels of Dyn A, Dyn B and nociceptin were determined in control, AD, PD and CVD subjects using RIA. Dyn A was significantly higher in the AD group relative to controls and each of the other diagnostic groups ($p<0.01$). PD and CVD groups did not differ significantly from each other and from the control group in respect to Dyn A levels. Dyn B and nociceptin were not significantly altered in the study groups (Figure 8). The levels of Dyn A correlated positively and significantly with the density of neuritic plaques ($R= 0.62$; $p<0.05$).

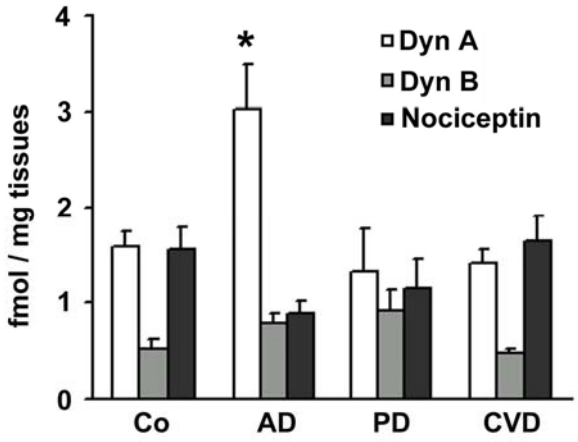

Figure 8. *Upregulation of Dyn A in Alzheimer disease*

These results are in accordance with a previous study showing elevated levels of Dyn A and Leu-enkephalin, endogenous ligands for κ- and δ-opioid receptors, respectively, in the frontal cortex in AD subjects (Risser D et al., 1996). As mentioned previously, under experimental or pathological conditions in which Dyn A levels are elevated, this peptide is excitotoxic and can have proapoptotic non-opioid actions in neurons and glia. The levels of soluble Aβ oligomers (4-60 fmol/mg; see Lue et al., 1999; McLean et al., 1999; Wang et al., 1999) and Dyn A (2-4 fmol/mg, see Figure 8) in the human cortex calculated per tissue weight are in the same range. The elevation of Dyn A levels in AD subjects and the correlation between Dyn A levels and neuritic

plaque density that we observed suggest that Dyn A in addition to soluble Aβ oligomers may play a role in AD pathogenesis.

Since the control and AD groups showed clear differences, we focused on mechanisms of Dyn A upregulation in AD. The precursor protein PDYN and processing convertase PC2, as well as the PC2 molecular chaperone 7B2, were analyzed by western blot. Their levels were normalized to the total amount of protein in each sample estimated with a membrane staining procedure. The levels of PDYN did not change in AD. The analysis of covariance revealed that the levels of PC2 were elevated in the AD group relative to the control group ($p<0.05$), suggesting a role of this enzyme in upregulation of Dyn A (Figure 9). 7B2 was detected as both precursor and mature forms. The levels of pro7B2 tended to decrease in AD ($p=0.07$). No differences were detected in the levels of 7B2 between the control and AD groups. No differences were detected in DABF activity, determined with radioligand-binding assay.

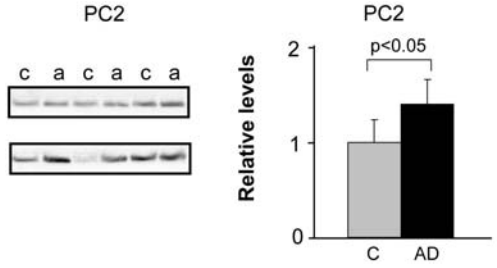

Figure 9. *Elevation of PC2 levels in Alzheimer disease.* Left panel shows representative western blots from control (c) and AD (a) subjects. Right panel demonstrates mean relative values of PC2 in control (C) and AD groups.

The novel findings of elevated PC2 levels in AD may be relevant for inferences regarding mechanism of Dyn A upregulation. That may occur due to activation of selective processing from PDYN by PC2.

Correlations of DABF and PDYN ($R= 0.77$; $p<0.01$), and DABF and PC2 ($R= –0.54$; $p<0.05$) were observed when the control and AD group were examined together, suggesting that there are specific interactions at the levels of expressions of these proteins or their functional interactions. A strong correlation of both pro7B2 ($R= –0.94$; $p<0.05$) and 7B2 ($R= –0.9$; $p<0.05$) with Clinical Dementia Rating score (CDR) was found in AD but not in control group. Since neither dynorphins nor nociceptin, which may be also processed by PC2, were associated with CDR, the pro7B2/7B2 – CDR

correlation suggests that other end-products of the enzymatic cascade regulated by the pro7B2 and 7B2 proteins may play a role in AD dementia.

5 CONCLUSIONS

1. In the current thesis we discovered a novel property of the opioid peptides Big Dyn and Dyn A to translocate across the plasma membrane of neuronal and non-neuronal cells. Dyn B and a central Big Dyn fragment 6-26 did not enter the cells. The translocated dynorphins were predominantly located in the cytoplasm where they were associated with the endoplasmic reticulum. They demonstrated rapid penetration kinetics with the steady-states levels being reached within 1-5 minutes after initiation of the reaction. Big Dyn showed higher translocation potential, while Dyn A and transportan-10, a prototypical CPP penetrated into cells with similar efficacy. The ability of dynorphins to translocate across the plasma membrane correlates well with their ability to induce non-opioid effects and suggests that their translocation is relevant for physiological processes such as the development of chronic pain.

2. Searching for non-opioid targets with which dynorphins can interact after their translocation into cells, we identified a novel binding factor (DABF) selective for Dyn A, Dyn A 2-17 and Big Dyn. DABF converts Dyn A into Leu-enkephalin and may function as an oligopeptidase that forms stable and specific complexes with Dyn A.

3. Generation of dynorphins may be regulated at the levels of gene transcription, translation or processing of the precursor protein PDYN. We provided pivotal evidence that PDYN is transported to and stored in axon terminals in the CA3 region of the hippocampus and the VTA in unprocessed form. PDYN was found to form insoluble oligomers stabilized by disulphide bonds that may represent the storage form of the protein in secretory granules in axon terminals and dendrites. Depolarization of neuronal cells stimulated dynorphin secretion and processing of PDYN into dynorphins and Leu-enkephalin-Arg^6. Activation of PDYN processing may represent a novel mechanism for the local regulation of synaptic transmission.

4. Dynorphins may be involved in pathogenesis of Alzheimer disease and other neurodegenerative disorders since they can induce cognitive impairments. We evaluated the status of the dynorphin system in postmortem samples from AD subjects and compared it to that in control subjects and those with PD or CVD. We found that Dyn A is selectively upregulated in AD and its content correlates with

neuropathological lesions. This correlation and the known non-opioid ability of Dyn A to induce neurodegeneration through glutamate receptors suggest a causal role for this peptide in AD pathogenesis.

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