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

Erzsébet Kató M.D.

Semmelweis University
Doctoral School of Pharmaceutical Sciences

Supervisor: András Z. Rónai, M.D., Ph.D.

Reviewers: Ildikó Világi, Ph.D.
Ákos Zsembery, M.D., Ph. D.

Head of Committee of Final Exam:
Gábor Varga, M.D., Ph.D., D.Sc.

Members of Committee of Final Exam:
Katalin Kékesi, Ph.D.
László Tóthfalusi, Ph.D.

Budapest
2010

FOREWORD

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 NTS–DVN, i.e. the "dorsal vagal complex", and the PVN/subPVN. 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 ^{3}H-NE, ^{3}H-D-Asp or ^{3}H-GABA and the modulation of release by peptide- and non-peptide drugs and also by neonatal pre-treatment with MSG.

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.

The isolated organ experiments served either i) 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 or ii) to characterize pharmacologically endomorphin derivatives and possible biosynthetic precursors to *de novo* endomorphin biosynthesis and iii) to develop a specific nociceptin antagonist which was a prerequisite for possible further experimentation at that stage.

ABBREVIATIONS

AUC - area under curve; β -FNA-β-funaltrexamine; DADLE - [D-Ala^{2}, D-Leu^{5}]enkephalin, DAMGO - [D-Ala^{2},MePhe^{4},Gly^{5}-ol]enkephalin; D-Asp - D-aspartate; DVC - dorsal vagal complex; DVN - dorsal motor nucleus of the vagus; hPVN/subPVN - hypothalamic paraventricular /subparaventricular nucleus; EMO - endomorphin; ES - electrical field–stimulation; GABA - γ-amino butyric acid; ^{3}H - tritiated; i.c. - intracisternal; i.c.v. - intracerebroventricular; KS - high potassium stimulation; K_e -

equilibrium dissociation constant; MSG - monosodium glutamate; MVD - mouse vas deferens; NE - norepinephrine; NOP - nociceptin opioid receptor; NTI - naltrindol NTS - nucleus tractus solitarius; ORL - opioid receptor like; RVD - rat vas deferens.

INTRODUCTION

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

The central autonomic integrative network is anatomically and functionally composed of several interconnected areas responsible for tonic, reflex and adaptive control of autonomic functions. The hypothalamus, as a center of neuroendocrine regulation, integrates autonomic with endocrine responses to maintain homeostasis. However, the first major relay station of the information going to different areas of the central autonomic network is 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. Furthermore, the involvement of central α_{2A}-adrenoceptor subtype might be suggested in the inhibition of gastric acid secretion whereas the central gastroprotective effect is likely to be mediated by α_{2B}-adrenoceptor subtype. Recently, an opioid component was proved in the antisecretory and mucosal protective effect of α_2-adrenoceptor agonists. The antisecretory and the mucosal protective effects of clonidine were reversed by the nonselective opioid receptor antagonist naloxone and the δ 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, i.e. in both cases the stimulation of α_2 receptors induces release of β-endorphin. High densities of α_2 receptors were found in several areas where an elevated density of opioid receptors has also been found among many others, in the DVC. Furthermore, superfusion of brainstem slices with α_2-agonists increased the release of β-endorphin immunoreactivity and electrolitic destruction of NTS abolished the antihypertensive effect of α_2 agonist clonidine in spontaneously hypertensive rats. 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, where β-endorphin as well as POMC containing nerve cell bodies have also been detected. In order to clarify the source of

the released β-endorphin, neonatal MSG treatment was used that had been reported to cause retinal damage and neuronal loss in and around the so called circumventricular organs with prominently high-percentage damage in hypothalamic arcuate nucleus neurons. Previously it was shown that δ-opioid receptor agonists applied either i.c. or i.c.v. had gastroprotective effect against acidified ethanol-induced gastric damage in the rat. After neonatal MSG treatment, not only the i.c. 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. To prove this assumption, we used the changes in the responses to L-glutamate or the $GABA_B$ receptor agonist baclofen as potential ‘‘markers’’ of local neuronal changes after neonatal MSG treatment.

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.

2. An α_{2B} adrenoceptor mediated action

α_{2B}-adrenoceptors have a highly restricted distribution both in the central nervous system and at the periphery. They are proved to play a pivotal role in the centrally mediated gastric mucosal protection in rats, furthermore, the site of action appears to be the dorsal vagal complex in the lower brainstem. We wished to find an experimental setting where the effects of α_{2B}-adrenoceptor stimulation can be studied. ST-91, the key agonist used in the present series, besides α_1-adrenoceptor agonism, has been shown to possess $\alpha_{2NON\text{-}A}$-, possibly, α_{2B}-adrenoceptor 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. 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.

3. The endomorphins

The two brain opioid tetrapeptides, EMO-1 and EMO-2 were isolated first in 1997 from bovine and human brain and they were recognized as to be highly potent and extremely selective partial agonists at μ-opioid receptors in vitro.

There were two particular features in endomorphin biochemistry /pharmacology that arouse our interest. One of them was their partial agonism. Here, I will present the results of a rarely used but sophisticated method for the calculation of parameters for characterizing partial and full agonism. The second and most puzzling feature in endomorphin biochemistry is that their biosynthetic route is still uncertain. We have proposed the possibility of a de novo biosynthetic pathway which is known in prokaryotes and used also in in vitro peptide synthetic chemistry by reversing peptide hydrolases 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. 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. Furthermore, we characterized pharmacologically the endomorphin-related substances which were detected, or might have been detected in the course of radiolabel incorporation and immunoanalyses, the endomorphins with a free carboxylic terminus (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) and the glycine-extended endomorphins (in the production of a C-terminally amidated end-product, the immediate, short-lived biosynthetic precursor must be the glycine-extended peptide). Furthermore, because we presumed that “de novo” 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.

4. The "Operational" calculation model

The operational model is practically a more straightforward way of analyzing agonism than the conventional null-equation approaches, however, its application is limited because of the assumptions that it makes about the postreceptor events as calibrates first the parameters of receptorial transducer function by constructing an equation for the [E]/[A] relation of a full agonist and this can, therefore, be fitted directly to experimental agonist concentration-effect data in order to estimate affinities and relative efficacies of agonists.

In this part of 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 met^2-endomorphin-2, in isolated mouse vas deferens by partial μ-opioid receptor pool inactivation strategy. Morpine, morphiceptin and the full agonist prototype enkephalin analogs DAMGO and DAMGA were used as reference substances.

5.The ORL-1/NOP receptor; Nociceptin

Following the molecular cloning of an opioid receptor-like (ORL-1) orphan receptor, its endogenous ligand, the heptadecapeptide nociceptin/orphanin FQ was discovered relatively quickly by two independent groups. Nociceptin receptor has become the fourth member within the opioid receptor subgroup of the G-protein coupled receptor family, and termed as NOP receptor Although NOP receptor protein shares high homology with opioid receptors, the nociceptin- and the opioid receptors are pharmacologically distinct entities, moreover, NOP receptor is not sensitive to the general opioid antagonist naloxone. To explore the physiological functions of novel endogenous mediators and their receptors, selective, pure and potent competitive antagonists are the most useful tools. 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 is considered a NOP receptor antagonist of fair selectivity but modest affinity and possessing also considerable partial agonist property. The hexapeptide hit, Ac-RYYRIK-NH_2, described by Dooley and her coworkers is a ligand of much higher affinity, however, the partial agonist character is still present.

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.

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 „operative" 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

METHODS

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.

1. MSG 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).

2. Brain slice preparation

2.1. Rat NTS-DVN slices

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. 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, approximately parasagittal prisms (i.e. 200 × ~500 × 1,000-2,500 μm) were cut. 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.

2.2. Rat PVN/subPVN slices

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, two triangles containing the PVN/subPVN were dissected. Two such pairs were pooled from two animals and used in a single loading and then the superfusion chamber.

3. Isolated organ preparations

3.1. Mouse vas deferens

Vasa deferentia from mice weighing 35-40 g were prepared, mounted and field-stimulated. 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.

3.2. Rat vas deferens

Vasa deferentia from rats weighing 140–160 g were dissected, mounted and an initial tension of 1 g was applied. 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.

3.3. Rat 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 animals weighing 250-350 g. Endothelium destruction was effected mechanically. For mounting modified "Edvinsson hook" was used and initial tension of 2 g was applied.

4. Superfused brain slice technique

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

Experimental paradigms

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.

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 AUC produced over the resting baseline (calculated as the mean of three pre-stimulation rates) by a given stimulation period was calculated.

In the analysis of the measurements we used the datas of 16 fractions with one stimulation period during fraction 12. For statistical comparisons one-way ANOVA followed by least significant difference test or student "t" test for grouped samples was used.

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

Three types of experiments were performed. In two of them 28 fractions were collected and two stimulation periods were used: 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.

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

Experimental paradigm

In general we followed the protocol used before with the following changes:

• to maintain neural connections we were interested in, instead of prisms, we prepared "whole" trapezoidal slices with approx 2.5-4 mm sides and 500 μm thickness, i.e. the preparation step before the parasagittal prisms.
• 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 viability of neurons 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.

Technical installations

• 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.
• 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
• Eight-track, programmable linear fraction collector

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

Experimental paradigms

In principle we followed the experimental settings described before 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.

We used 1mM β-alanine (glial GABA uptake inhibitor) containing Krebs' buffer for the equilibration (30 min) and loading with 1.5 μCi/0.5 mL ^{3}H-GABA (30 min). During the superfusion we added 0.1 mM nipecotic acid (GABA uptake inhibitor) and 0.1 mM amino-oxoacetic acid (GABA transaminase inhibitor) to the Krebs buffer.

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.

5. Isolated organ analysis

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

Experimental paradigms

When multiple concentrations of the same drug were administered, it was done in a cumulative manner. In aortic and mesenteric artery rings each

experiment was started by contraction with 10^{-6} M phenylephrine. 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. 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.

Data evaluation

Sigmoidal curve fitting was performed according to the Hill equation and the "A_{50}", "E_{max}" and "n_H" parameters were used for further statistical analyses. One-way ANOVA followed by Newman–Keuls Multiple Comparison Test was used for statistical analysis.

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

Experimental paradigms

Vasa deferentia were exposed to agonists for up to 2 min with the exception of Dmt-endomorphin-1, where exposure was 10–25 min. 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.

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 was used. According to the operational model, using Hill's equation, the E_{max} and n_H parameters for the full agonist prototypes were determined then these parameters were used to calculate the receptor constants for all the agonists by Leff's equation. ANOVA followed by Dunnett's test was used to compare efficacies, taking DAMGO as prototype full agonist for multiple comparisons. Student's ''t''-test was used to compare ''before–after'' data sets (paired arrangement). For correlation analysis, correlation coefficient (r) values were calculated.

5.3. The in vitro pharmacology of presumed endomorphin biosynthetic intermediates

For the pharmacological analysis of possible precursors-biosynthetic intermediates the MVD bioassay was used as described in previous sections. The only difference was that in these series NMRI mice were used.

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

CFLP mice were used for the MVD bioassay. For the antagonists the equilibrium dissociation constants (K_e) were calculated 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.

RESULTS AND DISCUSSION

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

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

In control rats L-glutamate increased field stimulation-induced release of ^{3}H-NE. 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 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 ^{3}H-NE release only in young adults. 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.

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

Nextly, we characterized the stimulation-induced (ES and KS) release of ^{3}H-NE and ^{3}H-D-Asp from rat NTS-DVN prisms preparation in vitro. Furthermore, we tested the Ca^{2+} dependency of the release of tritiated neurotransmitter by high potassium (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). This is in contrast with the marked Ca^{2+} dependent release of ^{3}H-NE by high K^+.
The release pattern of ^{3}H-NE from rat NTS-DVN slices is characteristic of a neurotransmitter. Moreover, there is an ambiguity as to the cellular compartment from which ^{3}H-D-Asp is released by 60 mM KCl.

1.3. EMO-1 and the modulation of stimulated ^{3}H-NE release in rat DVC *whole* trapezoidal slice preparation

Since neural connections are important for a meaningful analysis, we extended our previous studies in NTS-DVN prism preparation to *whole* slices. 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.
In trapezoidal NTS-DVN whole slices stimulated by high K^+ 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 K_2.
In contrast with previous measurements in NTS-DVN prisms 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, other, inhibitory neurons eg GABAergic, 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.

1.4. The release of ^{3}H-GABA from rat isolated PVN/subPVN slices. Comparison with release from NTS-DVN slices

In this subset of experiments we wished to compare the stimulation-induced release of ^{3}H-GABA from PVN/subPVN and NTS-DVN slices and to install some technical novelties to improve significantly the performance and precision of brain slice loading- and superfusion-system.
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. The experimental conditions for NTS/DVN should be improved to render the preparation suitable for studying properly tritiated GABA release.

2. Measurements related to α_{2A} and α_{2B} adrenoceptors

Comparing the α_2- adrenoceptor agonist potencies of xylazine and ST-91 in the presence of prazosine in RVD, ST-91 was almost 10 times more potent presynaptic inhibitor than xylazine. Knowing that the dominant prejunctional α adrenergic receptor subtype in vasa deferentia is $\alpha_{2A/D}$ and taking into account that the presence of 10^{-6} M prazosine excludes any significant agonist actions at the α_{2B}- or α_{2C}-subtypes whereas would permit effects at the $\alpha_{2A/D}$-subtype, we can confirm the $\alpha_{2A/D}$ agonist activity 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. In phenylephrine-precontracted rings, ST-91 inhibited isoproterenol-induced relaxation in a yohimbine-antagonized fashion, i.e. in α_1 independent manner. There is a known example of G_i-related negative coupling of α_{2B}-adrenoceptors to β_2-adrenoceptor stimulated adenylyl cyclise, however, it is possible though not proven that a similar negative coupling functions also in rat mesenteric artery.

3. The analysis of partial agonist properties of endomorphins and other μ-opioid receptor agonists

When calculating the agonist dissociation constants by either the null either the operational methods, the K_A values correlated significantly with each other (Pearson correlation coefficient $r=0.9998$, $p<0.0001$). 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.

4. The in vitro pharmacology of presumed endomorphin biosynthetic intermediates

The Gly-extended peptides developed their effect slowly (15-25 min) and the recovery was also prolonged, occasionally incomplete. The effect of E2-G was approximately 30 times more potent than E1-G, 20 times more potent than E2-OH (i.e. E2 with a free carboxylic C-terminus) but 15 times less potent than authentic E2. The opioid agonist action of E2-G was competitively antagonized by 10^{-6} M cyprodime, a μ-opioid receptor-preferring antagonist. On the other hand, NTI, an opioid receptor antagonist

with good preference for δ-opioid receptors displayed no antagonism against E2-G at 3 nM concentration of antagonist. There was a significant antagonism of E2-OH's effect by NTI. The inhibitory effect of 10^{-5} M E1-G could not be reversed by 30 nM NTI and only partially reversed by 10^{-6} M naloxone.

Regarding the relatively high agonist potency of Gly-extended EMO-2 and also the definite preference for μ-opioid receptors, we tested the possible conversion of glycylated peptide to authentic EMO-2 by PAM enzyme. Ascorbate had been described to stimulate whereas trans-styrylacetic acid to inhibit the enzyme. 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.

All the –Ser-/Ser(O-glucose)-extended Tyr-Pro derivatives were devoid of any significant opioid agonist activity. 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 EMO-2 biosynthesis.

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

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. The K_e calculated from the individual dose ratios and the corresponding antagonist concentration was 2.44 ± 0.15 nM. We showed that Ac-RYYRIK-ol is a competitive ORL-1 receptor antagonist of high affinity in the mouse vas deferens bioassay. The K_e 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.

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.

2. The effect of endomorphin-1 in 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 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. ^{3}H-D-Asp loaded a not "transmitter-like"neuronal pool whereas ^{3}H-NE and ^{3}H-GABA could be released by conventional stimuli (K^+ -induced depolarization, field electrical stimulation) in a transmitter-like manner.

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.

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.

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 and fulfilled the Schild-criteria for a competitive antagonism. It was by the traditional term, a "pure" antagonist showing no agonism at the concentration of 10^{-5} M.

ACKNOWLEDGEMENTS

This study was carried out at the Department of Pharmacology and Pharmacotherapy, Semmelweis University.

I would like to express my deep gratitude to all those who helped me during the course of my work, especially to:

Prof. Dr. Éva Szőke, head of the Doctoral School of Pharmaceutical and Pharmacological Sciences,

Prof. Dr. Kálmán Magyar, head of the Doctoral Program of Experimental and Clinical Pharmacology

Dr. András Z. Rónai, my tutor, who showed me the life of a researcher and guided me, since I was a medical student. He has taught me the way of thinking of a pharmacologist and helped me continually throughout my work,

Prof. Dr. Klára Gyires, the director of the department, who still supports me to continue my research activities,

Prof. Dr. Zsuzsanna Fürst, the former director of the department, who gave me the opportunity to start my doctoral work,

Dr. Mahmoud Al-Khrasani, for his theoretical and practical advices,

Dr. Pál Riba, for his critical review and helpful suggestions,

Mrs. Lilla Gábriel, for her assistance,

Jenő Balogh, Antal Gulyás, and the whole staff of the department,

and last but not least, to my family who have always stood beside me with unconditional love and supported me.

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: 2.231

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

Kató 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)