Interaction of peptidergic, biogenic amine and amino acid transmitters in the integration of central and peripheral autonomic functions. Pharmacological characterization of some opioid derivatives and of α_{2B} adrenoceptor

Ph.D. Doctoral Thesis

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1. List of abbreviations

ACN	acetonitril
ACTH	adrenocorticotrophic hormone
ANP	atrial natriuretic peptide
AUC	area under the curve
β-FNA	β-funaltrexamine
CB	cannabinoid
CCK	cholecystokinin
CHO	chinese hamster ovary
CNS	central nervous system
CRF	corticotrophin releasing factor
CYP	cyprodime hydrobromide
DADLE	[D-Ala^2, D-Leu^5]enkephalin
DAG	diacylglycerol
DAMGA	[D-Ala^2, MePhe^4, Gly^5-NH_2]enkephalin
DAMGO	[D-Ala^2,MePhe^4,Gly^5-ol]enkephalin
D-Asp	D-aspartate
DRG	dorsal root ganglia
DVC	dorsal vagal complex
DVN	dorsal motor nucleus of the vagus
EMO	endomorphin
ES	electrical field–stimulation
GABA	γ-amino butyric acid
^{3}H	tritiated
HPLC	high performance liquid cromatography
i.c.	intracisternal
i.c.v.	intracerebroventricular
KS	high potassium stimulation
LSD	least significant difference

MBH	mediobasal hypothalamus
MSG	monosodium glutamate
MSH	melanocyte stimulating hormone
MVD	mouse vas deferens
NPY	neuropeptide Y
NOP	nociceptin receptor
NE	norepinephrine
NTI	naltrindole hydrochloride
NTS	nucleus tractus solitarius
NTX	naltrexone hydrochloride
NX	naloxone
ORL	opioid receptor like
PAM	peptidyl - glycine - α - amidating monooxygenase enzyme
POMC	pro-opiomelanocortin
PVN	hypothalamic paraventricular nucleus
RIA	radio-immunoassay
RP-HPLC	reverse-phase high performance liquid cromatograhy
S.E.M.	standard error of mean
ST-91	2-[2,6-diethylphenylamino]-2-imidazoline
subPVN	hypothalamic subparaventricular nucleus
VIP	vasoactive intestinal polypeptide

2. Guide to the thematical structure of the thesis

My thesis is rather divergent, both thematically and methodologically. This diversity stems from two main factors. The primary determinant was the traditional methodological and thematical orientation of the pharmacological team I started working with, first as an undergraduate visiting researcher, later as a PhD worker. Our team had a prominent interest in neuropeptides, particularly opioids, with a far-reaching research history (to mention a few: Rónai et al., 1977a,b, 1979, 1981a, 1992; Gyires et al., 2000b; Gyires and Rónai 2001; Al-Khrasani et al., 2001; Rónai et al., 2001; 2002; Al-Khrasani et al., 2003; Rónai et al., 2009 etc.). Therefore, it was natural that our actual interest was aroused by the most recently identified new endogenous opioid peptide families, namely, the endomorphins (Zadina et al., 1997) and, in the broadest sense of „opioid" definition, the nociceptin/orphanin FQ family (Meunier et al., 1995; Reinscheid et al., 1995). The methodological background of the team was isolated organ technique and experimentation with isolated brain slices.

The second major determinant was the cooperation strategy of the team, involving cooperating parties from the Department of Pharmacology and Pharmacotherapy and also parties from other Departments either within or outside the Semmelweis University. As to the latter aspect, we had a standing cooperation with groups at the HAS/ELTE Research Group of Peptide Chemistry and the Institute of Biochemistry, HAS Biological Research Center, Szeged, specialised on peptide synthetic and/or peptide analytical chemistry. In these cases it was easy to establish a convergence in project coordination because the cooperation was based on our mutual interest in opioid peptides.

At our Department, one traditional research field was the systematical analysis of the factors providing a protection in gastric mucosa against different agressive agents/mechanisms, mostly in rats (Gyires et al., 2000c.). A part of these mechanisms is initiated in autonomic integrative areas within the central nervous system (CNS). One such a CNS area is the dorsal vagal complex in the medulla oblongata, comprising the area postrema, nucleus of the solitary tract and the dorsal motor nucleus of the vagus

(Gyires and Rónai, 2001a; Gyires et al., 2001b). An important core mechanism in the initiation of gastroprotection in the NTS-DVN is a complex interplay between α_{2B} subtype of adrenoceptors (Gyires et al., 2000a) and a number of neuropeptides, which are abundant in this area as compared to other parts of brainstem (Gyires et al., 2000b). In this case, again, the common factor of interest was that members of all opioid peptide families (the pro-opiomelanocortin (POMC)-derived β-endorphin, proenkephalin-, prodynorphin-, pronociceptin-derived peptides and endomorphins) as well as the major opioid receptor types (i.e. δ, μ and κ) are well-represented in the NTS-DVN (Van der Kooy, 1984; Diz et al., 1987). My task in this cooperation was, in part, to install slice preparation(s) prepared from rat NTS-DVN suitable for studying the stimulation-induced release of tritiated neurotransmitters/isomers (^{3}H-NE and ^{3}H-D-Asp). Because the functional definition of α_2-adrenoceptor subtypes is still full of ambiguities (Bylund et al., 1994; Calzada et al., 2001), my further, „etude-like" task was to find an isolated organ and an experimental setting where an α_{2B}-adrenoceptor-mediated action could be demonstrated (Kató et al., 2008).

Finally, in a brief cooperation with the Department of Physiology, Semmelweis University, I had an opportunity i) to install some technical novelties improving the loading- and transmitter release-generating conditions in our brain slice superfusion assembly and ii) to elaborate the loading and release conditions for an additional tritiated transmitter, γ-amino butyric acid in another autonomic integrative center in rat brain, in the hypothalamic PVN/subPVN complex.

The structure and duration of different project sections was governed i) by the actual requirements of research stage and ii) by the promise of success, i.e. by the intention to select and follow those avenues where the potential of discovery was high. This is the reason why, in the case of nociceptin, after having developed and characterized a useful receptor antagonist (Kocsis et al., 2004) the project was cut short at that stage whereas the analysis of partial agonist properties of endomorphins and the exploration of a possible *de novo* biosynthetic pathway for endomorphin-2 received distinguished attention.

3. Introduction

3.1. The NTS-DVN complex

3.1.1. General aspects

The central autonomic integrative network is anatomically and functionally composed of several interconnected areas responsible for tonic, reflex and adaptive control of autonomic functions (Benarroch, 1993; Benarroch, 1997). This network receives visceroceptive, humoral and environmental informations (Loewy, 1990) and contributes to autonomic (Spyer, 1990), endocrine (Swanson, 1991), behavioural motor (Bandler et al., 1991), emotional, attentional (Bechara et al., 2000) and pain-controlling responses (Lovick and Li, 1989). The structures forming the central autonomic network are distributed at the level of the cerebral cortex, basal forebrain, hypothalamus, midbrain, pons and medulla (Loewy, 1991). The insular and medial prefrontal cortices are involved at the highest level of integration of viscerosensory and visceromotor responses (Cechetto, 1987; Neafsey, 1990; Loewy, 1991). The central nucleus of the amygdala and the bed nucleus of the stria terminalis form a unit referred to as the extended amygdala that integrates autonomic, neuroendocrine, and behavioural responses to emotions (LeDoux, 2000). The hypothalamus, as a center of neuroendocrine regulation, integrates autonomic with endocrine responses to maintain homeostasis (Swanson, 1987). Catecholaminergic and noncatecholaminergic pathways convey viscerosensory and somatosensory signals to the hypothalamus and vice versa, the hypothalamus influences autonomic activities through humoral and neurohumoral pathways. Inputs to the hypothalamus arrive from the neocortex, limbic system, retina, brainstem and spinal cord, as well as outputs leave to the limbic system, brainstem and spinal cord. Actually, the hypothalamic afferents are both peptidergic (enkephalin, somatostatin, substance P, CCK, Glu-1, NPY, neurotensin, dynorphins) and catecholaminergic while the descending axons are mainly peptidergic (CRF, vasopressin, oxytocin, somatostatin, enkephalin, ACTH, β-endorphin, α-MSH,

neurotensin and cANP (for review see Palkovits, 1999). However, the first major relay station of the information going to different areas of the central autonomic network is the NTS-DVN complex.

The nucleus tractus solitarii-dorsal motor vagal nucleus complex in the brainstem is the most crucial area in the central integration of visceral functions. It receives the entire viscerosensory afferentation, it is the origin of major parasympathetic outflow to the periphery, it has bidirectional connections with the forebrain limbic circuitry, hypothalamic and pontine integrative areas as well as with the brainstem centers subserving sympathetic outflow (Van der Kooy et al., 1984, Diz et al., 1987; Palkovits, 1999). Moreover, alteration of cardiovascular, respiratory and gastrointestinal functions can be produced by electrostimulation or lesions at different areas of this complex circuitry (Hurley-Gius and Neafsey, 1986; Fisk and Wyss, 2000).

3.1.2. Anatomy of the NTS-DVN

Anatomically, the NTS has two divisions rostrocaudally: the rostral one third which functions primarily with gustatory and possible feeding information as it receives projections from the tongue and taste afferents associated with the Vth, VIIth, IXth and Xth cranial nerves and the caudal two third which plays a major role in the regulation of cardiovascular, respiratory, gastrointestinal and general visceral functions because it receives primary afferent inputs via the IXth and Xth cranial nerves from baroreceptors of the carotid sinus and aortic arch, chemoreceptors of the carotid body, as well as afferents from the heart, lungs, stomach and other viscera like pancreas, kidney.

3.1.3. Neuronal afferents to and efferents from the NTS-DVN. Neurotransmitters

The NTS is not a simple relay of messages coming from peripheral and central sources, but it is also an important center of physiological integration (Spyer, 1994). It receives neural afferents from:

1. the periphery via cranial nerves: the vagus, glossopharyngeal nerves as well as chorda tympani
2. the dorsal horn of cervical and thoracic segments of the spinal cord
3. the brainstem: a). the cardiovascular centers in the ventrolateral medulla,
   b). the reticular formation including the raphe, central gray, lateral reticular formation, the locus coeruleus and the parabrachial nucleus
   c). the area postrema
4. various forebrain areas: the medial and lateral prefrontal cortex, the hypothalamus (Van der Kooy, 1984), the amygdala (Schwaber et al., 1982) and the bed nucleus of the stria terminalis (Grijalva and Novin, 1990).

However, it is generally accepted that if the NTS receives an afferent from another neural area it has an efferent projection there as well. Thereby, the NTS fibers terminate:

1. on vagal efferent neurons in the ambiguus (Loewy and Burton, 1978) and dorsal vagal nuclei (Palkovits and Zaborszky, 1977). These connections on one hand form a short-loop neuronal link between vagal afferent and efferent fibers and on the other hand are essential for the transmission of information from the forebrain to the dorsal vagal nucleus which receives input, apart from the PVN, from forebrain structures mainly via the NTS.
2. on preganglionic efferent neurons in the intermediolateral cell column of the spinal cord
3. on the three catecholaminergic cell groups, present in the ventrolateral medulla: A1 and A5 noradrenergic and C1 adrenergic respectively.
4. in pontine structures such as locus coeruleus, raphe and parabrachial nuclei. The latter one is considered as a relay for visceral afferent coming from the NTS to the forebrain.
5. in forebrain structures including the hypothalamus, various parts of the limbic system such the amygdala or the bed nucleus of stria terminalis (Ricardo and Koh, 1978), the olfactory tubercle (Guevara-Aguilar et al., 1987) and the subfornical organ (Zardetto-Smith and Gray, 1987).

In the neural network of NTS-DVN have been found at least 30 neurotransmitters / neuromodulators. They arise from local neurons as evidenced by the presence of the neurotransmitters / neuromodulators in the cell bodies of the region and extrinsic sources, in fibers projecting in the region (Van Giersbergen et al., 1992).

The NTS contains a variety of cholinergic (Armstrong et al., 1990; Ruggiero et al., 1990), biogenic amine-ergic (Armstrong et al., 1982; Calza et al., 1985; Dahlström and Fuxe, 1964; Kalia et al., 1985a, 1985b; Pickel et al., 1989a), glutamatergic (Talman et al., 1984; Kaneko et al., 1989), GABA-ergic (Hwang and Wu, 1984; Meeley et al., 1985; Pickel et al., 1989b) and peptidergic (Leslie, 1985; Nakamura, 1988; Palkovits 1985, 1988, 1989) elements which have been recognised in neuronal perikarya localized in specific parts and subdivisions of the NTS. Projecting and local neurons can release about 20 neuropeptides in NTS (Palkovits, 1988; Kalia et al., 1984; Riche et al., 1990; Yamazoe et al., 1984) like Substance P (Ljungdahl et al., 1978;), Neuropeptide Y (Pickel et al., 1989c), neurotensin (Higgins et al., 1984), CCK (Kubota et al., 1983), enkephalins (Lee and Basbaum, 1984; Sawchenko et al., 1990), dynorphins (Fodor et al., 1990), β-endorphin (Palkovits et al., 1987), endomorphins (Zadina et al., 1999; Martin-Schield et al., 1999), nociceptin (Neal et al., 2001), somatostatin (Sawchenko et al., 1988), VIP (Palkovits et al., 1982), galanin (Melander et al., 1986), bombesin (Riche et al., 1990), inhibin-β (Sawchenko et al., 1990), ACTH (Palkovits et al., 1987), α-MSH (Palkovits et al., 1987), γ-MSH (Yamazoe et al., 1984), and angiotensin II (Healy et al., 1984). It is proposed that the catecholaminergic neurons constitute the bulk of solitary efferents where as peptidergic neurons may play a role in modulating the physiological state of the target nuclei. Norepinephrine and dopaminergic cells (A2 catecholaminergic cell group) are present throughout the rostrocaudal extent of the NTS; the highest number are observed in the comissural NTS. Epinephrine-containing cells (C1 cell group) are generally more rostral than A2 cells in the NTS. A number of neuropeptide such as enkephalin, dynorphin, CCK, galanin, neurotensin partly colocalises with catecholamine in A2 and C2 catecholaminergic neurons. Epinephrine and GABA may coexist in the comissural NTS, but they are more commonly detected in separate neurons.

Glutamate-containing axons are not associated with any particular cell group or function within the NTS and may be considered to innervate most if not every neurons

in the NTS. The main neurotransmitters of baroreceptors primary afferents are glutamate and substance P. Blockade of glutamate receptors in the NTS with Kyrenic acid abolishes the baroreceptor reflex (Leone and Gordon, 1989).

Finally, the most relevant conclusion for the present work is that the NTS-DVN complex is the richest in neuropeptides in the brainstem. Moreover, all the opioid peptide families (POMC-, enkephalin-, dynorphin- related peptides, endomorphins, nociceptin) as well as the major receptor types (μ, δ, κ, ORL-1) (Mansour et al, 1994) are present in the complex.

3.1.4. Opioid-adrenergic interactions in the NTS-DVN complex

We were and we are still interested in mechanisms involved in the central regulation of gastric acid secretion and of maintenance of gastric mucosal integrity. Literature data suggest that α_2-adrenoceptors play a pivotal role in these mechanisms (Blandizzi et al., 1995; Gyires et al., 1996). Furthermore, the involvement of central α_{2A}-adrenoceptor subtype might be suggested in the inhibition of gastric acid secretion (Blandizzi et al., 1995) whereas the central gastroprotective effect is likely to be mediated by α_{2B}-adrenoceptor subtype (Gyires et al., 2000a). Recently, an opioid component was proved in the antisecretory and mucosal protective effect of α_2-adrenoceptor agonists. The antisecretory (Müllner et al., 2001) and the mucosal protective (Gyires et al., 2000c) effects of α_2-adrenoceptor agonist clonidine were reversed by the nonselective opioid receptor antagonist naloxone and the delta opioid receptor selective antagonist naltrindole as well as by specific antiserum to β-endorphin. This might be analogous to several previous reports where the antihypertensive effect of some α_2-agonists has been suggested to be mediated by a β-endorphinergic mechanism (Farsang and Kunos, 1979; Kunos et al., 1981; Ramirez-Gonzales et al., 1983; Bentley et al., 1983; Van Giersbergen and De Jong, 1987; Li et al., 1996), i.e. in both cases the stimulation of α_2 receptors induces release of β-endorphin.

Although, there is a lot of evidence that the activation of brain α_2-adrenoceptors can influence the release of numerous neuropeptides (Puurunen, 1988; Baranowska, 1990; Gaumann et al., 1991; Lanzi et al., 1994; Yelken et al., 1999) the site of action in

few cases is known. High densities of α_2 receptors were found in several areas where an elevated density of opioid receptors has also been found (Pert et al., 1976; Atweh and Kuhar, 1977), among many others, in the nucleus of the solitary tract and dorsal motor nucleus of the vagus (Kuhar, 1982; Snyder, 1982; May et al, 1989). Furthermore, superfusion of brainstem slices with α_2-agonists increased the release of β-endorphin immunoreactivity (Kunos et al., 1981), and electrolitic destruction of NTS abolished the antihypertensive effect of α_2 agonist clonidine in spontaneously hypertensive rats (Rockhold and Caldwell, 1979). These findings suggest that the brainstem, particularly the NTS-DVN, is a possible site of β-endorphin release.

Outside the pituitary, prominently high concentration of β-endorphin is present in the hypothalamus, being synthesized by neurons residing in the arcuate nucleus. From here endorphin-containing descending projections reach the NTS (Finley et al., 1981), where β-endorphin as well as POMC containing nerve cell bodies have also been detected (Schwartzberg and Nakane, 1983; Joseph et al., 1983; Khachaturian et al., 1983; Palkovits and Eskay, 1987).

To obtain useful informations about neuronal pathways participating in different autonomic functions, surgical or chemical methods are preferred, used independently or in combinations. The local injection of an excitotoxin can cause a presumably selective lesion compared with surgical or electrolytic neuronal damage. Moreover, individual neurotoxic agents can have different effects on specific regions of the nervous system according to the precise amino acid receptors they activate.

Neonatal MSG treatment had been reported to cause retinal damage and neuronal loss in and around the so called circumventricular organs (Olney and Price, 1978) with prominently high-percentage damage in hypothalamic arcuate nucleus neurons (Olney and Price, 1978; Krieger et al., 1979; Mosqueda-Garcia et al., 1986; Pilcher and Joseph, 1986). In order to clarify the source of the released β-endorphin, which may originate either from a local neuronal pool of NTS-DVN or from descending projection originating in the hypothalamic arcuate nucleus (Palkovits et al., 1987), neonatal MSG treatment was used (Rónai et al., 2001). Following MSG treatment the immunoreactive β-endorphin level was reduced drastically in the mediobasal hypothalamus and slightly but significantly in the rostral, not in the caudal extent of NTS region (Table 1.).

Table.1. The immunoreactive β-endorphin contents in brain regions of rats treated neonatally with monosodium glutamate (Taken from Rónai et al., 2001)

Immunoreactive β-endorphin content (fmol / mg prot.)[a]				
Brain region[b]	Sham[c]		MSG[d]	
MBH	129.0 ± 4.3	(n=10)	62.3 ± 0.6***	(n=7)
NTS(r)	16.5 ± 0.7	(n=10)	12.6 ± 1.2*	(n=7)
NTS(c)	18.1 ± 1.2	(n=10)	13.9 ± 0.9	(n=7)

[a] Arithmetic mean ± S.E.M. values are listed, number of experiments appear in parenthesis

[b] Abbreviations: MBH: mediobasal hypothalamus; NTS: nucleus of the solitary tract, (r): rostral, (c): caudal, "commissural"

[c] In rats treated neonatally with hypertonic saline

[d]: MSG: in rats treated neonatally with monosodium glutamate

ANOVA followed by "t" test for grouped samples was used for statistical comparison. $p < 0.05$, *** $p < 0.001$

It was shown previously that δ-opioid receptor agonists applied either intracisternally (i.c.) or intracerebroventricularly (i.c.v.) had gastroprotective effect against acidified ethanol-induced gastric damage in the rat. Moreover, they were more potent given i.c. than i.c.v. suggesting again the brainstem as a site of action (Gyires and Rónai, 2001). Both δ- and μ-opioid receptors were described in NTS-DVN (Snyder, 1982; Mansour et al., 1995). However, it was not possible to prove the presence of δ-opioid receptors by whole-cell patch clamp recordings made from gastric projecting DVN neurons too (Browning et al., 2004). This might be explained by the lower sensitivity of the electrophysiologycal method compared with in vivo experiments detecting the functionality of the receptor.

After neonatal MSG treatment, not only the intracisternally applied clonidine but also the direct δ-opioid receptor stimulant DADLE lost its effectiveness in the maintenance of gastric mucosal integrity. Consequently, the obtained results could be

interpreted only by assuming that a local neuronal damage in the NTS-DVN complex was also caused (Rónai et al., 2001). Indeed, a morphological damage following neonatal MSG treatment have been reported in area postrema-subpostrema region (Olney et al., 1977; Karcsu et al., 1981, 1985; Jászai et al., 1998), we wished to find a measurable indication of destruction of the possible NTS-DVN source of β-endorphin. The measurement of stimulation-induced ^{3}H-NE from rat NTS-DVN slices has been used previously to characterize opioid receptor-mediated processes in the vagal complex (Arakawa et al., 1991; Al-Khrasani et al., 2003). Since the damage of neurons in the hypothalamic arcuate nucleus is prominent in the case of neonatal MSG treatment and therefore a considerable loss of β-endorphinergic projection to the vagal complex can be taken as granted, we decided against the use of opioid receptorial agents. Excitatory amino acid receptors are present in the great majority of neurons in the NTS-DVN complex (Miller and Felder, 1988; Raybould et al., 1989; Willis et al., 1996), and the local neuronal population bearing $GABA_B$ receptors is also substantial (Margeta-Mitrovic et al., 1999; Partosoedarso et al., 2001). Therefore, we used the changes in the responses to L-glutamate or the $GABA_B$ receptor agonist baclofen as potential ‘‘markers’’ of local neuronal changes.

Moreover, taking in consideration the role in the central regulation of several autonomic diseases like first of all gastric ulcer but also hypertension or obesity, nevertheless the receptor population and neuropeptide content of different brain regions, we choosed NTS-DVN to create a simple, useful, quick, reliable in vitro model to study the interactions between peptide- and non-peptide transmitters. Nextly, in collaboration with the Department of Physiology, Semmelweis University, we improved our brain slice superfusion technique in hypothalamic PVN/subPVN experiments. Therefore, we installed the rat NTS-DVN prism then the NTS-DVN/PVN slice preparation and studied the uptake and i). the stimulation induced (electrical field–stimulation, ES and high potassium, KS) release of ^{3}H-NE, ^{3}H-D-Asp from NTS-DVN prism preparation, ii). the modulation of stimulation induced release of ^{3}H-NE by an opioid peptide (endomorphin-1) in NTS-DVN slices and iii). the release of ^{3}H-GABA from NTS/PVN slices, in vitro.

3.2. An α_{2B}-adrenoceptor-mediated action

α_{2B}-adrenoceptors, as defined by Bylund et al. (1994) have a highly restricted distribution both in the central nervous system and at the periphery (Calzada and de Artinano, 2001). This property renders them an attractive target for drug research and development. One of the possible therapeutic profiles is a centrally mediated gastric mucosal protection described in rats (Gyires et al., 2000a) where the site of action appears to be the dorsal vagal complex in the lower brainstem. The centrally mediated antihypertensive effect of α_2-adrenoceptor agonists is attributed mainly to agonism at the α_{2A}-adrenoceptor subtype (Makaritsis et al., 1999b; MacMillan et al., 1996; MacDonald et al., 1997; Cobos-Puc et al., 2007). Therefore, α_{2B} selective agonists are expected to lack this property. Another promising area for the development of α_2 adrenergic receptor subtype selective agonists is the pain relief in neuropathic pain modalities (Cho et al., 1997; Yaksh, 1999; Malmberg et al., 2001; Duflo et al., 2002; Leiphart et al., 2003; Bantel et al., 2005). There is a bit of controversy around the changes in the spinal expression of α_2-adrenoceptor subtypes following peripheral nerve injury in rodent models of neuropathic pain (Cho et al., 1997 vs Leiphart et al., 2003 vs Bantel et al., 2005) and also whether α_{2A}-(Malmberg et al., 2001) or α_{2NON-A} (Duflo et al., 2002) receptor subtypes should be regarded as the primary targets for drug development. However, data obtained in genetically engineered mice rendered deficient in either the α_{2b}- or the α_{2c}-adrenoceptor subtype have indicated that α_{2B}-adrenoceptors may have a deleterious effect on the development of salt-induced hypertension (Makaritsis et al., 1999a) and unbalanced (i.e. selective) α_{2B}-adrenoceptor stimulation might enhance vascular resistance (Link et al., 1996). It is not settled whether the receptors responsible for these possible actions are found in the brain, in the kidney or in blood vessels.

We wished to find a vascular preparation and experimental setting where the effects of α_{2B}-adrenoceptor stimulation can be studied. The pharmacological characterization of α_2-adrenoceptor subtypes is hindered by the lack of conveniently selective ligands. ST-91 (2-[2,6-diethylphenylamino]-2-imidazoline), the key agonist used in the present series, besides α_1-adrenoceptor agonism, has been shown to possess α_{2NON-A}- (Doxey et al., 1981; Dowlatshahi andYaksh, 1997), possibly, α_{2B}-adrenoceptor

(Takano et al., 1992) agonist properties. The choice fell on the rat mesenteric artery because of the relatively high α_{2B}-adrenoceptor mRNA expression as compared to α_{2A} or α_{2C}-mRNAs (Phillips et al., 1997). The experimental setting was suggested by the high expression of α_{2B}-adrenoceptor mRNA in pregnant rat myometrium at term and the probably related persistent inhibition of β_2-adrenoceptor-stimulated adenylyl cyclase by α_2-adrenoceptor agonists in the same period (Mhaouty et al., 1995).

3.3. The endomorphins

The two brain opioid tetrapeptides, endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2, EMO-1) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2, EMO-2) were isolated first in 1997 from bovine (Zadina et al., 1997) and human (Hackler et al., 1997) brain. Their N-terminal opioid motif (Tyr-Pro-Trp/Phe--) differs considerably from the N-terminal sequences found earlier in opioid peptides endogenous to mammals and several other species, i.e. enkephalins (Hughes et al., 1975), endorphins (Bradbury et al., 1976; Li and Chung, 1976) and dynorphins/neo-endorphins (Goldstein et al., 1979; Chavkin et al., 1982) (Tyr-Gly-Gly-Phe) and also from the sequences of „amphibian skin" opioid peptides dermorphin and deltorphins (Erspamer et al., 1978; Erspamer et al., 1989; Kreil et al., 1989; Melchiorri et al., 1982) (Tyr-D-Ala / D-Met-Phe--).

Endomorphin-related non-neural peptides have been detected first in bovine milk and different blood digests. These were the β-casomorphins (Brantl et al., 1979), hemorphin (Brantl et al., 1986) and cytochrophin (Brantl et al., 1985). One casomorphin-related tetrapeptide sequence, designated as morphiceptin (Tyr-Pro-Phe-Pro-NH_2, Chang et al., 1981) has been claimed to occur naturally. It was found a highly selective though rather weak agonist of μ-opioid receptors. Following this trail, first Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2, Horvath and Kastin, 1989) then Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-NH_2, Érchegyi et al., 1992) were isolated from brain tissue. These peptides were antagonist-like rather than agonist-like ligands of μ-opioid receptors.

Endomorphins are highly potent and extremely selective partial agonists at μ-opioid receptors *in vitro* (Zadina et al., 1997; Alt et al., 1998; Hosohata et al., 1998; Rónai et al., 1998/99; Al-Khrasani et al., 2001). Endomorphins possess a relatively

moderate analgesic potency in the acute nociceptive tests given by central route as compared either to morphine or DAMGO, particularly if their relative *in vitro* potencies are also taken into consideration (Zadina et al., 1997; Rónai et al., 1998/99; Horváth, 2000; Przewlocki and Przewlocka, 2001). There is a prominent interspecies (rat versus mouse) and localisation (supraspinal versus spinal) dependency in these analgesic tests. Endomorphhins have more promising actions in some neuropathic pain models (Przewlocki and Przewlocka, 2001). Further, potentially advantageous pharmacological properties are the possible dissociation of analgesic and rewarding propensities in the rat (Wilson et al., 2000) and the moderate respiratory depression (Czapla et al., 2000; for review see Fichna et al., 2007).

There were two particular features in endomorphin biochemistry/pharmacology that arouse our interest. One of them was their partial agonism. In an early review (Horváth, 2000) a possible neurotransmitter role has been proposed for endomorphins in spite of the fact that partial agonism had been reported for them in the GTPγS binding stimulation assay (Alt et al., 1998; Hosohata et al., 1998). Theoretically, partial agonism is an unexpected property in a first messenger candidate. By using a simplified pharmacological approach (Al-Khrasani et al., 2001) we were able to demonstrate this partial agonism also pharmacologically. In my thesis, I will present the results of a more sophisticated method for the calculation of parameters for characterizing partial and full agonism (Rónai et al., 2006).

The second and most puzzling feature in endomorphin biochemistry is that their biosynthetic route is still uncertain. Advanced searches in genome and proteome databases (Wolfe et al., 2007; Terskiy et al., 2007) have failed to disclose genomic codes for endomorphin precursor protein(s). Still, they have a widespread distribution in mammalian central nervous system (Martin-Schild et al., 1999; Pierce and Wessendorf, 2000; for review see Wang et al., 2002), and, under special conditions, their release can be detected (Lisi and Sluka, 2006; Scanlin et al., 2008). We have proposed the possibility of a *de novo* biosynthetic pathway (Rónai et al., 2006), which is known in prokaryotes and used also in *in vitro* peptide synthetic chemistry by reversing peptide hydrolases (Clapés et al., 1995; Ye et al., 1998) but, with the exception of dipeptide carnosine and the tripeptide glutathione, is unprecedented in eukaryotes. However, when injecting tritiated Tyr-Pro dipeptide into the lateral cerebral ventricle of rats, we

have found the incorporation of label into two, chromatographically endomorphin-2 related tetrapeptide species in purified brain extracts (Rónai et al., 2006). Subsequently, we have detected endomorphin-2-like immunoreactivities in similarly processed rat brain extracts with the same RP-HPLC-gradient elution retention times where the label incorporation had previously been found by RP-HPLC – radiodetector combination (Szemenyei, Barna et al., 2008). My contribution in the early- and mid-stages of these efforts was to take part in the extraction procedure and the development of radio-immunoassay to endomorphins, and, more importantly, to characterize pharmacologically the endomorphin-related substances which were detected, or might have been detected in the course of radiolabel incorporation and immunoanalyses. These substances were, on the one hand, the endomorphins with a free carboxylic terminus because both the incorporation of radiolabel and the immunoreactivity appeared in the RP-HPLC elute with the retention time matching that of authentic EMO-2-OH standard. The other peptides to be characterized were the glycine-extended endomorphins (Szemenyei, Barna et al., 2008). It is known that in the course of traditional post-translational processing an amidated end-product is generated by peptidyl-glycine-α-amidating monooxygenase enzyme (PAM) (Eipper and Mains, 1991; Eipper et al., 1991). Assuming that in case of a *de novo* biosynthetic pathway the same rule applies to the production of a C-terminally amidated end-product, the immediate, short-lived biosynthetic precursors of endomorphins must be the glycine-extended pentapeptides. Furthermore, because until the quite recent stages (Rónai, Király et al., 2009) of our exploratory experiments for the elucidation of the *de novo* biosynthetic pathway of endomorphins, we presumed that the biosynthesis takes place intracellularly, we have designed serine-extended, O-glycosylated Tyr-Pro derivatives to promote the access of Tyr-Pro precursor to the presumed intracellular biosynthetic compartment. These derivatives were characterized both in the isolated organ bioassay and in the incorporation experiments with RIA detection.

3.4. The “operational” calculation model

All the chemical substances which are bound specifically to the receptors are the ligands. The ligands may be in competitive relationship with each other i.e. they may compete for the same binding site or one may allosterically modulate the binding of the other. According to the presently standing nomenclature (for review see Neubig et al., 2003), the binding site of receptor which interacts with the endogenous, first messenger ligand is the *primary* or *orthosteric binding site*, whereas all the other specific, functionally interacting binding sites are referred to as the *allosteric binding sites.* A ligand may be a pure antagonist (providing such a category can theoretically exist at all), partial agonist, full agonist or an inverse agonist. Pure antagonism is characterized only by the affinity of ligand (the ligand has virtually no ability to activate the receptor). Full agonism besides affinity is characterized also by the power of an agent to optimally activate the receptor upon binding (this is the „intrinsic efficacy” property of an agonist). Structural region(s) in a ligand responsible for binding (receptor recognition) is/are refferred to as the „address” domain(s) whereas the region(s) responsible for the receptor activation is/are designated as the „message” domain(s) (Schwyzer, 1980).

Partial agonism is a continuum, ranging from near-antagonist to near-agonist property. In a complex biological system it may be enough to activate fully only a fraction of receptors present to elicit full biological response (this biological system is said to contain a considerable receptor reserve with “spare” receptors). In a system with high receptor reserve even a partial agonist may produce a full biological response i.e. may behave, apparently, as a full agonist. In a system with low receptor reserve only the fullest possible activation of receptor pool will produce maximal biological response. In such a system a partial agonist –depending on its position in the partial agonist continuum- may produce only a fractional agonism or may behave, apparently, as a pure, competitive antagonist.

The most conventional method to characterize agonist affinity and efficacy by pharmacological means is the so called “null” method (Stephenson, 1956; Barlow and Andrews, 1967; Furchgott, 1978), an *indirect* way to treat experimental data; accordingly, using double-reciprocal plot of equieffective concentrations of agonists before and after the fractional receptor pool inactivation, we can calculate the residual

receptor fraction “q” and the agonist dissociation constant “K_A”. This method was developed avoiding the need to make assumptions about postreceptor events whereas the “operational” model of agonism calibrates first the parameters of receptorial transducer function by constructing an equation for the [E]/[A] relation of a full agonist; Providing an explicit definition of the relation between agonist concentration and pharmacologic effect, this can, therefore, be fitted *directly* to experimental agonist concentration-effect data in order to estimate affinities and relative efficacies of agonists.

The operational model is practically a more straightforward way of analyzing agonism (Black et al., 1985; Leff, 1990) than the conventional, null-equation approaches, however, its application is limited because of the assumptions that it makes about E/[A] curve shape.

In my doctoral thesis, using the “operational” model, I determine the partial agonism of endomorphins and their analogs (endomorphin-1-ol, 2',6'-dimethyltyrosine (Dmt)-endomorphin-1, endomorphin-2-ol and (D-Met^2)-endomorphin-2), in isolated mouse vas deferens by partial μ-opioid receptor pool inactivation strategy. Morpine, the first known, Tyr-Pro-related natural, μ-opioid receptor-selective agonist peptide, morphiceptin (Tyr-Pro-Phe-Pro-NH_2, Chang et al., 1981) and the full agonist prototype enkephalin analogs DAMGO (Kosterlitz and Paterson 1981) and its amide congener DAMGA (Kocsis et al., 2000) were used as reference substances.

3.5. The ORL-1/NOP receptor, nociceptin

3.5.1. History

After having identified the primary structure of δ opioid receptor protein by cloning the cDNA encoding for the receptor (Evans et al., 1992; Kieffer et al., 1992) the μ and κ opioid receptor types were cloned thereafter by using homology screening. By using similar homology screening strategies, several groups identified another cDNA clone termed as opioid receptor-like (ORL-1 human, Mollerau et al., 1994), rat opioid receptor-C (Fukuda et al., 1994) or mouse opioid receptor-C (Nishi et al., 1994). This

„orphan" receptor (i.e. at the time of identification with no known endogenous or exogenous ligand candidate) is highly homologous in amino acid sequence to the three known opioid receptor types and yet fails to show significant binding activities to major opioid ligands (Chen et al., 1994; Wang et al., 1994).

The endogenous ligand of ORL-1 receptor, a heptadecapeptide with the primary structure of Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln has been isolated and identified independently by two groups (Meunier et al., 1995; Reinscheid et al., 1995) from rat brain and pig hypothalamic extracts, respectively. The ligand, by a hint at its assumed role in nociception, was given the name of nociceptin (N or Noc) or was designated more prosaically in terms of its N- and C-terminal amino acids as orphanin FQ (OFQ).

The history of nociceptin research created an example of „inverse pharmacology" in the sense that the identification of receptor structure was followed by the identification of endogenous ligand candidate, and both are being followed presently by the creation of synthetic ligand pool necessary to establish the possible physiological and pathophysiological function(s) of this novel family of endogenous peptides and to initiate R&D strategies for finding the proper therapeutic targets.

3.5.2. Anatomy

The distribuiton of receptors was determined both in humans and rodents, using receptor binding autoradiography/histochemistry, OFQ-1-stimulated [^{35}S]GTPγS binding, probes directed to receptor protein or receptor mRNA (Bunzow et al., 1994; Mollerau et al., 1994; Fukuda et al., 1994; Lachowicz et al., 1995; Dun et al., 1997; Sim and Childers, 1997; Peluso et al., 1998; Houtani et al., 2000; for review, see Henderson and McKnight, 1997; Mogil and Pasternak, 2001). The „consensus" pattern shows high-, to moderate density in several cortical areas, particularly the pyriform cortex, dentate gyrus-hippocampus, lateral septum, nucleus accumbens, amygdala, subparafascicular thalamic nucleus, paraventricular, pre- and perioptic, anterior and ventromedial hypothalamic nuclei, central grey, pontine dorsal tegmentum, locus coeruleus, raphe nuclei particularly the dorsal raphe, brainstem auditory nuclei, the NTS, the spinal

trigeminal nucleus and the spinal cord, particularly the superficial layers of dorsal horn. The receptor is conspicuously absent from the cerebellum.

Not morphological but rather functional evidence suggests the presence of ORL-1/NOP receptors on peripheral sensory nerve endings and the neural elements of some autonomically innervated smooth muscle organs/tissues such as the longitudinal muscle strip/myenteric plexus of guinea-pig ileum, mouse colon, mouse-, rat- and rabbit vas deferens and several blood vessel beds (Wang et al., 1994; Berzetei-Gurske et al., 1996 Meunier, 1997; Nicholson et al., 1998; Bigoni et al., 1999; Menzies et al., 1999).

The receptor and ligand distribution in the CNS suggests that the system is likely to be involved in the modulation of nociception-related events, stress reactions, learning and memory, food, and water intake, reward functions, central autonomic integration, hormonal regulation and hearing.

3.5.3. Pharmacology

The ORL-1/NOP belongs to the G protein coupled superfamily of receptors, characterized by seven helical transmembrane regions. Because of the structural similarities of receptors, it is not surprising that the $G_{i/o}$-related signalling features by the ORL-1/NOP receptors share many similarities with the signalling mechanisms of other (μ, κ and δ) opioid receptors (Henderson and McKnight, 1997). However, the ligand binding selectivity profiles of ORL-1 and other opioid receptors are highly distinctive.

Using different radioligands, high affinity, saturable, sodium- and divalent cation sensitive ORL-1/NOP receptor binding was found in various cell lines expressing the receptor as well as in rodent brain membrane preparations. The binding parameters and the displacement characteristics were considerably influenced by the choice of labelled ligand. The primary ligands used were [^{125}I]- or [^{3}H]-(Tyr^{14})-nociceptin(1-17), [^{125}I]-(Tyr^{10})-nociceptin(1-11) or [^{3}H]-Ac-Arg-Tyr-Tyr-Arg-Trp-Lys-NH_2, a synthetic hexapeptide “screening hit” (Reinscheid et al., 1995, 1996; Dooley and Houghten, 1996; Pan et al., 1996; Ardati et al., 1997; Mathis et al., 1997, 1999; Albrecht et al., 1998; Thomsen et al., 2000; for review see Mogil and Pasternak, 2001). The binding

affinity of OFQ-1/nociceptin to transfected ORL-1/NOP receptor is characterized by a K_i/K_D of 30-80 pM range. Several groups reported similar ligand K_D values in transfected cells and brain membrane preparations (Albrecht et al., 1998; Thomsen et al., 2000) others (Dooley and Houghten, 1996; Mathis et al., 1997, 1999) found lower affinities in the brain.

The natural ORL-1/NOP receptor ligands i.e. nociceptin (1-17) and its bioactive fragments display affinity towards their own receptor but no appreciable affinity to other, classical opioid receptors such as μ, δ or κ; if anything, an extremely weak binding to the latter might take place. On the other hand, depending on the radioligand choice for the ORL-1/NOP receptor, some μ, κ and, to a much lesser extent, δ opioid receptor ligands may have displacing potencies at much higher concentrations that at their own respective receptors. Full length nociceptin is a high-affinity homologous displacer in the receptor binding assay when labelled heptadecapeptide derivative is used as the primary ligand. As the endogenous ligand, it is also the prototype full agonist.

The full peptide has potential sites for further processing to give rise to NOC/OFQ (1-13), (1-11) or (1-7). The main biodegradation routes are effected by aminopeptidase N and EC 3.4.24.14 enzyme activities, respectively (Montiel et al., 1997). However, there is a striking contrast with the biologically active cleavage product of dynorphin A (1-17), (dynorphin A (1-8)) (Corbett et al., 1982), which opioid peptide has the highest structural resemblance to nociceptin (1-17), as the degradative fragments (1-13), (1-11) and (1-7) show lost affinity by a factor of 30 in the case of (1-13) (Dooley and Houghten 1996) and 1000 times or even lesser activity then the parent peptide in the case of (1-11) and (1-7) respectively (Mathis et al., 1997).

Alanine scanning revealed the functional importance of amino acids in positions 1, 2, 4 and 8 (Dooley and Houghten, 1996). Deletion of Phe^1 (nociceptin (2-17)) caused, similarly to the feature well known for the des-Tyr^1-derivatives of classical opioid peptides, a more than 1000 fold loss in affinity. Tyr^1-nociceptin was high-affinity agonist at nociceptin receptor; however, the selectivity was considerably lost as reflected by a 100-fold increase in affinity for μ and κ, and, to a much lesser extent, δ opioid receptors. Replacement of Phe^1 or Phe^4 by leucine revealed that Phe^4 rather than Phe^1 is responsible for receptor activation (Guerrini et al., 1997). Therefore it was

stipulated that Phe-Tyr-Gly-Gly-Phe may serve as the common, "ancestral" address-and-message sequence for all opioid receptors including ORL-1/NOP, μ, κ and δ (Meunier et al., 1995). However nociceptin (6-17) retains a considerable affinity for the ORL-1/NOP receptors (only a 100-fold loss of affinity as compared to nociceptin (1-17)); more importantly, the fragment is capable of activating the receptor in the same concentration range (Butour et al., 1997). Therefore, nociceptin (1-17) may possess not just one but two message domains; this assumption is supported by the finding that a hexapeptide hit from positional scanning of combinatorial libraries of hexapeptides, Ac-Arg-Tyr-Tyr-Arg-Trp-Lys-NH_2 (Dooley et al., 1997) is a high affinity partial agonist at ORL-1/NOP receptors. This means that the peptide may show apparently pure competitive agonist properties in systems where the receptor reserve is low and may be a high-potency (even apparently full) agonist in systems where the receptor reserve is high. This emphasizes further the importance of basic residues in the formation of message core.

To explore the physiological functions of novel endogenous mediators and their receptors, selective, pure and potent competitive antagonists are the most useful tools. Naloxone gave such an impetus to opioid research (Kosterlitz and Watt, 1968), which was also expected from NOP receptor antagonists possessing similar pharmacological parameters. Contrary to the rapid identification of the natural agonist ligand for the NOP receptor, it took several years to find antagonist candidates for the NOP receptor system.

[Phe^1-ψ(CH_2–NH)Gly^2]nociceptin-(1–13)-NH_2 (FψGNC) is considered a NOP receptor antagonist of fair selectivity but modest affinity (Guerrini et al., 1998; Butour et al., 1998) and possessing also considerable partial agonist property (Menzies et al., 1999; Olianas et al., 1999). [$Nphe^1$]-nociceptin(1-13)-amide (Calo et al., 2000) is a selective, low affinity competitive antagonist whereas the hexapeptide hit Ac-Arg-Tyr-Tyr-Arg-Ile-Lys-NH_2 (Ac-RYYRIK-NH_2) described by Dooley and her coworkers is a ligand of much higher affinity (Dooley et al., 1997), however, the partial agonist character is still present (Berger et al., 1999). Importantly, this antagonist structure was found by pure combinatorial chemical methods, i.e., no assumption was made on the structure, metabolism or by any other means. Neither the sequence, nor the amino acids included in the structure are similar to the original nociceptin agonist structure (Table

1). One of the structural features of this compound is that its N-terminal amino group cannot be protonated since it is acetylated. Although the arginine or lysine side chain can be a substitute for the protonated N-terminal (Gacel et al., 1979), there are examples where opioid peptides lacking the protonated N-terminal amino group displayed adequate binding properties (Rónai et al., 1992; Rónai et al., 1993; Schiller et al., 2000; Lu et al., 2001; Weltrowska et al., 2003). Chemical modifications at the amino group of Tyr^1 in opioid peptides can also result in novel antagonist structures (Rónai et al., 1992; Rónai et al., 1993; Wan et al., 1999). A series of δ- and κ-opioid antagonists based on *N*-acyl peptide derivatives were described in our laboratories and others (Rónai et al., 1992; Rónai et al., 1993; Wan et al., 1999; Orosz et al., 1994; Orosz et al., 1995; Vig et al., 2003), which by analogy, might also have some effect on NOP receptor.

As a part of my PhD work, in collaboration with peptide chemistry research groups, I characterized by in vitro pharmacological bioassays a novel N-acyl hexapeptide (N-Ac-RYYRIK-ol), rich in positively charged amino acid side chains and containing both non-protonated N-terminus and a hydroxymethylene ($-CH_2OH$) moiety at the C-terminal and I compared its properties to known antagonists.

In present series of experiments it is demonstrated that Ac-RYYRIK-ol is a competitive NOP receptor antagonist of high affinity; its K_e value is comparable to that of naloxone at the opioid receptors in isolated organ preparation.

4. Aims

My doctoral work was aimed at:

1. testing the effect of neonatal MSG treatment on the field stimulation-induced release of ^{3}H-NE from NTS-DVN prism preparations and the differences in the responses to modulatory agents
2. exploring the differences in loading and release parameters of ^{3}H-NE and ^{3}H-D-Asp in NTS-DVN prism preparations
3. examining the effect of EMO-1 on field stimulation-induced release of ^{3}H-NE from NTS-DVN whole slice and prism preparations
4. establishing the loading and stimulus-induced release parameters of ^{3}H-GABA in PVN/subPVN and NTS-DVN whole slices
5. demonstrating an α_{2B}-adrenoceptor-mediated action by isolated organ technique.
6. analysing the partial agonist properties of endomorphins and other μ-opioid receptor agonists by using the „operational" calculation model
7. characterizing by in vitro pharmacological method the presumed endomorphin biosynthetic intermediates
8. characterizing a novel nociceptin antagonist by in vitro pharmacological approach

5. Materials and methods

5.1 Materials

All the substances used and not mentioned below were of analytical grade and were obtained either from Reanal Ltd (Hungary) or the Hungarian affiliation of Sigma-Aldrich Co. (St. Louis, MO, USA).

5.1.1. Superfused brain slice measurements

L(+)-ascorbic acid was obtained from Reanal Ltd., (Budapest, Hungary), (R)-baclofen, L-glutamic acid, AT-II, β-alanin, Nipecotic acid and Amino-oxoacetic acid from Sigma-Aldrich Ltd. (St. Louis, USA).

L-[ring-2, 5, 6,-3H]-norepinephrine (47.5 Ci/mmol) and D-[2, 3,-3H]- aspartic acid (16.2 Ci/mmol) was product of Perkin Elmer Inc. (Boston, USA). ^{3}H-GABA (specific activity 73.8 Ci/mmol) was purchased from NEN-Radiochemicals (Boston, MA).

Endomorphin-1 was synthetised by solid-phase procedure by L. Kocsis of Eötvös University, Budapest.

5.1.2. The α_{2B} adrenoceptor

ST-91 was synthesized by P. Mátyus of the Department of Organic Chemistry, Semmelweis University, Budapest, Hungary. Acetylcholine iodide, L-phenylephrine hydrochloride, xylazine (2-[2,6-dimethyl-phenylamino]-4H-5,6-di-hydrothiazine) hydrochloride and yohimbine hydrochloride were products of Sigma-Aldrich Co. (St. Louis, MO, USA). D,L-isopropylarterenol hydrochloride was obtained from Serva Feinbiochemica, (Heidelberg, Germany), prazosine hydrochloride from Pfizer Co. (New York, NY, USA) and terbutaline sulfate from AstraZeneca Co. (Sweden).

5.1.3. The “operational” calculation model

β-Funaltrexamine hydrochloride (β-FNA) was obtained from Tocris Cockson Ltd. (Bristol, UK), normorphine base and morphine sulfate from ICN Alkaloida Ltd. (Tiszavasvári, Hungary).

The syntheses of peptide amides were performed by solid-phase. The details of synthetic and analytical procedures are described elsewhere (Al-Khrasani et al., 2001; Kocsis et al., 2000; Lengyel et al., 2002; Rónai et al., 1998/1999). According to the analytical proofsheets received with each peptide batch, the peptides had the correct amino acid composition and an at least 95% HPLC purity; batches of Dmt-EMO-1 were also routinely subjected to mass spectrometry.

5.1.4. The pharmacological characterization of potential biosynthetic precursors of endomorphin-2

Endomorphins and endomorphin fragments/derivatives were synthesized at HAS Institute of Biochemistry, Biological Research Center, Szeged, by solid-phase method using the Merrifield procedure (Tömböly et al., 2001) applying the resin proper for free or amidated C-terminus, respectively. Cyprodime hydrobromide (CYP) was kindly provided by H. Schmidhammer of University of Innsbruck, Austria, naltrindole hydrochloride (NTI) by S. Hosztafi of ICN Alkaloida, Hungary whereas naloxone (NX) and naltrexone hydrochloride (NTX) were gifts from DuPont Pharmaceuticals, Geneva, Switzerland. DADLE and DAMGO were synthesized by A. Magyar, endomorphin-1-ol and -2-ol by L. Kocsis (Kocsis et al., 2000; Al-Khrasani et al., 2001) of HAS/ELTE Research Group of Peptide Chemistry, Budapest. Serine-extended Tyr-Pro derivatives and their O-glycosylated products (YPS, YPGS and their –Ser-O-glucose products) were synthesized by E. Szemenyei as described previously (Tömböly et al., 2002).

Labelled, high specific activity Tyr-Pro (~50 Ci/mmol) was produced by catalytical tritiation of 3`,5`-diiodo-Tyr-Pro precursor (Rónai et al., 2006). Radioiodination of EMO-2 was carried out by a revised version of the Chloramine T method under conditions that favour iodination of Tyr^1 (Szemenyei, Barna et al., 2008).

Both labelled derivatives were synthesized in and certified from Geza Tóth's laboratory, HAS Institute of Biochemistry, Biological Research Center, Szeged.

Trans-styrylacetic acid was obtained from Sigma-Aldrich Ltd.

5.1.5. Experiments related with a nociceptin antagonist

The synthesis of the used peptides was done by L. Kocsis and G. Orosz of HAS/ELTE Research Group of Peptide Chemistry, Budapest (Orosz et al., 1994, Kocsis et al., 2004).

5.2. Methods

5.2.1. General

Male Wistar/Wistar rats (adults or post-weaned groups) and CFLP or NMRI mice were kept in groups of 5-6 and groups of 8, respectively. Animals were housed in a temperature (22±0.5°C)- and humidity-controlled room with 12 h light/dark (08:00-20:00/20:00-08:00) cycle, and free access to tap water and standard rodent chow. Experiments were carried out between 10:00 and 15:00. The conditions of animal housing and experimentation followed the ethical guidelines set by the Ethical Board of Semmelweis University, based on European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. 32.§).

In general, the experiments were carried out in carbogen-aerated (O_2:CO_2=95:5) Krebs' solution with the following composition (in mM/L): NaCl 118.0, $NaHCO_3$ 25.0, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, glucose 11.0. The certain modifications made will be mentioned where is necessary at each experimental paradigms section.

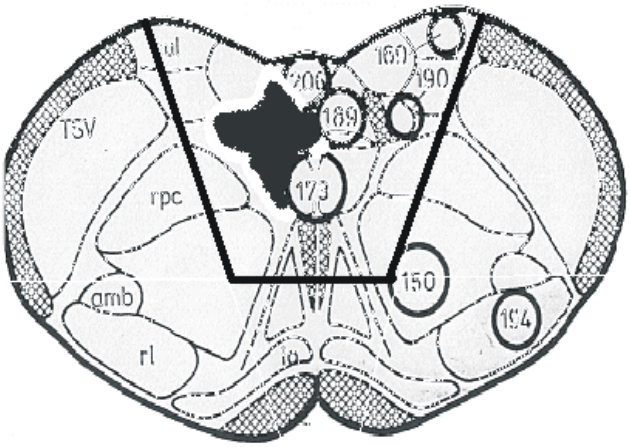
5.2.2. Brain slice preparations

5.2.2.1. Rat NTS-DVN slice/prism

The preparation was a modification (Al-Khrasani et al., 2003.) of the procedure described by K. Arakawa (Arakawa et al., 1991). The brain was removed and immersed immediately into 150 ml of cold (4°C) Krebs-bicarbonate buffer for 60 s.

The brainstem was dissected on a flat ice-battery covered by buffer-soaked filter-paper. One, approximately 500 μm thick coronal slice was cut immediately below, one at the level of and one above the obex (Fig. 1.)

Fig. 1. The preparation of rat nucleus tractus solitarii-dorsal motor vagal nucleus slices



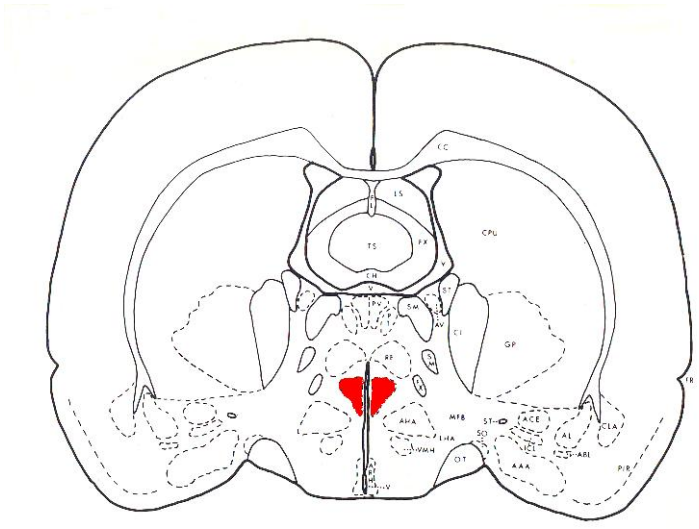
From the individual slices first a triangle with 3.0-3.5 mm sides was cut in the midline (apex down) and the apical part (approximately 1.5 mm) was removed. From the "truncated" triangles 200 μm thick, 1.0-2.5 mm long parasagittal *prisms* (i.e. 200 × ~500 × 1,000-2,500 μm) were cut by a MacIlwain tissue chopper. Prisms prepared from one rat were placed into the loading chamber containing 2.5 ml thermostated (36 °C), oxygenated (O_2:CO_2 = 95:5, i.e. carbogen) Krebs buffer containing 10^{-6} ascorbic acid.

To maintain neural connections we were interested in, we prepared *"whole" trapezoidal slices* in the third series of experiment, with approx 2.5-4 mm sides and 500 μm thickness, i.e. the preparation step before the parasagittal prisms.

5.2.2.2. Rat PVN/subPVN slice

For the dissection of hypothalamic PVN, we have followed first the "Microdissection of Individual Brain Nuclei and Areas" (Palkovits, 1986). Subsequently, upon a personal instruction from Prof. M. Palkovits, we included also the subPVN, which is rich in GABA-ergic elements. These instructions were also suitable to perform a simplified dissection pattern. In brief, on the supinely immobilized rat brain we made a coronal section approximately 0.5 mm rostrally to the virtual tangentional line connecting the apical part of olfactory tubercles. The next, parallel coronal section was made 0.5-0.8 mm behind the first one. From this slice, the red triangles shown in Fig. 2. were dissected. Two such pairs were pooled from two animals and used in a single loading and then the superfusion chamber.

Fig. 2. Dissection guide for hypothalamic paraventricular nucleus



5.2.3. Isolated organ preparations

5.2.3.1. Mouse vas deferens

Vasa deferentia from mice weighing 35-40 g were prepared, mounted and field-stimulated as described previously (Rónai et al., 1977a; Rónai et al., 1979, 1981b). In brief, a single vas per bath was mounted in Mg^{2+}-free Krebs' solution aerated with carbogen (O_2:CO_2=95:5) at 31°C under an initial tension of 0.1 g. The stimulation parameters were as follows: field stimulation of pairs (100 ms pulse distance) of rectangular impulses (1 ms pulse width, 9 V/cm i.e. supramaximal intensity) were repeated every 10 s.

5.2.3.2. Rat vas deferens

Vasa from male Wistar/Wistar rats weighing 140–160 g were dissected, cleaned by stripping and mounted as described previously (Rónai et al., 1981b; Vízi et al., 1983). After mounting, preparations were left without stretching for 10 min, then an initial tension of 1 g was applied, adjusted twice thereafter in the first 15 min, and field electrical stimulation was started. Stimulation parameters: rectangular pulses of alternate polarity, 1 ms pulse width, supramaximal intensity (9 V/cm voltage drop), 0.1 Hz frequency. 40 min equilibration was allowed before drug addition under stimulation, with 10 min washing cycles.

5.2.3.3. Aortic and mesenteric artery rings

Four aortic rings (approx. length 3.5 mm) and/or two mesenteric artery rings (2.5 mm) were prepared from the same rat (250-350 g). Endothelium destruction was effected mechanically, using "watchmaker's file" (aorta) or cut-tip, coarse-surfaced injection needle (mesenteric artery). For mounting, modified "Edvinsson hook" (Edvinsson et al., 1974) was used. Stepwise stretching paradigm was the same as for rat

vas deferens, the initial tension being 2 g; 40 min equilibrium was left after final stretching.

5.2.4. Neonatal monosodium glutamate treatment

Male Wistar/Wistar rat pups were treated subcutaneously with 4mg/g monosodium glutamate on postnatal days 2, 4, 6, 8 and 10 or matching volume of 10 % (w/v) NaCl as isosmotic control ("untreated" control littermates).

5.2.5. Superfused brain slice technique

5.2.5.1. Loading, perfusion and extraction conditions of ^{3}H-NE in NTS-DVN prism preparations of rats neonatally pretreated with MSG

5.2.5.1.1. Experimental paradigms

The experiments were carried out in male Wistar/Wistar rats weighing 180-260 g (young adults) or 460-580 g ("untreated" littermates of MSG-treated rats) and in rats treated neonatally with monosodium glutamate (350-530 g).

The slices were equilibrated for 30 min in 2.5 ml Krebs buffer containing 10^{-6}M ascorbic acid, aerated with carbogen at 36°C. The tissue was loaded with 2.5 μCi/2.5 ml of ^{3}H-NE (56.3 Ci/mmol) for 15 min. The slices were transferred to superfusion chambers where superfusion with carbogen-saturated buffer with no additives was started at least 10 min before the transfer. Perfusion rate was 1.0 ml/min for 50 min and 0.5 ml/min for 10 min before collection. Throughout the collection period the rate was maintained at 0.5 ml/min with 3 min fractions.

Two types of experiments were performed. In the first type we collected 26 fractions and used two stimulation periods, during fractions 12 and 22, respectively. In this type of experiment no drug was added. The second type involved the collection of 16 fractions with one stimulation period during fraction 12. Drugs were added after

fraction 7 and were present throughout the experiment thereafter. The parameters of stimulation were as follows: field stimulation, rectangular impulses of 2 ms pulse width, 50 mA intensity (constant current, alternate polarity). The impulses were delivered at 2 Hz frequency for 3 min, i.e. for the entire duration of the fraction(s) indicated.

Radioactivity remaining in the tissue at the end of experiments was extracted by homogenation in 1.0 ml of 0.1 n HCl. Homogenates were centrifuged and the radioactivity in the supernatant as well as in the collected samples was determined by liquid scintillation spectrometry.

5.2.5.1.2. Evaluation

Radioactivity present in the fractions was expressed as the percent of tissue content at the beginning of the actual fraction (i.e. as "fractional release"). To assess stimulation-induced ^{3}H-NE release, the area under the curve (AUC) produced over the resting baseline (calculated as the mean of three pre-stimulation rates) by a given stimulation period (i.e. between fractions 11-16 and optionally 21-26) was calculated. For the presentation of data arithmetic mean ± S.E.M. values were calculated. Tabulated data are given as geometric mean and 95 % confidence interval values. The statistical probes were applied to the logarithms of data sets because they passed the criteria for normal distribution whereas in the case of arithmetic sets some groups failed to do so. For statistical comparisons one-way ANOVA followed by least significant difference (LSD) test or student "t" test for grouped samples was used. In all the subsequent experiments either this kind of statistical probes were used or in the original "fractional release"-time course curves, Bonferroni test was performed, applied to a maximum of six data sets to evaluate the stimulation-induced point-to-point increments as compared to the last pre-stimulation value.

5.2.5.2. Loading, perfusion and extraction conditions of ^{3}H-NE and ^{3}H-D-Asp in NTS-DVN prisms

5.2.5.2.1. Experimental paradigms

The experimental paradigms were the same as in the part before with the following changes:

- we used modified Krebs buffer: KH_2PO_4 was replaced by NaH_2PO_4; KCl is set at 3.0 mM in "normal" Krebs' (composition in mmol/L: NaCl 119.7; $NaHCO_3$ 25; NaH_2PO_4 1.2; KCl 3.0; $CaCl_2$ 2.5; $MgSO_4$ 1.2; glucose 11). High potassium Krebs' buffer used for the stimulation contained 60 mM KCl and 62.7 mM NaCl.
- 10^{-6} M ascorbic acid was added in experiments with ^{3}H-NE but not with ^{3}H-D-Asp
- three types of experiments were performed. In two of them 28 fractions were collected and two stimulation periods were used: field electrical stimulation (ES) was applied at fractions 7 (S_1) and 17 (S_2) whereas 60 mM KCl (KS) for two fractions at 12, 13 (K_1) and at 24, 25 (K_2). During the third type of measurement, were collected 16 fractions and Ca^{2+} free Krebs containing 1 mM EGTA was started 21 min before adding 60 mM KCl.

5.2.5.2.2. Evaluation

To assess stimulation-induced ^{3}H-NE or ^{3}H-D-Asp release, the area under the curve (AUC) produced over the resting baseline in 5 fractions after the commencement of the stimulation was calculated. From the repeated ES as well as KS induced release, values S_2/S_1 respective K_2/K_1 ratios were obtained.

5.2.5.3. Loading, perfusion and extraction conditions of ^{3}H-NE in rat dorsal vagal complex *whole trapezoidal slice* preparation

5.2.5.3.1. Experimental paradigms

In the next subseries of experiments we made the following changes:

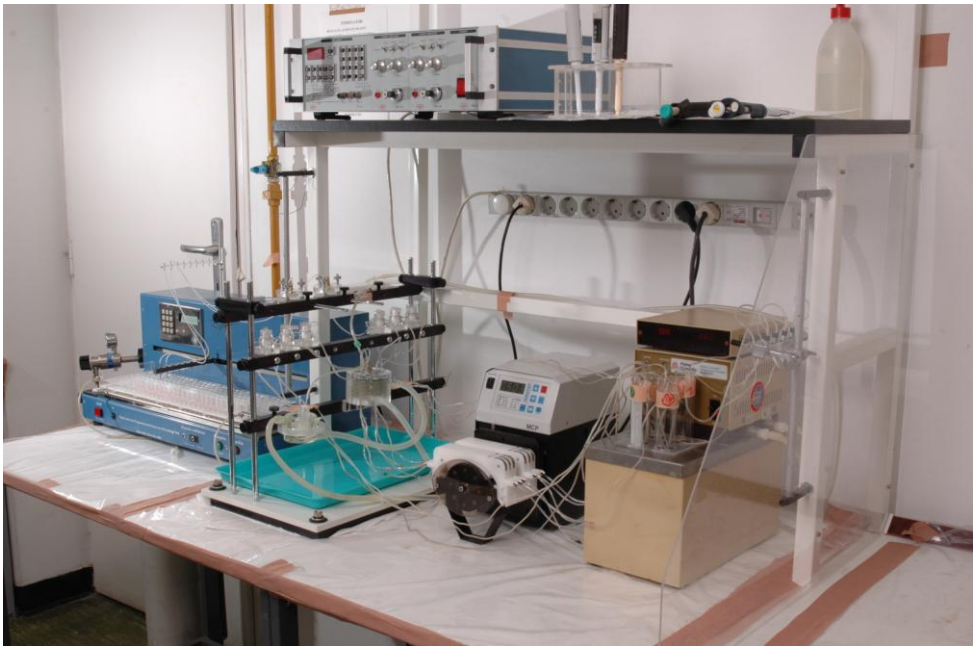
- to increase the drug uptake of the whole slices we loaded with 7μCi ^{3}H-NE for 45 min (instead of 2μCi ^{3}H-NE for 15 min)
- to shorten the experimental duration (increased by the longer loading), essential from the point of view of the neuronal viability we decreased the equilibration and the superfusion time 15 minutes each.

In the experiments, where field electrical stimulation was used we tested the effect of 10^{-5} EMO-1, applied 5 min before, during and 5 min after S_2.

5.2.5.3.2. Technical installations

The mechanical and electrical constituents of the system were built at our Department by Antal Gulyás and Péter Krucsó, to create the system parameters suiting our experimentation. The entire superfusion assembly is shown in Fig. 3.

Fig.3. The brain-slice superfusion assembly



Units (from left- to right): fraction collector, superfusion- and loading chambers, 8-channel perfusion pump, central circulating thermostate with 4 buffer containers, electrostimulator on the upper shelf

The novel technical installations in these subseries of experiments included a**:**

- Plexiglass thermostating block with 8 replaceable, small-calibre (0.8 mm ID) coils made of stainless steel for the simultaneous thermostating of 8 independent tubing systems (Fig. 4. unit “A”)
- Fast-switch perfusion-changing unit with approximately 70 µL dead space. This arrangement permitted the fast sequential change (within 5 s) of buffer with 3 different drugs / drug combinations (Fig. 4. unit “B”)
- Eight-track, programmable linear fraction collector (Fig. 4. unit “C”)

Fig. 4. unit “A”:Plexiglass thermostating block

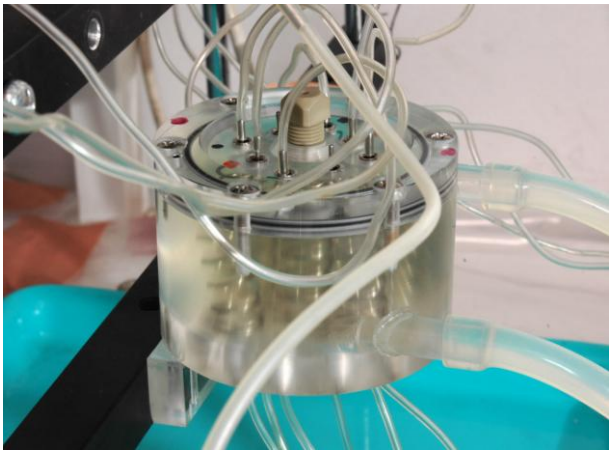


Fig.4. unit „B”: Fast-switch perfusion-changing unit, disassembled (left panel) or inbuilt (right panel) state

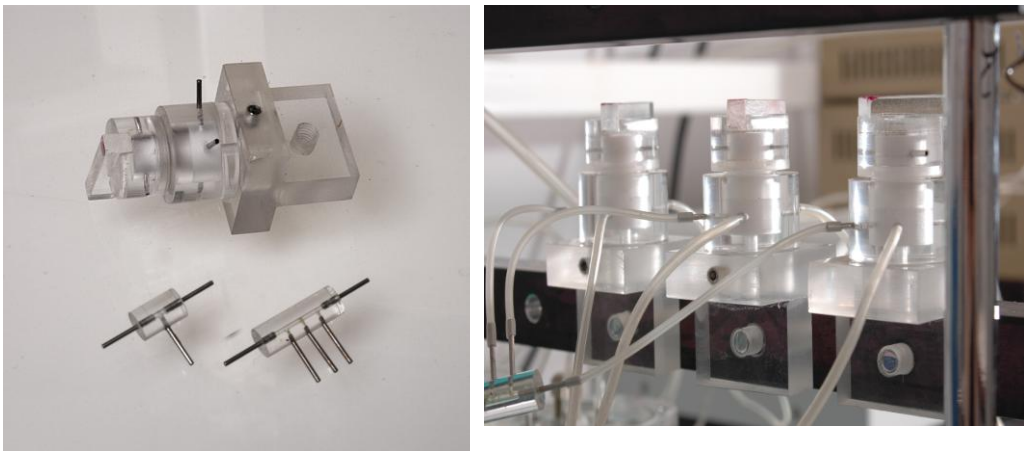


Fig.4. unit “C”: 8-track fraction collector

5.2.5.4. Loading, perfusion and extraction conditions of ^{3}H-GABA in rat isolated hypothalamic paraventricular/subparaventricvular (PVN/subPVN) slices

In principle we followed the experimental settings described in subsection 5.2.5.3.1., completed with additional improvements. We used small-volume, disposable loading chamber, which enabled us to load simultaneously and effectively four sets of tissue samples using small amounts of labelled substance. The chamber was constructed of an 1 mL Eppendorf vial, a truncated 1 mL disposable pipette tip and a polypropylene mesh of 200 μm grid size. Four of these chambers could be placed into and oxygenated in a common thermostated cup.

Furthermore, we determined the optimal loading conditions for PVN/subPVN in pilot experiments. The tested variations included label amount/chamber (1.25–3.125 μCi range), loading buffer volume (0.5–1.0 mL) and slice number per bath. The final protocol used was the following:

- after the preparation, two slice pairs/loading chamber were equilibrated for 30 min at 36 ºC in 0.5 ml Krebs buffer (3mM KCl, composition was the same as in the part 5.2.5.2.1.).
- afterward, we changed the solution for 1mM β-alanine (glial GABA uptake inhibitor) containing Krebs' buffer and equilibrated for further 30 min
- next, we added 1.5 μCi/0.5 mL ^{3}H-GABA and loaded for 30 min.
- then we transferred the slices to the superfusion chambers and started the superfusion with normal Krebs' solution with no additives for 30 min at 1 mL/min rate, thereafter for 15 min with Krebs' containing 0.1 mM nipecotic acid (GABA uptake inhibitor) and 0.1 mM amino-oxoacetic acid (GABA transaminase inhibitor)
- we switched to 0.5 mL/min rate for 15 min before collection. During the collection the rate was maintained at 0.5 ml/min with 3-min fractions.
- the stimulation periods were 2 min during fractions No 6. and 19. (K_1 and K_2) applied by "fast-switch" from 3mM K^+ to 30mM K^+ Krebs and back.
- in the PVN/subPVN experiments, we added 100 nM Angiotensin-II at the beginning of fraction 17 and the peptide was present in the buffer throughout the rest of experiment.

The same parameters were repeated in NTS/DVN slices.

5.2.6. Isolated organ analysis

5.2.6.1. Experiments related to α_{2A} or α_{2B}-adrenoceptors

5.2.6.1.1. Experimental paradigms

ST-91, isoproterenol and acetylcholine stock solutions were made freshly each experimental day. ST-91 (for 10^{-2} M stock) was dissolved in dimethylsulfoxide and made up to final volume with bi-distilled water (1:4 ratio). Isoproterenol stock and dilutions were prepared in 10^{-4} M ascorbic acid solution and were kept light-protected, similarly to acetylcholine stock (10^{-3} M).

When multiple concentrations of the same drug were administered, it was done in a cumulative manner both in rat vas deferens and the vascular preparations. In aortic and mesenteric artery rings each experiment was started by contraction with 10^{-6} M phenylephrine, and, to check the effectiveness of endothelium denudation, by addition of 10^{-6} M acetylcholine. Relaxation not higher than 12% was considered acceptable. After 30 min and repeated washing phenylephrine was applied again and after stabilization of contraction, the β-adrenoceptor stimulant isoproterenol or terbutaline (aorta) was added. In mesenteric artery only isoproterenol was used. After repeated washes the contraction–relaxation paradigm was repeated after 40 min, using the β-adrenoceptor agonist alone or in combination with other drug(s). α_2-adrenoceptor agonists were given to the organ bath 10 min after phenylephrine and 10 min before repeating the relaxation by isoproterenol. Buffer solutions containing different concentrations of α_2-adrenoceptor antagonist yohimbine were given to the reservoire. Change for the antagonist-containing buffer took place 30 min before phenylephrine contraction, i.e. equilibrium period before α_2-adrenoceptor agonist addition was altogether 40 min.

No reuptake or extraneural uptake inhibitor was used, to avoid possible interference with other drug interactions. To minimize distortions, the second

isoproterenol concentration–response curve (with no other drug addition) was used for comparison.

5.2.6.1.2. Recording, evaluation, data presentation

An ADInstruments Chart for Windows PC-assisted data acquisition system was used. "Macro" programs were written and run for the primary calculations, and the first sets of calculated data were collected in Excel files. From these, the data were exported to GraphPad Prism 3.0 or Sigmaplot 8.0 programs for further off-line evaluation and plotting. According to the recent IUPHAR recommendations (Neubig et al., 2003), whenever it was feasible, sigmoidal curve fitting was performed according to the Hill equation and the "A_{50}", "E_{max}" and "nH" parameters were used for further statistical analyses. In one experimental setting (see "Results" section) convergence and regression coefficient criteria excluded the application of Hill fitting. The best applicable sigmoidal fitting program was found by iterative runs. All the listed values are given as arithmetic mean ± S.E.M. One-way ANOVA followed by Newman–Keuls Multiple Comparison Test was used for statistical analysis.

5.2.6.2. The analysis of partial agonist properties of endomorphins and other μ-opioid receptor agonists by using the „operational" calculation model

5.2.6.2.1. Experimental paradigms

Vasa deferentia taken from CFLP mice weighing 35–45 g were prepared, mounted and stimulated. Following 30–40 min equilibration isolated vasa deferentia were exposed to agonists for up to 2 min with the exception of Dmt-endomorphin-1, where exposure was 10–25 min. Administration cycle was 12–18 min with three to four interim washes with the exception of Dmt-endomorphin-1 where it was 40–60 min with 8–12 washes.

Incubation conditions with β -FNA and the construction of before–after dose–response curves with the agonists were the same as used previously (Al-Khrasani et al., 2001). In brief, the agonist dose–response curves were taken non-cumulatively at 4–6 concentration levels; the vasa were then exposed to 5×10^{-7} M β -FNA for 30 min. The exposure was followed by a 60 min washout period with 12 washes then the agonist dose–response curves were re- taken at 4–6 pre-set concentration levels. The proper concentration range for each agonist was determined in a pilot experiment.

5.2.6.2.2. Evaluation

To obtain the parameters of agonist concentration–response (E/[A]) curves before and after β -FNA treatment non-linear curve fitting according to the Hill equation (three parameters, Eq. (1)) was used:

Eq. (1) $$\mathbf{E / E_{max} = [A]^{nH} / ([A]^{nH} + [A]_{50}^{nH})}$$

where ‘‘E’’ is the biological response (percent inhibitory effect), ‘‘E_{max}’’ the maximal effect, ‘‘[A]’’ the molar concentration of agonist, ‘‘$[A]_{50}$’’ the 50% effective concentration and ‘‘n_H’’ is the Hill slope. The ‘‘null’’ method, i.e. the double-reciprocal plot of equieffective concentrations of agonists before ([A], ordinate) and after ([A′], abscissa) β -FNA treatment (Furchgott and Bursztyn, 1967; Tallarida, 1982) was carried out as described previously (Al-Khrasani et al., 2001). From the slope and ‘‘y’’ intercept the residual receptor fraction ‘‘q’’ and agonist dissociation constant ‘‘K_A’’ were calculated. According to the operational model (Leff and Dougall, 1988; Leff et al., 1990) the ‘‘E_{max}’’ and ‘‘n_H’’ parameters for the full agonist prototypes were determined by using Eq. (1) then these parameters were used to determine the receptor constants for all the agonists by another equation

Eq. (2) $$\mathbf{E / E_{max}\, \tau^{nH}\, [A]^{nH} = (K_A + [A])^{nH} + \tau^{nH}\, [A]^{nH}}$$

where ‘‘τ’’ is the model definition of efficacy, ‘‘K_A’’ the agonist dissociation constant and other parameters as in Eq. (1). Agonist efficacies, were characterized uniformly by the $K_A/[A_{50}]$ ratios.

For pooled $[A_{50}]$ and K_A values geometric means and 95% confidence intervals (Fleming et al., 1972) were calculated otherwise arithmetic mean ± S.E.M. values were listed. Statistical probes were applied to the logarithms of data sets. ANOVA followed by Dunnett’s test was used to compare efficacies, taking DAMGO as prototype full agonist for multiple comparisons. Newman–Keuls post hoc test was applied in the case of residual receptor fraction (‘‘q’’, null method) values whereas Student’s ‘‘t’’-test was used to compare ‘‘before–after’’ data sets (paired arrangement). For correlation analysis, correlation coefficient (r) values were calculated (Snedecor and Cochran, 1994).

5.2.6.3. The in vitro pharmacology of presumed endomorphin biosynthetic intermediates

For the MVD bioassay of possible precursors-biosynthetic intermediates NMRI mice were used.

Methodology of supportive (background) informations

5.2.6.3.1. Extraction, purification and separation-detection

In the course of entire series of experiments, two kinds of extraction procedures were used, followed by principally similar purification-separation steps. The whole rat brains were extracted by a method quite similar to the one used originally by Zadina’s team (Zadina et al., 1997; Hackler et al., 1997), with the only difference that initial boiling and homogenation was replaced by mincing and powderizing under liquid nitrogen (Rónai et al., 2006; Szemenyei, Barna et al., 2008). The powderized tissue was taken up in 1.0 mL absolute ethanol and stored at -80 °C until the extraction. The stored samples were taken up in eight-fold amount of antioxidant sodium-metabisufite

($Na_2S_2O_5$, 0.08 % w/w) solution then acetonitrile (ACN) was added to yield 25 % (v/v) final acetonitrile concentration. After mixing overnight, the mixtures were centrifuged (26,000 g, 20 min) and the supernatants were extracted by solid-phase method, using 70% (v/v) ACN in the final step. The extracts were evaporated to dryness and dissolved in 2% ACN-98% TFA 0.1% (v/v) in water, and the samples were chromatographed on a Vydac C_{18} reverse-phase column (RP-HPLC). The mobile phase was mixed from 0.1 % (v/v) TFA in water and 0.08 % (v/v) TFA in ACN, and gradient elution was carried out from 2 to 40 % of organic modifier. Tandem UV- and radiodetection was used in the radiolabel-incorporation studies whereas 1-min fractions were collected (1 mL/min flow rate) for the radio-immunoassay. The final purification-separation steps and the RP-HPLC were carried out in Géza Tóth's laboratory, HAS Biological Reasearch Center, Szeged (Rónai et al., 2006; Szemenyei, Barna et al., 2008).

Another, simplified extraction-partial purification-desalting procedure was used in tissue extracts made from L4,5 isolated dorsal root ganglia (DRG) prepared from adult rats (Rónai, Király et al., 2009). The results of these experiments are not used in my thesis but the conclusions will be quoted in the discussion.

5.2.6.3.2. Radio-immunoassay

Antisera were raised in rabbits, using endomorphin-2 conjugated to keyhole limpet hemocyanin as antigen (Szemenyei, Barna et al., 2008). One basal and 3-6 booster injections of antigen solution were applied. On each occasion, 15-20 intracutaneous deposits of antigen solution, supplemented with Freund complete adjuvant, were made. The basal treatment was combined also with pertussis vaccine injection.

Radioiodinated (^{125}I) endomorphin-2 of high specific activity (approx. 2,000 Ci / mmol) was used as radiotracter. The detailed conditions of radio-immunoassay (RIA) were described by Szemenyei, Barna and their coworkers (2008).

5.2.6.4. The in vitro pharmacological characterization of a novel nociceptin antagonist

For the MVD bioassay of characterization of nociceptin and nociceptin derivatives CFLP mice were used.

For the antagonists the equilibrium dissociation constants (K_e) were calculated (Kosterlitz and Watt; 1968) according to the equation K_e=B/(DR-1) where B is the antagonist concentration in nM and DR (dose ratio) is the measure of the rightward shift of agonist dose-response curve by the actual concentration of antagonist. Competitive antagonism was determined by Schild analysis (Arunlakshana and Schild, 1959).

6. Results

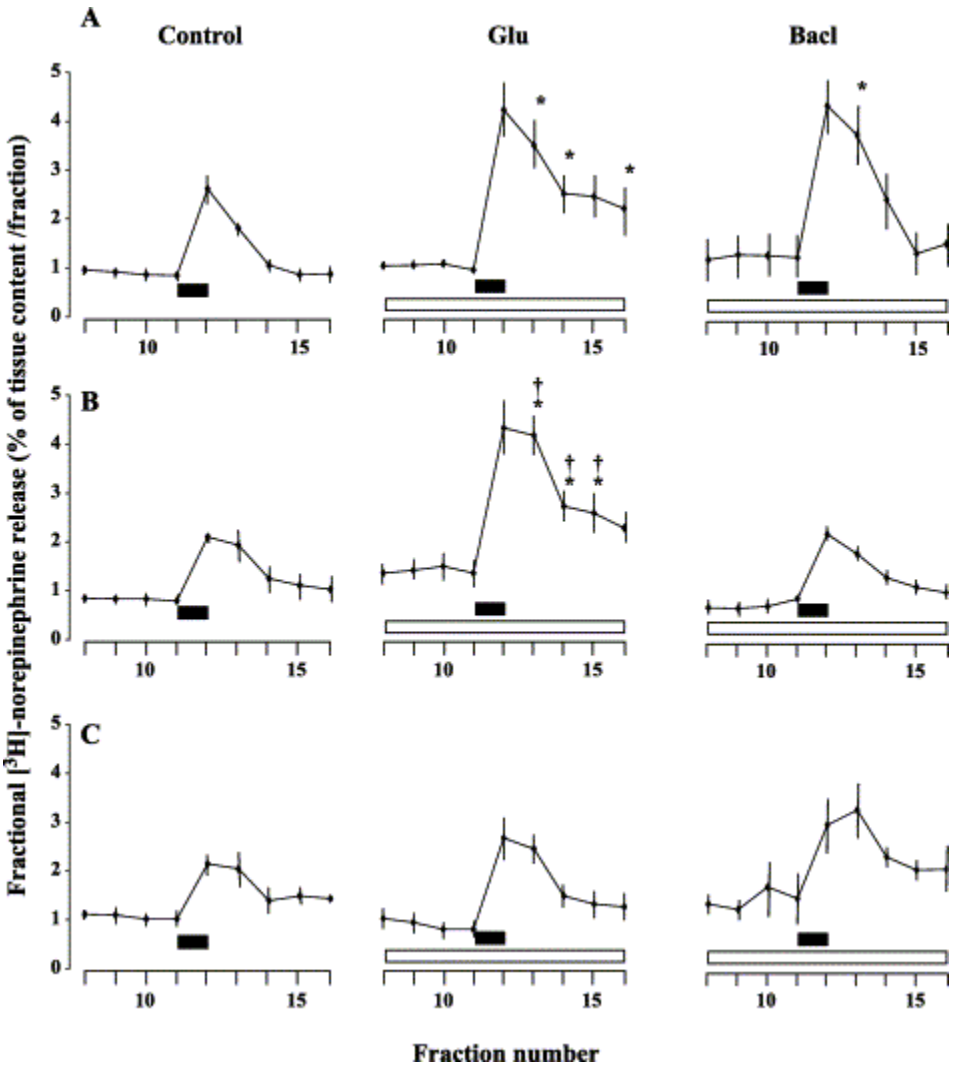
6.1. Experiments on NTS-DVN and PVN/subPVN prism/whole slice preparation

6.1.1. The effect of MSG treatment on the field stimulation-induced release of ^{3}H-NE from NTS-DVN prism preparations: differences in the responses to modulatory agents

When using two stimulation periods 30 min apart the S_2/S_1 ratios, calculated from the AUC-s of stimulation-induced release increments, were close to unity in the case of young as well as the untreated MSG littermate control rats (1.15±0.13, mean ± S.E.M., N=3 and 1.05±0.16, N=3, respectively) but not in the MSG-treated rats (0.62±0.13, N=3). Therefore, for a comparative study we could not choose the characterization of drug actions in terms of the alterations of S_2/S_1 ratios. Thus, we were compelled to use uniformly the single stimulation cycle, whereby the advantages of a paired experimental arrangement are missed.

The effects of L-glutamate and baclofen on the field stimulation-induced release of ^{3}H-NE in young adult control rats, MSG-treated rats and untreated littermates of MSG rats are shown in Fig. 5. The concentration of L-glutamate (e.g. Lehmann et al., 1983) and baclofen (e.g. Brooks et al., 1992) were chosen such as to fall into the medium- to high effective range in rat brain slice preparations. None of the agents tested affected the resting release of ^{3}H-NE. In both control groups but not in the MSG-treated rats L-glutamate caused a well-sustained increase in the stimulation-induced ^{3}H-NE release whereas baclofen increased the release only in young controls.

Fig. 5. The effect of L-glutamate and baclofen on the stimulation-induced release of ^{3}H-NE in rat nucleus tractus solitarii-dorsal vagal nucleus slices



Abscissa: Fraction number (3-min collection periods); Ordinate: Fractional release expressed as % of tissue ^{3}H-NE content released in a single fraction. Points represent the mean, vertical lines the S.E.M. values obtained in 3-6 independent experiments. Stimulation was applied for 3 min after fraction 11 as indicated by the dark bar. Drug incubation was started by fraction 8 i.e. they were present for 9 min before, during and after stimulation as indicated by the open bar. Drug concentrations: L-glutamate (Glu) = 10^{-3} M; Baclofen (Bacl) = 10^{-5} M. Panel A: Preparations taken from young adult rats (180-260 g). Panel B: Preparations taken from untreated littermates of MSG-treated rats

(460–580 g). Panel C: Preparations taken from rats treated neonatally with monosodium glutamate (350-530 g). The stimulation-induced increments in matching fractions were compared by one-way ANOVA followed by LSD test. *p<0.05 as compared to control; †p<0.05 as compared to baclofen.

The AUC values, calculated from the stimulation-induced increments are given in Table 2.

Table 2. The effect of excitatory amino acid and $GABA_B$ receptor agonists on the stimulation-induced release of ^{3}H-NE from rat NTS-DVN slices

Drug	Conc. (M)	Stimulation induced ^{3}H-NE release (percent of tissue content, AUC (11-16))		
		Young adults (180-260 g)	Untreated littermates (460-580 g)	MSG-treated rats (350-530 g)
None		2.52 (1.99-3.18, n=6)	2.73 (1.86-3.99, n=4)	2.83 (1.99-4.04, n=3)
L-Glutamate	10^{-3}	7,26** (4.90-10.8, n=3)	7.37* (5.45-9.95, n=5)	4.21 (3.10-5.72, n=4)
Baclofen	10^{-3}	5.67* (3.53-9.13, n=3)	2.90 (2.75-3.05, n=3)	3.66 (2.46-5.45, n=3)

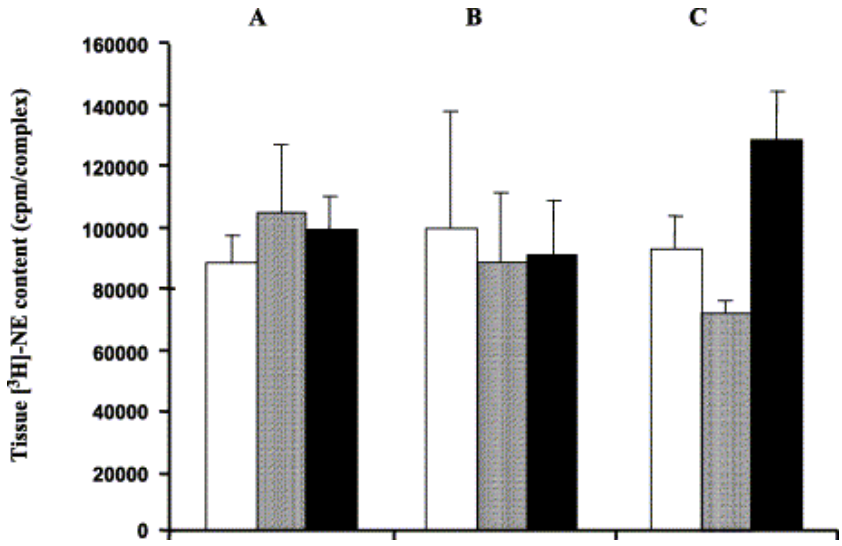
AUC: area under curve between fractions 11–16, geometric means and 95% confidence intervals.

**p<0.01

*p<0.05

The tritium content in NTS-DVN extracts obtained in the different experimental subgroups is presented in Fig. 6.

Fig. 6. The radioactivity in NTS-DVN extracts of young adult rats (A), untreated MSG littermates (B) and MSG-treated (C) rats



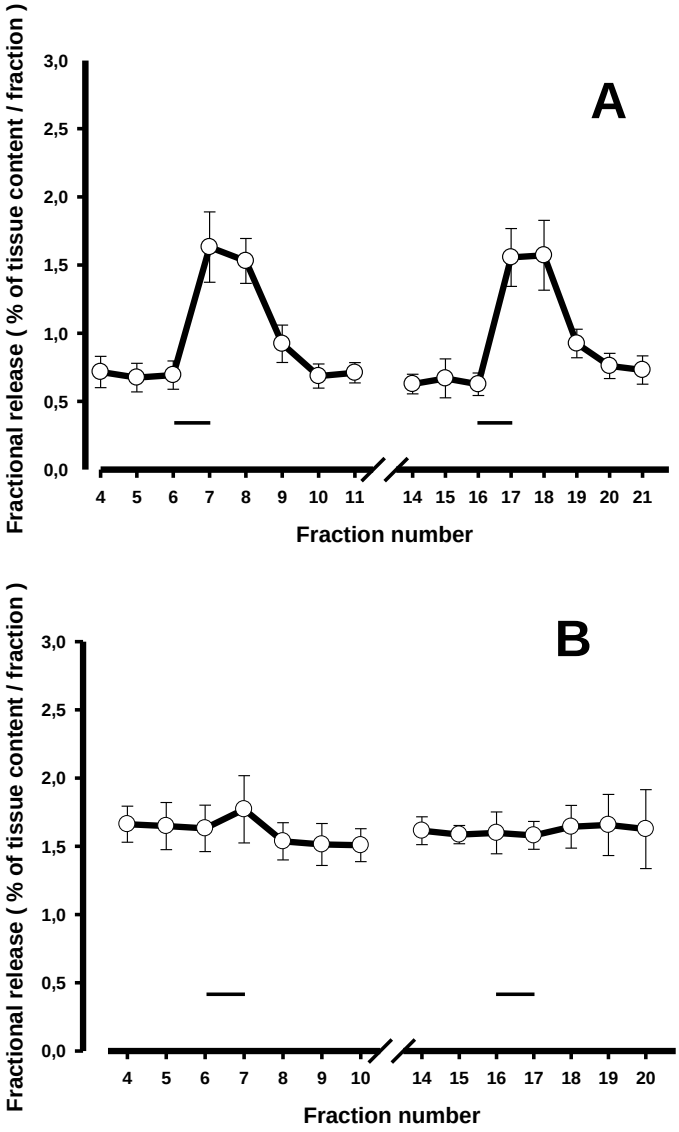
Legend:

Columns represent the arithmetic mean, vertical lines the S.E.M. values obtained in 3-6 independent experiments. Open columns: preparations receiving no drug in vitro; Hatched columns: preparations exposed to 10^{-5} M baclofen in vitro; Dark columns: preparations exposed to 10^{-3} M L-glutamate in vitro. No statistically significant differences were found by one-way ANOVA.

6.1.2. The study of stimulation induced release of ^{3}H-NE and ^{3}H-D-Asp in NTS-DVN prisms

We used two field-stimulation periods 30 min apart (S_1 and S_2); the S_2/S_1 ratio did not differ significantly from unity but only evoked release of ^{3}H-NE, not of ^{3}H-D-Asp could be induced (Fig. 7.)

Fig. 7. ^{3}H-NE (panel A) and ^{3}H-D-Asp (panel B) release induced by 3 min field electrical stimulation (dash line, S_1, S_2) from rat NTS-DVN prism preparation



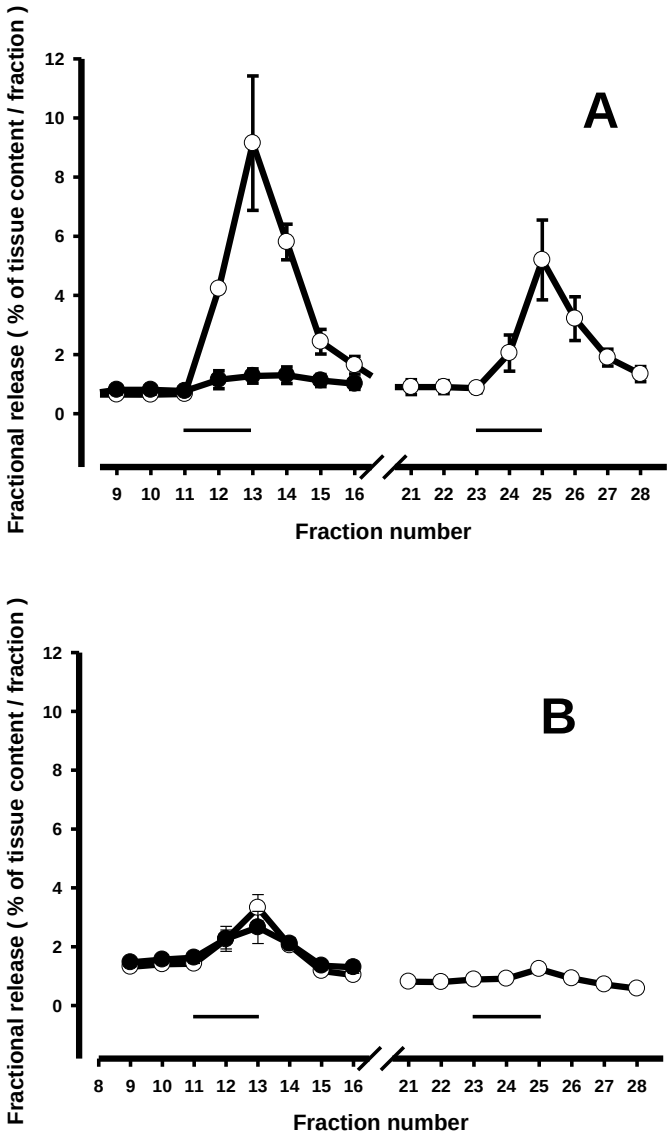
Legend

Abscissa: Fraction number (3 min collection periods); Ordinate: Fractional release expressed as % of tissue ^{3}H-NE/^{3}H-D-Asp content released in a single fraction. Points represent the mean, vertical lines the S.E.M. values obtained in 3-6 independent experiments. Field electrical stimulation was applied for 3 min during fraction 7 and 17 respectively, as indicated by the dark bars.

The K_2/K_1 ratio of 60 mM KCl induced ^{3}H-NE release was less then the unity (K_2/K_1=0.54). 60 mM KCl exposure for (nominally) 6 minutes released 3.93 ± 0.70 % (n=8) of tissue ^{3}H-D-Asp store above the resting baseline. Repeated challenge after 36 min released only 0.57 ± 0.12 % (n=4) (Fig. 8.)

^{3}H-NE released by 60 mM KCl was Ca^{2+} dependent whereas only an insignificant fraction of K^+ induced ^{3}H-D-Asp release may be Ca^{2+} dependent (Fig. 8. and Fig. 9.)

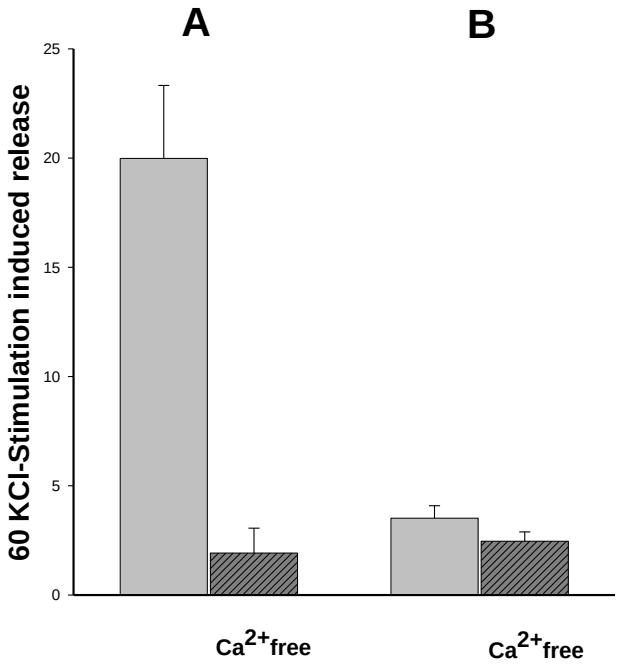
Fig.8. ^{3}H-NE (panel A) and ^{3}H-D-Asp (panel B) release induced by 60 mM KCl (dash line, K_1, K_2) in the presence (open circles) and in the absence (dark circles) of Ca^{2+}, from rat NTS-DVN prism preparation



Legend:

Abscissa: Fraction number (3 min collection periods); Ordinate: Fractional release expressed as % of tissue ^{3}H-NE/^{3}H-D-Asp content released in a single fraction. Points represent the mean, vertical lines the S.E.M. values obtained in 3-6 independent experiments. High K^+ (60mM KCl) stimulation was applied for 6 min during fractions 12, 13 and 24, 25 respectively, as indicated by the dark bars. Ca^{2+} free Krebs containing 1 mM EGTA, was started 21 min before adding 60 mM KCl.

Fig 9. The Ca^{2+} dependence of 60 mM KCl-induced release of ^{3}H-NE (columns A) and ^{3}H-D-Asp (columns B)



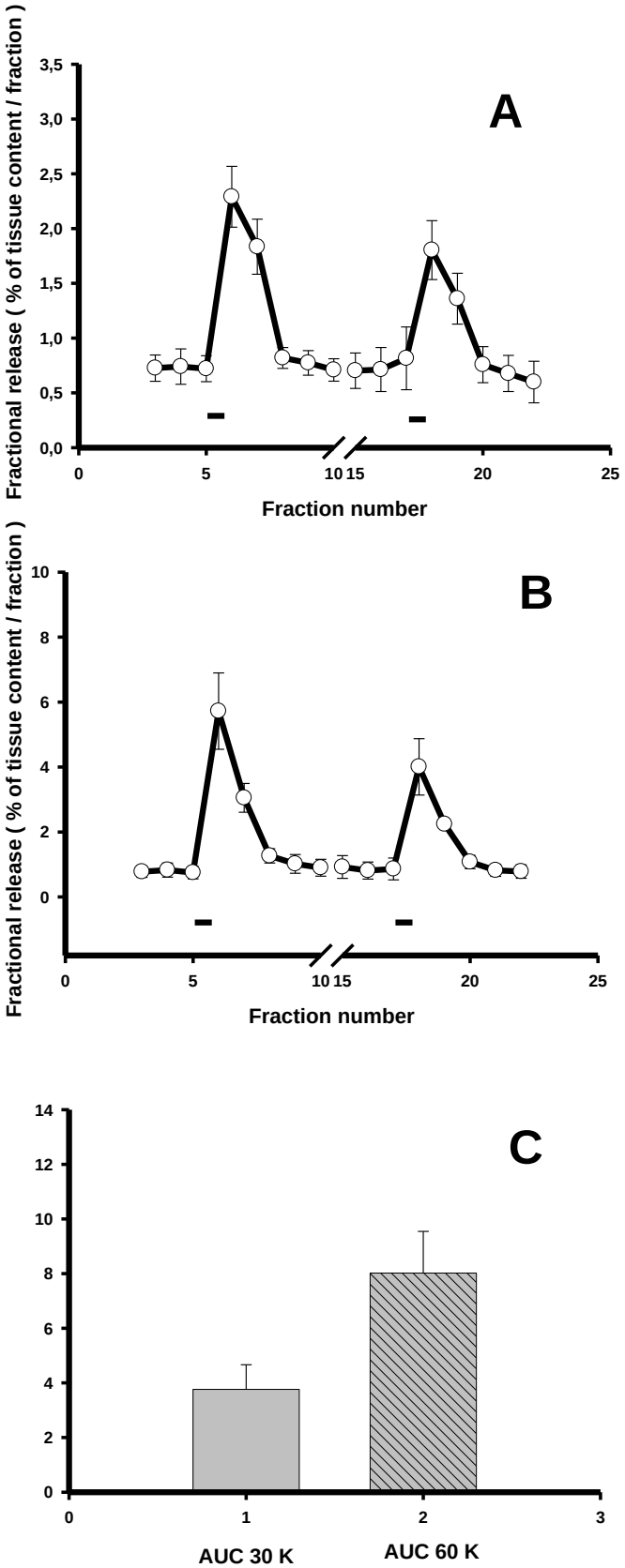
Legend:

Open columns: preparations receiving normal Krebs' buffer; Hatched columns: preparations exposed to Ca^{2+} free Krebs containing 1 mM EGTA, started 21 min before adding 60 mM KCl. Columns represent the arithmetic mean, vertical lines the S.E.M. values obtained in 3-6 independent experiments.

6.1.3. The modulation of stimulated ^{3}H-NE release by endomorphin-1 in rat dorsal vagal complex *whole trapezoidal slice* preparation

In trapezoidal NTS-DVN whole slices, stimulation by high K^{+} caused a concentration dependent increase in fractional ^{3}H-NE release. The K_2/K_1 ratios of ^{3}H-NE release induced by 30 mM and 60 mM KCl: 0,718 and 0,685 respectively, rendered this arrangement unsuitable for studying drug actions by introducing drugs before the second stimulation period (Fig. 10.).

Fig. 10. The high K^+, 30 mM (panel A) and 60 mM KCl (panel B) induced release of ^{3}H-NE in rat NTS-DVN whole slices

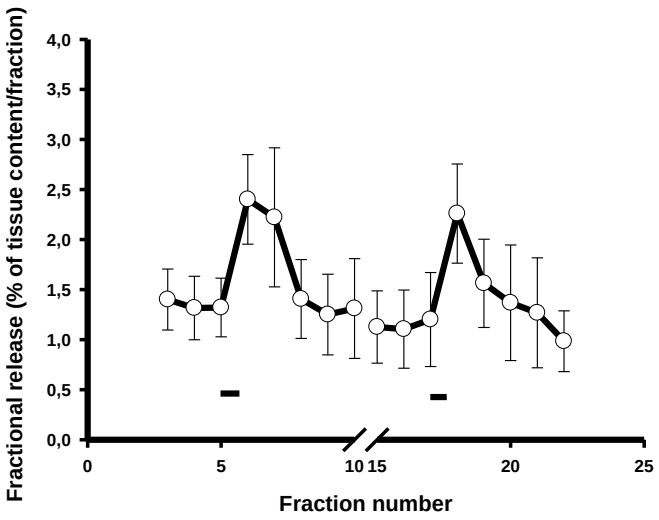


Legend:

The fractional release of ^{3}H-NE (percent of tissue content released in a single fraction) is plotted on the ordinate, the fraction number on the abscissa. High potassium (panel A: 30 mM KCl, panel B: 60 mM KCl) was applied for 2 min as indicated by the horizontal black bars. Stimulation was repeated after 34 min. Panel C: high K^{+} stimulation-induced release of ^{3}H-NE is expressed as AUC over the baseline. Open column represents stimulation by 30 mM KCl, whereas hatched column stimulation by 60 mM KCl.

The S_2/S_1 ratio approaching unity in the case of electrostimulation is suitable for studying drug actions by introducing drugs before S_2. (Fig. 11.)

Fig. 11. The field electrical stimulation induced ^{3}H-NE release in rat NTS-DVN whole slices



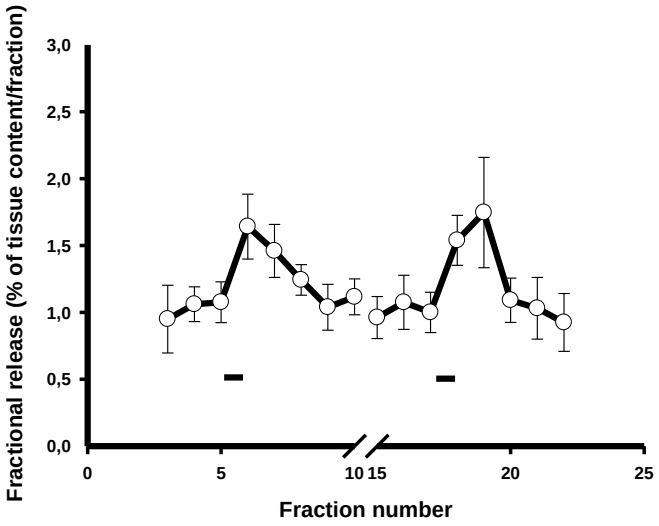
Legend:

The fractional release of ^{3}H-NE (percent of tissue content released in a single fraction) is plotted on the ordinate, the fraction number on the abscissa. Electrostimulation (field, rectangular 2 ms pulse width, 25mA current, 2Hz) was applied for 2 min as indicated by the horizontal black bars. Stimulation was repeated after 34 min.

Nextly, we tested the effect of 10^{-5} M EMO-1 on field electrical stimulation induced release of ^{3}H-NE. In contrast with previous measurements in NTS-DVN prisms

(Al-Khrasani et al., 2003), EMO-1 did not inhibit the ES induced ^{3}H-NE release in whole slice preparations (Fig. 12.).

Fig. 12. The effect of EMO-1 on field electrical stimulation induced release of ^{3}H-NE in rat NTS-DVN whole slices preparation



Legend:

The fractional release of ^{3}H-NE (percent of tissue content released in a single fraction) is plotted on the ordinate, the fraction number on the abscissa. Electrostimulation (field, rectangular 2 ms pulse width, 25mA current, 2Hz) was applied for 2 min as indicated by the horizontal black bars. Stimulation was repeated after 34 min. 10^{-5} M EMo-1 was present 5 min before, during and 5 min after S_2.

Table 3. The effect of EMO-1 on field electrical stimulation induced release of ^{3}H-NE in NTS-DVN prism and whole slice preparation

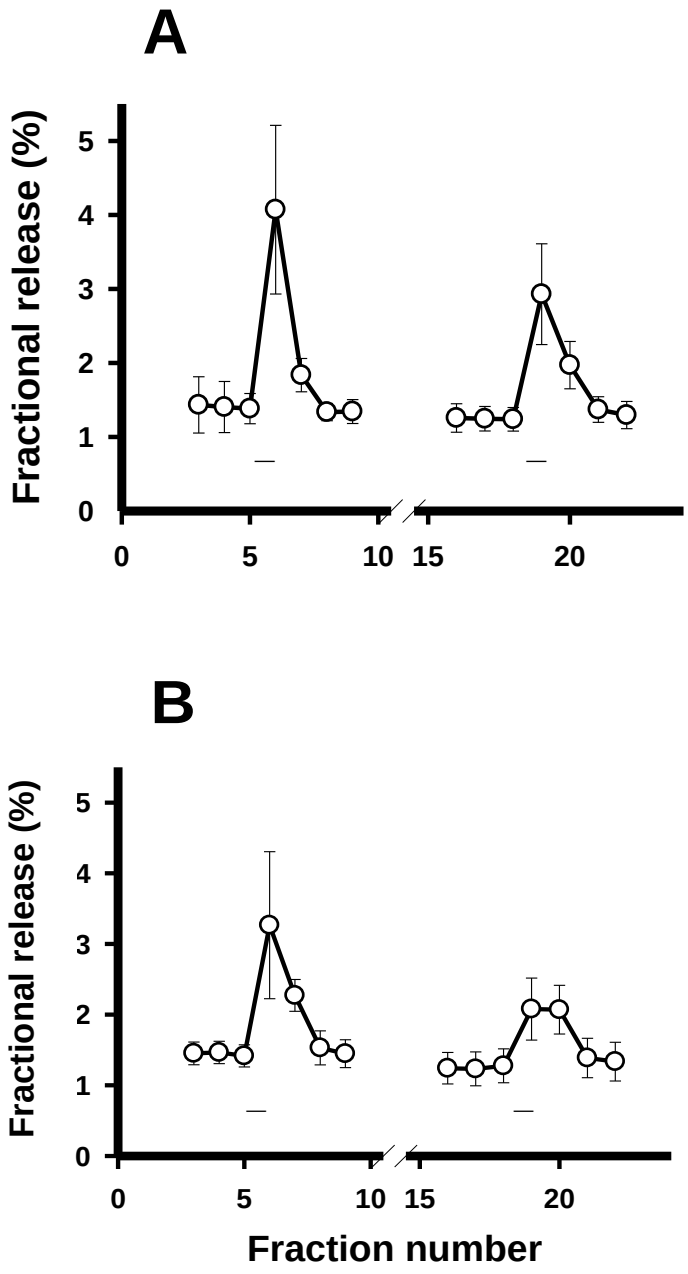
Drug	Conc (M)	S_2/S_1
None (c)	0	0.94 ± 0.13 (n=3)
EMO-1 (whole slices)	10^{-5}	0.96 ± 0.20 (n=3)
EMO-1 (prism)	10^{-5}	0.80 ± 0.34 (n=6)

Legend:
10^{-5} M EMo-1 was present 5 min before, during and 5 min after S_2.

6.1.4. The release of ^{3}H-GABA from rat isolated hypothalamic paraventricular / subparaventricvular (PVN/subPVN) slices. Comparison with the release from NTS-DVN slices

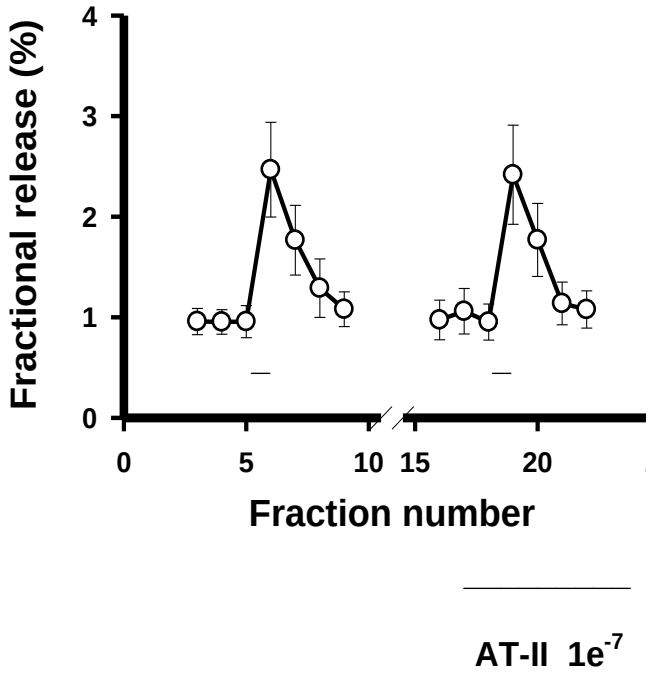
The loading appeared to be more efficient and the control K_2/K_1 ratio was closer to unity in PVN/subPVN as compared to NTS/DVN (Fig. 13. panels A and B, Table. 4.). Even in PVN/subPVN the pattern of stimulation-induced release was slightly different in the second stimulation period (Fig. 13. panel A) but the overall released amount was comparable to the amount obtained in the first period. Exposure of PVN/subPVN slices to 100 nM AT-II before and during the second stimulation period did not affect K_2/K_1 ratios (Fig. 14. and Table 4.). In NTS/DVN slices both the loading and the release during K_2 was less efficient under identical experimental conditions as compared to PVN/subPVN (Fig 13., panel B and Table 4.).

Fig. 13. The stimulation induced release of ^{3}H-GABA from rat PVN / subPVN (panel A) and NTS / DVN (panel B) slices.



Preparations were stimulated twice by raising KCl to 30 mM („fast-switch") for 2 min after fractions 5 and 18, respectively (indicated by dashes).

Fig. 14. The effect of angiotensin-II on the stimulation induced release of ^{3}H-GABA from rat PVN/subPVN slices



Preparations were stimulated twice by raising KCl to 30 mM („fast-switch") for 2 min after fractions 5 and 18 (indicated by dashes). Angiotensin-II, delivered at the beginning of fraction 17 by „fast-switch" was present in the buffer thereafter.

Table. 4. The loading and stimulation-induced release of ^{3}H-GABA in rat PVN/subPVN and NTS/DVN slices

Parameter	Added drug	PVN/subPVN	n	NTS/DVN	n
Load (cpm/mg_WTW) [a]	β-Ala 1 mM	7282.6 ± 1728.2	8	3570.9 ± 426.5	4
K_2/K_1 ratio [b]	None	0.922 ± 0.146	4	0.782 ± 0.078	4
K_2/K_1 ratio	AT-II 100 nM	0.952 ± 0.087	4	N.T.[c]	

Footnotes:

[a] WTW: wet tissue weight

[b] The stimulation-induced release (sum of fractional release values over the baseline) in the second stimulation period over the similar value obtained in the first stimulation period

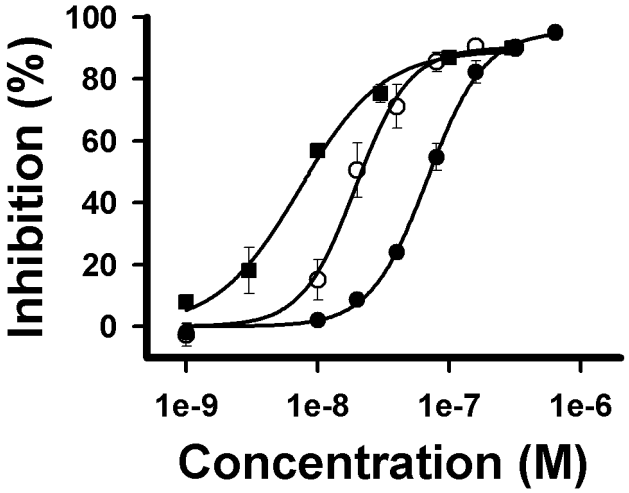
[c] N.T.: not tested

6.2. Measurements related to α_{2A} or α_{2B} adrenoceptors

6.2.1. Rat vas deferens

Owing to its known postsynaptic α_1-adrenoceptor agonist action, the presynaptic agonism by ST-91 at α_2-adrenoceptors in field-stimulated rat vas deferens could be determined only in the presence of 10^{-6} M prazosine (Fig. 15.). Comparing the α_2-adrenoceptor agonist potencies of xylazine and ST-91 obtained under identical conditions (i.e. both in the presence of prazosine), ST-91 was almost 10 times more potent presynaptic inhibitor than xylazine, the A_{50} values being $7.41\pm0.59\times10^{-9}$ M for the former and $7.06\pm0.56\times10^{-8}$ M for the latter (n=4 for both). The inhibitory actions of both agents could be antagonized by 10^{-6} M yohimbine (not shown).

Fig. 15. The presynaptic inhibitory effect of α_2-adrenoceptor agonists in field stimulated rat vas deferens



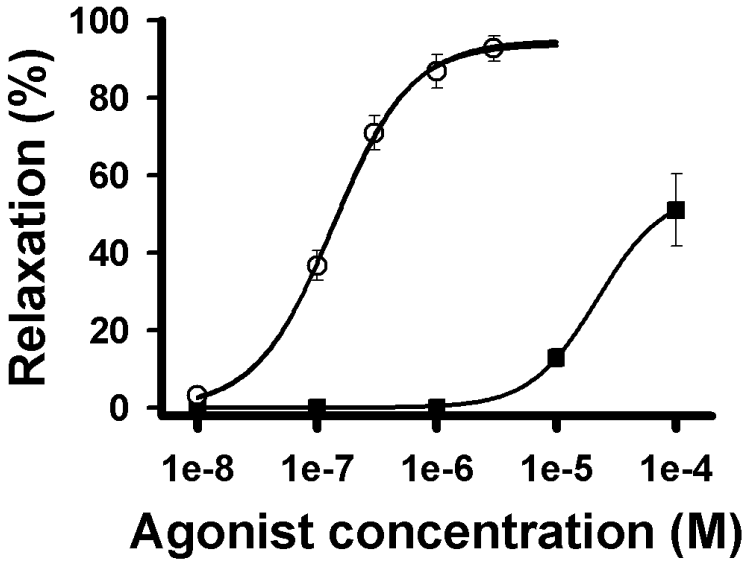
Legend

Points represent the arithmetic mean, vertical lines the S.E.M. values of 4–6 independent determinations. Symbols: the inhibitory effect of xylazine in the absence (open circles) or in the presence (black circles) of 10^{-6}M prazosine. Black squares: the inhibitory effect of ST-91 in the presence of 10^{-6} prazosine. The α_1-adrenoceptor antagonist was added to the buffer 30 min before cumulative agonist administration. Sigmoidal curve fitting was performed according to the Hill equation.

6.2.2. Rat aortic rings

In rat aortic rings precontracted with 10^{-6} phenylephrine, no exclusion was necessary because of the pharmacological indication of residual endothelium. Both isoproterenol and terbutaline exerted concentration-dependent relaxant effect. The former was more potent and acted as a full agonist whereas terbutaline was a partial agonist (Fig. 16.). Therefore, isoproterenol was used as relaxing agent also in mesenteric artery rings.

Fig. 16. The relaxing effect of β-adrenoceptor agonists in endothelium-denuded rat aortic rings precontracted with phenylephrine



Footnotes

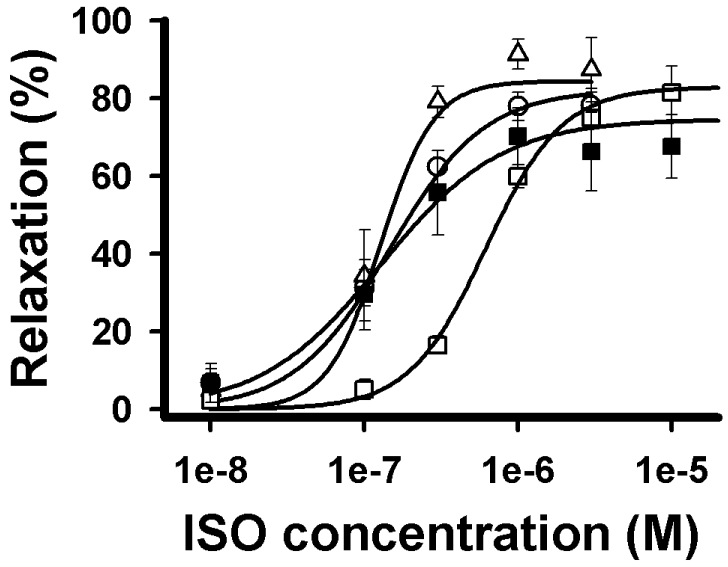
Points represent the arithmetic mean, vertical lines the S.E.M. values of 4–7 independent determinations. Symbols: the relaxing effect of cumulatively administered isoproterenol (open circles) or terbutaline (black squares). Sigmoidal curve fitting was performed according to the Hill equation.

6.2.3. Rat mesenteric artery rings

In pilot experiments, 10^{-6} M acetylcholine caused 86.3 ± 7.4% (n=8) relaxation in mesenteric artery rings with intact endothelium. In the present series, two preparations met the exclusion criterion (i.e. higher than 12% relaxation by acetylcholine). Phenylephrine elicited a reproducible, sustained contraction, suitable for studying the actions of drug combinations. Isoproterenol caused a concentration-dependent relaxation also in this preparation (Fig. 17.). 10^{-7} M xylazine did not affect the parameters of relaxant concentration–response curve of β-adrenoceptor agonist (Fig. 17., Table 5.). On the other hand, ST-91 gave a significant interaction with

isoproterenol-induced relaxation, both at 10^{-7} and 10^{-6} M of α_2-adrenoceptor agonist. At 10^{-7} M of ST-91 there was a rightward shift of isoproterenol dose−response curve with no E_{max} depression (Fig. 17., Table 5.). At 10^{-6} M the E_{max} depression was prevalent; both actions could be antagonized by the α_2-adrenoceptor antagonist yohimbine.

Fig. 17. The interaction of relaxant isoproterenol (ISO) and α_2-adrenoceptor ligands in endothelium-denuded rat mesenteric artery rings precontracted with phenylephrine



Points represent the arithmetic mean, vertical lines the S.E.M. values of 3–4 independent determinations. Symbols: the relaxing effect of cumulatively administered isoproterenol with no other drug addition (open circles), in combination with 10^{-7} M xylazine (open triangles), 10^{-7} üM ST-91 (open squares) and 10^{-7} M ST-91 plus 10^{-7} M yohimbine (black squares). α_2-adrenoceptor agonists were given 10 min before isoproterenol; yohimbine was present in the buffer from 40 min before ST-91 administration. Sigmoidal curve fitting was performed according to the Hill equation.

Table 5. The interaction of alpha-2 adrenoceptor ligands with isoproterenol in phenylephrine-contracted rat mesenteric artery rings

N°	Drug addition	Conc (M)	Parameters of isoproterenol relaxing dose-response curve[a]					
			A_{50} (M)	S	E_{max} (%)	S	nH	S
1	None (control)	-----	1.29±0.25 e-7 (4)		79.9±5.1		1.56±0.36	
2	Xylazine	10^{-7}	1.20±0.38 e-7 (3)	1 vs 2: ns	90.5±5.1	1 vs 2: ns	2.06±0.55	1 vs 2: ns
3	ST-91[b]	10^{-7}	6.81±1.40 e-7 (4)	1 vs 3: p<0.001 2 vs 3: p<0.001	84.7±7.9	1 vs 3: ns	1.97±0.35	1 vs 3: ns
4	ST-91 Yohimbine	10^{-7} 10^{-7}	1.51±0.45 e-7 (4)	1 vs 4: ns 3 vs 4: p<0.001	74.5±8.0	1 vs 4: ns 3 vs 4: ns	1.39±0.23	1 vs 4: ns 3 vs 4: ns
5	ST-91	10^{-6}	NA[c]		36.1±7.0 (4)	1 vs 5: p<0.01 3 vs 5: p<0.001	NA[c]	
6	ST-91 Yohimbine	10^{-6} 10^{-6}	0.90±0.27 e-7 (4)	1 vs 6: ns	68.6±7.6	1 vs 6: ns 5 vs 6: p<0.01	3.82±0.88	1 vs 6: p<0.05 4 vs 6: p<0.05

Footnotes:

[a]With the exception of setting N° 5, sigmoidal curve fitting was carried out according to the Hill equation. N° of experiments in parenthesis.

ANOVA followed by Newman-Keuls post hoc test was used for statistical comparison (S)

[b] ST-91: 2-[2,6-diethylphenylamino]-2-imidazoline

[c] not applicable

6.3. The analysis of partial agonist properties of endomorphins and other μ-opioid receptor agonists by using the „operational" calculation model

I determined the ''E_{max}'' and ''n_H,'' values for DAMGO and DAMGA using the Hill equation (Eq. (1)); these were then used for calculations in the operational procedure. According to a previous analysis (Al-Khrasani et al., 2001) both DAMGO and DAMGA appeared to fulfill the criteria of full agonism, therefore their E_{max} and ''n_H'' parameters were pooled. The pooled E_{max} value was 96.5 ± 2.8 (n=9) and the Hill slope 1.51 ± 0.08 (n=9) (see also Table 6.). When calculating the agonist dissociation constants by either the null either the operational methods (Table 7.), the K_A values correlated significantly with each other (Pearson correlation coefficient r=0.9998, $p<0.0001$). Furthermore, when using Dunnett's post hoc test for differentiating between full and partial agonism, using DAMGO as prototype full agonist, both methods gave a similar result. The test qualified morphine, EMO-1, EMO-1-ol, Dmt-EMO-1, EMO-2-ol and met^2-EMO-2 as partial agonists, gave intermediate parameters for normorphine, morphiceptin and EMO-2 whereas DAMGA ranked as a full agonist.

The residual receptor fraction (q) after β-FNA treatment was calculated by the null method (Table 7). There were significantly higher q values for Dmt-EMO-1 as compared to the majority of the other agonists; some comparisons yielded significant difference also for EMO-1.

Table 6. The dose-response curve parameters of μ-opioid agonists in the mouse vas deferens before and after β-funaltrexamine treatment

Agonist (n)	A_{50} (nM)[a]		E_{max} (%)[b]		Hill slope[b]	
	C[c]	F[c]	C[c]	F[c]	C[c]	F[c]
DAMGO (5)	46.9 (36.4-60.4)	482.7 (263.0-641.8)	96.3 ± 2.2	97.7 ± 1.9	1.62 ± 0.10	1.02 ± 0.04
DAMGA (4)	17.0 (12.9-22.2)	186.9 (153.3-227.9)	96.7 ± 5.6	98.5 ± 1.9	1.37 ± 0.11	1.04 ± 0.06
Morphine (4)	140.0 (111.3-176.0)	937.1 (671.1-1309)	81.6 ± 0.7	34.4 ± 3.4	1.52 ± 0.15	1.01 ± 0.09
Normorphine (4)	181.2 (133.7-245.6)	4202 (3570-4945)	94.3 ± 3.5	96.3 ± 3.0	1.40 ± 0.16	0.60 ± 0.04
Morphiceptin (4)	2619 (2381-2882)	43342 (25490-73696)	99.2 ± 1.7	91.9 ± 0.6	1.35 ± 0.06	0.83 ± 0.08
EMO-1 (6)	23.2 (16.4-32.9)	242.1 (170.3-344.3)	96.1 ± 2.8	95.2 ± 3.3	1.39 ± 0.13	0.48 ± 0.06
EMO-1-ol (4)	111.0 (82.1-150.1)	1450 (818.4-2570)	99.0 ± 0.5	67.9 ± 9.1	1.17 ± 0.07	0.46 ± 0.10
Dmt-EMO-1 (6)	1.48 (1.11-1.99)	4.54 (2.3-8.97)	99.8 ± 0.2	63.2 ± 7.7	1.57 ± 0.10	1.01 ± 0.24
EMO-2 (4)	17.2 (14.5-20.5)	127.1 (82.3-196.3)	99.5 ± 2.7	79.5 ± 3.1	1.61 ± 0.13	1.11 ± 0.04
EMO-2-ol (6)	26.3 (20.3-34.1)	212.1 (99.5-451.8)	98.5 ± 3.3	84.7 ± 2.9	1.52 ± 0.11	0.84 ± 0.10
met^2-EMO-2 (4)	42.2 (28.0-63.5)	192.5 (115.9-319.8)	89.5 ± 5.3	53.9 ± 8.6	1.19 ± 0.07	0.76 ± 0.10

Footnotes

The parameters were obtained from curve fitting by the Hill equation (three parameters).

[a] For the 50% effective concentration (A_{50}) geometric mean and 95 % confidence interval was given.

[b] For E_{max} and Hill slope arithmetic mean ± S.E.M. was given.

[c] C (control): before β-FNA; F: after 30 min incubation with 5×10^{-7} M β-FNA

Table 7. The receptor constants of μ-opioid agonists in the mouse vas deferens

Agonist (n)	K_A (nM)[a]		K_A/A_{50}[b]		q(%)[c]
	NULL	OPER	NULL	OPER	NULL
DAMGO (5)	727.7 (489.3-1220)	2412 (1281-4543)	17.4 ± 2.6	56.7 ± 11.3	17.7 ± 2.5
DAMGA (4)	355.4 (274.3-460.5)	1058 (583.3-1919)	21.7 ± 2.9	71.5 ±17.3	14.4. ± 1.5
Morphine (4)	574.2 (257.5-1280)	528.7 (462.7-604.1)	4.86 ± 1.39	3.99 ± 0.69	16.4 ± 4.6
Normorphine (4)	1748 (1542-1981)	3395 (2374-4855)	10.2 ± 1.7	20.5 ± 4.2	14.9 ± 2.0
Morphiceptin (4)	36244 (28967-45350)	63755 (49181-82649)	14.4 ± 2.0	25.1 ± 3.2	12.6 ± 0.6
EMO-1 (6)	57.8 (24.0-139.6)	135.3 (64.1-285.5)	3.19 ± 0.78	6.95 ± 1.57	40.1 ± 6.7
EMO-1-ol (4)	444.5 (220.5-895.9)	470.2 (325.9-678.4)	4.82 ± 1.48	4.72 ± 1.05	19.3 ± 6.2
Dmt-EMO-1 (6)	2.07 (0.53-8.04)	5.04 (3.23-7.86)	3.57 ± 1.71	3.69 ± 0.62	53.0 ± 12.6
EMO-2 (4)	247.5 (188.1-325.6)	356.0 (275.5-460.0)	14.7 ± 2.9	20.3 ± 2.5	13.3 ± 1.0
EMO-2-ol (6)	71.3 (35.0-145.3)	304.6 (229.3-404.5)	3.30 ± 0.79	12.9 ± 2.2	34.8 ± 4.1
met^2-EMO-2 (4)	104.5 (70.2-155.1)	160.4 (110.4-233.1)	2.86±0.68	5.03 ± 1.72	25.7 ± 2.8

Footnotes

The parameters were determined by the ‘‘null’’ method (based on the double reciprocal plot of equieffective agonist concentrations before and after β-FNA) or the ‘‘operational’’ (OPER) method (Leff et al., 1990a, 1990b). Statistics: for K_A/A_{50} ratios: by ANOVA followed by Dunnett’s multiple comparison test DAMGO vs. morphine, EMO-1, EMO-1-ol, Dmt-EMO-1, EMO-2-ol and met2-EMO-2: $p<0.001$ both by null and operational method; For residual receptor fraction ‘‘q’’: by ANOVA followed by Newman–Keuls test Dmt-EMO-1 vs. morphiceptin $p<0.01$, Dmt-EMO-1 vs. DAMGO,

DAMGA, morphine, normorphine and EMO-2 $p<0.05$; EMO-1 vs. morphiceptin and EMO-2 $p<0.05$.

[a] For the agonist dissociation constant (K_A) geometric mean and 95% confidence interval was given.

[b] For the efficacy-related K_A/A_{50} ratio arithmetic mean ± S.E.M. was given.

[c] The residual receptor fraction ‘‘q’’ was expressed in percent; arithmetic mean±S.E.M. was listed.

6.4. The characterization of potential biosynthetic precursors / intermediates of endomorphin-2

6.4.1. The results of background experiments

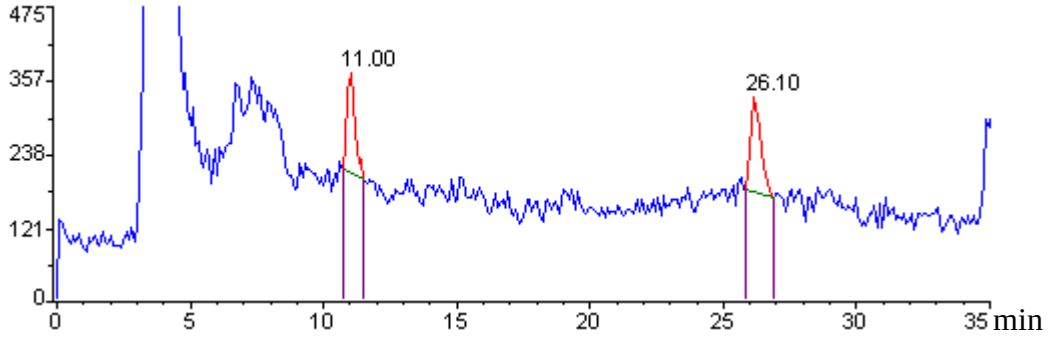
6.4.1.1. The incorporation of radiolabelled Tyr-Pro into EMO-2 related tetrapeptides

When injecting 20 μCi Tyr-Pro into the lateral cerebral ventricle of rats there was an incorporation of label into a peptide which had the same retention time (Fig. 18. panel A, radiodetection, upper part) as co-injected standard EMO-2-OH (UV detection, lower part). This finding was present in two out of four extracts made from the brains of animals sacrificed at 30 min but not at 15 (n=3) or 60 min (n=4) after injection. At 30 min post-injection of 200 μCi Tyr-Pro, there was robust label incorporation into a peptide which had the same retention time as standard EMO-2 (in 2 out of 4 rats, Fig. 18. panel B).

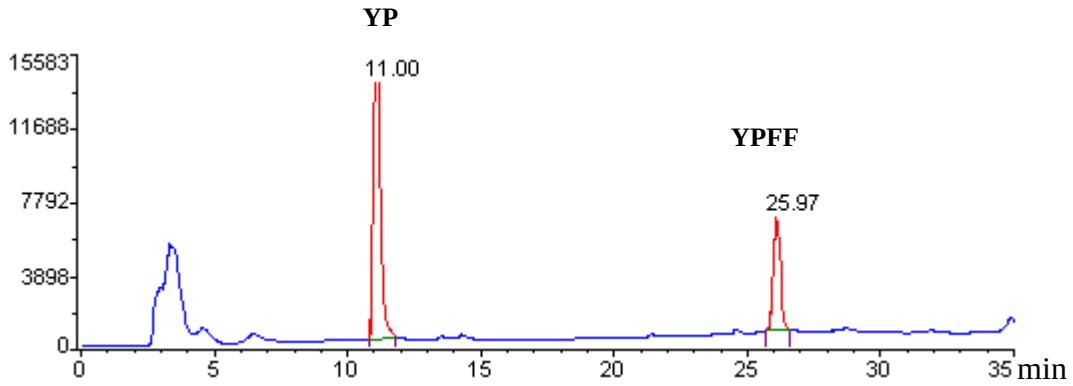
Fig. 18. The RP-HPLC analysis with online radiodetection of processed brain extracts obtained from rats injected intracerebroventricularly with 20 µCi (panel A) or 200 µCi ^{3}H-Tyr-Pro (panel B). (Taken from Rónai et al., 2006).

A

Cps

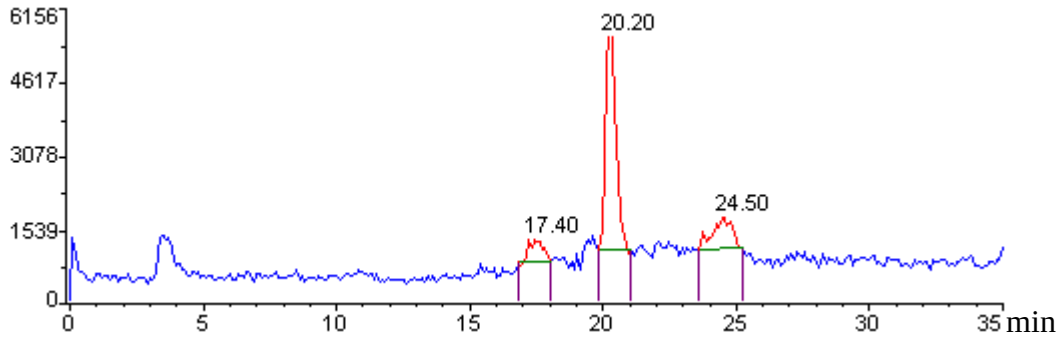


UV absorption

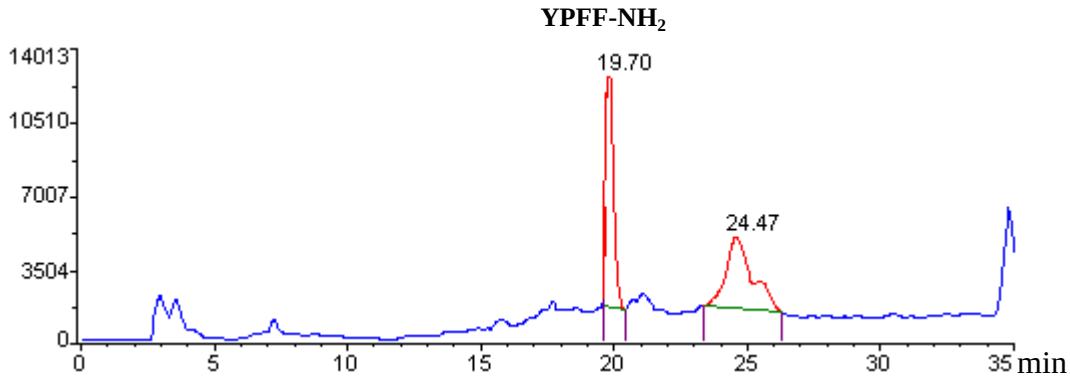


B

Cps



UV absorption



Legend:

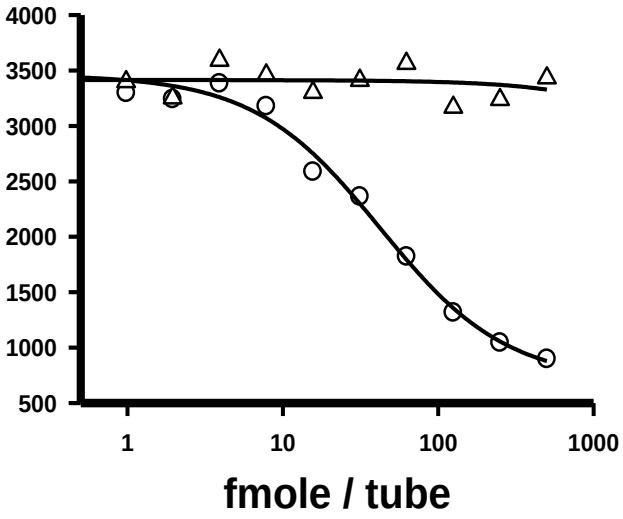
Reverse-phase HPLC analysis of purified rat brain extracts prepared from brains removed 30 min after intracerebroventricular injection of labeled Tyr-Pro. Radiodetection (upper parts in panels A, B) and UV (at 220 nm) detection (lower parts in panels A, B) were used. 4-4 rats were used both in the ‘‘20 μCi’’ (Nos. 1.5–1.8) and ‘‘200 μCi’’ series (Nos. 2.1.–2.4); chromatograms represent processed samples taken from rat No. 1.6 (panel A) and No. 2.4 (panel B). Chromatographic conditions: Vydac 218TP54 C18 reverse-phase column (250 × 4.6 mm, 5 μm) at a flow rate of 1 ml/min at ambient temperature. The mobile phase was mixed from 0.1% (v/v) TFA in water and 0.08% (v/v) TFA in ACN, and gradient elution was carried out from 2% to 40% of organic modifier within 30 min. In the UV chromatograms the co-injected standards appear with the exact retention times. The standards were prepared in 2% acetonitrile –

98% TFA 0.1% (v/v) in water to give 10–30 nmol amount in the injected volume, and 200 µl was injected onto the HPLC.

6.4.1.2. The properties of antisera used in RIA

Antisera raised in rabbit No 1 (R1) and No 4 (R4) were used for further experimentation. R1 antiserum recognized EMO-2 with a median sensitivity of 65.5±7.5 pg/tube (n=7), and did not recognize EMO-1 even at 500 pg/tube (representative displacement curves are shown in Figure 19., selectivity profile in Table 8).

Fig. 19. Representative calibration curves in R1 antiserum with endomorphin-1 (EMO-1) and endomorphin-2 (EMO-2)



Symbols: open triangles: EMO-1 displacer; open circles: EMO-2 displacer.

R4 antiserum recognized EMO-1 with a median sensitivity of 113.5±26.7 pg/tube (n=8) and also EMO-2 (46.3±11.3 % (n=4)) displacement at 500 pg/tube, although the displacement curve for the latter was rather shallow (Table 8.).

Neither antisera recognized N-terminal di- and tripeptide endomorphin fragments or endomorphins with a free C-terminal carboxylic function (i.e. EMO-1-OH and EMO-2-OH, resp), or reacted with [Met^5]- or [Leu^5]-enkephalins, [Met^5]-enkephalin-Arg^6,Phe^7, β-endorphin or [D-Ala^2]-dynorphin-A(1-17) (Table 8).

Table 8. **Displacement of radiotracer by natural opioid peptides, peptide fragments and synthetic analogs in R1 and R4 antisera**

Displacer	Addition (ng/tube)	Displacement (%)[a] in	
		R1 antiserum	R4 antiserum
Endomorphin-1	0.5	12.3 ± 6.7 (4)	70.9 ± 7.2 (5)
Endomorphin-2	0.5	79.9 ± 5.0 (5)	46.3 ± 11.3 (4)
Endomorphin-1-ol	100	15.0 ± 6.3 (3)	11.5 ± 3.2 (4)
Endomorphin-2-ol	100	69.0 ± 7.1 (4)	12.6 ± 1.5 (4)
Endomorphin-1-OH	100	8.5 ± 4.3 (4)	16.3 ± 4.9 (4)
Endomorphin-2-OH	100	6.9 ± 3.7 (4)	9.5 ± 2.6 (4)
YPW	100	11.4 ± 1.9 (3)	7.9 ± 0.4 (3)
YPF	100	4.7 ± 2.1 (3)	5.8 ± 2.8 (3)
YP	100	7.4 ± 2.8 (3)	6.2 ± 2.2 (3)
[Met^5]-enkephalin	100	1.3 ± 2.1 (3)	2.4 ± 0.6 (3)
[Leu^5]-enkephalin	100	0.5 ± 2.1 (3)±	2.9 ± 0.7 (3)
[Met^5]-enkephalin-Arg^6,Phe^7	100	11.6 ± 3.3 (3)	7.6 ± 1.0 (3)
[D-Ala^2]-dynorphin-A(1-17)	100	6.6 ± 3.4 (3)	3.6 ± 1.7 (3)
β-endorphin	0.25	5.0 ± 2.9 (3)	9.0 ± 0.8 (3)

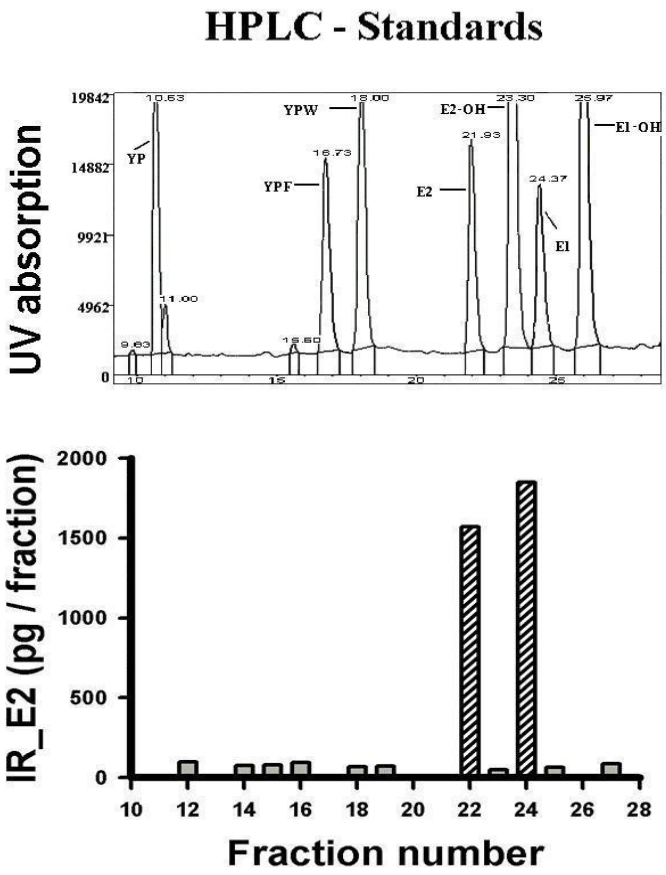
Footnote:

[a] Arithmetic mean ± S.E.M. values are listed, number of experiments appear in parenthesis.

6.4.1.3. EMO-2 immunoreactive peaks in rat brain extracts

When measuring immunoreactivities in rat brain extracts made and purified as in the case of label incorporation studies, we have found immunoreactivities by R1 antiserum in two fractions (Fig. 18.). One peak was present in the fraction where authentic EMO-2 standard eluted as detected by UV and the other where EMO-2-OH standard eluted. Please note that, fortunately, there is a „void" fraction between IR-EMO-2 and IR-„EMO-2-OH", therefore, the immunoreactivity in the second fraction could not have been attributed to „oversplit" EMO-2 from the previous one. Comparing the recognition profile of R1 antiserum with presently shown results it is now obvious that the second immunoreactive peak cannot be structurally identical to authentic EMO-2-OH. We also suspect though it is not yet proven, that the incorporation of label had taken place into an EMO-2-OH-like, though structurally distinct species.

Fig. 20. Endomorphin-2-like immunoreactivities found in RP-HPLC gradient separates of rat brain extract No. 1.



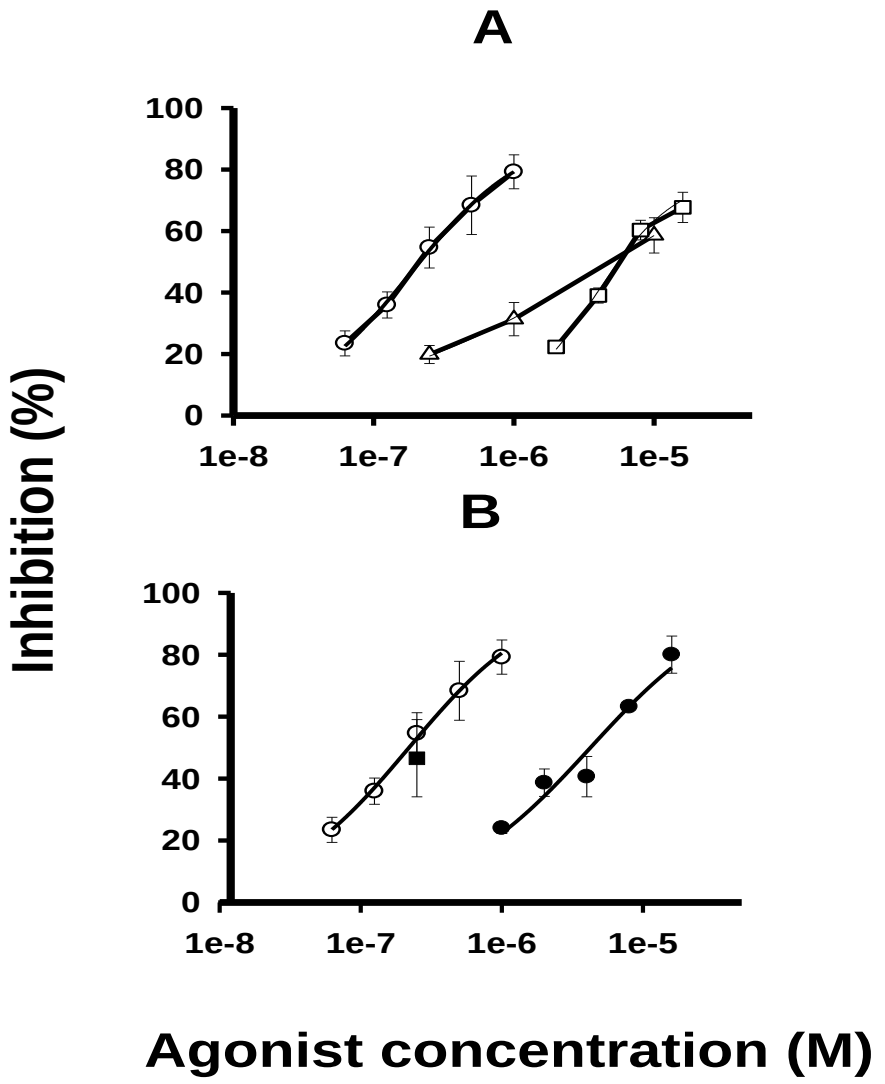
Legend:

The RP-HPLC gradient separation profiles of endomorphin standards and their fragments (upper panel). 0.01 mg amounts of peptides were injected. Chromatographic conditions: Vydac 218TP1010 C18 reverse-phase semipreparative column (250×10 mm, 10 μm) at a flow rate of 4 mL/min at ambient temperature with UV detection at 220 nm. The mobile phase was mixed from 0.1% (v/v) TFA in water and 0.08% (v/v) TFA in ACN, and gradient elution was carried out from 2% to 40% of ACN within 30 min. EMO-2-like immunoreactivities detected by R1 antiserum in the fractions prepared from rat brain extract No.1 (lower panel). The scales of panels were matched so that immunoreactivity at "22" represents peptide(s) with retention time(s) between 21–22 min etc.

6.4.2. The opioid properties of free carboxylic endomorphin-2, glycine-extended endomorphins and –Ser/-Ser-O-glucose Tyr-Pro derivatives in the MVD bioassay

In the MVD bioassay, both Gly-extended endomorphins inhibited field stimulation-induced contractions in a dose-dependent fashion. In contrast to DAMGO or the parent, authentic endomorphins, where the inhibitory actions developed in ≤3 min and the recovery after washout was rapid, the effect of Gly-extended peptides developed slowly (15-25 min) and the recovery was also prolonged, occasionally incomplete. The effect of EMO-2-G was approximately 30 times more potent than EMO-1-G, 20 times more potent than EMO-2-OH (i.e. EMO-2 with a free carboxylic C-terminus) but 15 times less potent than authentic EMO-2 (Fig. 21., panel A, Table 9.). Furthermore, the slope of EMO-1-G dose-response curve was lower as compared to EMO-2-G, the Hill slope being 0.48 for the former and 1.05 for the latter. The opioid agonist action of EMO-2-G was competitively antagonized by 10^{-6} M cyprodime (Schmidhammer et al., 1990), a μ-opioid receptor-preferring antagonist (Fig. 21., panel B, Table 10.). On the other hand, NTI, an opioid receptor antagonist with good preference for δ-opioid receptors (Portoghese et al., 1988) displayed no antagonism against EMO-2-G at 3 nM concentration of antagonist (Fig. 21., panel B, Table 10.). The K_e of CYP against EMO-2-G was significantly higher than against the μ-opioid receptor agonist prototype DAMGO, but the two values fell within the same order of magnitude (Table.10.). There was a significant antagonism of EMO-2-OH's effect by NTI; the contribution of δ-opioid receptorial component to the action of this peptide is supported also by the high K_e NTX against it (Table 10.). The inhibitory effect of 10^{-5} M EMO-1-G could not be reversed by 30 nM NTI and only partially reversed by 10^{-6} M naloxone (NX), the inhibitory effect of agonist being 59.2±3.2 % in the absence and 36.5±3.2 % (n=4) in the presence of NX.

Fig. 21. The inhibitory dose-response curves of EMO-1-G, EMO-2-G and EMO-2-OH in field-stimulated mouse vas deferens (panel A) and the antagonism of EMO-2-G by cyprodime and naltrindole (panel B)



Legend:

Symbols: open circles: EMO-2-G; open triangles: EMO-1-G; open squares: EMO-2-OH; dark circles: EMO-2-G in the presence of 10^{-6} M cyprodime; dark square: the effect of 2.5×10^{-7} M EMO-2-G in the presence of 3×10^{-9} M naltrindole. Points represent arithmetic mean, vertical lines the S.E.M. values of data sets obtained in 4-8 independent experiments

Table 9. The opioid agonist actions of endomorphin-related peptides and μ- and δ-opioid receptor agonist prototypes in isolated mouse vas deferens

Peptide	IC_{50} (nM)[a]	n
(D-Ala^2,MePhe^4,Gly^5-ol)enkephalin (DAMGO)	83.9 ± 20.1	10
Endomorphin-2	17.8 ± 1,10	5
Endomorphin-2-OH	6022.0 ± 586.0	8
Endomorphin-2-Gly-OH	272.1 ± 80.0	6
Endomorphin-1	24.8 ± 2.69	6
Endomorphin-1-Gly-OH	8318.0 ± 3519.0	7
(D-Ala^2,D-Leu^5)-enkephalin (DADLE)	0.78 ± 0.14	10

Footnote

[a] 50% inhibitory concentrations (i.e. [A] at E=50%). The IC_{50} values were determined either from curve fitting according to the Hill equation using the E_{max} = 100 constraint or from logarithmic regression. Arithmetic mean ± S.E.M. values are listed.

Table 10. Antagonism of endomorphin-2-related peptides and μ- and δ-opioid receptor agonist prototypes in isolated mouse vas deferens by cyprodime (CYP), naltrexone (NTX) and naltrindole (NTI)

Agonist peptide	Antagonism (K_e, nM)[a] by		
	Cyprodime	Naltrexone	Naltrindole
DAMGO	22.3 ± 2.24 (10)	0.33 ± 0.018 (6)	9.64 ± 0.68 (6)
Endomorphin-2-OH	N.T.[b]	6.40 ± 2.35 (4)	0.57 ± 0.05 (4)
Endomorphin-2-Gly-OH	83.89 ± 27.57 (4)	N.T.[b]	>>3.00 (4)
DADLE	1751.9 ± 361.6 (4)	8.02 ± 0.54 (6)	0.08 ± 0.02 (6)

Footnotes

[a] Equilibrium dissociation constants, calculated according to the equation K_e = [B] / (DR-1) (Kosterlitz and Watt, 1968) where [B] is the nanomolar concentration of antagonist and "DR" is the dose ratio. Preparations were equilibrated with the antagonists for 30 min (CYP, NTI) or 20 min (NTX) before re-taking the agonist dose-response curve. Arithmetic mean ± S.E.M. values are listed, number of experiments appear in parenthesis.

[b] Not tested

Regarding the relatively high agonist potency of Gly-extended EMO-2 and also the definite preference for μ-opioid receptors (see the detailed arguments in the Discussion), the possible conversion of glycylated peptide to authentic EMO-2 by PAM enzyme must have been taken into consideration also in MVD. Ascorbate had been described to stimulate whereas trans-styrylacetic acid to inhibit the enzyme (Bradbury et al., 1990; Eipper et al, 1983; Eipper and Mains, 1988). Testing a set concentration of EMO-2-Gly-OH ($2.5e^{-7}$ M) in ascorbic acid- or styrylacetic acid-containing buffer ($1e^{-5}$ M of both substances), no interaction was found: $2.5e^{-7}$M EMO-2-Gly-OH caused 40.678±10.06 % inhibition alone (n=7), 47.677±19.17 % in the presence of styrylacetic acid (n=4) and 33.64±15.622 % when ascorbic acid was added (n=3).

The lack of opioid agonism by –Ser- and –Ser(O-glucose)-derivatives of Tyr-Pro in MVD is shown in Table 11.

Table 11. The agonist properties of Tyr-Pro derivatives in MVD bioassay

Peptide	Concentration (M)	Inhibition (%).[a]	n
Tyr-Pro	$1e^{-4}$	28.44 ± 3.56	4
Tyr-Pro-Ser	$1e^{-4}$	19.11 ± 8.14	4
Tyr-Pro-Ser(O-glucose)	$1e^{-4}$	10.46 ± 8.73	4
Tyr-Pro-Gly-Ser	$1e^{-4}$	15.27 ± 8.86	4
Tyr-Pro-Gly-Ser(O-glucose)	$1e^{-4}$	14.10 ± 8.04	3
DAMGO [b]	$1e^{-7}$	65.35 ± 5.64	4

6.5. The in vitro pharmacological characterization of a novel nociceptin antagonist

The effects of nociceptin, two kinds of published NOP receptor antagonists (Guerrini et al., 1997; Guerrini et al., 1998) and some N-acyl oligopeptide derivatives were compared. The analogues were developed on the basis of Ac-RYYRIK-NH_2 structure (Dooley et al., 1997) and N-terminally modified short sequences possessing κ-opioid antagonist properties (Orosz et al., 1994; Orosz et al, 1995). Nociceptin showed a dose-dependent inhibitory effect in field-stimulated mouse vas deferens (MVD); the inhibitory potency can be taken as a measure of agonist activity in the bioassay. Before screening the antagonist candidates, a chosen concentration (160 nM) of nociceptin was utilized repeatedly in 20-25 min administration cycles over a period of 240 min; the magnitude of inhibitory effect was reproducible throughout. For the antagonist candidates a 30 min equilibration was allowed in MVD before retesting the effect of nociceptin. The bioassay data are summarized in Table 12. Nociceptin was a pure agonist in MVD, [D-Ala^2]-NC-(1–13)-NH_2 behaved as a weak partial agonist, whereas the κ-opioid receptor antagonist acylated peptides, displayed neither antagonism nor agonism at the NOP receptors, therefore no further studies were performed with them.

Table 12. The effects of nociceptin analogues in the mouse vas deferens bioassay

Peptide	Nociceptin antagonism/displacement K_e (nM)[a] in MVD	Agonist effect in MVD IC_{50} (nM)[b]
Nociceptin-OH FGGFTGARKSARKLANQ	no antagonism	77.5 ± 7.7 (28)
[D-Ala^2]-NC-(1-13)-NH_2 F-d-Ala-GFTGARKSARK-NH_2	3690 ± 830 (4)	2590 ± 470
[FψG]-NC-(1-13)-NH_2 FψGGFTGARKSARK-NH_2	215 ± 41 (5)	>10,000
Ac-RYYRIK-NH_2	28.2 ± 3.1 (6)	>1000
Ac-RYYRIK-ol	2.44 ± 0.15 (6)	>10,000

Legend:

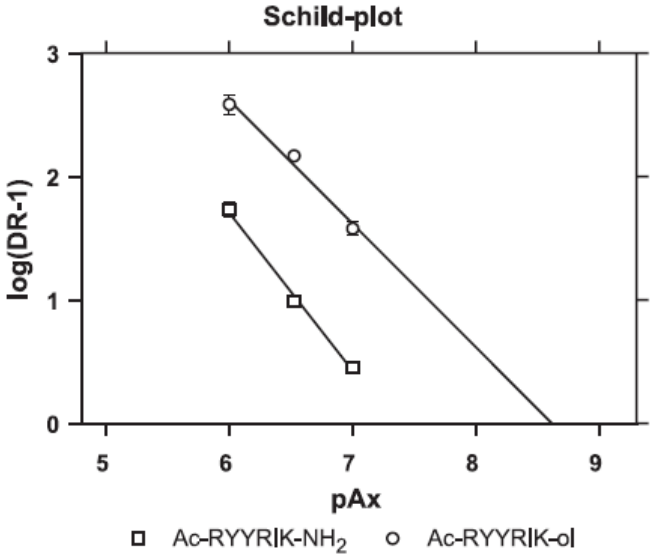
[a] Equilibrium dissociation constant; arithmetic mean ± S.E.M.

[b] 50% inhibitory concentration. The values in parenthesis refer to the number of determinations.

The new hexapeptide analogue Ac-RYYRIK-ol and its parent compound Ac-RYYRIK-NH_2, however, were able to antagonize the effects of nociceptin in a competitive manner. At a concentration range of 10^{-7}-10^{-6} M, Ac-RYYRIK-ol shifted the dose–response curves of nociceptin to the right with no maximum depression. The Schild-plot of antagonism had the characteristics of a competitive antagonist with a slope factor very close to the unity, i.e., the theoretical value (Fig. 22). pA_2 (x-axis intercept) was found to be 8.64 ± 0.019. The K_e calculated from the individual dose ratios and the corresponding antagonist concentration was 2.44±0.15 nM (n=6, geometric mean and 95% confidence interval) (see also Table 12). In the MVD assay, 10 μM Ac-RYYRIK-ol did not change the inhibitory action of the δ-opioid receptor agonist peptide DADLE suggesting NOP receptor selectivity of the antagonism. The

inhibitory effect of 0.8 nM DADLE was 71.6 ± 4% before and 79.7±3.6% after 10 min exposure to 10 μM Ac-RYYRIK-ol (n=3). After screening several *N*-acyl derivatives of the hexapeptide alcohol, such as pivaloyl-, Fmoc-, methanesulfonyl-, benzoyl-, phenylacetyl-, propionyl- and formyl- (not shown), the most active compound is appeared the Ac-derivative indicating the very sensitive nature of the N-terminal substitution. This might be due to the different steric requirements of the terminal acyl groups.

Fig. 22. Schild-plot of nociceptin antagonism by acetyl-hexapeptides in mouse vas deferens bioassay



Abscissa: The negative logarithm of molar concentration of the antagonist. Ordinate: The logarithm of (dose ratio-1) values. Linear regression analysis for Ac-RYYRIK-ol yielded the equation of: $y = -1.00 \pm 0.084x + 8.64 \pm 0.55$ (n=6, R^2=0.98).

7. Discussion

7.1. Experiments on NTS-DVN and PVN/subPVN prism/whole slice preparation

7.1.1.The effect of MSG treatment on the field stimulation-induced release of ^{3}H-NE from NTS-DVN prism preparations: differences in the responses to modulatory agents

The original aim of the study was to find a functional indication in vitro which would support our assumption based on in vivo experiments (Rónai et al., 2001) that neonatal MSG treatment causes a damage in the local circuitry of NTS-DVN neurons.

By measuring just one output function (i.e. the basal and stimulation-induced release of NE) the chances of detection of such damage are limited to altered function(s) with a bearing on the regulation of ^{3}H-NE release. There were no definite indications in our in vivo experiments as to the neurons actually damaged by MSG treatment whereas the morphological findings (Karcsu et al., 1981, Karcsu et al., 1985 and Jászai et al., 1998) pinpointed the area postrema-subpostrema region where neuronal damage did occur. To increase the probability of detection, we used agents which can act at several target receptors within the NTS-DVN complex. The complex has numerous subsets of excitatory amino acid (Miller and Felder, 1988, Raybould et al., 1989 and Willis et al., 1996) as well as $GABA_B$ (Margeta-Mitrovic et al., 1999 and Partosoedarso et al., 2001) receptors, in many cases with well-characterized location (Karcsu et al., 1981, Karcsu et al., 1985 and Varga et al., 1996).

The tritium content of NTS-DVN extracts from rats treated neonatally with MSG did not differ significantly from the radioactivities found in the untreated groups. This might be taken as an indication that no major morphological damage took place at the noradrenergic neurons.

In control rats L-glutamate failed to induce ^{3}H-NE release under resting conditions but increased field stimulation-induced release. In MSG-treated rats the

stimulatory effect of glutamate is lost. There is a morphologically verified damage of choline-acetyltransferase-containing neurons in the area postrema (Karcsu et al., 1985); these neurons are responsive to glutamate. Our data are insufficient to decide whether this or another subset of neurons is responsible for the loss.

The $GABA_B$ receptor agonist baclofen increased field stimulation-induced norepinephrine release only in young adults. Owing to the signal transduction pathways at $GABA_B$ receptors a stimulatory result after receptor activation can be brought about only by a disinhibitory mechanism, i.e. by the activation of inhibitory $GABA_B$ receptors located on inhibitory interneurons or on the terminals of inhibitory projections. The loss of $GABA_B$ receptor-mediated stimulation with aging indicates that during adulthood there is a functional maturation of neural circuitry within the NTS-DVN. One of the possible mechanisms is an input-related adaptive change in a subset of $GABA_B$ receptors within the complex. It should be born in mind that the age-matching controls couldn't be regarded as old animals; some groups even prefer using rats of that age range. Our results suggest that the pharmacological responsiveness of some central autonomic regulatory functions might be entirely different in these two, age-related subsets of adult rats.

7.1.2. The study of stimulation induced release of ^{3}H-NE and ^{3}H-D-Asp in NTS-DVN prisms

In these series of experiments we wished to characterize the stimulation-induced (electrical field-stimulation, ES and high potassium KS) release of ^{3}H-NE and ^{3}H-D-Asp from rat NTS-DVN prisms preparations, preloaded with 2.5 μCi of tritiated substances, in vitro. Furthermore, we tested the Ca^{2+} dependency of the release of tritiated neurotransmitter by high potassium (60 mM KCl).

Comparable quantities of ^{3}H-NE could be released by repeated (S_1, S_2) field electrical stimulation, whereas ^{3}H-D-Asp could be released only by high (60 mM) KCl.

In the case of ^{3}H-NE, the S_2/S_1 ratio, close to the unity, rendered suitable the field electrical stimulation release for studying drug actions introducing drugs before S_2,

contrary to the 60 mM KCl induced release where the K_2/K_1 ratio was inadequate for this.

Furthermore, the field electrical stimulation induced release of ^{3}H-D-Asp was insignificant. The high K^+ induced release of ^{3}H-D-Asp, beside the low release showed a remarkable Ca^{2+} independency; when using the conventional modification for extracellular Ca^{2+} removal (Ca^{2+}-free buffer + 1 mM EGTA). It appears, that only a small fraction of stimulated release is Ca^{2+} dependent.

In summary, we can say, that the release pattern of ^{3}H-NE from rat NTS-DVN slices is characteristic of a neurotransmitter. We were not able to install a similar methodological approach to measure ^{3}H-D-Asp release. Moreover, there is an ambiguity as to the cellular compartment from which ^{3}H-D-Asp is released by 60 mM KCl.

7.1.3. The modulation of stimulated ^{3}H-NE release by endomorphin-1 in rat dorsal vagal complex *whole trapezoidal slice* preparation

In the present series of experiments we extended our previous studies in NTS-DVN *prism* preparation to *whole slices* to develop a quick and easy model with maintained neural connections to study the functional role and the place of action of endogenous neuropeptides acting on neural elements involved in the physiological regulation and pathological shifts of autonomic functions. In a systematic attempt to locate neural element-neuropeptide receptor interaction, we chose ^{3}H-NE release modulation by μ-opioid receptors as the first model.

Endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2, EMO-1) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2, EMO-2) are highly selective and potent μ-opioid receptor agonists (Zadina et al., 1997). Although, they were proven to be partial agonists both biochemically (Alt et al., 1998; Narita et al., 1998; Lengyel et al., 2002) and pharmacologically (Al-Khrasani et al., 2001). Both endomorphins and μ-opioid receptors are present in the rat NTS-DVN (Martin-Schild et al., 1999; Mansour et al., 1994), moreover, endomorphins reduced the release of ^{3}H-NE from rat NTS-DVN *prisms* induced by field electrical stimulation (Al-Khrasani et al., 2003). Consequently,

we tested the effect of EMO-1 on stimulation induced ^{3}H-NE release from rat NTS-DVN *whole slices.*

However, the use of slices presented several potential drawbacks like viability, oxygenation, proper loading and release of labelled transmitters we concentrated first to solve these factors. Additionally, we developed a fast application system for the rapid change of several perfusion solutions. This series of experiments served also as an experimental validation of the new system.

In contrast with previous measurements in NTS-DVN prisms (Al-Khrasani et al., 2003), EMO-1 did not inhibit the ES induced ^{3}H-NE release in whole slice preparations. The ineffectiveness of EMO-1 might be due to technical reasons , like too short drug exposure or to a pharmacological one, such as the EMO-1's partial agonism. There may also be a physiological explanation comprising the possibility that besides noradrenergic (Pickel et al., 1998), other, inhibitory neurons (eg GABAergic, Browning et al., 2006) are also supplied with μ-opioid receptors in the NTS-DVN. Thus, due to the preserved neuronal connectivity in slices the apparent net effect of μ-opioid receptor agonists on ^{3}H-NE release might be zero.

As a next step, if even full μ-opioid receptor agonists would be ineffective in slices while using ES-induced ^{3}H-NE release, other tritiated transmitters should be introduced. Moreover, besides field electrical stimulation and high potassium, specific chemical stimuli, modelling vagal input (e.g. Glutamate, Substance P, CCK-8, galanin) or the destruction of descending limbic-hypothalamic projections, should also be tested.

7.1.4. The release of ^{3}H-GABA from rat isolated hypothalamic paraventricular / subparaventricvular (PVN/subPVN) slices. Comparison with the release from NTS-DVN slices

This sub-series of experiments were performed upon the initiation from and collaboration with the 1st Department of Physiology, Semmelweis University. They had initiated the project to find an isolated neural preparation, where the neuronal connectivity was, at least in part, preserved, and potentially suitable for the modelling of a trans-receptorial signalling pathway they had already characterized in transfected

CHO cells (Turu et al., 2007). In brief, they have demonstrated that a $G_{q/11}$ –coupled signalling pathway, initiated by AT_1 receptor stimulation, through the transformation of DAG second messenger by diacyl-glycerol lipase to 2-arachidonyl glycerol (an endogenous CB_1 cannabinoid receptor ligand) can be transferred to a $G_{i/o}$-coupled signalling route (i.e. to CB_1 receptors), either in an autocrine or paracrine manner. Hypothetically, this innervational constellation may converge on a GABA-ergic neuron in the PVN/subPVN complex in the rat (Freund et al., 2003; Castelli et al., 2007). My task in the sub-project was i) to install some technical novelties to improve significantly the performance and precision of brain slice loading- and superfusion-system and ii) to compare the properties of PVN / subPVN and NTS-DVN slices in terms of loading by tritiated GABA and the stimulation-induced release of ^{3}H-GABA from the two tissues slices.

Rat PVN/subPVN preparations accumulated and released ^{3}H-GABA more efficiently than did NTS/DVN slices. According to the „convergence hypothesis" exposure of PVN/subPVN preparations to AT-II should have reduced the stimulation-induced release of ^{3}H-GABA, which was definitely not the case in our experiments. Thus, either the hypothesis is invalid, or the „convergence" happens only in a smaller subpopulation of GABA-ergic neurons and the possible inhibition in these is not reflected in the „gross" release pattern. The experimental conditions for NTS/DVN should be improved to render the preparation suitable for studying properly tritiated GABA release.

7.2. The demonstration of an α_{2B}-adrenoceptor-mediated action

The effect of ST-91 in rat vas deferens cannot be fully reconciled with the standing opinions about the α adrenergic receptor profile of the agonist on the one hand and the presynaptic α_2-adrenoceptor subtype present in the preparation on the other hand. In an early publication (Doxey et al., 1981), the preponderance of postsynaptic α_1 adrenergic agonism was noted as opposed to the presynaptic α_2-adrenoceptor action. By following the same paradigm we have used for the demonstration of presynaptic, α_2-adrenoceptor-mediated inhibitory effect of norepinephrine in rat vas deferens (Vizi et

al., 1983), the potent presynaptic inhibition by ST-91 could also be substantiated in the presence of 10^{-6} M prazosine. By using correlative evidence in the rat (Connaughton and Docherty, 1990) and genetical engineering in mice (Rajamani et al., 2001) the dominant prejunctional α adrenergic receptor subtype in vasa deferentia of these two species was established as $\alpha_{2A/D}$. α_{2D}adrenoceptors are, presumably, the species variant of human recombinant α_{2a} receptors (Bylund et al., 1994; Calzada and de Artinano, 2001) in the rat. Taking into account the known pharmacological selectivity profile of prazosine at α_2-adrenoceptors (reviewed by Bylund et al., 1994) 10^{-6} M prazosine, as used presently, excludes any significant agonist actions at the α_{2B}- or α_{2C}-subtypes whereas would permit effects at the $\alpha_{2A/D}$-subtype. However, based on the differences between the central actions of α_{2A}-adrenoceptor-preferring agonist dexmedetomidine and ST-91 in rats (Dowlatshahi and Yaksh, 1997; Graham et al., 2000) ST-91 was rated as a non-α_{2A}-adrenoceptor agonist, and this rating could not be attributed to the pharmacokinetic differences between these two agonists (i.e. the lipophylicity of dexmedetomidine vs the hydrophylic properties of ST-91).

In rat mesenteric artery rings endothelium-denuded conditions were chosen for studying the possible interaction between α_2-adrenoceptor agonists and β_2-adrenoceptor-mediated relaxation. By this procedure we wished to minimize interference from endothelium-derived relaxing- or contracting agents possibly released by the drugs studied. In phenylephrine-precontracted rings, ST-91 inhibited isoproterenol-induced relaxation in a yohimbine-antagonized fashion and this action was not mimicked by the not subtype-selective α_2-adrenoceptor agonist xylazine. The antagonism by yohimbine was important, because the inhibitory ST-91→isoproterenol interaction might have been due to the α_1-adrenoceptor-stimulating effect of ST-91. To test the involvement of α_{2B}-adrenoceptors by more direct means such as the use of α_{2B}-adrenoceptor subtype-preferring antagonist ARC-239 was not feasible owing to the known high affinity of antagonist also to α_1-adrenoceptors (e.g. Honner and Docherty, 1999). There is a known example of G_i-related negative coupling of α_{2B}-adrenoceptors to β_2-adrenoceptor stimulated adenylyl cyclase (Mhaouty et al., 1995); it is possible though not proven that a similar negative coupling functions also in rat mesenteric artery.

Based on correlative evidence with ligand binding profiles, the postjunctional α_2-adrenoceptors mediating pressor responses in the pithed rat have been suggested

most closely resembling the α_{2D} subtype (Gavin and Docherty, 1996). However, data obtained in genetically engineered mice deficient in either α_{2b} or α_{2c} receptors have shown that the peripheral vasoconstrictor responses elicited by α_2-adrenoceptor agonists are probably mediated by the α_{2B}-adrenoceptor subtype (Link et al., 1996). Under physiological conditions the tonic activation of β_2-adrenoceptors in vascular smooth muscle is likely to be moderate. Therefore, under these conditions the inhibition by α_{2B}-adrenoceptor stimulation cannot be expected to result in systemic blood pressure responses. Genetical engineering (Link et al., 1996) or development of salt-induced hypertension (Makaritsis et al., 1999a) may alter tonic β_2-adrenoceptor activation or α_{2B}-adrenoceptor expression (or both) in vascular smooth muscle to the extent that their interaction may result in systemic pressor response.

Our in vitro experimental model, besides offering a possible explanation for the potentially deleterious circulatory effects of selective α_{2B}-adrenoceptor stimulation may also add a useful test system for studying $\alpha_{2NON\text{-}A}$-adrenoceptor subtype-mediated actions.

7.3. The analysis of partial agonist properties of endomorphins and other μ-opioid receptor agonists by using the „operational" calculation model

I used the "operational" evaluation/calculation method to obtain receptor constants for the μ-opioid agonists we set out to study and I compared the values with those calculated by the "null" method. The K_A values determined by the two methods correlated significantly with each other. In spite of the apparently higher resolution power of the ''operational'' method to differentiate between full and partial agonism, when using Dunnett's test for statistical evaluation and DAMGO as reference full agonist, both methods gave identical ranking patterns. Thus, we could confirm again the previously found full agonism for DAMGA and the partial agonism for EMO-1, EMO-1-ol, Dmt-EMO-1, EMO-2-ol and met^2-EMO-2. EMO-2 (as well as the reference agonists normorphine and morphiceptin) appear to have an efficacy moderately closer to full agonism. The fact that the two natural endomorphins may have distinct

pharmacological properties is potentially very interesting but by no means completely unexpected (Sakurada et al., 2000; Przewlocki and Przewlocka, 2001). It has been reported that the signal transduction patterns by EMO-1 and EMO-2 may have some distinctive features (Sánchez-Blázquez et al., 1999).

The partial agonism among the endomorphin derivatives and the theoretical residual receptor fraction (q) available for these ligands may also suggest some useful trends for medicinal chemistry. In a family of agents acting at the same receptors (in this case at opioid receptors), therapeutic target selectivity can be provided primarily by structural modifications aimed at altering the receptor type/subtype selectivity profile. However, partial agonism may offer a further factor for functional drug selectivity. All the functions where the spare receptor pool is high will be affected both by full and partial receptor agonists. On the other hand, the functions where the receptor reserve is low may remain unaffected by partial agonists. Therefore, μ-opioid receptor-mediated functions should be systematically mapped both in the CNS and at the periphery in terms of relative receptor reserve (Al-Khrasani et al., 2003). Then, the structural requirements of partial agonism for a ligand (in this case for the endomorphin derivatives) should be determined and, based on these informations, peptidomimetics could be designed.

7.4. The characterization of potential biosynthetic precursors/ intermediates of endomorphin-2

In the course of experiments aimed at proving the possibility of a *de novo* biosynthetic pathway for endomorphins the characterization of several endomorphin-related fragments and derivatives as well as of precursor candidates with improved penetration properties appeared to be necessary, in terms of their opioid properties in bioassays. The field-stimulated mouse vas deferens was chosen for bioassay because it contains all the three major types of „classical" opioid receptor types, δ-, μ- and κ (in the order of dominance both in CFLP and NMRI mouse strains).

Endomorphin derivatives with a free carboxylic function as well as glycine-extended endomorphin-1 proved to be weak opioid agonists whereas EMO-2-Gly had

fair opioid agonist potency and a definite preference for the μ-opioid receptor type, similarly to the parent natural peptide, i.e. EMO-2. Interactions with moderately μ-opioid receptor preferring antagonist (naltrexone, ~ 20-fold μ- over δ-, or κ-opioid receptor type preference) antagonist with a higher μ-opioid receptor preference (cyprodime, ~ 50-fold preference) and definitely δ-opioid receptor-preferring antagonist (naltrindole, 100-200-fold δ- over μ-opioid receptor type preference) were used for establishing the receptor type preference of agonism by EMO-2-Gly. As judged from K_e profile of antagonists against this peptide, in spite of free carboxylic C-terminus the type-selection is close to but not quite purely μ, whereas there is a definite δ-opioid receptor type selection in the case of EMO-2-OH. As it is apparent from the "background" experiments that although a probably endomorphin-related peptide appeared in the HPLC-gradient separation profile with retention time identical to endomorphin-2-OH standard, it cannot be structurally identical to it, because our antiserum did not recognize endomorphin-2 with a free carboxylic terminus.

On the other hand, the μ-receptor type preference has raised the possibility that the Gly-extended peptide might have been converted to EMO-2 by PAM enzyme also in MVD. Therefore, the interaction with the PAM stimulant ascorbate and the inhibitor trans-styrylacetic acid has been tested. In the original publication (Szemenyei, Barna et al., 2008) the Discussion has left open an alternative explanation for the lack of interaction. Namely, by assuming an intracellular compartment as the site of *de novo* endomorphin biosynthesis, the possibly saturating ascorbate supply and the lack of styrylacetic acid access to the compartment of biosynthesis have been offered as alternative explanations for the lack of interaction. In the light of the most recent findings made in another neural preparation, in isolated rat L4,5 dorsal root ganglion, we favor the probability of extracellular biosynthesis by a membrane ectoenzyme, dipeptidyl peptidase IV (Rónai, Király et al., 2009). Therefore, by extrapolating these results to the neural elements of MVD, we attribute the property of μ-receptor type preference to EMO-2-Gly itself.

All the –Ser-/Ser(O-glucose)-extended Tyr-Pro derivatives were devoid of any significant opioid agonist activity in the MVD bioassay. In fact, one of the first hints for a possible extracellular biosynthesis in the pilot experiments in isolated L4,5 rat dorsal

root ganglia came from the inefficiency of Tyr-Pro-Ser(O-glucose) to promote endomorphin-2 biosynthesis (Rónai, Király et al., 2009).

7.5. The in vitro pharmacological characterization of a novel nociceptin antagonist

For the pharmacological analyses of the action of a novel endogenous peptide mediator it is a principle that selective, high-affinity antagonists should be designed and synthesized at as early stage of these pursuits as possible. In search for potent nociceptin antagonist peptides several N-terminally modified compounds were synthesized and tested by in vitro pharmacological bioassay. Some of them displayed κ-opioid antagonist activities (Orosz et al., 1994; Orosz et al., 1995) and each has the common feature that their N-terminal amino group cannot be protonated. Because the κ-opioid agonist dynorphin A (1-13) was shown to interact with the nociceptin receptor (Zhang and Yu, 1995; Mogil and Pasternak, 2001) the κ -receptor antagonists mentioned above were also investigated. Further *N*-acyl peptides with considerable nociceptin receptor activities were obtained by screening synthetic combinatorial peptide libraries (Dooley et al., 1997). On the basis of one of the original Dooley's sequences (Ac-RYYRIK-NH_2), a related structure with more reduced C-terminal yielding, Ac-RYYRIK-ol, was synthesized. There are highly effective opioid peptides bearing C-terminal alcohol function, such as the prototype μ-agonist [D-Ala^2,MePhe^4,Gly^5-ol]enkephalin (DAMGO), or a closely related peptide with prolonged oral analgesic activity [D-Ala^2,MePhe^4,Met(O)^5-ol]enkephalin (Roemer et al., 1977). Recently our group published endomorphin-1-ol (Tyr-Pro-Trp-Phe-ol), a μ receptor selective tetrapeptide of high potency and higher efficacy (i.e. less partial agonism) as compared to the parent peptide, endomorphin-1 (Al-Khrasani et al., 2001).

Biological properties of Ac-RYYRIK-ol were compared with the effect of published NOP receptor antagonist structures, including the parent peptide Ac-RYYRIK-NH_2. The reference antagonists [Phe^1-ψ(CH_2–NH)Gly^2]-NC-(1–13)-NH_2 (Guerrini et al., 1998; Butour et al., 1998; Calo et al., 1998) and [D-Ala^2]-NC-(1–13)-

NH_2 (Montiel et al., 1997; Guerrini et al., 1997) were found to have only modest affinities with K_e/K_i values falling into the 10^{-7} M range.

We showed that in contrast to [Phe^1-ψ(CH_2–NH)Gly^2]-NC-(1–13)-NH_2, which is known to possess substantial agonist property (Menzies et al., 1999; Olianas et al., 1999), Ac-RYYRIK-ol is a competitive ORL-1 receptor antagonist of high affinity in the mouse vas deferens bioassay. Equilibrium dissociation constant value of Ac-RYYRIK-ol (2.44 nM) is as high as that of naloxone at the μ-opioid receptors in isolated organ preparations (3-4 nM). In 10^{-5} M concentration the peptide has neither significant agonist (nociceptin-like, i.e., partial agonist) activity nor opioid receptor antagonist property.

Although the initially disclosed pharmacology of nociceptin-related peptides generated some interest in pharmacologist circles and the presence of heptadecapeptide in the NTS/DVN region was also an attractive factor for our experimental profile, we did not pursue further this line. There was a general tapering off of interest in nociceptin-related research (including drug development efforts at pharmaceutical companies) partly because the not quite fortunate choice of "nociceptin" designation for OFQ-peptide. In our case, however, the major reason was that we gave preference to endomorphin-related pharmacological analyses.

8. Conclusions

1. The effect of glutamate and baclofen on the field stimulation-induced release of ^{3}H-NE from NTS/DVN prisms in rats of different age range. The effect of neonatal MSG treatment on these modulatory parameters.

Both glutamate and baclofen increased the field stimulation-induced release of ^{3}H-NE from NTS/DVN prisms prepared from young adult rats whereas the modulatory effect of baclofen was lost in rats of higher age range. Neonatal MSG treatment abolished the modulatory actions of both glutamate and baclofen. Although the underlying mechanism cannot be explained exactly, the results were analogous to the in vivo findings where the centrally mediated gastroprotective effects of α_2-adrenoceptor- and opioid agonists were different in young, old and neonatally MSG-treated rats (Rónai et al 2001; Gyires and Barna, 2002).

2. The ineffectiveness of endomorphin-1 in affecting the field stimulation-induced release of ^{3}H-NE from rat whole NTS/DVN slices. Comparison with the prism preparation.

10^{-5} M endomorphin-1, which has been found previously (Al-Khrasani et al., 2003) to reduce moderately the stimulation-induced release of ^{3}H-NE from rat NTS/DVN prisms, was ineffective in whole slices. The explanation might be technical (possibly reduced viability of whole slices) or physiological (the increased complexity of whole slices may result in zero net effect).

3. General conclusions from brain slice experiments using tritiated transmitters / isomers

3.1. Loading the chosen brain slices with either ^{3}H-NE or ^{3}H-GABA gave neural pools from which the labelled transmitter could be released by conventional (field electrical or K^{+}-depolarization) stimulations in a manner which originated from true

transmitter-like pools. The inability to stimulate release by field stimulation and the Ca^{2+}-independency of depolarization-induced release upon loading with ^{3}H-D-aspartate indicated that the labelled isomer was present mostly in non transmitter-like pool.

3.2. The whole slice preparation offered no advantage over the "prism" preparation in rat NTS/DVN

3.3. The technical improvements gave good results in loading parsimony and also in the sharp, well-defined responses to depolarizing stimuli

3.4. There is a contradictory, technical-physiological pitfall in an effort to use a brain slice composed of an integrative center and to expect unambiguous results from drug interactions in the conventionally stimulated, labelled transmitter-loaded slices. The informational value of such experiments may be improved either i) by using more specific chemical stimuli (e.g. CCK-8, substance P, galanin etc.) ii) to use further subdivided regions of preparations or to combine the conventional release techniques with iii) chemical neuronal ablations such as neonatal MSG treatment or iv) trans-sections of fiber tract(s) supplying the dissected area

4. An isolated organ model for studying α_{2B}-adrenergic receptor-mediated action.

By studying the interaction of general α_2-adrenoceptor agonist xylazine and the non- α_{2A}-adrenoceptor agonist ST-91 with isoproterenol in phenylephrine-contracted, endothel-denuded rat mesenteric arteries, I created a useful tool for demonstrating α_{2B}-adrenergic receptor-mediated action. ST-91 inhibited isoproterenol-induced relaxations in a yohimbine-antagonizable manner whereas the general α_2-adrenoceptor agonist xylazine failed to do so.

5. The *in vitro* pharmacology of possible biosynthetic intermediates, precursors and endogenous variants of endomorphin-2

5.1. The potential immediate biosynthetic precursors, the glycine-extended endomorphin-1 and -2 exhibited a divergent *in vitro* pharmacology. In the MVD bioassay EMO-2-Gly had a remarkably potent agonist activity at a receptor type closely

related to or identical with the μ-type whereas EMO-1-Gly was a weak, probably partial agonist.

5.2. The free carboxylic derivatives of endomorphin were weak agonists in the same bioassay. The antagonism of the effect of EMO-2-OH by naltrindole and naltrexone indicated a significant agonism at the δ-opioid receptor type. Although a peptide with identical retention time as standard EMO-2-OH appeared in the RP-HPLC gradient separates of rat brain extracts, the experiments with specific antiserum to EMO-2 proved that the peptide is not identical with authentic EMO-2.

5.3. The extension of presumed biosynthetic precursor of EMO-2, Tyr-Pro dipeptide with Ser or Ser(O-glucose) with or without Gly spacer(s) to improve intracellular penetration resulted in derivatives devoid of agonist activity in the MVD bioassay. One of the first indications for an extracellular biosynthesis had emerged from the inefficiency of Tyr-Pro-Ser(O-glucose) to promote EMO-2 biosynthesis in rat isolated L4,5 spinal dorsal root ganglia (Rónai, Király et al., 2009).

6. The *in vitro* pharmacological characterization of a novel nociceptin antagonist

N-Ac-RYYRIK-ol peptide was found a high-affinity ORL-1 antagonist in the MVD bioassay, approximately ten times surpassing the affinity of the parent, C-terminally amidated peptide (Dooley et al., 1997). The novel peptide fulfilled the Schild-criteria for a competitive antagonism and was, by the traditional term, a "pure" antagonist (i.e. not a partial agonist) showing no agonism at the concentration of 10^{-5} M. The successful structural modification further supported the notion that i) an N-acylated opioid structure, where the nitrogen cannot be protonated (Rónai et al., 1992) can serve as an antagonist ligand with good affinity and ii) C-terminal alcoholic function (i.e. a peptide alcohol) can result in at least as good ligand as an amidated derivative, as it has been demonstrated for several agonists in the opioid peptide family such as FK-33 824-peptide (Roemer et al., 1977), DAMGO (Kosterlitz and Paterson, 1981) and the –ol-derivatives of endomorphins (Al-Khrasani et al., 2001; Kocsis et al., 2000).

9. Summary

The experiments covered by my thesis were related, either directly or indirectly, to the analysis of the interaction of neuropeptides and non-peptide neurotransmitters involved in the regulation of autonomic functions. The experiments were carried out partly in rat brain slice/prism preparations, prepared from areas considered as autonomic integrative centers; these were the nucleus tractus solitarii–dorsal motor nucleus of the vagus (NTS–DVN, "dorsal vagal complex") and the hypothalamic paraventricular-subparaventricular nucleus. In these series, my task was to explore the advantages/disadvantages of whole slice versus "prism" preparations and find technical/experimental conditions to obtain more complex informations in the experimental settings. I studied the stimulation-induced release of tritiated norepinephrine (^{3}H-NE), D-aspartate (^{3}H-D-Asp) or γ-amino butyric acid (^{3}H-GABA) and the modulation of release by peptide- and non-peptide drugs and also by neonatal pre-treatment with monosodium glutamate (MSG). The isolated organ experiments served either i) to develop a specific nociceptin antagonist which was a prerequisite for possible further experimentation at that stage or ii) to characterize pharmacologically endomorphin derivatives and possible biosynthetic precursors to *de novo* endomorphin biosynthesis and iii) to develop a specific bioassay for α_{2B} adrenoceptors, which is the adrenergic receptor subtype crucially involved in the initiation of an opioid peptide-mediated gastroprotection in the NTS–DVN (Gyires et al., 2000a, 2000c).

In brain slice/prism experiments it was established that

1) ^{3}H-NE and ^{3}H-GABA could be released by conventional stimuli (K^+ -induced depolarization, field electrical stimulation) in a manner suitable for experimentation.

2) Whole slice preparations offered no advantage over "prisms". Endomorphin-1, which has been found previously to reduce moderately but effectively stimulation-induced ^{3}H-NE release from NTS–DVN prisms, was ineffective in whole slices.

3). Reducing the β-endorphinergic input to NTS–DVN by neonatal MSG treatment increased the informational value of experiments carried out in isolated "prism" preparations.

10. Összefoglaló

Doktori munkám során, azon neuropeptid- és nem peptid transzmitter rendszerek kölcsönhatásait vizsgáltam, amelyeknek a vegetatív funkciók integrálásában illetve szabályozásában van kiemelt szerepük. A kísérletek egy részét patkányagy NTS-DVM és hPVM/szubPVM régiókból kipreparált összefüggő (teljes) szelet és paraszagittális hasábokon végeztem. Ezen központi idegrendszeri magvak autonom integratív területek is egyben. Feladatom többek között a különböző (teljes szelet/hasáb) preparálási módok működési előnyeinek és hátrányainak vizsgálata illetve olyan technikai/experimentális feltételek megtalálása volt, amelyek a készítmények „jelképző" sajátságait kedvezőbbé tehették és erre kiegészítő farmakológiai eljárást is alkalmaztam. Konvencionális ingerlési módokat (elektromos téringerlés, K^+-depolarizáció) alkalmazva, a kiváltott ^{3}H-NA, ^{3}H-D-Asp vagy ^{3}H-GABA felszabadulásokat mértem valamint ezek modulációját neuropeptidek, nem peptiderg hatóanyagok illetve újszülöttkori MSG kezelés által. Izolált szerveken végzett kísérleteim segítségével i). egy nagy hatékonyságú, kompetitív nociceptin antagonista kifejlesztésében vettem részt, ii). endomorfinok, analógjaik illetve feltételezett bioszintetikus intermedier előanyagaik in vitro farmakológiai jellemzését végeztem el illetve iii). kifejlesztettem egy izolált-szervi kísérletes modellt, amely az α_{2B} mediált hatások vizsgálatára alkalmas. Az α_{2B} adrenerg receptor altípus szerepet játszik az opioid peptidek által mediált centrális gyomornyálkahártya védelemben; a hatás helye feltételezhetően a NTS-DVM (Gyires és mtsai., 2000a, 2000c).

Agyszelet kísérleteim eredményei alapján megállapítható, hogy:

1). Konvencionális ingerlési paraméterekkel, ^{3}H-NA és ^{3}H-GABA kísérletesen felszabadítható volt.

2). Téringerléssel kiváltott ^{3}H-NA felszabadulás modulációjának mérése tekintetében a teljes NTS-DVM szelet-preparátum nem mutat előnyöket a hasábokkal szemben. EMO-1-nek, hasábokon mért korábbi eredményekkel ellentétben, nem volt ^{3}H-NA felszabadulást gátló hatása teljes szelet preparátumokon.

3). Újszülöttkori MSG kezelés növelte a felszabadulási mintázatot mérő kísérlet információ tartalmát.

11. References

Albrecht E, Samovilova NN, Oswald S, Baeger I, Berger H. (1998) Nociceptin (orphanin FQ): high-affinity and high-capacity binding site coupled to low-potency stimulation of guanylyl-5'-O-(gamma-thio)-triphosphate binding in rat brain membranes. J Pharmacol Exp Ther, 286(2):896-902.

Al-Khrasani M, Orosz G, Kocsis L, Farkas V, Magyar A, Lengyel I, Benyhe S, Borsodi A, Rónai AZ. (2001) Receptor constants for endomorphin-1 and endomorphin-1-ol indicate differences in efficacy and receptor occupancy. Eur J Pharmacol, 421(1):61-7.

Al-Khrasani M, Elor G, Yusuf Abbas M, Rónai AZ. (2003) The effect of endomorphins on the release of ^{3}H-norepinephrine from rat nucleus tractus solitarii slices. Regul Pept, 111(1-3):97-101.

Alt A, Mansour A, Akil H, Medzihradsky F, Traynor JR, Woods JH. (1998) Stimulation of guanosine-5'-O-(3-[35S]thio)triphosphate binding by endogenous opioids acting at a cloned mu receptor. J Pharmacol Exp Ther, 286(1):282-8.

Arakawa K, De Jong W, Mulder AH, Versteeg DH. (1991) The electrically stimulated release of [3H]noradrenaline from nucleus tractus solitarii slices in vitro is modulated via mu-opioid receptors. Eur J Pharmacol, 192(2):311-6.

Ardati A, Henningsen RA, Higelin J, Reinscheid RK, Civelli O, Monsma FJ Jr. (1997) Interaction of [3H]orphanin FQ and 125I-Tyr14-orphanin FQ with the orphanin FQ receptor: kinetics and modulation by cations and guanine nucleotides. Mol Pharmacol. 51(5):816-24.

Armstrong DM, Ross CA, Pickel VM, Joh TH, Reis DJ. (1982) Distribution of dopamine-, noradrenaline-, and adrenaline-containing cell bodies in the rat medulla

oblongata: demonstrated by the immunocytochemical localization of catecholamine biosynthetic enzymes. J Comp Neurol, 212(2):173-87.

Armstrong DM, Manley L, Haycock JW, Hersh LB. (1990) Co-localization of choline acetyltransferase and tyrosine hydroxylase within neurons of the dorsal motor nucleus of the vagus. J Chem Neuroanat, 3(2):133-40.

Arunlakshana O, Schild HO. (1959) Some quantitative uses of drug antagonists. Br J Pharmacol Chemother, 14(1):48-58.

Atweh SF, Kuhar MJ. (1977) Autoradiographic localization of opiate receptors in rat brain. I. Spinal cord and lower medulla. Brain Res, 124(1):53-67.

Bandler R, Carrive P, Zhang SP. (1991) Integration of somatic and autonomic reactions within the midbrain periaqueductal grey: viscerotopic, somatotopic and functional organization. Prog Brain Res, 87:269-305.

Bantel C, Esisenach JC, Duflo F, Tobin JR, Childers SR. (2005). Spinal nerve ligation increases α_2-adrenergic receptor G-protein coupling in the spinal cord. Brain Res. 1038, 76–82.

Baranowska B. (1990) The effect of clonidine on hormone release mediated through activation of opiate receptors. Cardiovasc Drugs Ther, 4(4):1113-7.

Barlow HB, Andrews DP. (1967) Sensitivity of receptors and receptor "pools". J Opt Soc Am, 57(6):837-8.

Bechara A, Damasio H, Damasio AR. (2000) Emotion, decision making and the orbitofrontal cortex. Cereb Cortex, 10.3:295-307.

Benarroch EE. (1993) The central autonomic network: functional organization, dysfunction, and perspective. Mayo Clin Proc. 68(10):988-1001.

Benarroch EE. (1997) Overview of the Organization of the Central Autonomic Network. In: Beanrroch EE, Armonk NY (Eds.) Central Autonomic Network: Functional Organisation and Clinical Correlations. Futura Publishing Company Inc, 3-28.

Bentley GA, Newton SH, Starr J. (1983) Studies on the antinociceptive action of alpha-agonist drugs and their interactions with opioid mechanisms. Br J Pharmacol, 79(1):125-34.

Berger H, Albrecht E, Wallukat G, Bienert M. (1999) Antagonism by acetyl-RYYRIK-NH_2 of G protein activation in rat brain preparations and of chronotropic effect on rat cardiomyocytes evoked by nociceptin/orphanin FQ. Br J Pharmacol, 126(3):555-8.

Berzetei-Gurske IP, Schwartz RW, Toll L. (1996) Determination of activity for nociceptin in the mouse vas deferens. Eur J Pharmacol, 302(1-3):R1-2.

Bigoni R, Giuliani S, Calo' G, Rizzi A, Guerrini R, Salvadori S, Regoli D, Maggi CA. (1999) Characterization of nociceptin receptors in the periphery: in vitro and in vivo studies. Naunyn Schmiedebergs Arch Pharmacol, 359(3):160-7.

Black JW, Leff P, Shankley NP, Wood J. (1985) An operational model of pharmacological agonism: the effect of E/[A] curve shape on agonist dissociation constant estimation. Br J Pharmacol, 84(2):561-71.

Blandizzi C, Natale G, Colucci R, Carignani D, Lazzeri G, Del Tacca M. (1995) Characterization of alpha 2-adrenoceptor subtypes involved in the modulation of gastric acid secretion. Eur J Pharmacol, 278(2):179-82.

Bradbury AF, Smyth DG, Snell CR. (1976) Lipotropin: precursor to two biologically active peptides. Biochem Biophys Res Commun, 69(4):950-6.

Bradbury AF, Mistry J, Roos BA, Smyth DG. (1990) 4-Phenyl-3-butenoic acid, an in vivo inhibitor of peptidylglycine hydroxylase (peptide amidating enzyme). Eur J Biochem, 189(2):363-8.

Brantl V, Teschemacher H, Henschen A, Lottspeich F. (1979) Novel opioid peptides derived from casein (beta-casomorphins). I. Isolation from bovine casein peptone. Hoppe Seylers Z Physiol Chem, 360(9):1211-6.

Brantl V, Gramsch C, Lottspeich F, Henschen A, Jaeger KH, Herz A. (1985) Novel opioid peptides derived from mitochondrial cytochrome b: cytochrophins. Eur J Pharmacol, 111(2):293-4.

Brantl V, Gramsch C, Lottspeich F, Mertz R, Jaeger KH, Herz A. (1986) Novel opioid peptides derived from hemoglobin: hemorphins. Eur J Pharmacol, 125(2):309-10.

Brooks PA, Glaum SR, Miller RJ, Spyer KM. (1992) The actions of baclofen on neurones and synaptic transmission in the nucleus tractus solitarii of the rat in vitro. J Physiol, 457:115-29.

Browning KN, Kalyuzhny AE, Travagli RA. (2004) Mu-opioid receptor trafficking on inhibitory synapses in the rat brainstem. J Neurosci, 24(33):7344-52.

Browning KN, Zheng Z, Gettys TW, Travagli RA. (2006) Vagal afferent control of opioidergic effects in rat brainstem circuits. J Physiol, 575(Pt 3):761-76.

Bunzow JR, Saez C, Mortrud M, Bouvier C, Williams JT, Low M, Grandy DK. (1994) Molecular cloning and tissue distribution of a putative member of the rat opioid receptor gene family that is not a mu, delta or kappa opioid receptor type. FEBS Lett, 347(2-3):284-8.

Butour JL, Moisand C, Mazarguil H, Mollereau C, Meunier JC. (1997) Recognition and activation of the opioid receptor-like ORL 1 receptor by nociceptin, nociceptin analogs and opioids. Eur J Pharmacol, 321(1):97-103.

Butour JL, Moisand C, Mollereau C, Meunier JC. (1998) [Phe^1-ψ(CH_2–NH)Gly^2]nociceptin-(1–13)-NH_2 is an agonist of the nociceptin (ORL1) receptor. Eur J Pharmacol, 349(1):R5-6.

Bylund DB, Eikenberg DC, Hieble JP, Langer SZ, Lefkowitz RJ, Minneman KP, Molinoff PB, Ruffolo RR Jr, Trendelenburg U. (1994) International Union of Pharmacology nomenclature of adrenoceptors. Pharmacol Rev, 46(2):121-36.

Caló G, Guerrini R, Bigoni R, Rizzi A, Bianchi C, Regoli D, Salvadori S. (1998) Structure-activity study of the nociceptin(1-13)-NH_2 N-terminal tetrapeptide and discovery of a nociceptin receptor antagonist. J Med Chem, 41(18):3360-6.

Caló G, Guerrini R, Bigoni R, Rizzi A, Marzola G, Okawa H, Bianchi C, Lambert DG, Salvadori S, Regoli D. (2000) Characterization of [$Nphe^1$]-nociceptin(1-13)-NH_2, a new selective nociceptin receptor antagonist. Br J Pharmacol, 129(6):1183-93.

Calzà L, Giardino L, Grimaldi R, Rigoli M, Steinbusch HW, Tiengo M. (1985) Presence of 5-HT-positive neurons in the medial nuclei of the solitary tract. Brain Res, 347(1):135-9.

Calzada CB, de Artinano AA. (2001) Alpha-adrenoceptor subtypes. Pharmacol Res, 44:195-208.

Castelli MP, Piras AP, Melis T, Succu S, Sanna F, Melis MR, Collu S, Grazia Ennas M, Diaz G, Mackie K, Argiolas A. (2007) Cannabinoid CB1 receptors in the paraventricular nucleus and central control of penile erection: immunocytochemistry, autoradiography and behavioral studies. Neuroscience, 147(1):197-206.

Cechetto DF. (1987) Central representation of visceral function. Fed Proc, 46(1):17-23.

Chang KJ, Lillian A, Hazum E, Cuatrecasas P, Chang JK. (1981) Morphiceptin (NH4-tyr-pro-phe-pro-COHN2): a potent and specific agonist for morphine (mu) receptors. Science, 212(4490):75-77.

Chavkin C, James IF, Goldstein A. (1982) Dynorphin is a specific endogenous ligand of the kappa opioid receptor. Science, 215(4531):413-5.

Chen F, Cooney AJ, Wang Y, Law SW, O'Malley BW. (1994) Cloning of a novel orphan receptor (GCNF) expressed during germ cell development. Mol Endocrinol, 8(10):1434-44.

Cho HJ, Kim DS, Lee NH, Kim JK, Lee KM, Han KS, Kang YN, Kim KJ. (1997) Changes in the α_2-adrenergic receptor subtypes gene expression in rat dorsal root ganglion in an experimental model of neuropathic pain. NeuroReport, 8:3119–3122.

Clapés P, Torres JL, Adlercreutz P. (1995) Enzymatic peptide synthesis in low water content systems: preparative enzymatic synthesis of [Leu]- and [Met]-enkephalin derivatives. Bioorg Med Chem, 3(3):245-55.

Cobos-Puc LE, Villalon CM, Sanchez-Lopez A, Lozano-Cuenca J, Pertz HH, Gornemann T, Centurion D. (2007) Pharmacological evidence that alpha$_2$A-and alpha2C-adrenoceptors mediate the inhibition of cardioaccelerator sympathetic outflow in pithed rats. Eur J Pharmacol, 554:205–211.

Connaughton S, Docherty JR. (1990) No evidence for differences between pre- and postjunctional alpha 2-adrenoceptors in the periphery. Br J Pharmacol, 99:97–102.

Corbett AD, Paterson SJ, McKnight AT, Magnan J and Kosterlitz HW (1982) Dynorphin$_{1-8}$ and Dynorphin$_{1-9}$ are ligands for the κ-subtype of opiate receptor. Nature 299: 79-81

Czapla MA, Gozal D, Alea OA, Beckerman RC, Zadina JE. (2000) Differential cardiorespiratory effects of endomorphin 1, endomorphin 2, DAMGO, and morphine. Am J Respir Crit Care Med, 162(3 Pt 1):994-9.

Dahlström A, Fuxe K. (1964) Localization of monoamines in the lower brain stem. Experientia, 20(7):398-9.

Diz DI, Barnes KL, Ferrario CM. (1987) Functional charasteristics of neuropeptides in the dorsal medulla oblongata and vagus nerve. Fed Proc, 46(1): 30-35.

Dooley CT, Houghten RA. (1996) Orphanin FQ: receptor binding and analog structure activity relationships in rat brain. Life Sci, 59(1):PL23-9.

Dooley CT, Spaeth CG, Berzetei-Gurske IP, Craymer K, Adapa ID, Brandt SR, Houghten RA, Toll L. (1997) Binding and in vitro activities of peptides with high affinity for the nociceptin/orphanin FQ receptor, ORL1. J Pharmacol Exp Ther, 283(2): 735-41.

Dowlatshahi P, Yaksh TL. (1997) Differential effects of two intraventricularly injected alpha 2 agonists, ST-91 and dexmedetomidine, on electroencephalogram, feeding, and electromyogram. Anesth Analg, 84:133–138.

Doxey JC, Frank LW, Hersom AS. (1981) Studies on the pre-and postjunctional activities of alpha-adrenoreceptor agonists and their cardiovascular effects in the anaesthetized rat. J Auton Pharmacol, 1:157–169.

Dun NJ, Dun SL, Hwang LL. (1997) Nociceptin-like immunoreactivity in autonomic nuclei of the rat spinal cord. Neurosci Lett, 234(2-3):95-8

Edvinsson L, Nielsen KC, Owman C. (1974) Influence of initial tension and changes in sensitivity during amine-induced contractions of pial arteries in vitro. Arch Int Pharmacodyn Ther, 208:235–242.

Eipper BA, Mains RE, Glembotski CC. (1983) Identification in pituitary tissue of a peptide alpha-amidation activity that acts on glycine-extended peptides and requires molecular oxygen, copper, and ascorbic acid. Proc Natl Acad Sci U S A., 80(16):5144-8.

Eipper BA, Mains RE. (1988) Peptide alpha-amidation. Annu Rev Physiol, 50:333-44.

Eipper BA, Mains RE. (1991) The role of ascorbate in the biosynthesis of neuroendocrine peptides. Am J Clin Nutr, 54(6 Suppl):1153S-1156S.

Eipper BA, Perkins SN, Husten EJ, Johnson RC, Keutmann HT, Mains RE. (1991) Peptidyl-alpha-hydroxyglycine alpha-amidating lyase. Purification, characterization, and expression. J Biol Chem, 266(12):7827-33.

Érchegyi J, Kastin AJ, Zadina JE. (1992) Isolation of a novel tetrapeptide with opiate and antiopiate activity from human brain cortex: Tyr-Pro-Trp-Gly-NH2 (Tyr-W-MIF-1). Peptides, 13(4):623-31.

Erspamer V, Melchiorri P, Erspamer CF, Negri L. (1978) Polypeptides of the amphibian skin active on the gut and their mammalian counterparts. Adv Exp Med Biol, 106:51-64.

Erspamer V, Melchiorri P, Falconieri-Erspamer G, Negri L, Corsi R, Severini C, Barra D, Simmaco M, Kreil G. (1989) Deltorphins: a family of naturally occurring peptides with high affinity and selectivity for delta opioid binding sites. Proc Natl Acad Sci U S A., 86(13):5188-92.

Evans CJ, Keith DE Jr, Morrison H, Magendzo K, Edwards RH. (1992) Cloning of a delta opioid receptor by functional expression. Science, 258(5090):1952-5.

Farsang C, Kunos G. (1979) Naloxone reverses the antihypertensive effect of clonidine. Br J Pharmacol, 67(2):161-4.

Ferguson AV, Marcus P, Spencer J, Wallace JL. (1988) Paraventricular nucleus stimulation causes gastroduodenal mucosal necrosis in the rat. Am J Physiol, 255(5 Pt 2):R861-5.

Fichna J, Janecka A, Costentin J, Do Rego JC. (2007) The endomorphin system and its evolving neurophysiological role. Pharmacol Rev, 59(1):88-123.

Finley JC, Lindström P, Petrusz P. (1981) Immunocytochemical localization of beta-endorphin-containing neurons in the rat brain. Neuroendocrinology, 33(1):28-42.

Fisk GD, Wyss JM. (2000) Descending projections of infralimbic cortex that mediate stimulation-evoked changes in arterial pressure. Brain Res, 859(1):83-95.

Fleming WW, Westfall DP, De la Lande IS, Jellett LB. (1972) Log-normal distribution of equiefective doses of norepinephrine and acetylcholine in several tissues. J Pharmacol Exp Ther, 181(2):339-45.

Fodor M, Csiffáry A, Kiss P, Palkovits M. (1990) Dynorphin A-containing neural elements in the nucleus of the solitary tract of the rat. Light and electron microscopic immunohistochemistry. Brain Res, 522(2):251-8.

Freund TF, Katona I, Piomelli D. (2003) Role of endogenous cannabinoids in synaptic signaling. Physiol Rev, 83(3):1017-66

Fukuda K, Kato S, Mori K, Nishi M, Takeshima H, Iwabe N, Miyata T, Houtani T, Sugimoto T. (1994) cDNA cloning and regional distribution of a novel member of the opioid receptor family. FEBS Lett, 343(1):42-6.

Furchgott RF, Bursztyn P. (1967) Comparison of dissociation constants and of relative efficacies of selected agonists acting on parasympathetic receptors. Ann NY Acad Sci, 144:882-899.

Furchgott RF. (1978) Pharmacological characterization of receptors: its relation to radioligand-binding studies. Fed Proc, 37(2):115-20.

Gacel G, Fournié-Zaluski MC, Fellion E, Roques BP, Senault B, Lecomte JM, Malfroy B, Swerts JP, Schwartz JC. (1979) Conformation and biological activities of hexapeptides related to enkephalins: respective roles of the ammonium and hydroxyl groups of tyrosine. Life Sci, 24(8):725-31.

Gavin K, Docherty JR. (1996) Investigation of the subtype of alpha 2-adrenoceptor mediating pressor responses in the pithed rat. Eur J Pharmacol, 318:81–87.

Gaumann DM, Tassonyi E, Rivest RW, Fathi M, Reverdin AF. (1991) Cardiovascular and endocrine effects of clonidine premedication in neurosurgical patients. Can J Anaesth, 38(7):837-43.

Goldstein A, Tachibana S, Lowney LI, Hunkapiller M, Hood L. (1979) Dynorphin-(1-13), an extraordinarily potent opioid peptide. Proc Natl Acad Sci U S A., 76(12):6666-70.

Graham BA, Hammond DL, Proudfit HK. (2000) Synergistic interactions between two alpha(2)-adrenoceptor agonists, dexmedetomidine and ST-91, in two substrains of Sprague–Dawley rats. Pain, 85:135–143.

Grijalva CV, Novin D. (1990) The role of the hypothalamus and dorsal vagal complex in gastrointestinal function and pathophysiology. Ann N Y Acad Sci, 597:207-22.

Guerrini R, Calo G, Rizzi A, Bianchi C, Lazarus LH, Salvadori S, Temussi PA, Regoli D. (1997) Address and message sequences for the nociceptin receptor: a structure-activity study of nociceptin-(1-13)-peptide amide. J Med Chem, 40(12):1789-93.

Guerrini R, Calo G, Rizzi A, Bigoni R, Bianchi C, Salvadori S, Regoli D. (1998) A new selective antagonist of the nociceptin receptor. Br J Pharmacol, 123(2):163-5.

Guevara-Aguilar R, Donatti-Albarran OA, Solano-Flores LP, Wayner MJ. (1987) Nucleus of the tractus solitarius projections to the olfactory tubercle: an HRP study. Brain Res Bull, 18(5):673-5.

Gyires K, Blandazzi C, Del Tacca M. (1996) Analysis of the inhibiting activity of presynaptic α2-adrenoceptors against NSAID-induced gastric mucosal lesions in the rat. Immunopharmacology, 4:173-180.

Gyires K, Müllner K, Rónai AZ. (2000a) Functional evidence that gastroprotection can be induced by activation of central alpha(2B)-adrenoceptor subtypes in the rat. Eur J Pharmacol, 396(2-3):131-5.

Gyires K, Rónai AZ, Mullner K, Furst S. (2000b) Intracerebroventricular injection of clonidine releases beta-endorphin to induce mucosal protection in the rat. Neuropharmacology, 39: 961-968.

Gyires K, Müllner K, Fürst S, Rónai AZ. (2000c) Alpha-2 adrenergic and opioid receptor-mediated gastroprotection. J Physiol Paris, 94(2):117-21.

Gyires K, Rónai AZ. (2001a) Supraspinal delta- and mu-opioid receptors mediate gastric mucosal protection in the rat. J Pharmacol Exp Ther, 297: 1010-1015.

Gyires K, Müllner K, Rónai AZ. (2001b) Activation of central opioid receptors may induce gastric mucosal defence in the rat. J Physiol Paris, 95(1-6):189-96

Gyires K, Barna I. (2002) Differences in gastroprotective processes in 6- to 8- and 14- to 16-week-old rats. Dig Dis Sci, 47(12):2775-82.

Hackler L, Zadina JE, Ge LJ, Kastin AJ. (1997) Isolation of relatively large amounts of endomorphin-1 and endomorphin-2 from human brain cortex. Peptides, 18(10):1635-9.

Healy DP, Rettig R, Printz MP. (1984) Evidence of an endogenous Ang II system in the rat nucleus tractus solitarius. Clin Exp Hypertens A, 6(10-11):1743-7

Henderson G, McKnight AT. (1997) The orphan opioid receptor and its endogenous ligand--nociceptin/orphanin FQ. Trends Pharmacol Sci, 18(8):293-300.

Higgins GA, Hoffman GE, Wray S, Schwaber JS. (1984) Distribution of neurotensin-immunoreactivity within baroreceptive portions of the nucleus of the tractus solitarius and the dorsal vagal nucleus of the rat. J Comp Neurol, 226(2):155-64.

Honner V, Docherty JR. (1999) Investigation of the subtypes of alpha1-adrenoceptor mediating contractions of rat vas deferens. Br J Pharmacol, 128:1323–1331.

Horvath A, Kastin AJ. (1989) Isolation of tyrosine-melanocyte-stimulating hormone release-inhibiting factor 1 from bovine brain tissue. J Biol Chem, 264(4):2175-9.

Horvath G. (2000) Endomorphin-1 and endomorphin-2: pharmacology of the selective endogenous mu-opioid receptor agonists. Pharmacol Ther, 88(3):437-63.

Hosohata K, Burkey TH, Alfaro-Lopez J, Varga E, Hruby VJ, Roeske WR, Yamamura HI. (1998) Endomorphin-1 and endomorphin-2 are partial agonists at the human mu-opioid receptor. Eur J Pharmacol, 346(1):111-4.

Houtani T, Nishi M, Takeshima H, Sato K, Sakuma S, Kakimoto S, Ueyama T, Noda T, Sugimoto T. (2000) Distribution of nociceptin/orphanin FQ precursor protein and receptor in brain and spinal cord: a study using in situ hybridization and X-gal histochemistry in receptor-deficient mice. J Comp Neurol, 424(3):489-508.

Hughes J, Smith TW, Kosterlitz HW, Fothergill LA, Morgan BA, Morris HR. (1975) Identification of two related pentapeptides from the brain with potent opiate agonist activity. Nature. 1975 Dec 18;258(5536):577-80.

Hurley-Gius KM, Neafsey EJ. (1986) The medial frontal cortex and gastric motility: microstimulation results and their possible significance for the overall pattern of organization of rat frontal and parietal cortex. Brain Res, 365(2):241-8.

Hwang BH, Wu JY. (1984) Ultrastructural studies on catecholaminergic terminals and GABAergic neurons in nucleus tractus solitarius of the rat medulla oblongata. Brain Res, 302(1):57-67.

Jászai J, Farkas LM, Gallatz K, Palkovits M. (1998) Effects of glutamate-induced excitotoxicity on calretinin-expressing neuron populations in the area postrema of the rat. Cell Tissue Res, 293(2):227-33.

Joseph SA, Pilcher WH, Bennett-Clarke C. (1983) Immunocytochemical localization of ACTH perikarya in nucleus tractus solitarius: evidence for a second opiocortin neuronal system. Neurosci Lett, 38(3):221-5.

Kalia M, Fuxe K, Hökfelt T, Johansson O, Lang R, Ganten D, Cuello C, Terenius L. (1984) Distribution of neuropeptide immunoreactive nerve terminals within the subnuclei of the nucleus of the tractus solitarius of the rat. J Comp Neurol, 222(3):409-44.

Kalia M, Fuxe K, Goldstein M. (1985a) Rat medulla oblongata. III. Adrenergic (C1 and C2) neurons, nerve fibers and presumptive terminal processes. J Comp Neurol, 233(3):333-49.

Kalia M, Fuxe K, Goldstein M. (1985b) Rat medulla oblongata. II. Dopaminergic, noradrenergic (A1 and A2) and adrenergic neurons, nerve fibers, and presumptive terminal processes. J Comp Neurol, 233(3):308-32.

Kaneko T, Itoh K, Shigemoto R, Mizuno N. (1989) Glutaminase-like immunoreactivity in the lower brainstem and cerebellum of the adult rat. Neuroscience, 32(1):79-98.

Kató E, Lipták L, Shujaa N, Mátyus P, Gyires K, Rónai AZ. (2008) alpha2B-adrenoceptor agonist ST-91 antagonizes beta2-adrenoceptor-mediated relaxation in rat mesenteric artery rings. Eur J Pharmacol, 580(3):361-5.

Karcsú S, Tóth L, Jancsó G, Poberai M. (1981) Na-glutamate sensitive neurons in the area postrema of the rat, Acta Histochem, 68(2):181-7.

Karcsú S, Jancsó G, Kreutzberg GW, Tóth L, Király E, Bácsy E, László FA. (1985) A glutamate-sensitive neuronal system originating from the area postrema terminates in and transports acetylcholinesterase to the nucleus of the solitary tract. J Neurocytol, 14(4):563-78.

Khachaturian H, Lewis ME, Watson SJ. (1983) Enkephalin systems in diencephalon and brainstem of the rat. J Comp Neurol, 220(3):310-20.

Kieffer BL, Befort K, Gaveriaux-Ruff C, Hirth CG. (1992) The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. Proc Natl Acad Sci U S A, 89(24):12048-52.

Kocsis L, Benyhe S, Borsodi A, Rónai AZ, Al-Khrasani M, Magyar A. (2000) Endomorphin related peptide alcohols: structure-activity studies. In: Martinez J, Fehrentz JA (Eds.). Peptides 2000, Paris:EDK, 823.824.

Kocsis L, Orosz G, Magyar A, Al-Khrasani M, Kató E, Rónai AZ, Bes B, Meunier JC, Gündüz O, Tóth G, Borsodi A, Benyhe S. (2004) Nociceptin antagonism: probing the receptor by N-acetyl oligopeptides. Regul Pept, 122(3):199-207.

Kosterlitz HW, Watt AJ. (1968) Kinetic parameters of narcotic agonists and antagonists, with particular reference to N-allylnoroxymorphone (naloxone). Br J Pharmacol Chemother, 33(2):266-76.

Kosterlitz HW, Paterson SJ. (1981) Tyr-D-Ala-Gly-MePhe-NH$(CH_2)_2$OH is a selective ligand for the μ-opiate binding site. Br J Pharmacol, 73:299.

Kreil G, Barra D, Simmaco M, Erspamer V, Erspamer GF, Negri L, Severini C, Corsi R, Melchiorri P. (1989) Deltorphin, a novel amphibian skin peptide with high selectivity and affinity for delta opioid receptors. Eur J Pharmacol, 162(1):123-8.

Krieger DT, Liotta AS, Nicholsen G, Kizer JS. (1979) Brain ACTH and endorphin reduced in rats with monosodium glutamate-induced arcuate nuclear lesions. Nature, 278(5704):562-3.

Kubota Y, Inagaki S, Shiosaka S, Cho HJ, Tateishi K, Hashimura E, Hamaoka T, Tohyama M. (1983) The distribution of cholecystokinin octapeptide-like structures in the lower brain stem of the rat: an immunohistochemical analysis. Neuroscience, 9(3):587-604.

Kuhar MJ. (1982) Receptors for clonidine in brain: insights into therapeutic actions. J Clin Psychiatry, 43(6 Pt 2):17-9.

Kunos G, Farsang C, Ramirez-Gonzales MD. (1981) beta-Endorphin: possible involvement in the antihypertensive effect of central alpha-receptor activation. Science, 211(4477):82-4.

Lachowicz JE, Shen Y, Monsma FJ Jr, Sibley DR. (1995) Molecular cloning of a novel G protein-coupled receptor related to the opiate receptor family. J Neurochem. 64(1):34-40.

Lanzi R, Lapointe M, Gurd W, Tannenbaum GS. (1994) Evidence for a primary involvement of somatostatin in clonidine-induced growth hormone release in conscious rats. J Endocrinol, 141(2):259-66.

LeDoux JE. (2000) Emotion circuits in the brain. Annu Rev Neurosci, 23:155-84.

Lee HS, Basbaum AI. (1984) Immunoreactive pro-enkephalin and prodynorphin products are differentially distributed within the nucleus of the solitary tract of the rat. J Comp Neurol, 230(4):614-9.

Leff P, Dougall IG. (1988) Estimation of opioid receptor agonist dissociation constants with beta-chlornaltrexamine, an irreversible ligand which also displays agonism. Br J Pharmacol, 95(1):234-40.

Leff P, Prentice DJ, Giles H, Martin GR, Wood J. (1990a) Estimation of agonist affinity and efficacy by direct, operational model-fitting. J Pharmacol Methods, 23(3):225-37.

Leff P, Harper D, Dainty IA, Dougall IG. (1990b) Pharmacological estimation of agonist affinity: detection of errors that may be caused by the operation of receptor isomerisation or ternary complex mechanisms. Br J Pharmacol, 101(1):55-60.

Lehmann J, Schaefer P, Ferkany JW, Coyle JT. (1983) Quinolinic acid evokes [3H]acetylcholine release in striatal slices: mediation by NMDA-type excitatory amino acid receptors. Eur J Pharmacol, 96(1-2):111-5.

Leiphart JW, Dills CV, Levy RM. (2003) Decreased spinal alpha2a-and alpha2c-adrenergic receptor subtype mRNA in a rat model of neuropathic pain. Neurosci Lett, 349:5–8.

Lengyel I, Orosz G, Biyashev D, Kocsis L, Al-Khrasani M, Rónai A, Tömböly C, Fürst Z, Tóth G, Borsodi A. (2002) Side chain modifications change the binding and agonist properties of endomorphin 2. Biochem Biophys Res Commun, 290(1):153-61.

Leone C, Gordon FJ. (1989) Is L-glutamate a neurotransmitter of baroreceptor information in the nucleus of the tractus solitarius? J Pharmacol Exp Ther, 250(3): 953-62.

Leslie RA. (1985) Neuroactive substances in the dorsal vagal complex of the medulla oblongata: nucleus of the tractus solitarius, area postrema, and the dorsal motor nucleus of the vagus. Neurochem Int, 7:191-212.

Li CH, Chung D. (1976) Isolation and structure of an untriakontapeptide with opiate activity from camel pituitary glands. Proc Natl Acad Sci U S A., 73(4):1145-8.

Li SJ, Scanlon MN, Járai Z, Varga K, Gantenberg NS, Lazar-Wesley E, Kunos G. (1996) Alpha-2-Adrenergic activation of proopiomelanocortin-containing neurons in the arcuate nucleus causes opioid-mediated hypotension and bradycardia. Neuroendocrinology, 63(3):275-83.

Link RE, Desai K, Hein L, Stevens ME, Chruscinski A, Bernstein D, Barsh GS, Kobilka BK. (1996) Cardiovascular regulation in mice lacking alpha2-adrenergic receptor subtypes b and c. Science, 273:803–805.

Lisi TL, Sluka KA. (2006) A new electrochemical HPLC method for analysis of enkephalins and endomorphins. J Neurosci Methods, 150(1):74-9.

Ljungdahl A, Hökfelt T, Nilsson G. (1978) Distribution of substance P-like immunoreactivity in the central nervous system of the rat--I. Cell bodies and nerve terminals. Neuroscience, 3(10):861-943.

Loewy AD, Burton H. (1978) Nuclei of the solitary tract: efferent projections to the lower brain stem and spinal cord of the cat. J Comp Neurol, 181(2):421-49.

Loewy AD. (1990) Central autonomic Pathways. In: Central Regulation of Autonomic Functions. Ed.Loewy AD & Spyer KM. New York: Oxford University Press, 88-103.

Loewy AD. (1991) Forebrain nuclei involved in autonomic control. Prog Brain Res, 87:253-68.

Lovick TA, Li P. (1989) Integrated function of neurones in the rostral ventrolateral medulla. Prog Brain Res, 81:223-32.

Lu Y, Nguyen TM, Weltrowska G, Berezowska I, Lemieux C, Chung NN, Schiller PW. (2001) [2',6'-Dimethyltyrosine]dynorphin A(1-11)-NH2 analogues lacking an N-terminal amino group: potent and selective kappa opioid antagonists. J Med Chem. 44(19):3048-53.

MacDonald E, Kobilka BK, Scheinin M. (1997) Gene targeting–homing in on α2-adrenoceptor-subtype function. Trends Pharmacol Sci, 18:211–219.

MacMillan LB, Hein L, Smith MS, Piascik MT, Limbird LE. (1996) Central hypotensive effects of the alpha2a-adrenergic receptor subtype. Science, 273:801–803.

Makaritsis KP, Handy DE, Johns C, Kobilka B, Gavras I, Gavras H. (1999a) Role of the alpha2B-adrenergic receptor in the development of saltinduced hypertension. Hypertension, 33:14–17.

Makaritsis KP, Johns C, Gavras I, Altman JD, Handy DE, Bresnahan MR, Gavras H. (1999b) Sympathoinhibitory function of the alpha(2A)-adrenergic receptor subtype. Hypertension, 34:403–407.

Malmberg AB, Hedley LR, Jasper JR, Hunter JC, Basbaum AI. (2001) Contribution of α2 receptor subtypes to nerve injury-induced pain and its regulation by dexmedetomidine. Br J Pharmacol, 132:1827–1836.

Mansour A, Fox CA, Burke S, Meng F, Thompson RC, Akil H, Watson SJ. (1994) Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: an in situ hybridization study. J Comp Neurol, 350(3):412-38.

Margeta-Mitrovic M, Mitrovic I, Riley RC, Jan LY, Basbaum AI. (1999) Immunohistochemical localization of GABA(B) receptors in the rat central nervous system. J Comp Neurol, 405(3):299-321.

Martin-Schild S, Gerall AA, Kastin AJ, Zadina JE. (1999) Differential distribution of endomorphin 1- and endomorphin 2-like immunoreactivities in the CNS of the rodent. J Comp Neurol, 405(4):450-71.

Mathis JP, Ryan-Moro J, Chang A, Hom JS, Scheinberg DA, Pasternak GW. (1997) Biochemical evidence for orphanin FQ/nociceptin receptor heterogeneity in mouse brain. Biochem Biophys Res Commun. 230(2):462-5.

Mathis JP, Goldberg IE, Letchworth SR, Ryan-Moro JP, Pasternak GW. (1999) Identification of a high-affinity orphanin FQ/nociceptin(1-11) binding site in mouse brain. Synapse, 34(3):181-6.

May CN, Dashwood MR, Whitehead CJ, Mathias CJ. (1989) Differential cardiovascular and respiratory responses to central administration of selective opioid agonists in conscious rabbits: correlation with receptor distribution. Br J Pharmacol, 98(3):903-13.

Mhaouty S, Cohen-Tannoudji J, Bouet-Alard R, Limon-Boulez I, Maltier JP, Legrand C. (1995) Characteristics of the alpha 2/beta 2-adrenergic receptor-coupled adenylyl cyclase system in rat myometrium during pregnancy. J Biol Chem 270:11012–11016.

Meeley MP, Ruggiero DA, Ishitsuka T, Reis DJ. (1985) Intrinsic gamma-aminobutyric acid neurons in the nucleus of the solitary tract and the rostral ventrolateral medulla of the rat: an immunocytochemical and biochemical study. Neurosci Lett, 58(1):83-9.

Melander T, Hökfelt T, Rökaeus A. (1986) Distribution of galaninlike immunoreactivity in the rat central nervous system. J Comp Neurol, 248(4):475-517.

Melchiorri P, Erspamer GF, Erspamer V, Guglietta A, De Castiglione R, Faoro F, Perseo G, Piani S, Santangelo F. (1982) Synthetic peptides related to the dermorphins. II. Synthesis and biological activities of new analogues. Peptides, 3(5):745-8.

Menzies JR, Glen T, Davies MR, Paterson SJ, Corbett AD. (1999) In vitro agonist effects of nociceptin and [Phe^1-ψ(CH_2–NH)Gly^2]nociceptin-(1–13)-NH_2 in the mouse and rat colon and the mouse vas deferens. Eur J Pharmacol, 385(2-3):217-23.

Meunier JC, Mollereau C, Toll L, Suaudeau C, Moisand C, Alvinerie P, Butour JL, Guillemot JC, Ferrara P, Monsarrat B. (1995) Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. Nature, 377(6549):532-5.

Meunier JC. (1997) Nociceptin/orphanin FQ and the opioid receptor-like ORL1 receptor. Eur J Pharmacol, 340(1):1-15.

Miller BD, Felder RB. (1988) Excitatory amino acid receptors intrinsic to synaptic transmission in nucleus tractus solitarii. Brain Res, 456(2):333-43.

Mogil JS, Pasternak GW. (2001) The molecular and behavioral pharmacology of the orphanin FQ/nociceptin peptide and receptor family. Pharmacol Rev, 53(3):381-415.

Mollereau C, Parmentier M, Mailleux P, Butour JL, Moisand C, Chalon P, Caput D, Vassart G, Meunier JC. (1994) ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization. FEBS Lett, 341(1):33-8.

Montiel JL, Cornille F, Roques BP, Noble F. (1997) Nociceptin/orphanin FQ metabolism: role of aminopeptidase and endopeptidase 24.15. J Neurochem, 68(1):354-61.

Mosqueda-Garcia R, Eskay R, Zamir N, Palkovits M, Kunos G. (1986) Opioid-mediated cardiovascular effects of clonidine in spontaneously hypertensive rats: elimination by neonatal treatment with monosodium glutamate. Endocrinology, 118(5):1814-22.

Müllner K, Gyires K, Furst S. (2001) Involvement of the opioid system in the central antisecretory action of alpha-2 adrenoceptor agonists in rat. J Physiol Paris, 95(1-6):209-14.

Nakamura K. (1988) The nucleus tractus solitarii and neuropeptides. In: Saito H, Parvez H, Parvez S, Nagatsu T. (eds.) Progress in Hypertension. Utrecht, The Netherlands: VSP, 1:261-297.

Narita M, Mizoguchi H, Oji GS, Tseng EL, Suganuma C, Nagase H, Tseng LF. (1998) Characterization of endomorphin-1 and -2 on [35S]GTPgammaS binding in the mouse spinal cord. Eur J Pharmacol, 351(3):383-7.

Neafsey EJ. (1990) Prefrontal cortical control of the autonomic nervous system: anatomical and physiological observations. Prog Brain Res, 85:147-65; discussion 165-6.

Neal CR Jr, Akil H, Watson SJ Jr. (2001) Expression of orphanin FQ and the opioid receptor-like (ORL1) receptor in the developing human and rat brain. J Chem Neuroanat, 22(4):219-49.

Neubig RR, Spedding M, Kenakin T, Christopoulos A; International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. (2003) International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on terms and symbols in quantitative pharmacology. Pharmacol Rev, 55(4):597-606.

Nicholson JR, Paterson SJ, Menzies JR, Corbett AD, McKnight AT. (1998) Pharmacological studies on the "orphan" opioid receptor in central and peripheral sites. Can J Physiol Pharmacol, 76(3):304-13.

Nishi M, Takeshima H, Mori M, Nakagawara K, Takeuchi T. (1994) Structure and chromosomal mapping of genes for the mouse kappa-opioid receptor and an opioid receptor homologue (MOR-C). Biochem Biophys Res Commun, 205(2):1353-7.

Olianas MC, Maullu C, Ingianni A, Onali P. (1999) [Phe^1-ψ(CH_2–NH)Gly^2]nociceptin-(1–13)-NH_2 acts as a partial agonist at ORL1 receptor endogenously expressed in mouse N1E-115 neuroblastoma cells. Neuroreport, 10(5):1127-31.

Olney JW, Rhee V, Gubareff TD. (1977) Neurotoxic effects of glutamate on mouse area postrema. Brain Res, 120(1):151-7.

Olney JW, Price MT. (1978) Excitotoxic amino acids as neuroendocrine probes. In: McGeer EG, Olney JW, McGeer PL 8Eds.), Kainic acids as a tool in neurobiology, Raven Press, New York, pp. 239-264.

Orosz G, Rónai AZ, Bajusz S, Medzihradszky K. (1994) N-terminally protected penta- and tetrapeptide opioid antagonists based on a pentapeptide sequence found in the venom of Philippine cobra. Biochem Biophys Res Commun, 202(3):1285-90.

Orosz G, Rónai AZ, Kátay E, Medzihradszky K. (1995) Kappa opioid receptor antagonists. Peptides 1994, (Proceedings of the 23rd European Peptide Symposium, Braga). Leiden: Braga, ESCOM; pp. 607-8.

Palkovits M, Záborszky L. (1977) Neuroanatomy of central cardiovascular control. Nucleus tractus solitarii: afferent and efferent neuronal connections in relation to the baroreceptor reflex arc. Prog Brain Res, 47:9-34.

Palkovits M, Léránth C, Eiden LE, Rotsztejn W, Williams TH. (1982) Intrinsic vasoactive intestinal polypeptide (VIP)-containing neurons in the baroreceptor nucleus of the solitary tract in rat. Brain Res, 244(2):351-5.

Palkovits M. (1985) Distribution of neuroactive substances in the dorsal vagal complex of the medulla oblongata. Neurochem Int, 7:213-219.

Palkovits M. (1986) Microdissection of Individual Brain Nuclei and Areas. Book: General Neurochemical Techniques, Volume 1, pages: 1-17.

Palkovits M, Eskay RL. (1987) Distribution and possible origin of beta-endorphin and ACTH in discrete brainstem nuclei of rats. Neuropeptides, 9(2):123-37.

Palkovits M, Mezey E, Eskay RL. (1987) Pro-opiomelanocortin-derived peptides (ACTH/beta-endorphin/alpha-MSH) in brainstem baroreceptor areas of the rat. Brain Res, 436(2):323-38.

Palkovits M. (1988) Neuronal circuits in central baroreceptor mechanism. In: Saito H, Parvez S, Nagatsu T. Progress in Hypertension. Utrecht, The Netherlands: VSP, 1:387-409.

Palkovits M. (1989) Neuropeptides and peptidergic transmission in the nucleus of the solitary tract: their role in the cardiovascular regulation. In: Nijkamp FP, de Wied D. Hypertension, Brain Catecholamines and Peptides. Amsterdam: Elsevier, 299-310.

Palkovits M. (1999) Interconnections between the neuroendocrine hypothalamus and the central autonomic system. Geoffrey Harris Memorial Lecture, Kitakyushu, Japan, October 1998. Front Neuroendocrinol, 20(4):270-95.

Pan YX, Xu J, Pasternak GW. (1996) Cloning and expression of a cDNA encoding a mouse brain orphanin FQ/nociceptin precursor. Biochem J, 315 (Pt 1):11-3.

Partosoedarso ER, Young RL, Blackshaw LA. (2001) GABA(B) receptors on vagal afferent pathways: peripheral and central inhibition. Am J Physiol Gastrointest Liver Physiol, 280(4):G658-68.

Peluso J, LaForge KS, Matthes HW, Kreek MJ, Kieffer BL, Gavériaux-Ruff C. (1998) Distribution of nociceptin/orphanin FQ receptor transcript in human central nervous system and immune cells. J Neuroimmunol, 81(1-2):184-92.

Pert CB, Kuhar MJ, Snyder SH. (1976) Opiate receptor: autoradiographic localization in rat brain. Proc Natl Acad Sci U S A., 73(10):3729-33.

Pickel VM, Chan J, Milner TA. (1989a) Ultrastructural basis for interactions between central opioids and catecholamines. II. Nuclei of the solitary tracts. J Neurosci, 9(7):2519-35.

Pickel VM, Chan J, Milner TA. (1989b) Cellular substrates for interactions between neurons containing phenylethanolamine N-methyltransferase and GABA in the nuclei of the solitary tracts. J Comp Neurol, 286(2):243-59.

Pickel VM, Chan J, Massari VJ. (1989c) Neuropeptide Y-like immunoreactivity in neurons of the solitary tract nuclei: vesicular localization and synaptic input from GABAergic terminals. Brain Res, 476(2):265-78.

Pickel VM, Aicher SA, Aoki C, Cheng PY, Nirenberg MJ. (1998) Catecholamines, opioids, and vagal afferents in the nucleus of the solitary tract. Adv Pharmacol, 42:642-5.

Pierce TL, Wessendorf MW. (2000) Immunocytochemical mapping of endomorphin-2-immunoreactivity in rat brain. J Chem Neuroanat, 18(4):181-207.

Pilcher WH, Joseph SA. (1986) Differential sensitivity of hypothalamic and medullary opiocortin and tyrosine hydroxylase neurons to the neurotoxic effects of monosodium glutamate (MSG). Peptides, 7(5):783-9.

Portoghese PS, Sultana M, Takemori AE. (1988) Naltrindole, a highly selective and potent non-peptide delta opioid receptor antagonist. Eur J Pharmacol, 146(1):185-6.

Przewłocki R, Przewłocka B. (2001) Opioids in chronic pain. Eur J Pharmacol, 429(1-3):79-91.

Puurunen J. (1988) Stimulation of central alpha-2 adrenoceptors inhibits gastric secretion in rats by releasing vasopressin. Naunyn Schmiedebergs Arch Pharmacol, 337(2):164-8.

Rajamani K, Leong S, Lavelle A, Docherty JR. (2001) Prejunctional actions of methylenedioxymethamphetamine in vas deferens from wild-type and alpha(2A/D)-adrenoceptor knockout mice. Eur J Pharmacol, 423:223–228.

Ramirez-Gonzalez MD, Tchakarov L, Garcia MR, Kunos G. (1983) β-endorphin acting on brainstem is involved in the antihypertensive action of clonidine and α-methyldopa in rats. Circ Res, 53: 150-157.

Raybould HE, Jakobsen LJ, Novin D, Taché Y. (1989) TRH stimulation and L-glutamic acid inhibition of proximal gastric motor activity in the rat dorsal vagal complex. Brain Res, 495(2):319-28.

Reinscheid RK, Nothacker HP, Bourson A, Ardati A, Henningsen RA, Bunzow JR, Grandy DK, Langen H, Monsma FJ Jr, Civelli O. (1995) Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. Science, 270(5237):792-4.

Reinscheid RK, Ardati A, Monsma FJ Jr, Civelli O. (1996) Structure-activity relationship studies on the novel neuropeptide orphanin FQ. J Biol Chem, 271(24):14163-8.

Ricardo JA, Koh ET. (1978) Anatomical evidence of direct projections from the nucleus of the solitary tract to the hypothalamus, amygdala, and other forebrain structures in the rat. Brain Res, 153(1):1-26.

Riche D, De Pommery J, Menetrey D. (1990) Neuropeptides and catecholamines in efferent projections of the nuclei of the solitary tract in the rat. J Comp Neurol, 293(3):399-424.

Rockhold RW, Caldwell RW. (1979) Effect of lesions of the nucleus tractus solitarii on the cardiovascular actions of clonidine in conscious rats. Neuropharmacology, 18(4):347-54.

Roemer D, Buescher HH, Hill RC, Pless J, Bauer W, Cardinaux F. (1977) A synthetic enkephalin analogue with prolonged parenteral and oral analgesic activity. Nature, 268:547-9.

Rónai AZ, Berzétei I, Bajusz S. (1977a) Differentiation between opioid peptides by naltrexone. Eur J Pharmacol, 45(4):393-4.

Rónai A, Gráf L, Székely I, Dunai-Kovács Z, Bajusz S. (1977b) Differential behaviour of LPH-(61-91)-peptide in different model systems: comparison of the opioid activities of LPH-(61-91)-peptide and its fragments. FEBS Lett, 74(2):182-4.

Rónai AZ, Berzétei I. (1979) Similarities and differences of opioid receptors in different isolated organs. Endorphins'78. Budapest, Hungary: Akadémia Könyvkiadó, p. 237-57.

Rónai AZ, Székely JI, Berzétei I, Miglécz E, Bajusz S. (1979) Tetrapeptide-amide analogues of enkephalin: the role of C-terminus in determining the character of opioid activity. Biochem Biophys Res Commun, 91(4):1239-49.

Rónai AZ, Berzétei IP, Székely JI, Miglécz E, Kurgyis J, Bajusz S. (1981a) Enkephalin-like character and analgesia. Eur J Pharmacol, 69(3):263-71.

Rónai AZ, Berzétei IP, Kurgyis J. (1981b) Opioid effects in developing rat vas deferens. Cell Mol Neurobiol, 1(4):335-42.

Rónai AZ, Botyánszki J, Hepp J, Medzihradszky K. (1992) A novel opioid structure which accepts protonated as well as non-protonated nitrogen: a family of pure, delta receptor selective antagonists. Life Sci, 50(18):1371-8.

Rónai AZ, Botyánszki J, Magyar A, Borsodi A, Benyhe S, Medzihradszky K. (1993) Novel, delta-opioid receptor-selective peptide antagonists: demonstration of a possible opioid interaction in the absence of a protonated nitrogen and attempts to locate the protonated nitrogen site. Arch Int Pharmacodyn Ther, 323:114-25.

Rónai AZ, Timár J, Makó E, Erdö F, Gyarmati Z, Tóth G, Orosz G, Fürst S, Székely JI. (1998/1999) Diprotin A, an inhibitor of dipeptidyl aminopeptidase IV(EC 3.4.14.5) produces naloxone-reversible analgesia in rats. Life Sci, 64(2):145-52.

Rónai AZ, Gyires K, Barna I, Müllner K, Palkovits M. (2001) Neonatal monosodium glutamate treatment abolishes both delta opioid receptor-induced and alpha-2 adrenoceptor-mediated gastroprotection in the lower brainstem in rats. J Physiol Paris. 95(1-6):215-20.

Rónai AZ, Gyires K, Barna I, Müllner K, Reichart A, Palkovits M. (2002) Gyrus cinguli transection abolishes delta-opioid receptor-induced gastroprotection and alters alpha 2 adrenoceptor activity in the lower brainstem in rats. Brain Res, 947(1):90-9.

Rónai AZ, Szemenyei E, Kató E, Kocsis L, Orosz G, Al-Khrasani M, Tóth G. (2006) Endomorphin synthesis in rat brain from intracerebroventricularly injected [3H]-Tyr-Pro: a possible biosynthetic route for endomorphins. Regul Pept, 134(1):54-60.

Rónai AZ, Al-Khrasani M, Benyhe S, Lengyel I, Kocsis L, Orosz G, Tóth G, Kató E, Tóthfalusi L. (2006) Partial and full agonism in endomorphin derivatives: comparison by null and operational model. Peptides, 27(6):1507-13.

Szemenyei E, Barna I, Mergl Z, Keresztes A, Darula Z, Kató E, Tóth G, Rónai AZ. (2008) Detection of a novel immunoreactive endomorphin 2-like peptide in rat brain extracts. Regul Pept, 148(1-3):54-61.

Rónai AZ, Király K, Szebeni A, Szemenyei E, Prohászka Z, Darula Z, Tóth G, Till I, Szalay B, Kató E, Barna I. (2009) Immunoreactive endomorphin 2 is generated extracellularly in rat isolated L4,5 dorsal root ganglia by DPP-IV. Regul Pept, 157(1-3):1-2.

Ruggiero DA, Giuliano R, Anwar M, Stornetta R, Reis DJ. (1990) Anatomical substrates of cholinergic-autonomic regulation in the rat. J Comp Neurol, 292(1):1-53.

Sakurada S, Hayashi T, Yuhki M, Fujimura T, Murayama K, Yonezawa A, Sakurada C, Takeshita M, Zadina JE, Kastin AJ, Sakurada T. (2000) Differential antagonism of endomorphin-1 and endomorphin-2 spinal antinociception by naloxonazine and 3-methoxynaltrexone. Brain Res, 881(1):1-8.

Sánchez-Blázquez P, Rodríguez-Díaz M, DeAntonio I, Garzón J. (1999) Endomorphin-1 and endomorphin-2 show differences in their activation of mu opioid receptor-

regulated G proteins in supraspinal antinociception in mice. J Pharmacol Exp Ther, 291(1):12-8.

Sawchenko PE, Benoit R, Brown MR. (1988) Somatostatin 28-immunoreactive inputs to the paraventricular and supraoptic nuclei: principal origin from non-aminergic neurons in the nucleus of the solitary tract. J Chem Neuroanat, 1(2):81-94.

Sawchenko PE, Arias C, Bittencourt JC. (1990) Inhibin beta, somatostatin, and enkephalin immunoreactivities coexist in caudal medullary neurons that project to the paraventricular nucleus of the hypothalamus. J Comp Neurol, 291(2):269-80.

Scanlin HL, Carroll EA, Jenkins VK, Balkowiec A. (2008) Endomorphin-2 is released from newborn rat primary sensory neurons in a frequency- and calcium-dependent manner. Eur J Neurosci, 27(10):2629-42.

Schiller PW, Berezowska I, Nguyen TM, Schmidt R, Lemieux C, Chung NN, Falcone-Hindley ML, Yao W, Liu J, Iwama S, Smith AB 3rd, Hirschmann R. (2000) Novel ligands lacking a positive charge for the delta- and mu-opioid receptors. J Med Chem, 43(4):551-9.

Schmidhammer H, Smith CF, Erlach D, Koch M, Krassnig R, Schwetz W, Wechner C. (1990) Synthesis and biological evaluation of 14-alkoxymorphinans. 3. Extensive study on cyprodime-related compounds. J Med Chem, 33(4):1200-6.

Schwaber JS, Kapp BS, Higgins GA, Rapp PR. (1982) Amygdaloid and basal forebrain direct connections with the nucleus of the solitary tract and the dorsal motor nucleus. J Neurosci, 2(10):1424-38.

Schwartzberg DG, Nakane PK. (1983) ACTH-related peptide containing neurons within the medulla oblongata of the rat. Brain Res, 276(2):351-6.

Schwyzer R. (1980) Structure and function in neuropeptides. Proc R Soc Lond B Biol Sci, 210(1178):5-20

Sim LJ, Childers SR. (1997) Anatomical distribution of mu, delta, and kappa opioid- and nociceptin/orphanin FQ-stimulated [35S]guanylyl-5'-O-(gamma-thio)-triphosphate binding in guinea pig brain. J Comp Neurol, 386(4):562-72.

Snedecor GW, Cochran WG. (1994) Statistical methods. Ames: Iowa State University Press

Snyder SH. (1982) A multiplicity of opiate receptors and enkephalin neuronal systems. J Clin Psychiatry, 43(6 Pt 2):9-12.

Spyer KM. (1990) The Central Nervous Organization of Reflex Circulatory Control. In. Loewy AD & Spyer KM. (Eds.). Central Regulation of Autonomic Functions. New York: Oxford University Press, 168-88.

Spyer KM. (1994) Annual review prize lecture. Central nervous mechanisms contributing to cardiovascular control. J Physiol, 474(1):1-19.

Stephenson RP. (1956) A modification of receptor theory. Br J Pharmacol Chemother, 11(4):379-93.

Swanson LW. The hypothalamus. In Björklund A, Hökfelt T, Swanson LW (Eds.) Handbook of Chemical Neuroanatomy, vol. 5, Part I., Integrated Systems of the CNS, Elsevier: Amsterdam, 1-124.

Swanson LW. (1982) The hypothalamus. In: Bjorklund AJ, Hokfelt T, Swanson LW (Eds.). Integrated Systems of the CNS, Part I. Hypothalamus, Hippocampus, Amygdala, Retina, Handbook of Chemical Neuroanatomy, Vol. 5, Elsevier, Amsterdam, 1-124.

Swanson LW, Sawchenko PE. (1983) Hypothalamic integration: organization of the paraventricular and supraoptic nuclei. Annu Rev Neurosci, 6:269-324.

Swanson LW. (1991) Biochemical switching in hypothalamic circuits mediating responses to stress. Prog Brain Res, 87:181-200.

Szemenyei E, Barna I, Mergl Z, Keresztes A, Darula Z, Kató E, Tóth G, Rónai AZ. (2008) Detection of a novel immunoreactive endomorphin 2-like peptide in rat brain extracts. Regul Pept, 148(1-3):54-61.

Tallarida RJ. (1982) The use of drug-receptor affinity measures in the differentiation of receptors. Fed Proc, 41(7):2323-7.

Talman WT, Granata AR, Reis DJ. (1984) Glutamatergic mechanisms in the nucleus tractus solitarius in blood pressure control. Fed Proc, 43(1):39-44.

Terskiy A, Wannemacher KM, Yadav PN, Tsai M, Tian B, Howells RD. (2007) Search of the human proteome for endomorphin-1 and endomorphin-2 precursor proteins. Life Sci, ;81(23-24):1593-601.

Thomsen C, Valsborg JS, Platou J, Martin J, Foged C, Johansen NL, Olsen UB, Madsen K. (2000) [^{3}H]AC-RYYRWK-NH_2, a novel specific radioligand for the nociceptin/orphanin FQ receptor. Naunyn Schmiedebergs Arch Pharmacol, 362(6):538-45.

Tömböly Cs, Dixit R, Lengyel I, Borsodi A, Tóth G. (2001) Preparation of specifically tritiated endomorphins. J Labelled Compd Radiopharm, 44:355-363.

Tömböly C, Péter A, Tóth G. (2002) In vitro quantitative study of the degradation of endomorphins. Peptides, 23(9):1573-80.

Turu G, Simon A, Gyombolai P, Szidonya L, Bagdy G, Lenkei Z, Hunyady L. (2007) The role of diacylglycerol lipase in constitutive and angiotensin AT1 receptor-stimulated cannabinoid CB1 receptor activity. J Biol Chem, 282(11):7753-7.

Van der Kooy D, Koda LY, McGinty JF, Gerfen CR, Bloom FE. (1984) The organization of projections from the cortex, amygdala, and hypothalamus to the nucleus of the solitary tract in rat. J Comp Neurol, 224(1):1-24.

Van Giersbergen PLM, De Jong W. (1987) Antagonism by naltrexone of the hypotension and bradycardia induced by α-methyldopa in conscious normotensive rats. J. Pharmacol, Exp Ther, 244:341-347.

Van Giersbergen PLM, Palkovits M, De Jong W. (1992) Involvement of neurotransmitters in the nucleus tractus solitarii in cardiovascular regulation. Physiol Rev, 72(3):789-824.

Varga K, Lovas G, Palkovits M, Kunos G. (1996) Ethanol inhibition of stress-related tachycardia involves medullary NMDA receptors. Eur J Pharmacol, 310(2-3):145-53.

Vig BS, Murray TF, Aldrich JV. (2003) A novel N-terminal cyclic dynorphin A analogue cyclo(N,5)[Trp(3),Trp(4),Glu(5)] dynorphin A-(1-11)NH(2) that lacks the basic N-terminus. J Med Chem, 46(8):1279-82.

Vizi ES, Ludvig N, Ronai AZ, Folly G. (1983) Dissociation of presynaptic alpha 2-adrenoceptors following prazosin administration: presynaptic effect of prazosin. Eur J Pharmacol, 95: 287–290.

Wan Q, Murray TF, Aldrich JV. (1999) A novel acetylated analogue of dynorphin A-(1-11) amide as a kappa-opioid receptor antagonist. J Med Chem, 42(16):3011-3.

Wang JB, Johnson PS, Imai Y, Persico AM, Ozenberger BA, Eppler CM, Uhl GR. (1994) cDNA cloning of an orphan opiate receptor gene family member and its splice variant. FEBS Lett, 348(1):75-9.

Wang QP, Zadina JE, Guan JL, Shioda S. (2002) Morphological studies of the endomorphinergic neurons in the central nervous system. Jpn J Pharmacol, 89(3):209-15.

Weltrowska G, Lu Y, Lemieux C, Chung NN, Schiller PW. (2003) A potent cyclic opioid peptide antagonist lacking an N-terminal amino group. Biopolymers, 71:P315.

Willis A, Mihalevich M, Neff RA, Mendelowitz D. (1996) Three types of postsynaptic glutamatergic receptors are activated in DMNX neurons upon stimulation of NTS. Am J Physiol, 271(6 Pt 2):R1614-9.

Wilson AM, Soignier RD, Zadina JE, Kastin AJ, Nores WL, Olson RD, Olson GA. (2000) Dissociation of analgesic and rewarding effects of endomorphin-1 in rats. Peptides, 21(12):1871-4.

Wolfe D, Hao S, Hu J, Srinivasan R, Goss J, Mata M, Fink DJ, Glorioso JC. (2007) Engineering an endomorphin-2 gene for use in neuropathic pain therapy. Pain ,133(1-3):29-38.

Yaksh TL. (1999) Spinal systems and pain processing: development of novel analgesic drugs with mechanistically defined models. Trends Pharmacol Sci, 20:329–337

Yamazoe M, Shiosaka S, Shibasaki T, Ling N, Tateishi K, Hashimura E, Hamaoka T, Kimmel JR, Matsuo H, Tohyama M. (1984) Distribution of six neuropeptides in the nucleus tractus solitarii of the rat: an immunohistochemical analysis. Neuroscience, 13(4):1243-66.

Yelken B, Dorman T, Erkasap S, Dundar E, Tanriverdi B. (1999) Clonidine pretreatment inhibits stress-induced gastric ulcer in rats. Anesth Analg, 89(1):159-62.

Ye YH, Tian GL, Xing GW, Dai DC, Chen G, Li CX. (1998) Enzymatic syntheses of N-protected Leu-enkephalin and some oligopeptides in organic solvents. Tetrahedron, 54(41): 12585-12596.

Zhang SW, Yu L. (1995) Identification of dynorphins as endogenous ligands for an opioid receptor-like orphan receptor. J Biol Chem, 270:22772-6.

Zadina JE, Hackler L, Ge LJ, Kastin AJ. (1997) A potent and selective endogenous agonist for the mu-opiate receptor. Nature, 386(6624):499-502.

Zadina JE, Martin-Schild S, Gerall AA, Kastin AJ, Hackler L, Ge LJ, Zhang X. (1999) Endomorphins: novel endogenous mu-opiate receptor agonists in regions of high mu-opiate receptor density. Ann N Y Acad Sci, 897:136-44.

Zardetto-Smith AM, Gray TS. (1987) A direct neural projection from the nucleus of the solitary tract to the subfornical organ in the rat. Neurosci Lett, 80(2):163-6.

12. List of publications

Publications related to the dissertation:

Kató E, Lipták L, Shujaa N, Mátyus P, Gyires K, Rónai AZ. (2008) Alpha 2B adrenoceptor agonist ST-91 antagonizes beta 2 adrenoceptor-mediated relaxation in rat mesenteric artery rings. Eur J Pharmacol, 580(3):361-5. IF: 2.787

Rónai AZ, **Kató E**, Al-Khrasani M, Hajdu M, Müllner K, Elor G, Gyires K, Fürst S, Palkovits M. (2004) Age and monosodium glutamate treatment cause changes in the stimulation-induced [3H]-norepinephrine release from rat nucleus tractus solitarii-dorsal vagal nucleus slices. Life Sciences, 74(13): 1573-80. IF: 2.158

Kocsis L, Orosz G, Magyar A, Al-Khrasani M, **Kató E**, Rónai AZ, Bes B, Meunier JC, Gunduz O, Tóth G, Borsodi A, Benyhe S. (2004) Nociceptin antagonism: probing the receptor by N-acetyl oligopeptides. Regul Pept, 122(3):199-207. IF: 2.531

Rónai AZ, Al-Khrasani M, Benyhe S, Lengyel I, Kocsis L, Orosz G, Tóth G, **Kató E**, Tóthfalusi L. (2006) Partial and full agonism in endomorphin derivatives: Comparison by null and operational model**.** Peptides, 27(6):1507-13. IF: 2.701

Rónai AZ, Szemenyei E, **Kató E**, Kocsis L, Orosz G, Al-Khrasani M, Tóth G. (2006) Endomorphin synthesis in rat brain from intracerebroventricularly injected [^{3}H]-Tyr-Pro: a possible biosynthetic route for endomorphins. Regul Pept, 134(1):54-60. IF: 2.442

Szemenyei E, Barna I, Mergl Z, Keresztes A, Darula Z, **Kató E**, Tóth G, Rónai AZ. (2008) Detection of a novel immunoreactive endomorphin 2-like peptide in rat brain extracts. Regul Pept, 148(1-3):54-61. IF: 2.276

Rónai AZ, Király K, Szebeni A, Szemenyei E, Prohászka Z, Darula Z, Tóth G, Till I, Szalay B, **Kató E**, Barna I. (2009) Immunoreactive endomorphin 2 is generated extracellularly in rat isolated L4,5 dorsal root ganglia by DPP-IV. Regul Pept, 157(1-3):1-2. IF: 2.276

Publications not related to the dissertation:

Ligeti M, Gunduz O, Magyar A, **Kató E**, Rónai AZ, Vita C, Varga I, Hudecz F, Tóth G, Borsodi A, Benyhe S. (2005) Synthesis and biological studies of nociceptin derivatives containing the DTPA chelating group for further labeling with therapeutic radionuclides. Peptides, 26(7):1159-66. IF: 2.731

Wirkner K, Stanchev D, Milius D, Hartmann L, **Kató E**, Zádori ZS, Mager PP, Rubini P, Nörenberg W, Illes P. (2008) Regulation of the pH sensitivity of human P2X receptors by N-linked glycosylation. J Neurochem, 107(5):1216-24. IF: 4.5

Abstracts published in journals

Kato E, Stanchev D, Hartman L, Milius D, Illes P, Wirkner K. (2008) The pH sensitivity of the hP2X3 receptor is regulated by N-linked glycosylation. Naunyn-Schmiedeberg's Arch. Pharmacol, 377(S1): 20. IF: 2.83

Kató E, Al-Khrasani M, Szemenyei E, Tóth G. (2006) *In vitro* opioid properties of Tyr-Pro related peptides-from Tyr-Pro to endomorphins. FEBS J, 273(S1): 278-279. IF: 3.033

Tóth G, Keresztes A, Szemenyei E, Rónai AZ, **Kató E**, Szücs M, Fülöp F. (2006) Design, synthesis and radiolabelling of novel endomorphin analogues containing unnatural α- and β-amino acids. FEBS J, 273(S1): 69-70. IF: 3.033

Al-Khrasani M, Erdo F, Székely J, Timár J, Gyarmati Zs, Szemenyei E, Tóth G, **Kató E**, Rónai AZ. (2006) Analgesic effect of Tyr-Pro-related peptides without EC 3.4.14.5 inhibitor. FEBS J, 273(S1):747. IF: 3.033

Szemenyei E, Rónai A, **Kató E**, Al-Khrasani M, Tóth G. (2006) Detection of tritiated, endomorphin-related products in rat brain extracts after ^{3}HTyr-Pro treatment. FEBS J, 273(S1):311. IF: 3.033

Kató E, Rónai AZ, Gyires K, Palkovits M, Brassai A. (2002) Gyomornyálkahártya integritását fenntartó egyes centrális mechanizmusok neurofarmakológiai tanulmányozása. EME Orvostudományi Értesítő, Kolozsvár, 75(4):471-474.

Conferences

Oral and poster presentations

49th Annual Meeting of „Deutsche Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie", Mainz, Germany, 11-13 March, 2008

Kato E, Stanchev D, Hartman L, Milius D, Illes P, Wirkner K. The pH sensitivity of the hP2X3 receptor is regulated by N-linked glycosylation. (poster)

5th Joint Meeting on Medicinal Chemistry, Portoroz, Slovenia, 17-21 June, 2007

Kató E, Lipták L, Mátyus P, Gyires K, Rónai AZ. Alpha 2B adrenoceptor agonist ST-91 antagonizes beta 2 adrenoceptor-mediated relaxation in rat mesenteric artery rings (poster)

31st FEBS Congress, Istanbul, Turkey, 24-19 June 2006

Kató E, Al-Khrasani M, Szemenyei E, Tóth G. *In vitro* opioid properties of Tyr-Pro related peptides-from Tyr-Pro to endomorphins. (poster)

Tóth G, Keresztes A, Szemenyei E, Rónai AZ, Kató E, Szücs M, Fülöp F. Design, synthesis and radiolabelling of novel endomorphin analogues containing unnatural α- and β-amino acids. (oral presentation)

Al-Khrasani M, Erdo F, Székely J, Timár J, Gyarmati Zs, Szemenyei E, Tóth G, Kató E, Rónai AZ. Analgesic effect of Tyr-Pro-related peptides without EC 3.4.14.5 inhibitor. (poster)

Szemenyei E, Rónai A, Kató E, Al-Khrasani M, Tóth G. Detection of tritiated, endomorphin-related products in rat brain extracts after ^{3}HTyr-Pro treatment. (poster)

IInd Spring Experimental Symposium of Hungarian Society for Experimental and Clinical Pharmacology, Pécs, Hungary, June, 2006

Rónai A., Szemenyei E., Kató E., Al-Khrasani M., Tóth G. Endomorfinok bioszintézise: egy lehetséges de novo bioszintetikus út, (oral presentation)

Kató E., Al-Khrasani M., Szemenyei E., Tóth G., Rónai A.Z. Tyr-Pro peptidek in vitro farmakológiája, (poster-P12)

Al-Khrasani M., Kató E., Szemenyei E., Tóth G., Rónai A. Z. Tyr-Pro-peptidek analgetikus hatása patkányon, (poster-P11)

Workshop of Peptide Chemistry Section of Hungarian Academy of Sciences, Balatonszemes, Hungary, May, 2006

Al-Khrasani M, Szemenyei E, Kató E, Kocsis L, Magyar A, Orosz G, Tóth G, Rónai AZ. The opioid effects of endomorphins in vitro and in vivo models (oral presentation)

Rónai AZ, Szemenyei E, Kató E, Al-Khrasani M, Tóth G. Egy lehetséges patkányagyi de novo endomorphin bioszintetikus út, Tyr-Pro dipeptid prekurzorból, (oral presentation)

European Opioid Conference, Salamanca, Spain, 2006

Kató E, Rónai AZ. The modulation of stimulated [3H]NE release by endomorphin 1 in rat dorsal vagal complex prism and slice preparation (poster P39)

European Opioid Conference, Visegrád, Hungary, 2004

Gündüz Ö, Kocsis L, Sipos F, Orosz G, Magyar A, Al-Khrasani M, Kató E, Rónai AZ, Farkas J, Tóth G, Borsodi A, Benyhe S. Searching for nociceptin antagonists among N-acetylated hexapeptide alcohols (oral presentation, O5)

Kató E, Al-Khrasani M, Guy E, Abbas MY, Orosz G, Kocsis L, Magyar A, Tóth G, Rónai AZ. The release of tritiated norepinephrine and D-aspartate from rat vagal nucleus slices and its modulation by μ-opioid receptor agonists (poster-P22)

Al-Khrasani M, Kató E, Orosz G, Kocsis L, Magyar A, Tóth G, Rónai AZ. Partial and full agonism in the rat vas deferens with particular reference to μ-receptor selective peptides (poster-P2)

Ligeti M, Magyar A, Kató E, Rónai AZ, Gündüz Ö, Benyhe S, Vita C, Hudecz F. Synthesis and biological studies of nociceptin derivative containing DTPA chelating group (poster-P29)

Workshop of Peptide Chemistry Section of Hungarian Academy of Sciences, Balatonszemes, Hungary, May, 2003

Al-Khrasani M, Elor G, Kató E, Rónai AZ. The effect of endomorphins and DAMGO on the release of 3H-norepinephrine from rat brain slices in vitro (oral presentation)

Annual Meeting of Transylvanian Museum Society, Targu Mures, April, 2002

Kató E, Rónai AZ, Gyires K, Palkovits M, Brassai A. Gyomornyálkahártya integritását fenntartó egyes centrális mechanizmusok neurofarmakológiai tanulmányozása (oral presentation)

IXth. Scientific Congress of Undergraduate Researchers, Targu Mures, March, 2002

Kató E. Elektromos stimuláció hatására felszabadult noradrenalin mérése, patkányagy nucleus tractus solitarii-nucleus dorsalis nervi vagi régiójából (oral presentation)

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