# SZENT ISTVÁN UNIVERSITY

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# Opioids and Opioid Receptors in the Rodent Hippocampus: Distribution, Synaptology, and Connection with the Local GABAergic Circuitry

**PhD Dissertation**


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# LIST OF ABBREVIATIONS

**ABC**: Avidin-biotin peroxidase complex

**AC**: Adenylate cyclase

**ACTH**: Adenocorticotrop hormone

**App**.: Appendix

**CA**: Cornu Ammonis

**CNS**: Central nervous system

**DAB**: 3,3-diaminobenzidine-tetrahydrochloride

**δ**: delta

**DG**: Dentate gyrus

**Dyn-A; Dyn-B**: Dynorphin A; B

**EKC**: Ethylketocyclazocine

**ε**: epsilon

**GPCR**: G-protein coupled receptor

**GA**: Glutaraldehyde

**GABA**: γ-amino butiric acid

**GAR**: Goat-anti-rabbit

**κ**: kappa

**KOR**: Kappa opioid receptor

**KOR-IR**: Kappa opioid receptor immunoreactive/immunoreactivity

**Leu-enk**: Leucine-enkephalin

**LTP**: Long term potentiation

**mAb-KA8**: monoclonal anti-kappa opioid receptor antibody produced by the KA8 hybridoma cell line

**Met-enk**: Methionine-enkephalin

**μ**: mu

**NGS**: Normal goat serum

**NMDA**: *N*-methyl *D*-aspartate

**NPY**: Neuropeptide-Y

**$OsO_4$**: Osmium-tetroxide

**PB**: Phosphate buffer

**PBS**: Phosphate buffered saline

**POMC**: Proopiomelanocortin

**rEr**: Rough surfaced endoplasmatic reticulum

**σ**: sigma

**SOM**: Somatostatin

**TB**: Tris buffer

**TBS**: Tris buffered saline

# Opioids and Opioid Receptors in the Rodent Hippocampus: Distribution, Synaptology, and Connection with the Local GABAergic Circuitry


## Summary

Endogenous opioid peptides have been implicated as inhibitory peptides in the central nervous system. In the hippocampal formation, however opioids have an excitatory effect on principal neurons. A number of physiological experiments have shown that this effect is elicited by a reduction of GABA-mediated inhibitory transmission. These opioid peptides are known to have a powerful effect on hippocampal inhibition. The possible endogenous source of these peptides and their relationship to inhibitory interneurons still remain to be identified.

In our studies we investigated the morphological and structural characteristics of the hippocampal opioidergic elements in a number of rodents widely used in laboratory experiments, their coexistence with other classical and non-classical transmitters, their target selectivity, and the distribution of their receptors at the light and electron microscopic level. Beside the most prominent mossy fibre system, we revealed opioid-containing varicose fiber-system innervating principal and non-principal neurons. These fibres mainly formed pericellular baskets around non-principal (rat, mouse) or principal cells (mouse, guinea-pig, hamster). The electron microscopic studies showed that part of the hippocampal opioidergic terminals also contain GABA and establish contact with dendrites and somata of inhibitory interneurons, whereas others are GABA-negative and make asymmetrical synapses.

We also examined the distribution of the kappa-type opioid receptor (KOR). The guinea-pig hippocampus did not exhibit KOR immunoreactivity, however KOR immunopositive neuronal cell bodies, proximal dendrites and occasionally glial processes surrounding neuronal somata were labelled in the hilus of the dentate gyrus and in the oriens layer of the CA1 area of the rat, hamster and gerbil. The shape of these interneurons was fusiform or multipolar. From among the known interneuron subtypes, somatostatin- (SOM) and neuropeptide Y- (NPY) immunoreactive hippocampal interneurons show similar morphology and distribution. With the help of double immunocytochemical labelling, we provided direct evidence that the majority of the interneurons are immunoreactive for SOM and/or NPY also express the κ-opioid receptor.

The target selectivity of opioid peptide containing terminals and co-expression of opioid receptors with other neuropeptides suggests a highly specific opioidergic control of hippocampal synaptic plasticity. The involvement of specific subsets of GABAergic neurons in hippocampal opiate effects provide evidence that endogenous opioids can indirectly modulate the activity of principal cells and play a crucial role in the normal and pathological activity of the hippocampal formation.

# Opioidok és receptoraik a rágcsáló hippocampusban: megoszlás, szinaptológia és kapcsolat a lokális GABAerg rendszerrel

## Összefoglalás

Az endogén opioidok elsősorban gátló hatású neurotranszmitterként váltak ismertté a központi idegrendszerben. A hippocampalis formatioban azonban hatásuk ezzel ellentétes, a különböző opioid hatású szerek és peptidek serkentőleg hatnak a principális sejtekre. Számos fiziológiai vizsgálat arra hívja fel a figyelmet, hogy a megfigyelt, opioidoknak tulajdonított serkentő hatás a helyi GABAerg gátló rendszer hatásfokának csökkentése révén alakul ki. A feltételezések szerint az endogén opioid peptidek igen hatásosan képesek befolyásolni a hippocampalis gátlási folyamatokat. Ezen gátlási szabályozás megértéséhez azonban ismernünk kell az opioid peptidek endogén forrását és a gátló interneuronokhoz fűződő viszonyukat.

Vizsgálataink során a hippocampalis opioid rendszer elemeinek morfológiai és strukturális jellemzőit tártuk fel számos, a laboratóriumi kísérletekben leggyakrabban használt rágcsáló fajban. Megállapítottuk az endogén opioid peptidek viszonyát más, klasszikus és nem klasszikus neurotranszmittert tartalmazó rendszerekhez, morfológiailag jellemeztük preferált célprofiljaikat, a szinapszisok specificitását valamint az egyik opioid receptor altípus, a kappa-opioid receptor megoszlását mind fény, mind elektronmikroszkópos szinten.

A moharost-rendszeren kívül – amely a legprominensebb opioid tartalmú pálya a hippocampusban – számos varikózus, opioid tartalmú rostot figyeltünk meg, amelyek mind principális, mind nem-principális sejtekkel alakítottak ki szinaptikus kapcsolatokat. Ezen rostok elsősorban pericelluláris kosár-szerű elrendeződésben vettek körül nem-principális (egér, patkány), ill. principális sejteket (egér, tengerimalac, aranyhörcsög). Az elektron-mikroszkópos vizsgálataink szerint az opioid immunpozitív rostok részben GABA tartalmúak és szimmetrikus szinaptikus kapcsolatot hoznak létre gátló interneuronok dendritjeivel és sejttesteivel, míg másik részükben nem mutatható ki a GABA és aszimmetrikus szinapszisokkal kapcsolódnak cél-profiljaikhoz.

További kísérleteink során a κ-opioid receptor (KOR) tartalmú idegelemek megoszlását vizsgáltuk, és azt találtuk, hogy interneuronok sejttestjein és proximális dendritjein, ill. helyenként az ezeket körülvevő glianyúlványokban fordul elő ez a receptor altípus, elsősorban a gyrus dentatus hilusában valamint a CA1 régió oriens rétegében, a tengerimalac kivételével az összes vizsgált rágcsálóban. Ezen interneuronok morfológiai jellemzői, valamint elhelyezkedésük arra engedett következtetni, hogy ezen sejtek a somatostatin (SOM) vagy neuropeptide-Y (NPY) tartalmú interneuronok egy alcsoportja lehet. Kettős immunhisztokémiai jelölési technikákkal bebizonyítottuk, hogy a SOM ill. NPY tartalmú interneuronok nagy része expresszálják a κ-opioid receptort.

Az opioid peptid tartalmú pályák szelektivitása és az opioid receptort expresszáló specifikus interneuron populációk jelenléte a hippocampusban alátámasztják azt a feltételezést, miszerint az opioidok alapvető mediátorai a hippocampalis szinaptikus plaszticitásnak. E hatásaikat a GABAerg interneuronok közvetítő szerepével, indirekt módon is kifejthetik a hippocampus normál és kóros aktivitásmintázatainak kialakításában.

# 1. INTRODUCTION

Opioids gain more and more attention nowadays not only in the field of neurobiology but also at the social level. The current interest in the drug problem in the society has stimulated investigators to search more for the causes and results of opiate addiction, as well as possible improvement and treatment. Therefore it is of utmost importance that we develop a better understanding of the mechanisms underlying behaviours controlled or modulated by the opiates.

In the central nervous system (CNS) neuropeptides are produced by neurons which also release classical neurotransmitters. Neurons – in an activity dependent manner – may release a mixture of transmitters, consisting of one or more small classical transmitters and one or more neuropeptides (Grobecker 1983). The functional consequence of this model is that interactions between neurotransmitters can take place at the level of biosynthesis and neuronal release. Endogenous opioid peptides coexist and are co-released with other (classical) transmitters. Based on this coexistence, it has been suggested that they play a role in neuromodulation and interneuronal communication and are, thus, involved in neural plasticity and in some pathological conditions (Palkovits 1995).

Nowadays, perhaps one of the most exciting research fields is the brain area of memory and learning - the hippocampus. Although we are still far from fully understanding how the opiates are involved in these processes, significant efforts have been made in the last decades. It seems that the opiate system is involved in learning and memory (Gallagher 1988). Understanding a system should always begin with the identification of its basic elements and its place in the neuronal circuitry. With the increasing tool-arsenal of neuroanatomy, we are able to precisely identify the fundamental elements of a system, even if it is as composite as the hippocampus.

## 1.1 The neuronal circuitry of the hippocampus

Of the various parts of the limbic system that are thought to be involved in learning and memory, the hippocampal formation has been attracting the greatest attention. This may be due to its anatomical organisation, with clearly distinct pathways that connect one group of neurones to the next.

These excitatory connections can be summarized as follows: information from neocortical association areas is passed on to the hippocampus via the perforant path, a major afferent pathway from the entorhinal cortex. This pathway terminates in the molecular layer of the dentate gyrus (DG), which contains, mainly, the dendrites of the granule cells, the principal cell type of the DG. The granule cells then send their axons (the mossy fibres) to innervate the hilus and CA3 field of the hippocampus. The mossy fibres run most prominently in the stratum lucidum, adjacent the pyramidal cell layer, and establish synapses with the proximal dendrites of CA3 pyramidal cells. The CA3 pyramidal neurons have connections, via the Schaffer collaterals, with the CA1 pyramidal neurons, which then project out of the hippocampus (Fig. 1). The major transmitter in each component of this pathway is an excitatory amino acid, glutamate.

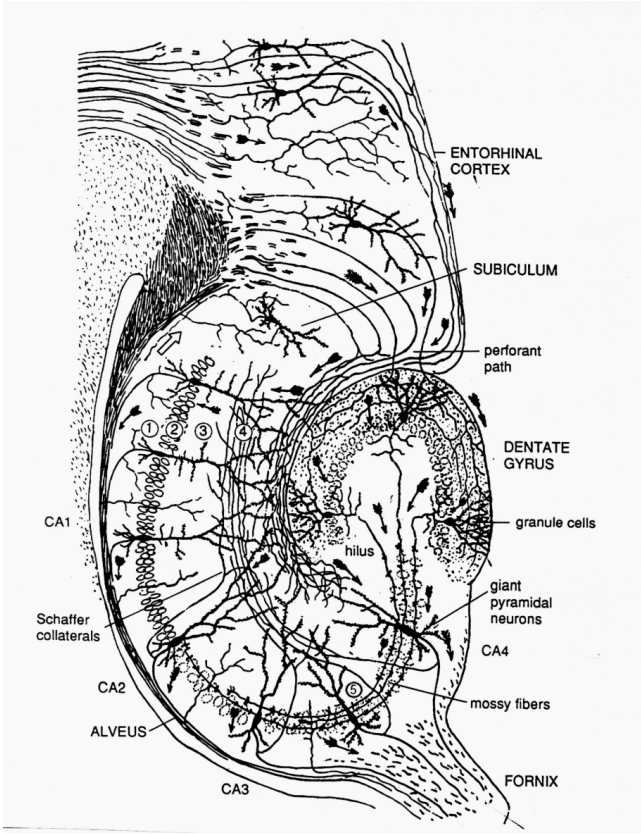


**Fig.1**. **Basic excitatory circuits and neuronal elements of the hippocampus**, modified from Ramón y Cajal, 1911. The hippocampal Cornu Ammonis (CA) can be divided into stratum oriens (1), stratum pyramidale (2); stratum radiatum (3), and stratum lacunosum moleculare. In the CA3 subfield only, the stratum lucidum (5) can be distinguished, which contains the axons of the granule cells, the mossy fibres. The perforant path input terminates in the outer two third of the molecular layer of the DG and in the lacunosum-moleculare layer of the CA subfields as shown. Granule cells in the dentate gyrus send mossy fibres to the CA3 pyramidal cells. The giant pyramidal cells in CA3 project to CA1 pyramidal cells via the Schaffer collaterals, to other CA3 pyramidal cells, and through the commissure via the alveus to the contralateral hippocampus. The commissural inputs and the various interneurons are not illustrated here.

In addition there is a monosynaptic projection from the entorhinal cortex directly to the CA1 and CA3 pyramidal cells which, while less dense than the perforant path, possibly shows greater basal level of activity (Morris and Johnston 1995). It has been proposed that the granule cells are not active tonically and that, under normal conditions, the stimulation of CA1 neuron is a result of activity from the direct entorhinal pathway. The DG granule cells and CA3 pyramidal neurons, however, do become active in certain circumstances, and use-dependent potentiation from the perforant, mossy-fibre and Schaffer-collateral projections would allow greater access to the hippocampus through the major, trisynaptic pathway. Those mechanism that have influence in the modulation of synaptic plasticity in these regions are, therefore, likely to be of great importance for hippocampal function. High frequency stimulation of the presynaptic fibres in each of these areas produces a long-lasting increase in the response of the postsynaptic activity. This phenomenon is called long term potentiation (LTP) and has been investigated extensively as being one of the neuronal

activities responsible for learning and memory (Bliss and Gardner-Medwin 1971; Bliss and Lomo 1973; Bliss et al. 1983; Racine et al. 1983). LTP is a long-lasting enhancement of synaptic transmission observed following brief trains of high-frequency stimulation. Because of its associative property, its persistent nature, and the fact that it is especially prominent in hippocampal circuits, LTP is also widely viewed as a neuronal model of learning and memory. Schaffer-collateral LTP appears to be the result of the stimulation of the NMDA class of glutamate receptors, which then elicit a number of pre- and postsynaptic modifications (Bliss and Collingridge 1993), however LTP in the mossy fibre synapses is not dependent on the activation of NMDA receptors.

There are numerous subcortical nuclei, which contain neurons that send their axons to the hippocampal formation. These pathways include GABAergic and cholinergic projection from the basal forebrain (medial septal area) (Leranth and Frotscher 1987; Freund and Antal 1988), serotonergic and non-serotonergic from the dorsal and median raphe nuclei (Lidov et al. 1980; Kosofsky and Molliver 1987), noradrenergic projection from the locus coeruleus (Frotscher and Leranth 1988), just to name a few. Common feature of these projections that they arise from a small number of neurons; however they are able to exert a powerful control over the activity patterns in the hippocampus and other cortical areas as well. This effective control is due to the selective innervation of GABAergic inhibitory interneurons. These interneurons can in turn regulate large population of principal cells. Therefore, the subcortical afferents further emphasize the importance of GABAergic inhibition in the neuronal circuitry of the hippocampus (Buzsaki 1984; Freund et al. 1990; Freund 1992).

From a neurochemical point of view the hippocampal neuronal circuits, in addition to glutamate, also contain a great deal of other neuroactive substances. In this respect, the hippocampal interneurons show a more obvious variability, although the excitatory pathways are also containing various co-transmitters and modulators.

The synaptic organisation of the hippocampus demonstrates that the surface domain of its principal cells is subdivided not only by extrinsic but also intrinsic inputs into several functional domains. Distinct GABAergic interneurons innervate the soma, axon initial segment or dendritic zones of pyramidal and granule cells (Halasy and Somogyi 1993a; Han et al. 1993; Buhl et al. 1994). The precise placement and target-selectivity of GABAergic synapses on the neuronal surface predicts distinct functional roles of interneurons. Many roles, including feed-forward, feed-back, tonic and lateral inhibition, etc., have been suggested, requiring distinct populations of GABAergic cells (for review see McBain and Fisahn 2001). To assign distinct functions to groups of interneurons requires a precise definition of their identity, based on functionally relevant criteria such as synaptic input/output characteristics and molecular markers. One of the most frequently used functional neuroanatomical feature for determining cell identity has been (i) the expression of neurochemical markers, such as neuroactive peptides and $Ca^{2+}$ -binding proteins, and (ii) their neurotransmitter receptor expression (for review see Freund and Buzsaki 1996).

GABAergic inhibitory cells provide perisomatic inhibition from synapses on pyramidal neuron somata (basket cells) and axon initial segments (axo-axonic cells), whereas others display circumscribed innervation of pyramidal neuron dendrites (Buhl et al. 1994; Cobb et al. 1995; Miles et al. 1996; Cobb et al. 1997). Because of this axonal segregation, these classes of interneurons exert distinct functional effects on pyramidal neurons in the hippocampus: namely the perisomatic inhibition affects the output of pyramidal cells, whereas dendritic inhibition inferences the input.

The hippocampal excitatory neuronal network is therefore under control of inhibitory interneurons that govern many of the firing characteristics of the pyramidal cell activity. From an anatomical point of view there are many different kinds of GABAergic interneurons (Gulyas et al. 1993; Halasy et al. 1996a; Vida et al. 1998). The dendritic architecture of these interneurons reflects the spatial availability of afferent inputs, their axonal arborisation vary with respect to targeting different domains on their postsynaptic target cells, and their axonal terminal field is precisely co-aligned with afferent excitatory inputs.

## 1.2 Opioids and opioid peptides – history and background

Opium, obtained from the plant, *Papaver somniferum* (Fig. 2) the most ancient psychoactive drug, having been used for at least 5000 years. In 1803 Sertürner isolated morphine, the opium's active ingredient. Since the mid 19th century, morphine has been used as an analgesic in medical practice. Scientific research on the opiates exploded in the early and mid 1970's, with the demonstration of specific opiate receptors and, shortly thereafter, the identification and purification of the enkephalins, the prototypical endogenous opioid ligand (Hughes 1975; Hughes et al. 1975a; Hughes et al. 1975b).

**Fig 2. Picture of a poppy plant** (*Papaver somniferum*) As one of the most ancient 'culture plants' Poppies have been a companion to humanity since its infancy during the upper Neolithic period. According to archeological studies, remains of Poppies have been found in prehistoric settlements in central Europe, Switzerland, Southern Germany and Southern England which date to at least 4000 BC. The so called latex is present within the tissues of the whole plant, but is most prolific and potent in the capsules prior to the ripening of the seed. This juice, commonly referred to as 'raw Opium', has been known about and utilized for thousands of years (after *www.poppies.org*).

The catalyst for this research was the discovery of specific receptor sites which interacted with morphine to produce analgesic effects (reduction in pain perception), then to be blocked by the opioid antagonist, naloxone (Snyder and Matthysse 1975; Kosterlitz and

Hughes 1977; Simon and Hiller 1978b; Snyder 1978). Their reasoning was the following: if the analgesic effects of morphine resulted from interactions with specific receptor sites, there must be endogenous opioid-like substances that bind to and activate these specific receptors located within neural tissue (Terenius and Wahlstrom 1975; Simon and Hiller 1978a). Upon performing bioassays coupled with receptor binding studies, investigators demonstrated that brain did, in fact, contain endogenous compounds that can act specifically on opioid receptors.

The wealth of information generated in the past quarter century on opioid receptors and their ligands is truly astounding (for review see Brownstein 1993). New studies on the biochemistry and pharmacology of this fundamental vertebrate regulatory system emerge weekly. The implications of understanding the opioid system are broad, ranging from medicine, both clinical and psychiatric, and to society at large, in terms of opiate addiction per se, and in terms of the opioid system's involvement in addiction in general.

## 1.3 Source of endogenous opioids

The various classes of endogenous opioids are all synthesized from three distinct precursor proteins, proopiomelanocortin (POMC), proenkephalin (proenkephalin A), and prodynorphin (proenkephalin B). The major site of production of POMC derived peptides is the anterior pituitary. Additionally, three distinct brain cell groups located in either the arcuate nucleus of the medial basal hypothalamus, or in the nucleus of the solitary tract and nucleus commissuralis have been determined to be responsible for production of POMC derived peptides. Proenkephalin derived peptides are distributed throughout several endocrine and CNS structures. Peripheral locations include the adrenal medulla and gastrointestinal tract, whereas, central distribution of proenkephalin derived peptides include the lateral hypothalamus, paraventricular hypothalamic nucleus, and periaqueductal gray area. Prodynorphin derived peptides can be found in the gut, posterior pituitary, and brain (Khachaturian et al. 1983; Akil et al. 1984). Each of these proteins contains specific sequences of both opioid and non-opioid peptides within their structures. Non-opioid peptides contained within POMC include the pituitary hormones corticotropin (ACTH), alfa-lipotropin, and melanocyte-stimulating hormone (alfa-MSH). One copy of leu-enkephalin and six copies of met-enkephalin have been identified in proenkephalin. Prodynorphin is a precursor which gives rise to many opioid peptides incorporating leu-enkephalin as a fragment within their sequence of amino acids. These include dynorphin A, dynorphin B (rimorphin), and neoendorphins (Khachaturian et al. 1983; Hollt 1992)

## 1.4 Opioid Receptor Classification

The existence of specific opiate receptors had long been supposed; but biochemical proof for their existence was lacking until 1973. In experiments using radiolabeled naloxone, an opioid receptor antagonist, Pert and his co-workers showed regional variation in high affinity and stereospecific binding. In addition, the binding affinity of opiates was correlated with physiological potency (Hollt 1986). Their data proved that there are specific opiate receptors; and since then, much progress has been made in understanding the pharmacology and structure of these receptors. Various types and sub-types of opioid receptors have been characterized and their genes cloned (Pert et al. 1974; Meng et al. 1993; Yasuda et al. 1993; Minami et al. 1994; Raynor et al. 1994; Simonin et al. 1994).

The existence of multiple opioid receptor types was first shown pharmacologically through the differential binding of morphine and its derivatives in chronic spinal dogs (Martin et al. 1976; Bunzow et al. 1995). These experiments, led to the conclusion that there were three opioid receptor types: μ (mu) for morphine, κ (kappa) for ketocyclazocine, and σ (sigma) for

SKF 10047. Two other receptors were proposed during the next few years: δ (delta), with a high affinity for the enkephalins, was first found in the mouse vas deferens; and ε (epsilon), believed to be the beta-endorphin binding site in the rat vas deferens Subsequent studies revealed that sigma is the only receptor mentioned above which is not antagonized by naloxone; it is therefore no longer considered an opioid receptor (Pert and Snyder 1973; Gilbert and Martin 1976). The μ-, κ-, and δ-opioid receptors are the most widely distributed, and consequently the most studied; these are considered to be the three major opioid receptor types (Pert and Snyder 1975).

## 1.5 Opioid Receptors: Signal Transduction and Structure

The role of membrane bound G-proteins (guanine-nucleotide binding proteins) in receptor-mediated signal transduction has been known for a long time. Substantial evidence indicates that G-proteins act as signal transducers by coupling receptors to effectors (for review see (Mansour et al. 1986). G-proteins are heterodimers, they consist of several subunits, each subunit having different function in signal transduction. The alfa-subunit binds to the receptor molecule, moreover it has the GTPase activity, and toxin sensitivity (cholera or pertussis), as well. Beta- and gamma-subunits form a heterodimer, which can modulate the activity of other effector molecules (e.g. enzymes and ion channels). According to the type of the alfa sububnit the mediated effect can be stimulation (Gαs) or inhibition (Gαi and Gαo) of adenylate cyclase (AC) (Gilman 1987).

The μ-, δ-, and κ-opioid receptors are all G-protein coupled (GPCR) and they have seven transmembrane domains (Spiegel 1987). The amino acid sequences of the different types show in an average 60% identity, with the extracellular domains being notably less similar than the transmembrane and intracellular domains (Reisine and Bell 1993). Potential sites for N-glycosylation have been found in the N-terminal (extracellular) domain of the three receptor types; also, a conserved cysteine residue in the C-terminal domain (intracellular) has been noted as a potential palmitoylation site in all three.

## 1.6 Opioid Receptor Modes of Action

All three subtypes have been shown to lead to the inhibition of adenylate cyclase (AC). Recent studies have suggested that in some cases opioid receptors may actually result in adenylate cyclase stimulation. Both effects on adenylate cyclase could be prevented by pertussis toxin, indicating that the opioid receptors are coupled to the Gi(o) protein (Minami and Satoh 1995).

The inhibitory effect of opioid receptors on cellular excitability and neurotransmitter release is due, at least in part, to the *inhibition of voltage dependent calcium channels*. Voltage induced calcium influx at axon terminals is involved in the stimulation of neurotransmitter release. It has been shown in vitro that activation of μ-, δ-, and κ-opioid receptors leads to a reduction in voltage-dependent calcium currents in a number of cell preparations. Pertussis toxin blocks this reduction, again showing Gi(o) involvement. A cell hyperpolarization resulting from an influx of potassium ions (Minami and Satoh 1995) is also important in the intracellular actions of opioid receptor stimulation. This pertussis toxin sensitive effect has been shown for all three major opioid receptor types.

Activation of the opioid receptors has also been shown to release calcium from internal stores. This occurs because the stimulation of the opioid receptors leads to the stimulation of phospholipase C by the beta-gamma G-protein subunit, which then leads to inositol phosphate formation, and $Ca^{2+}$ release. As mentioned above, opioid receptor activation usually leads to an inhibitory response (Satoh and Minami 1995; Murthy and Makhlouf 1996); however, the

opioid stimulated internal calcium release suggests that the opioid receptor may mediate excitatory effects, as well (Wu et al. 1998).

## 1.7 Opioid Receptor Distributions

Opioid receptors are widely distributed in the CNS. They are found in the dorsal horn, trigeminal nerve, locus coeruleus, nucleus tractus solitarius and the brain stem, area postrema, superior colliculus, posterior pituitary, hypothalamus, amygdala, nucleus accumbens, and striatum, just to name the most important ones. The different receptor subtypes show regional variation. μ-opioid receptors are found in cortical layers I and IV, the caudate putamen, amygdala, thalamus, periaqueductal gray matter, median raphe, hypothalamus, and hippocampus. δ-opioid receptors are found in the cortical layers, II, III, and V, as well as in the caudate putamen, amygdala, pontine and septal nuclei, olfactory bulbs and tubercle. Kappa-receptors exist in cortical layers V and VI, caudate putamen, amygdala, thalamus, hypothalamus, substantia nigra, nucleus accumbens, nucleus tractus solitarius, parabrachial nucleus, and zona incerta (Mansour et al. 1986; Mansour et al. 1987; Minami and Satoh 1995). These are specific subsets of opioid receptors attributed to certain neuron types.

## 1.8 Opioids in the Hippocampus: Anatomy and Physiology

A number of studies are dealing with the origin and distribution of opioid-containing nerve fibres, cells and paths in the hippocampus (Tielen et al. 1982; Roberts et al. 1984; Mansour et al. 1994a). Enkephalin immunoreactivity in the rat hippocampus was found in the hilus and in a narrow zone in the suprapyramidal part of the CA3 region outlining the mossy fibre system. The intensity of labelling increased in septotemporal direction. Apart from the dentate granule cells giving rise to the mossy fibre system, some interneurons in the rat hippocampus also show enkephalin immunoreactivity (Gall et al. 1981; Fredens et al. 1984) and enk- or dyn- immunopositive fibres from extrinsic sources terminating in the distal part of the dentate molecular layer and stratum lacunosum-moleculare of the CA1 region are the lateral part of the entorhinal cortex, the lateral temporo-ammonic tract (Chavkin et al. 1985; Blasco-Ibanez et al. 1998). The opioid immunoreactive elements were widely studied in various species (Gall et al. 1981; Corrigall 1983; Mcginty et al. 1983; Fredens et al. 1984; Roberts et al. 1984; Merchenthaler et al. 1986; Holm et al. 1993), and their presence, amount and distribution were established to be species-, or even strain-specific (McLean et al. 1987; van Daal et al. 1989). However a detailed comparative approach has not been performed so far. Since endogenous opioids play a regulatory role within the hippocampal formation both in physiological and pathological conditions, it is important to know the distribution of the opioidergic elements, cells and pathways and identify their exact anatomical position and origin. From this anatomical information we can better understand the functional role of the endogenous opioids in the hippocampal formation.

Among others opioid peptides were shown to exert a profound effect on hippocampal synaptic plasticity and therefore on LTP, as well (van Daal et al. 1989; Martinez and Derrick 1996). The involvement of opioids and opioid receptors in neurological disorders, such as epilepsy, stroke, Alzheimer's disease etc. has also been reported (Hiller et al. 1987; Morris and Johnston 1995). Powerful opioid effects have been demonstrated on the inhibitory processes of the hippocampus in electrophysiological, pharmacological, and behavioural studies (Siggins and Zieglgansberger 1981; Sagratella et al. 1996; Sandin et al. 1998; Svoboda et al. 1999). So it is interesting that the hippocampal formation contains a relatively low amount of opioid receptors, and the level of endogenous opioid ligands is also low, compared to other brain areas (Nicoll et al. 1980). Moreover endogenous opioids

decrease the excitability of neurons in most areas of the central nervous system (CNS), in the hippocampal formation; however, opioids have an excitatory effect on principal neurons. A number of physiological experiments have shown that this effect is elicited by a reduction in GABA-mediated inhibitory transmission (Corrigall 1983; Cohen et al. 1992). Moreover, certain opioid agonists and the stimulation of paths containing opioids modify the synchronized excitation of the hippocampal pyramidal cells, and the endogenous opioids can regulate the plasticity of the inhibitory connections by their disinhibitory effects (Zieglgansberger et al. 1979).

Unfortunately, very little was known about the role of the GABAergic inhibitory cells in the above mentioned regulation. GABAergic inhibitory neurons represent only 6-12% of the neurons of the hippocampus and the DG (Woodson et al. 1989; Xie and Lewis 1995); nevertheless, these cells can regulate large proportion of principal cells by their extensive local axonal (Gulyas et al. 1993; Han et al. 1993; Aika et al. 1994; Buhl et al. 1994). A certain decrease in GABAergic inhibition may result in epileptic bursts (Schwartzkroin and Prince 1980; Herron et al. 1985; Dingledine et al. 1986; Freund and Buzsaki 1996)Very little was known about the molecular and structural basis of the effects of opioid peptides on the GABAergic cells, although these might play a role in epileptiform seizures by increasing the excitability of principal cells.

The opioid site of action, the opioid receptors, are also few in number, however in many respect they are strategically important in the normal and pathological function of the hippocampus. Powerful opioid effects have been demonstrated on the inhibitory processes of the hippocampus and morphological evidence supporting the synaptic connection between opioidergic axon terminals and GABAergic interneurons in the rat hippocampus has also been provided (Commons and Milner 1996; Blasco-Ibanez et al. 1998; Cossart et al. 2001).

The distribution of opioid receptors throughout the hippocampal formation seems to be very varied, depending on the species and the method used for their visualization (Mansour et al. 1987; Fuzesi et al. 1997). In the CA1 region of the hippocampus the μ-opiate receptor was shown to be present on the somata and dendrites of GABAergic, non-pyramidal cells (McLean et al. 1987). δ-opioid receptors were also demonstrated in interneurons and pyramidal cells in the rat hippocampus (Bausch et al. 1995a), and on GABAergic axon terminals surrounding the somata of pyramidal neurons (Commons and Milner 1997). Moreover, Svoboda and his co-workers demonstrated (Bausch et al. 1995b) that opioid receptor expression defines morphologically distinct classes of hippocampal interneurons, mediating diverse types of inhibition to principal cells by innervating different domains of their cell surface. It was also shown, that each type of the opioid receptors share common effector mechanisms (Svoboda et al. 1999), therefore all types of opioid receptor mediate inhibitory effect to their postsynaptic targets.

κ-receptor is was shown to be the densest in and around the principal cell layers in the rat hippocampus using receptor autoradiography. Kappa receptors were also demonstrated by immunocytochemistry in the brain and spinal cord of rat and guinea-pig in neuronal somata and dendrites, and occasionally in axons in many brain areas (Svoboda et al. 1999). Drake et al. used a rabbit antibody raised against a synthetic peptide from the carboxyl terminus of the cloned κ receptor in the guinea-pig hippocampus (Arvidsson et al. 1995). The labeling was confined to unmyelinated axons and axon terminals forming asymmetric synapses. Another monoclonal antibody (mAb-KA8) raised against a frog brain κ receptor preparate recognizing selectively the κ receptor with preference for the $kappa_2$ subtype, was produced and characterized by Maderspach and her co-workers (Drake et al. 1996). The antibody was used

in the young rat and chicken brain revealing kappa receptor immunoreactivity associated to glial elements, postsynaptic densities of synapses and microtubules of dendrites (Maderspach et al. 1991).

Kappa opioid effects were found to be principally inhibitory in the hippocampus (Maderspach et al. 1995). Kappa opioids, such as dynorphin, can inhibit excitatory neurotransmission in the hippocampus via activation of κ opioid receptors, in addition, KOR agonists are highly effective against limbic seizures (Wagner et al. 1993). KOR receptor-evoked cellular responses were demonstrated to inhibit glutamate release at mossy fibre synapses (Simonato and Romualdi 1996).

## 2. AIMES AND SCOPES

The involvement of the opioids in learning and memory processes, moreover their unique disinhibitory effect in the hippocampal formation raised our attention. We supposed that similarly to the subcortical afferents, opioids may target interneurons, and they exert excitation on the hippocampal principal cells via the inhibition of certain types of interneurons. From this point of view the distribution and fine structural characteristics of the endogenous opioids and their receptors have not been thoroughly studied yet. Therefore, the aims of our studies were:

- to survey systematically the occurrence of immunocytochemically detectable opioid peptides in the hippocampal formation of rodents widely used in laboratory experiments;
- to reveal the ultrastucture of opioiderg varicosities and synapses;
- to establish their target profile, with specific attention to GABAergic and non-GABAergic postsynaptic targets;
- to clarify and compare the distribution and species-specificity of the κ-type opioid receptor in the hippocampus of four rodent species based on light microscopic observation;
- to describe the cellular and subcellular localization and the fine structure of the cells expressing κ-opioid receptor in the rat;
- to provide direct morphological evidence with the co-localization of the receptor and neuropeptides, that κ opioid receptor is associated to a distinct subset of inhibitory interneurons.

# 3. MATERIALS AND METHODS

The experiments were carried out on 22 adult Wistar rats (*Rattus norvegicus*), eight domestic mice (*Mus musculus*), seven golden hamsters (*Mesocricetus auratus*), seven guinea-pigs (*Cavia porcellus*) and six gerbils (*Meriones unguiculatus)* of both sexes. Animal housing, and all experimental procedures, followed the relevant provisions and general recommendations of the current Hungarian Animal Protection legislation. The experiments were approved by a local Animal Ethics Committee.

## *3.1 Tissue preparation*

### *3.1.1 Perfusion*

The animals were perfused under pentobarbital or Ketamin/Xylazin anaesthesia through the left ventricle of the heart with 4% paraformaldehyde, 0.1% glutaraldehyde, and/or 3.75% acrolein, and 5-15% of a saturated solution of picric acid in 0.1 M phosphate buffer (PB) (pH 7.4; 500 ml/rat). The fixation was preceded by a short rinse with NaCl (50-100 ml/rat), not more than 3 min.

### *3.1.2 Post-fixation*

The brain was taken out immediately after the perfusion and stored for 1-2 h in the same fixative (4$^{o}$C) under agitation. Then 30-70 μm thick vibratome sections were cut from the hippocampus at the coronal plane.

## *3.2 Pre-embedding immunohystochemistry*

### *3.2.1 Preembedding immunocytochemistry for light- and electronmicroscopy*

Sections were kept in 10, 20 and 30% saccharose in 0.1 M PB for cryoprotection, and then freeze-thawed in liquid nitrogen in order to increase the penetration of the antibodies. This was followed by three washes in 0.1 M PB, and treatment of 1% Na borohydride for 30 min if the fixative contained glutraldehyde. After a short rinse in three changes of 0.1 M TRIS-buffered saline (TBS), non-specific immunoreactivity was blocked with 20% normal goat serum for 45 min at room temperature.
The sections were incubated with the following primary antibodies:

- supernatant of the KA8 hybridoma cell line diluted in 1:2 in (TBS)(Gannon and Terrian 1992);
- polyclonal anti-5-leucine-enkephalin, Amersham, Code 7;
- polyclonal anti-5-metionine-enkephalin, Incstar Corp., Code 519;
- polyclonal anti-5-met-enkephalin antibody, Peninsula Laboratories, Inc. Code IHC 8602;
- polyclonal anti-5-leu-enkephalin antibody, Peninsula Laboratories, Inc. Code IHC 8601;
- polyclonal anti-dynorphin-A antibody, Peninsula Laboratories, Inc. Code IHC 8730;
- polyclonal dynorphin-B antibody, Peninsula Laboratories, Inc. Code IHC 8731

for 48 h at 4$^{o}$C. Following several rinses in TBS, biotinylated rabbit anti-mouse (DAKO, 1:50) or goat anti-rabbit IgG (Vector) was used as a secondary antibody for 5 h at room

temperature. This was followed by several rinses in 1% NGS and incubation in Avidin Biotin peroxidase Complex (1:100 for both components, Vectastain, Elite Kit) overnight. The immunopositive structures were visualised with 3,3-diaminobenzidine-tetrahydrochloride (DAB) after numerous washes in 0.05 M Tris buffer at pH 7.6. Sections for light microscopy were then mounted on gelatine-coated glass slides, dehydrated in ascending ethanol series, kept in xylene, and covered with cover slips in DPX (Fluka). Sections for electron microscopy were post-fixed in 1% $OsO_4$ for 30-45 min and contrasted with 70% ethanol saturated with uranyl acetate. After complete dehydration in ascending ethanol series and propylene oxide, sections were mounted on slides in Durcupan ACM resin (Fluka). The sections were viewed under a light microscope, areas of interest were selected and re-embedded for ultrathin sectioning. Ultrathin serial sections were cut, mounted on single-slot Formwar-coated grids, contrasted with lead citrate, examined and photographs were taken in a JEOL 100 C electron microscope

### *3.2.2 Pre-embedding double immunocytochemistry for co-localisation of neuropeptides and the KOR*

was performed on free-floating sections, as described previously, combining the silver intensification of the DAB precipitation (Maderspach et al. 1991) and the silver intensification of colloidal gold (Liposits et al. 1990) for the visualization of the immunoreactions. These methods gave the best results in following combinations: on sections for the co-localization of SOM and the receptor, κ opioid receptors (monoclonal anti-kappa opioid receptor antibody; Pharmingen, USA, Code 60501A) were first visualized with the silver-intensified colloidal gold (AURION, The Netherlands, Code 110.022; AURION, Code No.: 500.011) method, then SOM- (polyclonal anti-somatostatin antiserum; DiaSorin, Code 20067) immunohistochemistry was performed and visualized with the conventional ABC-DAB visualization method, as described above.

The silver enhancement reaction is based on the gold particle catalysed reduction of $Ag^+$ to metallic silver using photographic developing compounds as electron source. The silver intensification of the colloidal gold was done according to the instructions of manufacturers. Briefly: after the incubation in the primary antiserum sections were washed in TBS and non-specific background labelling (e.g.: residual aldehyde activity, multipoint hydrophobic moieties and positive charges with high molecular weight compounds, and hydrophilic interactions with competing molecules in the incubation and washing solutions) was reduced by a blocking solution containing 0.8% BSA, 0.1% gelatine for 30 min. Diluted in the same blocking solution the secondary antiserum was put on the sections (Amersham GAR-gold, 10 nm) in 1:50 dilution and incubated for 6 hours in room temperature. Thereafter, sections were washed twice with TBS for 10 min each, postfixed in 2% glutaraldehyde in TBS for 10 min and washed with distilled water. Using the AURION R-Gent SE-LM silver enhancement reagents (Product code: 500.011) gold particles were developed in size at room temperature in a 1:1 mixture of the developer and enhancer. The intensification process was performed under continuous light microscopic control. When the enhancement was complete (i.e. the silver particles became visible in the light microscope without background labeling) the specimens were washed extensively with distilled water and a second immunoperoxidase procedure could be performed.

NPY (polyclonal anti-NPY antiserum; DiaSorin, Code No.: 22940) was visualized with the DAB-silver intensification method followed by KOR immunohistochemistry using common DAB reaction for the visualization.

The best dual immuno-labelling result was achieved by using different visualisation methods for the co-localisation of the neuropeptides and the receptor. In case of the somatostatin, the DAB immunoprecipitate did not alter the amount and quality of the previously bound immunogold particles. In this way the co-localisation of the KOR and the neuropeptide SOM could be easily demonstrated. However, the DAB precipitation of the NPY immunoreaction often removed the previously attached gold particles that resulted in a less sensitive and selective co-localisation of the two markers. The distinctive feature of the DAB immunoprecipitation of the NPY immunoreaction (i.e. the brown, patch-like grains in the cell body and in the axon) made it possible to silver intensify the end product of the first immunoreaction turning the DAB-polymer into a black immunoprecipitate. The selective intensification of the DAB-chromogen increased the sensitivity by the modification of the chromogen itself (van de Plas and Leunissen 1993). The silver postintensified black DAB grains could be easily distinguished from the non-intensified brown DAB. In this case the second marker was the KOR, labelled with the brown DAB.

Briefly, the silver-gold intensification of the DAB-chromogen was performed as follows: The immunostained vibratome sections were placed into a 10% solution of thioglycolic acid for 2 h. Thereafter, they were rinsed in a 2% sodium acetate solution for 2 h (8 changes, 15 min in each). The sections were then placed into a special silver-containing developer (for details of compounds see Liposits 1990) until the brown colour of the DAB labelled structures turned black. The progress of development was checked under light microscopic control. The reaction was terminated by transferring the sections into 1% acetic acid solution for 5 min. Thereafter, the sections were rinsed in 2% sodium acetate again for 10 min. Sections were placed then into 0.05% gold chloride solution ($HAuCl_4x4H_2O$, dissolved in distilled water and used at $4^oC$) for 10 min to tone the silver. The gold chloride was removed by rinsing the sections in 2% sodium acetate for 10 min. Finally sections were placed into a 3% sodium thiosulfate solution for 10 min. to remove any unbound silver and rinsed in sodium acetate and in PBS.

## *3.3 Post-embedding immunogold labeling*

### *3.3.1 Postembedding immunocytochemical (immunogold) demonstration of GABA in resin embedded ultrathin sections.*

Ultrathin sections from Durcupan (Fluka) resin embedded blocks, which were previously reacted to identify leu-enk or met-enk with preembedding immunocytochemistry, were mounted on single-slot Formwar-coated grids. Sections were never allowed to dry off during procedure. Grids were placed on drops on parafilm in a wet chamber (i.e. Petri dish). Nickel or gold grids were used, since acids used for etching and osmium removal interact with copper grids. The Durcupan resin was etched from the surface of the sections with 1% periodic acid followed by three dips in Millipore filtered distilled water. Thereafter, removing of osmium with 2% sodium metaperiodate and three dips in filtered distilled water was succeeded. After washing in TBS, the sections were blocked in 1% ovalbumin for 30 min and dipped in distilled water twice. This was followed by incubation with the primary antiserum to GABA, Code No. 9 (Gallyas et al. 1982) at a dilution of 1:2000 in TBS containing 1% normal goat serum (NGS) at least for 90 min and two dips in distilled water. Before incubating the sections with the 1 nm colloidal gold-coated IgG protein (dilution 1:200, Nanoprobes, USA) they were incubated with Gold-GAR buffer (Tris buffer containing 0.5% Tween 20 detergent and 1% bovine serum albumin (BSA)). The 1 nm gold particles bound to the tissue were visualized with silver intensification, according to the manufacturer's instructions

(Nanoprobes, USA). After immunostaining sections were contrasted with aqueous solution of uranyl acetate for 30 min and lead citrate for 2 min.

In control experiments the primary antibodies were omitted from the incubation solution, and replaced with normal serum of the animal in which the primary antiserum was raised. In such sections no immunostaining was observed.

# 4. RESULTS

In our series of experiments we first made a comparative study on the hippocampi of several species widely used in laboratory studies (Wistar rats (*Rattus norvegicus*), domestic mice (*Mus musculus*), golden hamsters (*Mesocricetus auratus*), guinea-pigs (*Cavia porcellus*) and gerbils (*Meriones unguiculatus*)) in order to reveal the distribution and localization of the opioidergic elements in the hippocampus of different rodent species and to identify specific opioid-containing synaptic systems.

## 4.1 Distribution of the opioidergic elements in the hippocampus of the rodents

Four opioid peptides were localized in the hippocampus of the studied species, at least in some sublayers of the hippocampal formation (see Table1.). In our experiments immunopositive cell bodies were rarely observed. In agreement with previous data, the most obvious opioid immunoreactivity was localized in the mossy fibre system in the hippocampi of the studied species (rat, mouse, hamster, and guinea-pig). In addition, a varicose immunoreactive fibre system was observed in several regions of the hippocampi, well distinguishable from the mossy fibre system.

The axons of this network were thinner and their varicosities smaller - though obvious - than those of the mossy fibres. The following differences were found in the distribution of immunopositive elements:

### *4.1.1 Enkephalin peptides*

Using primary antisera from various sources, the distribution of the two enkephalin peptides was found to be very similar within a species; however the staining intensity of met-enk was generally stronger than that of leu-enk.
In the mouse and rat hippocampus, the intensively labelled mossy fibre system (App 1. Fig. 1A) was the most obvious, circumscribing the hilus of the dentate gyrus and the stratum lucidum of the CA3 area. The immunolabelling was very dense in some of the mossy fibres (App. 1. Fig. 1B).
The non-mossy enk-immunoreactive varicose fibre system was most pronounced in strata radiatum and lacunosum-moleculare of the CA1 and CA3 region both in the rat (App. 1 Fig. 1C) and mouse (App. 1. Fig. 1E). The axons seemed either to follow immunonegative dendrites, or encircled blood vessels. Enk-immunopositive axons often formed pericellular baskets around immunonegative - presumably interneuronal - cell bodies at the border of strata radiatum and lacunosum-moleculare (Fig. 3A-C). The pyramidal layer did not contain immunoreactive fibres in the CA1 region of the rat, and only few boutons were detected in this layer in the mouse as well. The density of the varicose enkephalinergic fibre system increased rostrocaudally (App 1. Fig. 1E).

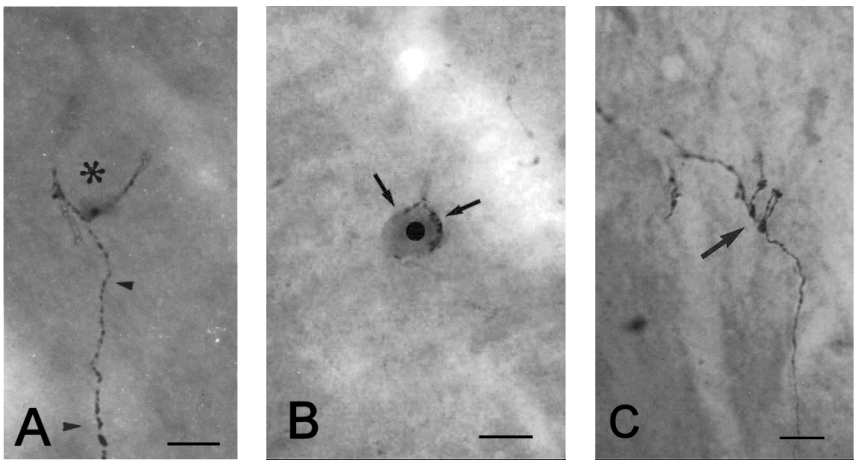


**Fig. 3. A, B.** The leu-enk-immunopositive boutons form pericellular baskets (arrows) around immunonegative non-pyramidal somata (asterisk or black dot) in the CA1 stratum lacunosum-moleculare of the rat hippocampus. **C.** Met-enk-immunoreactive varicose fibres (arrow) in the CA1 stratum radiatum of the rat hippocampus. Scale bar: A: 10μm; B: 20 μm; C: 10 μm.

In the hippocampus of the hamster and guinea-pig, there was only a weak mossy-fibre labelling detected with met-enk, and a moderate staining with leu-enk. Enk-immunopositive elements occurred in every single layer of the hamster hippocampus. Varicose enk-immunoreactive fibres were localized both in the CA1 and CA3 regions. Weakly leu-enk-immunopositive cell bodies and pericapillary enkephalinergic boutons were also found in the stratum radiatum (App. 1 Fig. 2C). The somata of pyramidal cells were also surrounded by enkephalinergic boutons. This arrangement was most obvious in the hamster, and detected only with leu-enk in the guinea-pig. In the hilus of the guinea pig, enkephalinergic mossy fibres were found in a narrow band under the granule cell layer (plexiform layer). This arrangement is different from those described in other species earlier. These fibres were presumably the collaterals of the granule cell axons. The intensity of the immunoreactivity increased from the hilus to the CA3, along the mossy fibre system. Non-pyramidal neurons were also occasionally surrounded by enkephalinergic boutons in the guinea-pig hippocampus. In the stratum radiatum of the CA1 region a unique arrangement of enk-immunopositive axons was observed: these varicose immunoreactive axons run parallel to the radiatum-pyramidale border, and with each other in various focus-depths, thus they met perpendicularly the apical dendrites of the pyramidal cells (App. 1 Fig. 2B). This arrangement was present exclusively in the guinea pig hippocampus.

### *4.1.2 Dynorphin peptides*

Dynorphins were detected as the dominant opioids in the mossy fibre system of guinea pig and hamster. In the guinea-pig dyn-B gave the strongest immunoreaction (Fig. 4B), whereas in the hamster dyn-A staining was more pronounced. Similarly to the staining pattern with enkephalin, the immunopositive mossy fibres formed a very dense narrow band under the granule cell layer in the dentate gyrus of the guinea pig hippocampus, and a more diffusely stained central part was distinguishable in the hilus (Fig. 4B). In both species varicose nerve fibres entered the principal cell layers (including granule-, as well as CA3 pyramidal cells), very presumably being recurrent collaterals of the mossy fibres, and formed pericellular baskets around them (Fig. 4A). Apart from the mossy fibre system, each layers of the hippocampus contained few dyn-B-immunoreactive axons with large varicosities which could

be followed from the stratum oriens up to the stratum lacunosum-moleculare in the hamster hippocampus.

In the rat and mouse hippocampus neither dyn-A nor dyn-B showed strong immunoreactivity. These peptides were present mainly in the mossy fibre system. The hilus of the gyrus dentatus was evenly filled by immunopositive elements. At the border of granule cells and hilus there were no immunopositive fibres and no pericellular arrangement was observed in the CA1area unlike in the hamster and guinea pig. However a weak perinuclear labelling was observed in the somata of the granule cells.

In the fields of str. radiatum and str. lacunosum-moleculare none of the dynorphin peptides showed immunoreactivity neither in the rat, nor in the mouse hippocampus. Occassionally, dyn-positive fibres were detected in the stratum oriens and some dyn-A-immunoreactive bouton-like punctae were visible in the stratum pyramidale of the CA1 region in the mouse hippocampus.

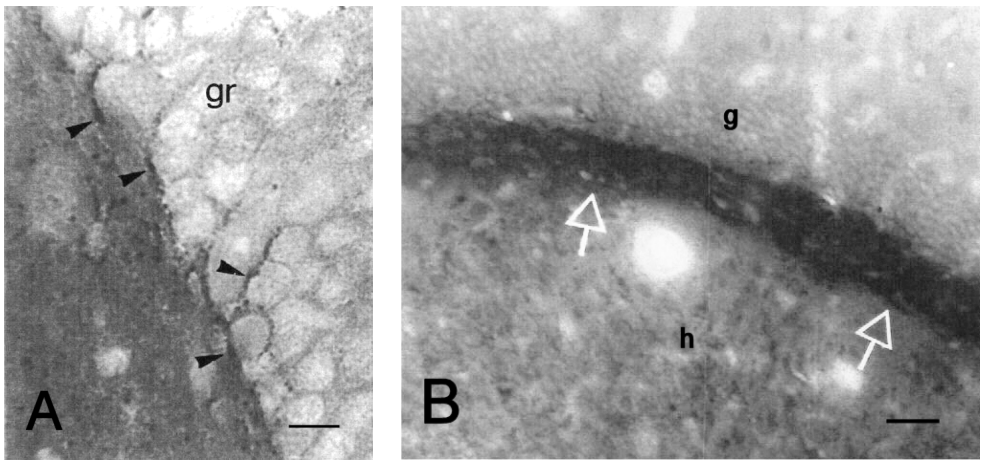


**Fig 4. Characteristics of the hippocampal opioidergic system of the golden hamster and guinea-pig. A**: Dyn-A-immunopositive varicose fibres enter the granule cell layer (g) and form pericellular baskets around the immunonegative somata of granule cells (asterisks) in the dentate gyrus of the hamster hippocampus. h=hilus. **B**. The arrangement of dyn-B-immunopositive mossy fibre system in the dentate gyrus of the guinea-pig hippocampus. Note the strongly immunopositive narrow strip (arrows) under the granule cell layer (g), and the more diffusely stained central part in the hilus (h). Scale bars: A: 18 μm; B: 110 μm.

These results are based on light microscopic observations. In order to reveal the fine-structure of the opioid immunopositive varicosities and synapses and to establish the sites of their synaptic connections with GABAergic and non-GABAergic postsynaptic targets we carried out ultrastructural investigations. For this study we chose the rat, being the most studied laboratory animal. Moreover, previous studies on the GABAergic interneuronal system (Somogyi et al. 1985; Halasy et al. 1996a) provided data about the local GABAergic circuitry of the rat hippocampus, as well.

**Table 1. Occurrence of opioid peptides in the hippocampal formation of four species**

| Species | Area | Opioid peptide | | | |
|---|---|---|---|---|---|
| | | Met-enk | Leu-enk | Dyn-A | Dyn-B |
| **Rat** | DG | ** | * | ** | * |
| | CA1 oriens | * | * | * | * |
| | CA1 pyr | n | n | n | n |
| | CA1 rad | ** | * | n | n |
| | CA1 lm | ** | * | n | n |
| | CA3 oriens | * | * | * | * |
| | CA3 pyr | n | n | n | n |
| | CA3 luc | ** | * | ** | ** |
| | CA3 rad | * | * | n | n |
| **Mouse** | DG | *** | * | * | * |
| | CA1 oriens | * | n | * | n |
| | CA1 pyr | n | n | * | * |
| | CA1 rad | * | * | * | n |
| | CA1 lm | ** | * | * | n |
| | CA3 oriens | * | n | * | n |
| | CA3 pyr | * | * | n | n |
| | CA3 luc | ** | * | * | n |
| | CA3 rad | * | n | n | n |
| **Hamster** | DG | * | ** | *** | ** |
| | CA1 oriens | * | * | ** | * |
| | CA1 pyr | * | * | * | * |
| | CA1 rad | ** | * | * | * |
| | CA1 lm | ** | ** | ** | ** |
| | CA3 oriens | * | * | * | * |
| | CA3 pyr | ** | ** | * | * |
| | CA3 luc | ** | ** | ** | ** |
| | CA3 rad | * | * | * | * |
| **Guinea pig** | DG | * | ** | * | *** |
| | CA1 oriens | * | * | * | * |
| | CA1 pyr | n | * | n | n |
| | CA1 rad | * | * | n | * |
| | CA1 lm | n | n | n | n |
| | CA3 oriens | * | * | * | n |
| | CA3 pyr | n | * | * | n |
| | CA3 luc | * | * | * | *** |
| | CA3 rad | n | * | * | n |

*Abbreviations: DG=dentate gyrus; pyr=stratum pyramidale; rad=stratum radiatum; lm=stratum lacunosum-moleculare; n=not detected; *=few; **=moderate; ***=many.*

## 4.2 Opioid-GABA connection in the rat hippocampus

The localisation and distribution of the opioid peptide immunopositive axons in strata oriens, radiatum, and lacunosum moleculare of the CA1 area in the light microscope suggested, that these axons may innervate interneurons. In order to prove the existence of

real opioid-GABA synapses, we re-embedded areas containing these opioid immunopositive axons for electron microscopy.

In agreement with our previous observation, enk-immunoreactivity was not only localized in the mossy fibre system, but also in clearly distinguishable varicose axons. The varicose axons were most abundant at the border of CA2/CA3 region. In the stratum radiatum of the CA1 area relatively few fibres showed immunopositivity. In the stratum lacunosum-moleculare of the CA1 region immunopositive axons often encircled immunonegative cell bodies forming pericellular baskets (Fig. 3A,B).

According to the electron microscopic examinations the varicosities (boutons) showing enk-like immunoreactivity made type II. (symmetric) synapses with the surrounding immunonegative elements (Fig. 5; Fig 6.). The boutons contained numerous agranular vesicles (average diameter about 50 nm) and a few large dense core vesicles (average diameter about 100 nm). The immunoprecipitation was not uniformly distributed: the dense core vesicles were always more strongly labelled than the agranular vesicles (Fig. 6 A,C). The immunopositive synaptic boutons usually contained 1-3 mitochondria.

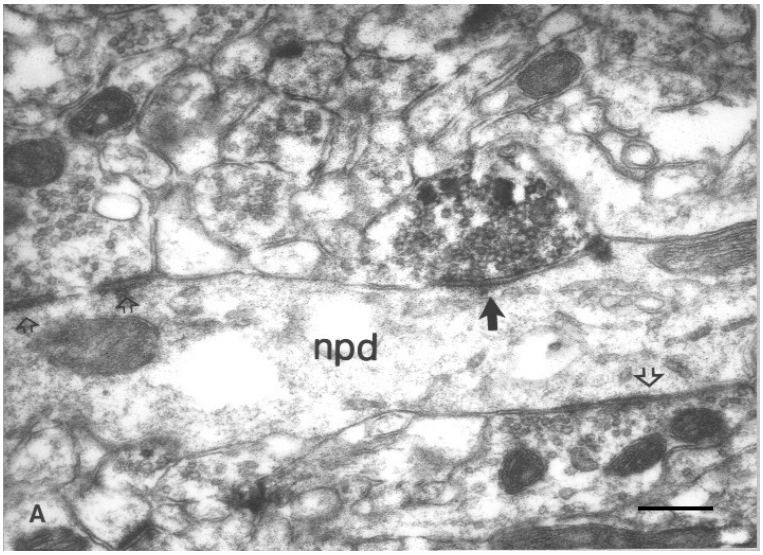


**Fig. 5. Electron micrograph of a leu-enk immunopositive synaptic varicosity at the border of stratum lacunosum-moleculare and radiatum of the CA1 region.** Axodendritic synapse (arrow) between an immunopositive terminal and a non-pyramidal dendrite (npd). Open arrows show two non-labelled axon terminals converging onto the same postsynaptic target. Scale bar: 0.25 μm.

The targets of enk-immunoreactive synaptic varicosities were cell bodies, dendritic shafts and dendritic spines both in strata radiatum and lacunosum-moleculare. The position of the postsynaptic cells in the hippocampus and their morphological features (abundant rough endoplasmatic reticulum (RER) in the perikaryon, indented nucleus) were identical to those of inhibitory GABAergic interneurons. There were a number of dendrites with non-spiny, smooth surfaces among the postsynaptic targets, on which numerous other, non-immunoreactive synapses converged (Fig. 5, Fig. 6A). These morphological features are also characteristic of inhibitory interneuron dendrites.

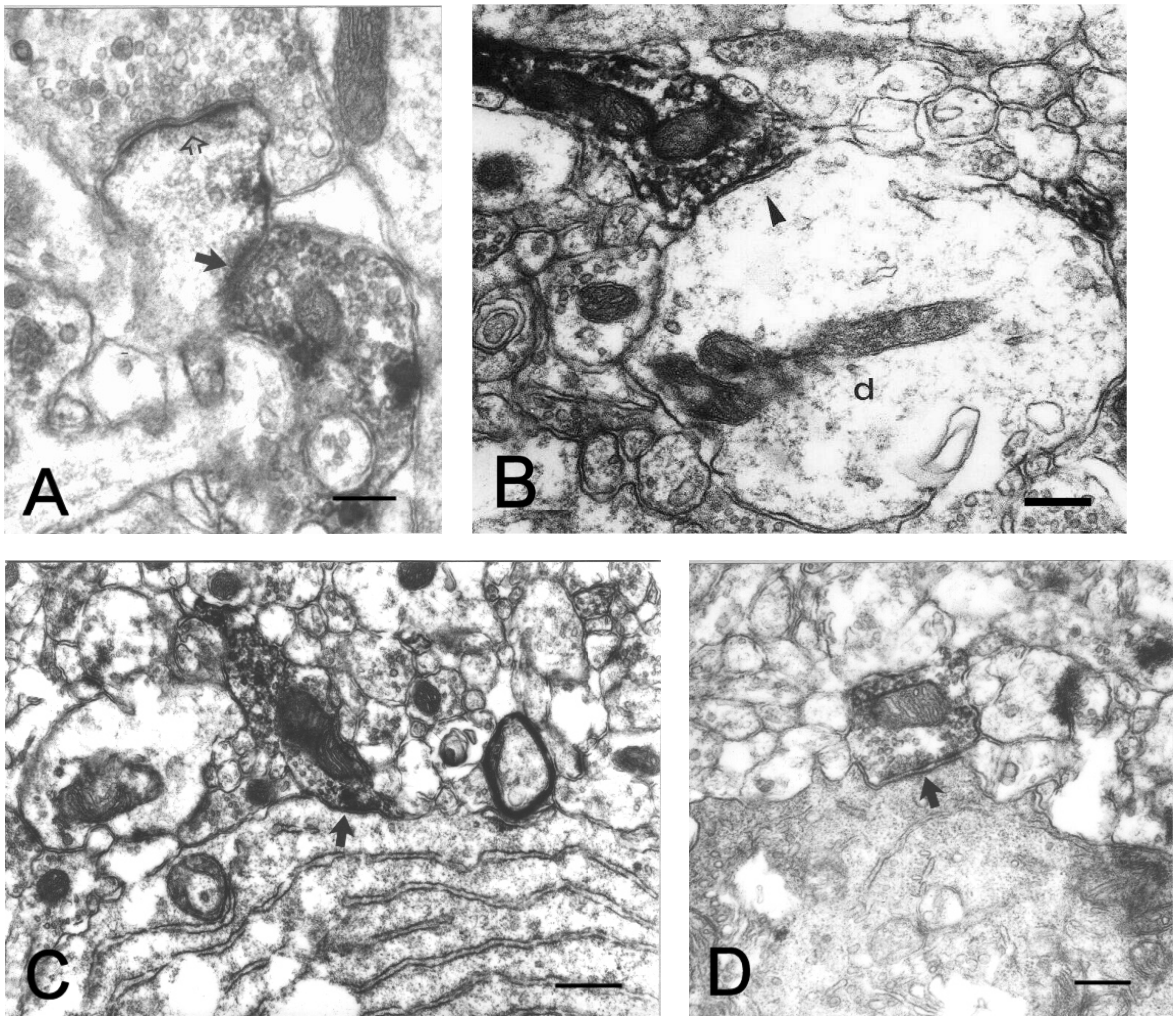


**Fig. 6. Electron micrograph of leu-enk immunopositive synapses at the border of stratum lacunosum-moleculare and radiatum of the CA1 region. A, B.** Axodendritic synapses (arrows) between an immunopositive terminal and a non-pyramidal dendritic spine (A) and shaft (d) (B). Open arrows show non-labelled axon terminals converging onto the same postsynaptic target. **C, D.** Axosomatic synapse (arrows) of immunopositive terminals on non-pyramidal somata. (Note the abundant rough endoplasmic reticulum characteristic of inhibitory interneurons). Scale bars: A: 0.12 µm; B: 0.3µm; C: 0.4 µm; D: 0.2 µm.

In order to support the inhibitory nature of the postsynaptic profiles we used postembedding immunogold reaction to show the presence of GABA. The preembedding immunocytochemical localization of enkephalins was followed by postembedding GABA immunogold reaction and confirmed the inhibitory nature of a considerable proportion of the postsynaptic targets (Fig. 7). An analysis of a postsynaptic target sample of leucine enkephalin-immunopositive boutons (n=40) showed that the postsynaptic targets were mainly dendritic shafts followed by somata and dendritic spines in both strata lacunosum-moleculare and radiatum (Fig. 8).

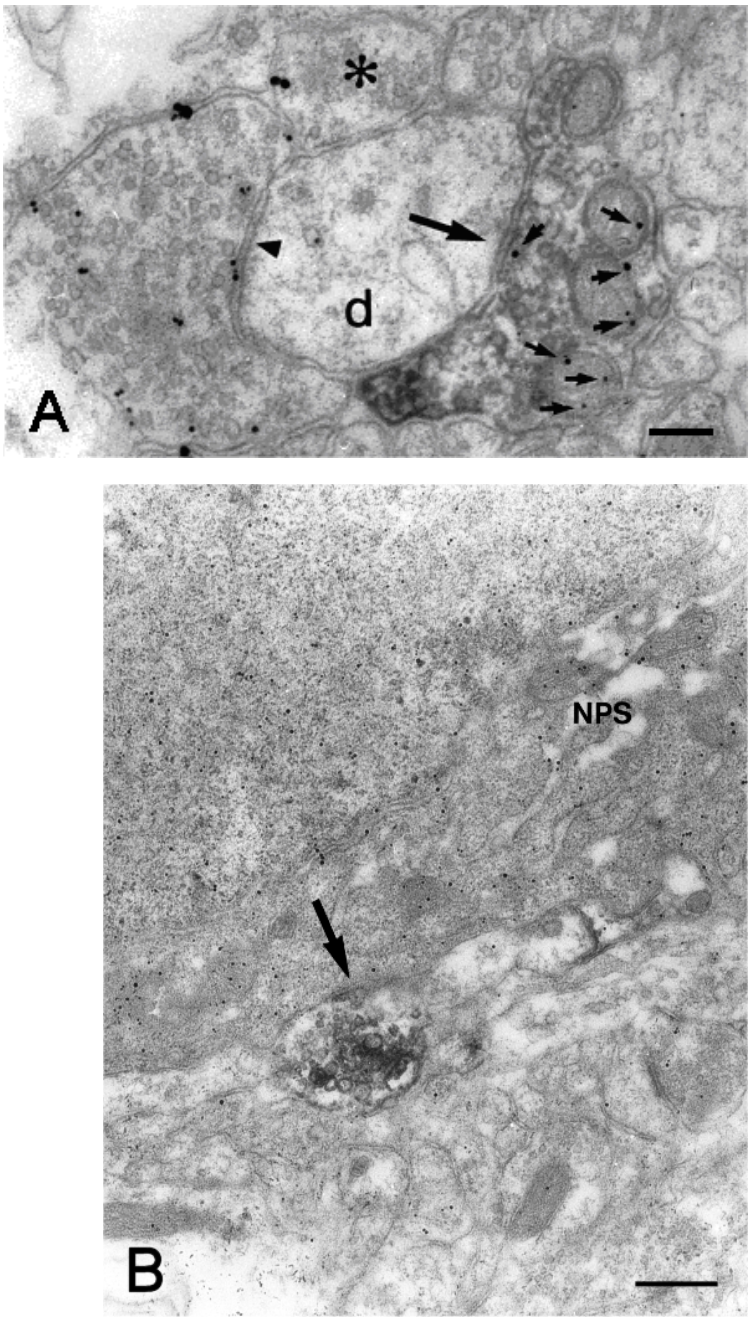


**Fig. 7. A.** Axodendritic synapse between a leu-enk immunopositive soma and GABA-negative dendrite (arrow). The enk-immunopositive soma is GABA-positive according to the presence of the immunogold particles over the mitochondria. Note that a GABA-positive- but enk-negative synapse (arrowhead) also converging on the same dendrite. **B.** Axosomatic synapse (arrow) between a leucine enkephalin-positive bouton and a GABA-immunopositive soma (NPS). Note that the enk-immunopsitive bouton in this case is GABA-negative, however there is no mitochondia. The high density of silver-intensified gold particles over the cell body directly proves its GABAergic nature (see also App. 2 Fig. 2C). Scale bars: A: 0.13 μm; B: 0.5 μm.

All somatic targets and about the half of the dendritic shafts (47%) were non-pyramidal, the remaining 53% and all the dendritic spines belonged to pyramidal cells. In a subpopulation

of the studied presynaptic boutons GABA and leu-enk could be co-localized whereas others were enk-positive only.

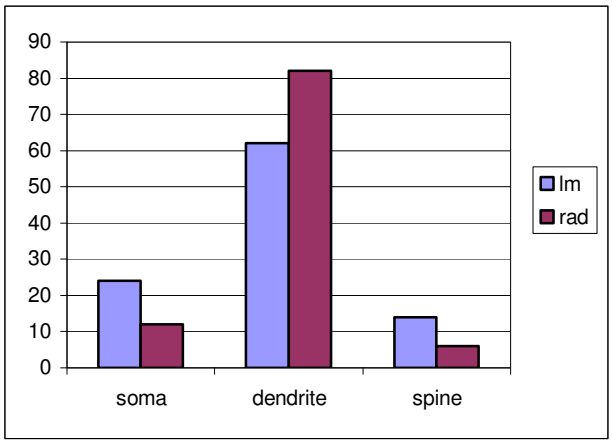


**Fig 8.** The percentage of postsynaptic target distribution of enkephalinergic synaptic boutons in two layers of the CA1 region of the rat hippocampus. *rad=stratum radiatum; lm=stratum lacunosum-moleculare*

## 4.3 Distribution of the kappa-type opioid receptor in the hippocampus

In the CA1 region of the hippocampus, numerous studies have confirmed the presence of μ- and δ-opioid receptors on GABAergic interneurons (see *Discussion* for references), two of the target receptors of the endogenous opioid peptides. In order to understand the hippocampal opioid-receptor system in its integrity, we have examined the hippocampal distribution and localisation of a less known opioid receptor, the κ-type.

KOR-IR was studied in the hippocampi of four species. The following species-specific morphological features were revealed:

**Rat**: KOR-IR was localised mainly postsynaptically within cell bodies and in their proximal dendrites in the subiculum (Fig. 9A) in strata pyramidale, oriens of CA1 region (Fig. 9B,C), and in the hilus of the dentate gyrus (Fig. 9D). The immunprecipitate completely filled the proximal dendrites and interneuron-like cell bodies in the majority of the labelled profiles. In some cases the immunoprecipitate was attached exclusively to the membrane of the labelled cells, whereas the rest of the cytoplasm was not labelled (Fig. 9A). The majority of the immunoreactive cells was found in the stratum oriens in the caudal part of the hippocampus. In the control sections no immunopositive elements were detected. Two types of staining patterns were observed: in the majority of the labelled cells the perinuclear cytoplasmic region and proximal dendrites contained observable amount of DAB immunoprecipitate. Less frequently, only the outlines of the cells were circumscribed by the immunoprecipitate (Fig. 9A; App. 3 Fig 1F). Morphologically two groups of labelled cells were distinguishable: elongated fusiform cells, horizontally arranged at the alveus-oriens border (App. 3 Fig. 1E), and multipolar cells, mainly in the stratum oriens and in the stratum pyramidale (Fig. 9C). Immunoreactive bouton-like punctae were also present, especially in the alveus. In the stratum radiatum few elongated cells showed immunoreactivity, each with its long axis parallel to the apical dendrites of pyramidal cells. Occasionally KOR-immunoreactive boutons could also be

seen surrounding weakly immunopositive somata, in a pericellular basket-like manner. This arrangement was most often seen at the oriens-pyramidale border.

**Guinea pig**: In the guinea pig hippocampus no labelling for the κ-type opioid receptor could be observed with the KOR-antibody mAb-KA8. Immunoreactive neuronal elements were not detected in any layers of the hippocampal formation (Fig.9 E,F).

**Hamster**: KOR-IR occurred in every single layer of the hippocampus and subiculum except the stratum lacunosum-moleculare of the CA1 area. The hippocampus of this species contained the strongest KOR-immunoreactivity in a variety of nerve elements. In the stratum oriens of the CA1 area (Fig. 10A,C), in the dentate gyrus (Fig. 10D) several - mainly fusiform - KOR-IR interneurons were detected. Among the pyramidal cells in the CA3 area varicose immunoreactive axon-like elements were observed (Fig. 10B). This type of labelling most probably represents presynaptically localized receptors.

**Gerbil**: KOR-IR was present primarily in the CA1 area in both the stratum oriens and the pyramidal layer (Fig. 10F), moreover in the subiculum. According to their morphological characteristics these cells represent a subset of the interneuron population of these areas. Some labelled cells resembling to basket- or axo-axonic cells were present in the pyramidal layer as well. A smaller amount of κ-opioid receptor containing cells was present in the stratum radiatum of the CA3 area (Fig. 10E).

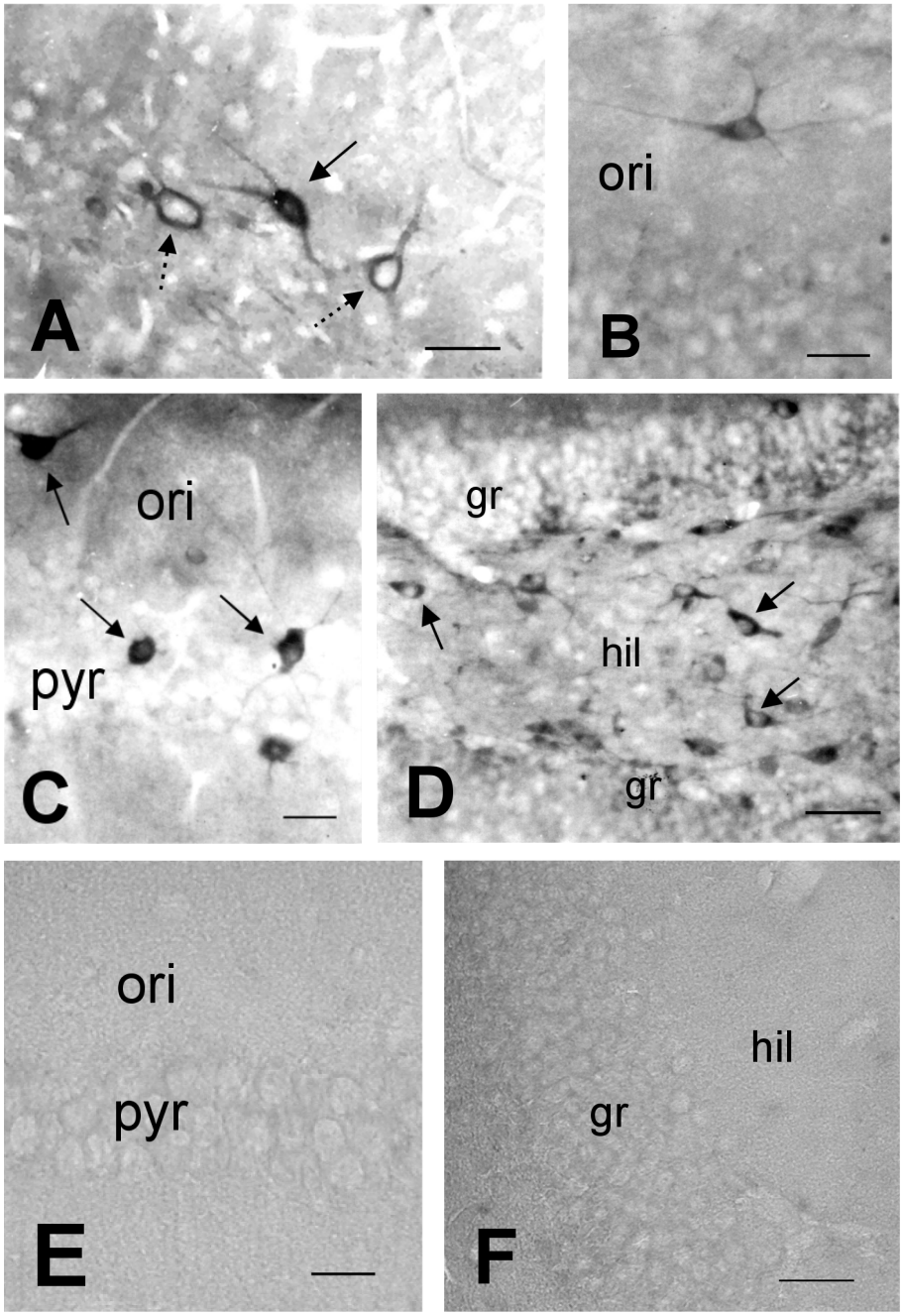


**Fig. 9. Kappa opioid receptor immunoreactive elements in the rat and guinea-pig hippocampus**: **A:** Pericellular κ opioid receptor immunoreactivity around two cell bodies (dashed arrows) in the rat subiculum. In the middle neuron (black arrow) the immunoprecipitate evenly fills the cell body and the main dendrites. Scale bar: 30 μm. **B:** A fusiform interneuron in the stratum oriens (ori) in the CA1 area of the rat hippocampus. Scale bar: 30 μm. **C:** Immunoreactive cell bodies in the pyramidal (pyr) and oriens (ori) layer of the CA1 area of the rat hippocampus (arrows). Scale bar: 32 μm. **D:** mAb-KA8-IR in the hilar (hil) neurons of the rat (arrows) (gr=stratum granulosum). Scale bar: 55 μm. **E:** Kappa receptor immunoreactivity can be observed neither in the pyramidal layer (pyr) nor in the oriens layer (ori) in the guinea pig hippocampus. Scale bar: 35 μm. **F:** The lack of immunoreactive elements characterises the guinea pig dentate gyrus as well (gr=stratum granulosum; hil=hilus). Scale bar: 43 μm.

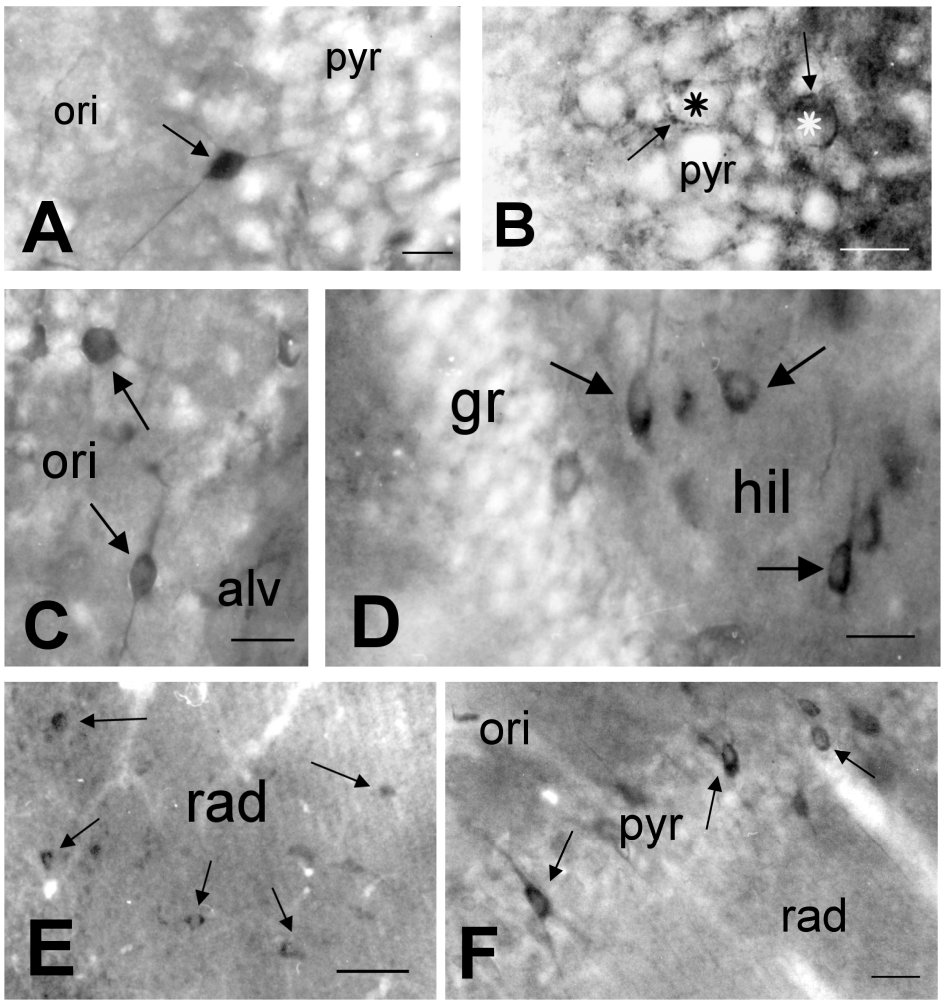


**Fig. 10. Morphology of the κ opioid receptor immunreactivity in the hamster and gerbil hippocampus: A.** Kappa-opioid receptor immunopositive neuron in the oriens layer of the CA1 area of the hamster hippocampus (arrow). (ori=stratum oriens, pyr=stratum pyramidale) Scale bar: 30 μm. B: Varicose axons around the pyramidal cells (asterisks) in the CA3 area of the hamster hippocampus show mAb-KA8-IR (arrows). Scale bar: 32 μm. C: Two strongly immunostained interneurons at the oriens/alveus border (ori, alv) of the CA1 area of the hamster (arrows). Scale bar: 30 μm. D: mAb-KA8-immunopositive fusiform cell bodies (arrows) in the hilus (hil) of the dentate gyrus (gr=stratum granulosum) of the hamster. Scale bar: 33 μm. E: Kappa-opioid immunopositive neurons (arrows) in the radiatum layer (rad) of the CA3 area of the gerbil hippocampus Scale bar:80 μm. F: Cell bodies located in the CA1 subiculum border show immunoreactivity in the gerbil. Scale bar: 35 μm.

### 4.4 Fine-structure of the KOR expressing interneurons in the rat hippocampus

In the electron microscope the immunoreactive cells showed the morphological features of inhibitory interneurons, such as indented nuclei and abundant rough surfaced endoplasmic reticulum cisternae (rEr) (Fig. 11A). Finely granulated DAB-precipitate was visible associated to free ribosomes and polyribosomes in the cytoplasm of the labelled cells (Fig. 11D). Non-labelled neurons of the same area did not contain precipitate in their perikarya (Fig. 11B). It is noteworthy, that the labelled cells differed from the non-labelled ones in the amount of cytoplasmic cell organelles: e.g. the cytoplasm of the latter type contained less rEr, but many more mitochondria (Fig. 11B).

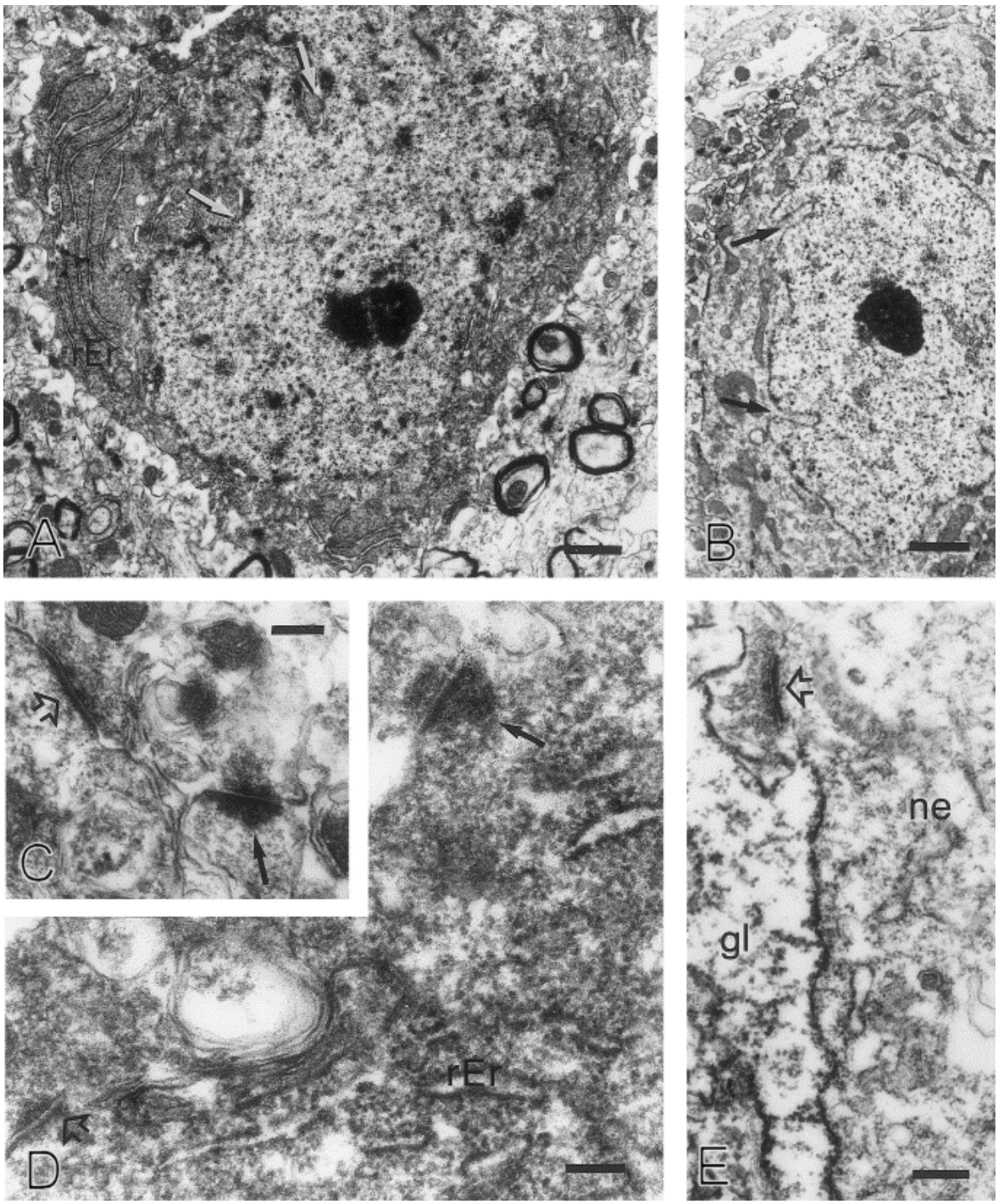


**Fig. 11. Electron microscopy of the KOR-immunolabelling: A.** Fine structure of an immunoreactive soma in the stratum oriens showing the morphological properties of an inhibitory interneuron, such as abundant rough surfaced endoplasmic reticulum (rEr) and indented nucleus (white arrows). Scale bar: 1 µm. **B.** An immunonegative perikaryon in the stratum oriens: the cytoplasm is free of immunprecipitate. Arrows show indentations on the nucleus. Scale bar: 1.5 µm. **C.** An axospinous asymmetrical synapse (arrow) has a postsynaptic density with increased width as compared with another asymmetrical synapse (open arrow) in the same field. Scale bar: 0.2 µm. **D.** An axosomatic synapse on the immunopositive soma exhibits an unusually wide postsynaptic density (arrow), whereas another seems to be normal (open arrow). The immunoprecipitate is also very dense at the outer surface of the rough surfaced endoplasmic reticulum cisternae (rEr). Scale bar: 0.1 µm. **E.** A fine structural detail from a cell also shown in Fig. 9A (dashed arrows), reveals that the KOR immunoprecipitate labels the inner membrane surface of glial processes (gl) surrounding the neuronal soma (ne) and an axosomatic synapse (open arrow). Scale bar: 0.3 µm

The fine structural examination of an immunopositive cell surrounded by immunopositive boutons revealed a true synaptic connection between the two immunoreactive structures (Fig. 11D). A substantial increase in the width and density of the postsynaptic membrane specialization was also observed at these axosomatic synapses. However, there were other, conventional axosomatic synapses on the same perikaryon, without labelling (Fig. 11D). Some, but not all, axospinous and axodendritic synapses also had similarly increased postsynaptic densities (Fig. 11C).

The neuronal somata with the `circumscribed'-type labelling (see also in Fig. 9A) were also re-embedded and examined in the electron microscope. It appeared that not the neuron itself, but a glial sheath, completely surrounding the perikaryon and the proximal dendrites, contained the immunoprecipitate which was attached to the inner surface of the glial plasma membrane (Fig. 11E).

### 4.5 Co-expression of neuropeptides and the kappa opioid receptor in hippocampal interneurons

The localisation (e.g. stratum oriens of CA1 region and hilus of the DG) and morphological features of the above described KOR-immunopositive cells closely resembled those of the interneuron subpopulation that contain somatostatin (SOM) or neuropeptide Y (NPY). Since these neuropeptides are used to classify subsets of inhibitory interneurons, we wanted to examine, whether SOM- and/or NPY- cells indeed express the κ-opioid receptor.

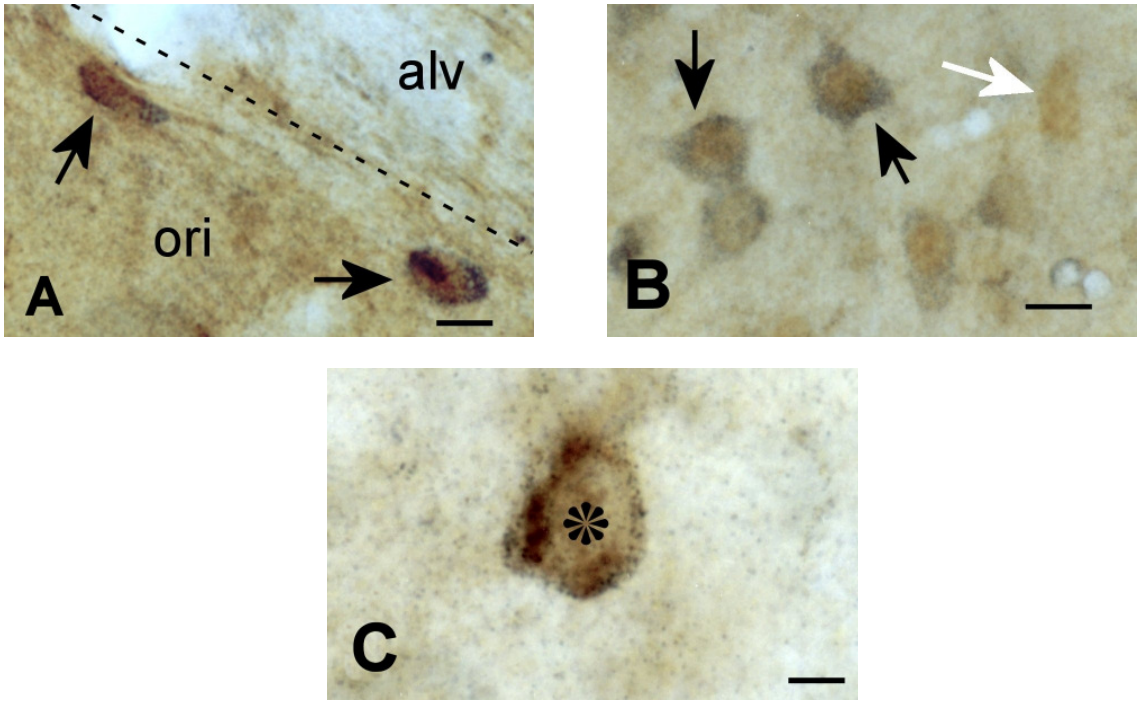


**Fig. 12. A.** Fusiform interneurons (arrows) double-labelled for SOM (brown immunoprecipitate) and the κ opioid receptor (black granules) at the border of stratum oriens (ori) and alveus (alv). Scale bar: 15µm **B.** Multipolar SOM cells in the hilus are also immunoreactive for KOR (arrows) (labelling as above). White arrow points at a cell weakly labelled for SOM only. Scale bar: 15µm **C.** Higher magnification of a double-labelled cell (asterisk) in the CA1stratum oriens, where the association of immunogold particles to the cell membrane indicating KOR, is clearly distinguishable from the brown SOM-immunoprecipitate unevenly distributed around the nucleus. Scale bar: 5µm

In the first series of our double labelling experiments, the silver-intensified gold labelling for the visualization of the κ-receptor was combined with the conventional brown DAB reaction, revealing SOM-immunopositivity. We found cells double-labelled for both markers, as well

as cells being exclusively SOM- or KOR- immunopositive.

The cytoplasm of double-labelled cells was brown, and, in addition, the contours of these cells were surrounded by fine granulated grey-to-black immunogold particles referring to the presence of κ receptors (Figs 12A-C). In the CA1 area, the stratum oriens contained the majority of the double-labelled cells, and these interneurons belonged mainly to the fusiform subpopulation (Fig. 12A). At the same time, in the hilar region of the dentate gyrus, mainly multipolar neurons were found to be double-labelled (Fig. 12B). Due to the silver intensification, the size of the gold particles was uneven. They were consistently attached to the internal surface of the cell membrane and to the peripheral cytoplasmic region of the interneuronal somata, whereas the brown SOM-immunoprecipitate was unevenly distributed in the perikaryon (Fig. 12C). The dendrites of the somatostatinergic neurons were poorly identifiable. The overlap between the SOM- and KOR-immunopositivity was not complete: there were single-labelled cells immunopositive for only one of the two examined antigens in both subregions (Fig 12B; Fig. 14A).

For the co-localization of the κ receptor and NPY we used another combination of staining methods: in this case the brown colour indicated the presence of KOR, whereas the black patchy staining was due to the presence of NPY (Fig. 13A-C). In the dentate hilus several fusiform and multipolar NPY interneurons exhibited KOR immunoreactivity (Fig.13A,C). No double-labelled cells were observed within the granule cell layer and in the molecular layer of the dentate gyrus. In the CA1 area the distribution and morphology of the double-labelled cells was identical with that of the KOR/SOM co-localization, namely, mainly fusiform cells showed immunoreactivity for both of the studied markers (Fig 13B).

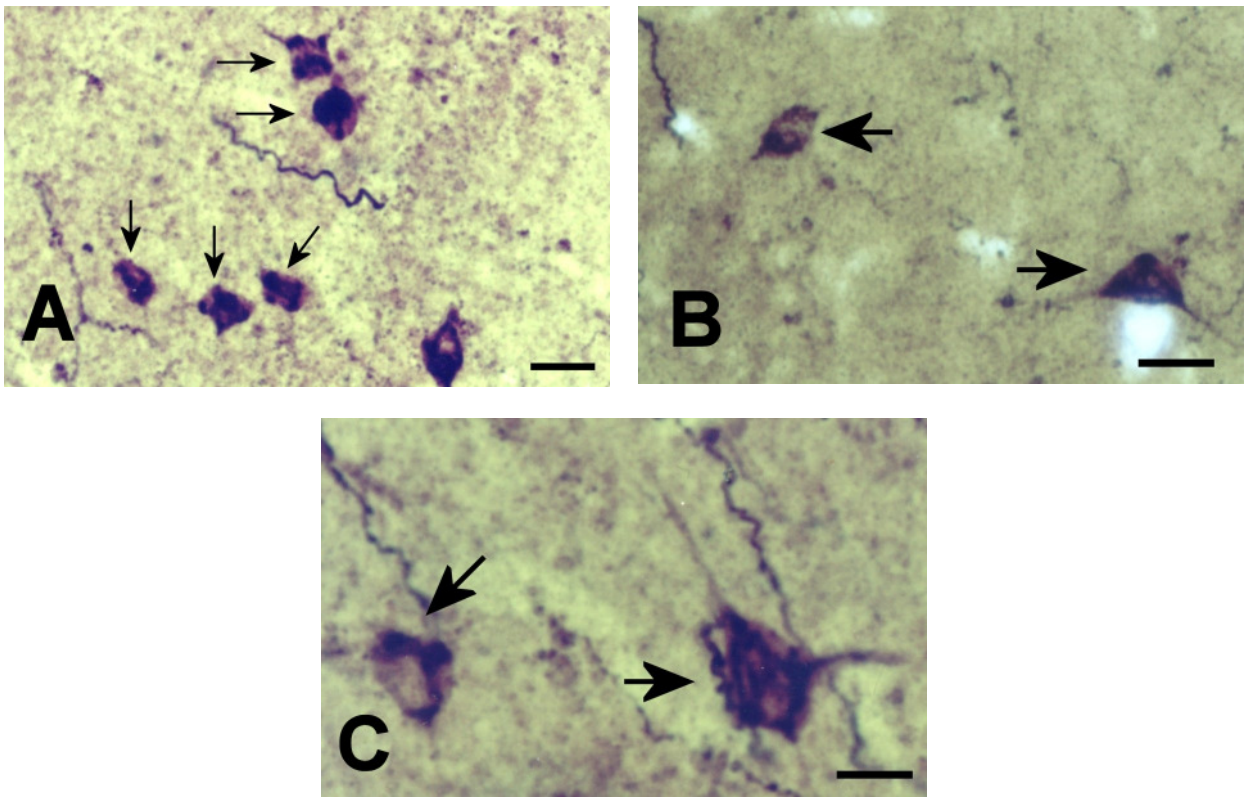


**Fig. 13. A.** Interneurons double-labelled for NPY (dark blue immunoprecipitate) and KOR (brown immunoprecipitate) (arrows) in the hilus (hil). Scale bar: 20μm **B.** Fusiform interneurons double-labelled for NPY and KOR in stratum oriens (arrows) (labelling as in F). Scale bar: 23μm **C.** At higher magnification, the patchy dark blue NPY-immunoprecipitate is well distinguishable from the lighter brown precipitate, indicating the presence of KOR in the perikaryon of the same cell (arrows). Scale bar: 11μm

The overlap between KOR immunoreactive and NPY containing cells was not complete in either hippocampal subregions, although the majority of the KOR immunoreactive cells were double-labelled (Fig. 14B). No KOR immunoreactivity was observed in axonal profiles or in distal dendrites, whereas the NPY staining revealed several dendritic segments and varicose axonal branches as well (Fig 14A-C).In the hilus 77 % of the SOM cells and a little more than the half (51%) in the CA1 stratum oriens, were double labelled (Fig. 15A). In the hilus, 56% of the NPY containing neurons and almost two third (64%) in the CA1 oriens layer showed KOR-immunoreactivity (Fig. 15B). The number of the SOM positive hilar cells in a 40 µm-thick section was 37±4 in an average, while the number of the NPY positive cells was 41±7. The CA1 stratum oriens and pyramidale (from the CA2 up to the subicular region) contained 65±11 SOM immunopositive cells and 74±11 NPY immunopositive positive cells at –4 mm caudal from the Bregma.

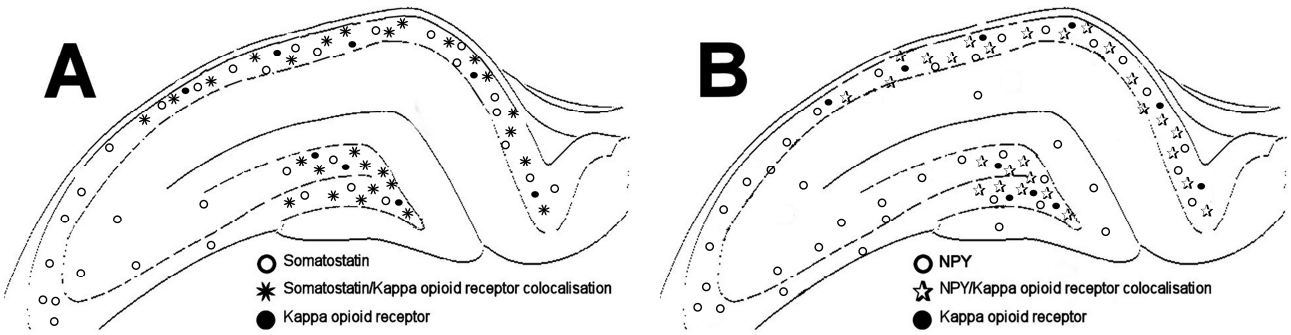


**Fig. 14. A schematic graph of the distribution of hippocampal interneurons immunopositive for SOM and/or KOR (A), and for NPY and/or KOR (B).** Please note thatthe labels represent the appropriate location and proportion not the absolute number of labelled cells.

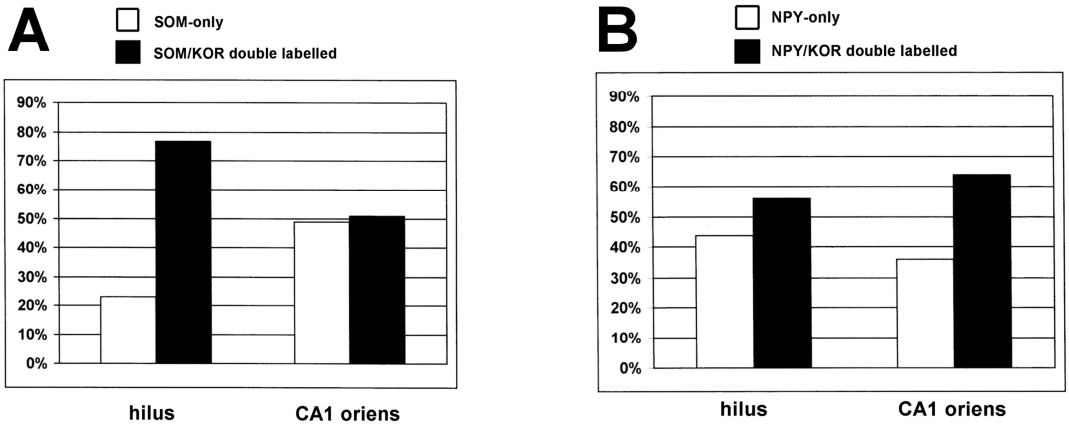


**Fig. 15. The percentage of double and single labelled interneurons in two areas of the hippocampus. A.** Comparison of SOM and SOM/KOR labelled interneurons **B.** Comparison of NPY and NPY/KOR labelled interneurons.

# 5. DISCUSSION

Our work demonstrates that opioid-peptide containing terminals establish multiple cellular interactions through which opioids may function to modulate hippocampal activity. This modulation of excitatory activity may be direct (targeting principal cells) or indirect (modulating local-circuit interneurons). In addition, we revealed that the anatomical basis of opioid modulation is species-specific. The indirect modulation of principal cells of the hippocampal formation may occur through associations with GABAergic inhibitory interneurons, which provide an anatomical substrate for opioid mediated disinhibition in the hippocampus. The cell-specific expression of opioid receptors suggests that interneurons can selectively be activated by the different opioids under different physiological circumstances.

## 5.1 Methodological considerations

*Lack of opioid immunopositive cell bodies*. In our experiments opioid immunopositive cell bodies were not observed, although many studies have reported the presence of opioid containing neurons in different subfields of the hippocampal formation (Gall et al. 1981; Buhl et al. 1994; Blasco-Ibanez et al. 1998). This seemingly contradictory observation may be due to the lack of colchicine treatment. Colchicine is able to block the axonal transport, therefore the peptide neurotransmitters are remaining within the cell body. Under normal conditions, peptides can only be produced ribosomally in the cell bodies followed by packaging in the Golgi apparatus (Sherman et al. 1989), and replacement after release occurs via axonal transport from the cell bodies to the nerve endings. Exceptions were also reported, when the mRNS is transported to nerve endings, and the final conversion take place at the termination zone. In the case of hippocampal opioid peptides the somata of the granule cells or even the interneurons did not show immunoreactivity, because the somata synthesize the opioid precursor and the final conversion to the active transmitter takes place in the terminals only (Tielen et al. 1982). The lack of opioid-immunopositive cell body staining therefore must be considered false negative in our experiments.

*Specificity of the anti κ opioid receptor antibody*. The mAb KA8 antibody was raised applying the hybridoma technology (Tielen et al. 1982) against a solubilized frog brain κ opioid receptor fraction, which was free from μ and δ opioid receptors. The monospecificity and the recognition-specificity of the antibody were tested carefully by the methods of specific radioligand binding, comparative gel filtration, immuno-precipitation and immunoblotting (Kohler and Milstein 1975). It was established, that the mAb KA8 effectively competes for the binding sites with the κ agonists EKC (ethylketocyclazocine) and dynorphine, as well as antagonists such as norbinaltorphimine and the non-selective naloxone, but much less readily with the agonists U 50488 and U 69593. Thus, it was characterized as a κ opioid-selective antibody with preference to the $kappa_2$ subtype.

*Specificity of the KOR immunostaining*. We carried out correlated light and electron microscopic immunocytochemistry. When choosing the fixative mixture we had to compromise between the detectability of the antigen (the maximal retaining of the native conformation of the receptor protein) and the possible best tissue conservation. In order to find the best concentration we tried several combinations of paraformaldehyde and glutaraldehyde in a series of experiments. The fixative referred in the *Materials and Methods* section was chosen, because the low concentration of glutaraldehyde (0.1%) did not reduce

the intensity of the immunostaining and the amount of immunolabelled structures did not change either, as compared to the application of 4% paraformaldehyde alone. The result was a specific staining of the described structures, with low background and at the same time in the electron microscope the tissue preservation was improved.

Because the mAb-KA8 antibody has not previously been used in the hippocampal formation of rat, we used other brain areas as positive controls in order to check the specificity of immunostaining. The antibody has been used previously for the localization of the receptor in the human frontal cortex (Maderspach et al. 1991), in the young rat and chick brain and cultured neuronal and glial cells (Schmidt et al. 1994; Maderspach et al. 1995; Gurwell et al. 1996). Our positive controls included the frontal cortex, where pyramidal neurons were shown to exhibit immunopositivity similar to the human (Knapp et al. 1998), and the hypothalamic nuclei, where the labelling was restricted to the perinuclear cytoplasm and proximal dendrites in neurons, both in our material and in the previous study (Schmidt et al. 1994).

## 5.2 The distribution of opioid-immunopositive nerve elements in the hippocampal formation

The amount and distribution of opioid-immunopositive axons were heterogeneous in the studied species. In accordance with previous studies, met-enk was found to be the dominant opioid peptide in the rat and the mouse. The distribution of immunopositive nerves was similar to earlier results (Maderspach et al. 1995). The pericellular baskets formed by varicose enk-immunopositive axons in strata lacunosum-moleculare and radiatum were the most interesting findings. We were able to provide direct morphological evidence that the opioid-containing boutons are in synaptic contact with GABAergic interneurons. These experiments were carried out parallel with those of Commons and Milner (1984), and our results were in good agreement with theirs.

Apart from the mossy fibres, dynorphin peptides were not very abundant in the rat and mouse according to our experiments. However the amount of these peptides is known to be strongly dependent on the functional state, as well as the strain, or genotype of the experimental animal (Commons and Milner 1996).

In the hamster hippocampus an abundant and intimate direct connection was revealed between enkephalinergic varicose fibres and the principal cells, especially pyramidal cells. Similar arrangement was characteristic of the dynorphin-immunopositive nerve fibres, in each studied species except the rat, suggesting a direct effect of dyn-peptides on the principal cells.

The arrangement of the parallelly running leu-enk-immunopositive axons in the CA1 stratum radiatum of the guinea-pig hippocampus suggests that the boutons of these axons, crossing the apical pyramidal dendrites, may establish “en passant” type axodendritic synapses. Further electron microscopic studies are needed to confirm the existence of this direct type of connection between the enkephalinergic boutons and the pyramidal dendrites.

## 5.3 The origin of the hippocampal opioidergic elements

The hippocampal opioidergic fibres have both extrinsic and intrinsic sources. An extrinsic source is the entorhinal cortex (Tielen et al. 1982; van Daal et al. 1989), which is thought to contribute to the enk-immunopositive plexus in stratum lacunosum-moleculare. Another projection enters the hippocampus via the fornix (Fredens et al. 1984).

The axons of granule cells, the mossy fibres are the main intrinsic sources of hippocampal opioids. Interestingly, the somata of granule cells did not show strong immunoreactivity, because the somata synthesize the opioid precursor and the final conversion to the active transmitter takes place in the terminals only (Roberts et al. 1984).

A subset of inhibitory interneurons may also serve as another intrinsic source of enkephalins (Gall et al. 1981; Tielen et al. 1982). We failed to detect such cell bodies (see *methodological considerations*), except in the hamster, where only few weakly-stained enk-immunopositive somata were present. A recent study of Blasco-Ibanez et al. (1998) established that the enkephalinergic neurons of the CA1 area, also containing VIP and calretinin, belong to the subset of "disinhibitory" interneurons specialized to innervate exclusively other interneurons.

Without using tract-tracing methods combined with opioid immunocytochemistry, extrinsic and intrinsic opioidergic boutons cannot be distinguished. However, fine structural studies show certain morphological heterogeneity among hippocampal enkephalinergic boutons (Blasco-Ibanez et al. 1998). Our electron microscopic results showed that a part of the hippocampal enk-positive boutons also contain GABA, and establish symmetrical synapses, whereas others are GABA-negative and make asymmetrical synapses, as follows.

### 5.4 Connection between the opioid-peptide containing terminals and GABAergic nerve elements

Our ultrastuctural findings clearly prove that the opioidergic system is closely related to the GABAergic network in the hippocampus. The enkephalinergic synaptic boutons terminated both on pyramidal and non-pyramidal postsynaptic targets. Similarly to other extrinsic sources (Commons and Milner 1995) the extrinsic enkephalinergic axonal system may act via the hippocampal GABAergic interneurons. In this indirect way, compensating their low axonal density with the extensive axonal arborization of the local circuit cells, these enkephalinergic axons can influence large populations of principal cells.

A large number of interneuronal somata are located at the border region of stratum radiatum and lacunosum-moleculare of the CA1 area (Lacaille and Schwartzkroin 1988; Freund and Buzsaki 1996). Recently, numerous different groups of inhibitory interneurons were anatomically and physiologically identified innervating distinct membrane domains of the CA1 pyramidal cells and also the granule cells in the DG (Halasy and Somogyi 1993b; Vida et al. 1998) These cells are definitely among the postsynaptic targets of enkephalinergic boutons just like the few interneurons situated in the stratum radiatum.

The ultrastructural examination of the enkephalin-immunopositive boutons showed a heterogeneous vesicle population, indicating a co-transmitter, or neuromodulator role of the opioid peptides. We could demonstrate the presence of GABA in a subpopulation of enk-positive terminals. Since the majority of hippocampal interneurons are GABAergic (Buhl et al. 1994), the double labeled, enk- and GABA-positive boutons are good candidates for axon terminals of the enk-immunopositive intrinsic interneurons (Freund and Buzsaki 1996). Our findings suggest that similarly to other inhibitory interneurons, the boutons of the leu-enk-positive subpopulation of GABAergic interneurons may directly target the pyramidal cells. Thus, the enk-immunopositive boutons synapsing on spiny dendritic shafts and dendritic spines may represent one of the intrinsic parts of the hippocampal opioidergic system. The proportion of these in our random sampling seems to be considerable, but taking into account, that the number of pyramidal cells in the CA areas represents the overwhelming majority (90% at least) of the entire cell population (Gall et al. 1981), the

relative weight of this innervation is much lower than that of the extrinsic one. In addition, the co-existence of GABA and opioids in these terminals suggests that they use GABA as the main transmitter and the opioids probably have a modulatory role. They were shown to be released upon high frequency stimulation only. Thus they may be able to change the inhibitory effect of GABA, but it cannot change the inhibition itself.

Numerous studies have confirmed, that opioids act by inhibiting inhibitory interneurons (Nicoll et al. 1980; Dingledine et al. 1986; Aika et al. 1994), and that opioids directly hyperpolarize interneurons, and presynaptically inhibit GABA release (Siggins and Zieglgansberger 1981). These inhibitory effects were described to be mediated by μ- and δ-opiate receptors (Cohen et al. 1992; Miller and Lupica 1994), and the presence of these receptors on interneurons in the hippocampus were also presented (Lupica and Dunwiddie 1991; Mansour et al. 1994b; Commons and Milner 1997) The presence of κ-opiate receptor in interneurons hasn't been proved yet, although κ-opioid receptor mediated effects also suggested interneuronal involvement in the activity pattern of the hippocampal principal cell.

### 5.5 Kappa-opioid Receptor in the rodent hippocampus

Labelled cells were present in all species except the guinea pig in the stratum oriens of the CA1 region of the hippocampus. Since only GABAergic inhibitory interneurons have been published to be present in this layer, it can be hypothesised that these cells also represent a subpopulation of GABAergic interneurons. It is also observable, that there is a fusiform cell population, which express the KOR. The localisation and distribution of this type of cells is very similar to that of the somatostatin containing neurons. Correspondingly in the dentate gyrus of the rat and hamster a KOR immunopositive cell population exists that also represents morphological similarities to the hilar somatostatin containing neurons. Both cell populations have an axonal arborization in connection with the termination zone of the entorhinal efferents, which represents the main opioidergic input of the hippocampal formation. Thus, the location of the receptors suggests that they play a key role in the regulation of the neuronal transmission mediated by this input.

It is notable that a morphologically uniform κ-opioid receptor immunopositive cell population exists in the subiculum of the hippocampus in all investigated species except the guinea pig. Recently it has been proposed, that the subiculum probably contains chemical sites at which antipsychotic drugs could act specifically to produce appropriate physiological effects. This is consistent with the hypothesis that the subiculum is a potential site of action for certain antipsychotic drugs (Mansour et al. 1987). The KOR immunopositive cells may modify or mediate the incoming and/or outgoing entorhinal information transmitted by dynorphin.

In the gerbil the majority of the KOR cells are located in this area, thus it can be proposed that these subicular neurons play a key role in the mediation of the protective effect of κ agonists in forebrain ischemia resulting in a lower level of neuron loss.

We failed to detect mAb-KA8-IR elements in the guinea pig hippocampus. This may be due to the fact, that here previously presynaptic $\kappa_1$ type-opioid receptors were described (Greene 1996). Zukin and his co-workers have shown that the U 69,593-sensitive, high-affinity $\kappa_1$ site predominates in the guinea pig brain, and the U 69,593-insensitive, low affinity $\kappa_2$ site predominates in the rat brain (Drake et al. 1996). Thus, being $\kappa_2$ specific, with our antibody we did not manage to detect immunoreactivity in the guinea-pig hippocampus.

Another explanation for the lacking KOR-immunostaining is that the monoclonal antibody could not recognize the 'guinea pig variant' of the κ opioid receptor that is present in the guinea pig hippocampus. This receptor is 90% identical to the mouse and rat κ receptors, with the greatest level of divergence in the N-terminal region (Zukin et al. 1988). Our monoclonal antibody may not recognize an N-terminal amino-acid sequence that is different from that of the rat or mouse receptor resulting in no immunolabelling.

It is relatively well known that opioids may influence glial cell activity, presumably related to glia-neuron communication. Beside our electron microscopical studies others in other brain areas have also shown (Xie et al. 1994) that the pericellular κ opioid receptor immunolabelling around principal cells are indeed localised in a glial sheet that covers these neurons suggesting a very close glia-neuron interaction of opioids. In addition Eriksson et al. (1995) demonstrated that κ-opioid receptors are coupled to L-type $Ca^{2+}$ channels on hippocampal astrocytes *in vitro*. This arrangement may represent a mechanism contributing to the depressant action of opioids on synaptic plasticity via the manipulation of the accessibility of the extracellular calcium necessary for presynaptic transmitter release. We could visualize this kind of glia-neuron arrangements in the rat and hamster hippocampus in many cases.

The hamster hippocampus not only contains the highest amount of opioid peptide (Eriksson et al. 1993) but also the κ-opioid receptors are the most abundant, from among the other three species. This may have functional relationship to the fact, that the hamster is a hibernator. Changes in the amount of opioid peptides and receptors in this part of the limbic system were reported to be involved in hibernation and the adaptation of thermal stress in hibernating rodent species (Racz et al. 1998).

## 5.6 Subcellular localization of KOR: comparison with other results

Most of the studies dealing with the subcellular localization of the κ-opioid receptors found them in postsynaptic position. With the mAb KA8 antibody immunoreactivity was detected in neuronal membranes at extrasynaptic sites and postsynaptic densities of synapses associated to microtubules of dendrites, free ribosomes and rEr in the cytoplasm, furthermore attached to glial membranes in discontinuous arrangement (Maderspach and Nemeth 1993; Maderspach et al. 1995; Gurwell et al. 1996; Bourhim et al. 1997; Knapp et al. 1998). With the same antibody, in the present study we also found similar, mainly postsynaptic, and occasionally glial localization. Another antiserum raised against a synthetic peptide corresponding to the C terminal sequence of the $kappa_1$ receptor, resulted in similarly postsynaptic subcellular localization of the κ-opioid receptor in somata and dendrites in the rat and guinea pig brain (Schmidt et al. 1994).

Drake et al. (1995) used a polyclonal antiserum raised also against the C terminal peptide sequence of the cloned κ receptor in the guinea pig hippocampus. They did not find κ receptor labelling in the CA1 region of the guinea-pig hippocampus, but they were able to localize the receptor exclusively presynaptically in other hippocampal areas. This discrepancy may be due to the differences of the experimental circumstances: firstly, the animal species used was not the same (rat versus guinea pig), secondly, the primary antibody/antiserum derived from different sources. As we have mentioned in the previous chapter, the antibody we used recognizes preferentially the $kappa_2$ subtype (Drake et al. 1996), whereas in the guinea pig hippocampus the presynaptic $\kappa_1$ receptor is dominating. On the other hand, $\kappa_2$ is the predominant subtype in the rat hippocampus (Maderspach et al. 1991; Unterwald et al. 1991; Wagner et al. 1991).

## 5.7 Co-expression of SOM, NPY and KOR in a subpopulation of hippocampal interneurons

The interneurons of the stratum oriens were shown to belong to a sub-population also containing somatostatin (SOM) as a co-transmitter to GABA (Zukin et al. 1988). Our colocalization experiments also confirm an overlap between the KOR-immunopositive and the SOM-containing neuron population in the stratum oriens. We provided here direct evidence that the interneurons expressing the receptor in a considerable proportion belong to the SOM- and/or NPY-immunopositive subpopulation.

Köhler et al. (1982) co-localized the two neuropeptides in the same interneuron population, but the overlap was found to be partial only. Since we have not carried out SOM/NPY co-localization, the extent of the overlap between the cell groups characterised by these neurochemical markers cannot be established either. In our material we also observed cells labeled exclusively with either the receptor, or one of the two neuropeptides. In the dentate gyrus only large and medium sized multipolar and fusiform hilar cells were double labelled. The morphology and distribution of the double-labelled cells suggests, that here, a specific interneuron population that contain both neuropeptides, might express the KOR.

Intracellular filling studies (Kohler et al. 1987; Toth and Freund 1992) proved that the somatostatinergic subpopulation of interneurons can be further divided into two groups: local circuit SOM-positive neurons have their axonal arborisation and synaptic contacts in the distal dendritic region of the principal cells both in the dentate gyrus (Han et al. 1993), and hippocampus proper (Halasy and Somogyi 1993b), in conjunction with the entorhinal excitatory input. The other group of SOM cells take part either in the commissural (Gulyas et al. 1993) or the hippocamposeptal projection (Alonso and Kohler 1982; Leranth and Frotscher 1987). At present, it is an open question whether the κ receptors are expressed by both, or exclusively by one of the two SOM immunoreactive cell groups. Since the majority of SOM cells is known to project commissurally, among these double-labelled neurons may be commissural neurons participating in lateral inhibition (Toth and Freund 1992).

The role of SOM- and NPY-containing interneurons in the hippocampal neuronal network has been extensively studied (Vezzani et al. 1996; Katona et al. 1999; van Hooft et al. 2000; Boyett and Buckmaster 2001). The axons of these interneurons selectively innervate the distal apical dendrites of the pyramidal cells and here the inhibitory SOM/NPY cell synapses interfere with excitatory input originating from the entorhinal cortex and the thalamus. These so called *Oriens-Lacunosum Moleculare* (O-LM) interneurons are activated after CA1 pyramidal neuron discharge only (feedback, or recurrent inhibitory cells). This suggests that SOM- and/or NPY- containing inhibitory interneurons in the stratum oriens, when activated, can selectively reduce the excitatory input from entorhinal cortex to CA1. On the contrary, the inhibition of these interneurons by κ-opioid receptor activation may result in enhanced entorhinal activation of the CA1 pyramidal neurons similarly as it has been described previously concerning the μ- and δ-opioid receptor (Kunkel et al. 1994).

## 5.8 Other opioid receptor types in the hippocampus: relationship between μ-, δ-, and κ-opioid receptors

The labelled cells were most numerous in the stratum oriens and DG of the rat hippocampus. Because only GABAergic inhibitory interneurons have been shown to be present in this layer, it is likely that the cells showing κ receptor immunoreactivity also belong to this group.

Although there were labelled cells in the stratum pyramidale, this layer is known to contain at least three types of interneurons in addition to pyramidal cells (Buhl et al. 1994; Halasy et al. 1996a; Svoboda et al. 1999). Our fine structural findings confirm the inhibitory character of the KOR-immunoreactive cells.

Interestingly, a cell population similar to that described in this work, was found to exhibit δ-opioid receptor-immunoreactivity (Halasy et al. 1996b). To date, it has not been clarified, whether this is the same, partially overlapping, or a distinct cell population. Jordan and Devi (1999) have recently reported that κ receptors selectively dimerize with δ-, but not with μ-opioid receptors in co-expression experiments (Commons and Milner 1997). The ligand binding properties of these heterodimers were reported to be virtually identical to those of the $kappa_2$ subtype. In the light of these new results, it is interesting to speculate that the same neuron population was labelled but the two antibodies recognized different binding sites.

Others found the μ-opioid receptor in somatodendritic and axonal compartments of inhibitory interneurons in the hippocampus, as well (Bausch et al. 1995b; Jordan and Devi 1999). Thus, it seems that all the three main types of opioid receptors are present on a certain subpopulation of hippocampal interneurons, each being activated under different physiological circumstances. The fact that local circuit inhibitory interneurons are equipped with a variety of opioid receptors provides further evidence for their salient role in the local microcircuitry.

## 5.9 Transmitter-receptor mismatch

Kappa opioid receptors bind primarily the prodynorphin gene-derived peptides as endogenous ligands. Apart from the mossy fibre system, dynorphin peptides are present in very few varicose fibres in the hippocampus, as we detected by immunocytochemistry at light microscopic level (Mansour et al. 1995). Drake et al. localized dynorphin opioids in large dense-core vesicles at electron microscopic level (Racz et al. 1998). These organelles are too small to be visualized in the light microscope. Fine structural studies indicating the presence of dynorphins in large dense-core vesicles in axon terminals of the CA1 area have not been carried out yet. Thus, we can only suppose, that these endogenous ligands -- although in low amount -- may be present in the stratum oriens. Moreover, astrocytes were shown to contain opioid mRNA in vitro (Drake et al. 1994) and they may serve as an additional source of possible endogenous opioid ligands in vivo as well. On the other hand, the κ receptors identified in astrocyte cultures by the mAb KA8 were shown to be highly sensitive (Hauser et al. 1990), thus, the release and binding of endogenous ligands can activate them in a very low concentration. If the sensitivity of the receptors demonstrated in vivo is similar to the one measured in vitro, this would compensate the low amount of detectable endogenous ligands in the CA1 area. However, the elucidation of these questions needs further experiments.

It is also notable that opiate receptors can bind all endogenous opioid peptide-forms, only with different affinity. Under continuously changing physiological circumstances these κ opioid receptors could be activated by any of the opioid peptides present. In addition, physiological properties of dynorphin release showed that it is very probable that dynorphin could act via volume transmission and can diffuse to the targets, which enables the peptide to activate κ receptors within 50-100 μm from the site of release (Gurwell et al. 1996).

## 5.10 Functional implications

The actual activity pattern of hippocampal principal cells and the probability of LTP is strongly influenced by the enkephalins and dynorphin peptides interacting with the glutamate in the mossy fibre system (Gannon and Terrian 1992; Chavkin 2000).

Non-principal cells are also affected by the opioids (Blasco-Ibanez et al. 1998; Terman et al. 2000). A number of afferent projections use the inhibitory interneurons (Freund et al. 1990; Freund and Gulyás 1991; Halasy et al. 1992; Acsady et al. 1993; Svoboda et al. 1999), since this is an efficient indirect way to influence the activity of large principal cell populations. The disinhibitory effect of opioids probably contributes to the formation of epileptic discharges as well (Cain and Corcoran 1985; Leranth and Frotscher 1987) mediated by opioids through μ-, δ- and κ-opioid receptors.

In general, the level of opioid activity is dramatically changing depending on the physiological state of the tissue, leading to the conclusion that opioids are among the primary mediators of the hippocampal plasticity (Henriksen et al. 1983).

In the dentate hilus and CA3 area dynorphin peptides released upon high frequency stimulation, and acting through κ receptors, inhibit the release of excitatory amino acids from the perforant path and mossy fibre terminals. Therefore dynorphins may reduce the probability of LTP formation (Morris and Johnston 1995). Hippocampal dynorphin B injection to the CA3 region was shown to impair spatial learning in rats, and the effect was mediated by κ receptors (Terman et al. 1994). Pharmacological evidence for functional κ receptors on hippocampal pyramidal neurons in the CA1 area, and a direct action of opioids on the principal cells was provided by Madamba and his co-workers (Sandin et al. 1998). We could demonstrate κ receptor-like immunoreactivity in the postsynaptic densities of some axospinous and axodendritic synapses. Because spines are characteristic of pyramidal cell dendrites, our findings provide a morphological support to the κ-mediated direct effect demonstrated by them. However, the presence of κ receptors in pyramidal cells does not exclude their presence in interneuron membranes either. Our fine structural colocalization studies on the enkephalinergic system showed that similarly to the findings of Commons and Milner (1997) enkephalinergic terminals establish synapses partially with GABAergic, but also with GABA-negative postsynaptic targets.

The hippocampal interneurons are in connection with a high number of principal cells as well as with each other (Freund and Buzsáki, 1996) playing a key role in the electrical activity of this brain area. It was shown that several inputs using a wide variety of neurotransmitters are in synaptic contact exclusively with the local inhibitory cells. The hippocampal opioidergic system seems to follow this pattern of innervation. As it was mentioned, the interneuron population identified in our experiments has an axonal arborization in the stratum lacunosum-moleculare. Via these interneurons, which are equipped with a variety of opioid receptors, the endogenous opioids may modify the direct entorhinal input to the CA1 area.

## 5.11 Pathological consequences

In certain form of temporal lobe epilepsy, it has been shown that the loss of SOM-positive inhibitory interneurons causes the hyperactivity of CA1 pyramidal cells (Madamba et al. 1999). The fact that κ opioid receptors are present in these interneurons may account for the neuroprotective role of endogenously released dynorphin on CA1 pyramidal cells, (Birch et al. 1991; Hong et al. 1993; Cossart et al. 2001). In the hilus, similar phenomena have been observed in connection with the mossy fibre reorganisation, which accompanies temporal lobe epilepsy (Genovese et al. 1994). Other studies revealed that κ opioid receptor-mediated effects are neuroprotective in ischaemic insult of the hippocampus (Tang 1985; Houser et al. 1990). Since κ opioid receptors are exclusively confined to the above subfields of the hippocampal formation, and interneurons of this area are involved in the pathological processes, we suggest that the opioid effect on the principal cells of the area is mediated indirectly, via SOM and/or

NPY-immunopositive interneurons.

Physiological and pharmacological studies are needed to reveal the exact functional role of opioid receptors on inhibitory interneurons, but the immunohistochemical co-localisation of the neuropeptide markers and opioid receptors suggest a very close interaction and connection between the two systems. The expression of the κ opioid receptor by SOM and NPY neuropeptide containing interneurons represent a highly specific involvement of the opioids in the hippocampal inhibitory processes. The presence of κ opioid receptors on a specific subset of GABAergic neurons suggest that opioids can indirectly modulate the activity of principal cells and may play a crucial role in the normal and pathological activity of the hippocampal formation. Interneurons involved in the hippocampal plastic neuronal processes can thus be a potential target of future therapeutic research, since they could be the site of action of many drugs effective against limbic disorders.

### 5.12 Concluding remarks

We analyzed the distribution, species-specificity, and fine structural localization of opioid peptides and their receptors in the hippocampal region of the brain in the hope that we contribute to a more precise understanding how the opiates influence the activity pattern of the area involved in learning and memory. Further studies are yet required to establish the full range of possibilities. However it is clear now, that opioids act very specifically on hippocampal local-circuit GABAergic interneurons in order to fine tune large population of principal cells. It can be speculated, that opioids in the hippocampus are acting to switch activity states at numerous targets. In summary, our data provided insight to structural properties underlying mechanisms of action of opioids in the hippocampus by revealing multiple potential sites of action.

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