

**ENDOGENOUS LIGANDS IN PAIN CONTROL AT THE SPINAL LEVEL:
AGMATINE, ENDOMORPHIN-1 AND KYNURENIC ACID**

Ph. D. thesis

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Introduction

Transmission of nociceptive information is subject to modulation at several levels of the neuraxis including the dorsal horn of the spinal cord {7736}. Afferent impulses arriving in the dorsal horn initiate inhibitory mechanisms, which limit the effect of subsequent impulses. Inhibition occurs through the effect of local inhibitory interneurons, descending pathways from the brain and inhibitory neurotransmitters/neuromodulators released from the primary sensory neurones.

In the dorsal horn, incoming nociceptive messages are modulated by endogenous ligands acting on $\alpha 2$ -adrenoceptors, GABA_{A-B}, glutamate, opioid (μ , δ and κ), 5-HT₂₋₃, NK-1, adenosine and 5-HT_{1B} receptors located pre- and/or postsynaptically.

The aim of the thesis was to investigate the antinociceptive properties of three endogenous ligand – agmatine, endomorphin-1, kynurenic acid - acting on different receptor types at the spinal level using an inflammatory pain test in awake rats.

Agmatine possesses modest affinity for $\alpha 2$ -adrenoceptors, as well as I1 and I2 sites, like clonidine. Complicating the interpretation of its influence upon nociceptive processing, agmatine behaves as an inhibitor of NOS, expresses antagonist properties at NMDA receptors and block the nicotinic cation channel. It has been shown in 1996 that systemic administration of agmatine enhances morphine analgesia and also blocks tolerance to opioids in mice, and it attenuated all of the signs of the morphine abstinence syndrome in rats. Up to now another study had evaluated its spinal effects using mice and rats. According to their findings intrathecally administered to rodents decreased hyperalgesia accompanying inflammation, normalized the mechanical hypersensitivity in the absence of antinociceptive effects in acute pain test.

Endomorphins, discovered in 1997, exhibit the highest affinity and specificity for the μ -opioid receptors of any compound found so far. The endomorphin family includes two peptides: endomorphin-1 (Tyr-Pro-Trp-Phe-NH₂) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH₂). The endomorphins distribution in nociceptive pathways and in regions of high μ -opioid receptor density implicates them as particularly important for the modulation of pain, which is verified by *in vivo* studies. There are several studies on the antinociceptive properties of EM-1 in different animal models. The administration of endomorphin-1 resulted antinociception, although it has low efficacy, short-lasting effect and tolerance was also observed.

Degradation of the essential amino acid tryptophan along the kynurenine pathway yields kynurenic acid. Kynurenic acid is the only endogenous antagonist acting at the glycine as well as the NMDA recognition sites of the NMDA receptor complex. In larger concentrations (0.1-1 mM) also antagonize the AMPA and kainate receptors. Recently it was proposed that kynurenic acid may be a potent noncompetitive antagonist of acetylcholine $\alpha 7$ nicotinic receptors (IC₅₀~7 μ M). Several data suggest the beneficial effect of kynurenic acid as an anticonvulsant, but there are only a few studies about its role in the pain perception. Although Ganong et al. in 1983 have already suggested that kynurenic acid influenced on the pain transmission at the spinal level, to date only three studies were performed in rodents to investigate the antinociceptive effect of kynurenic acid in rodents after intrathecal administration with inconsistent results.

Goals of the study

However, there are several methods and drugs for pain therapy, there is still not

available the most appropriate one. Using pharmacological agents we have to calculate with different side-effects, toxicity and tolerance. These problems are associated with using the most of the synthetic drugs. However, there are several techniques employed to decrease side-effects: 1) local drug administration, 2) the application of endogenous ligands/substances, 3) receptor-specific drug administration, 4) the use of low dose combinations of several agents that produce the same therapeutic effects as a single drug applied in a higher dose.

In order to obtain the most effective way of pain relief, we applied the above mentioned techniques in the same experimental set-ups. Exactly, we administered the combination of several endogenous ligands locally, applied them into the subarachnoid space. The aim was to investigate the antinociceptive potency of different endogenous ligands and their interactions at the spinal level. Since endogenous ligands generally have lower specificity and affinity for their receptors, their effectivity compared to the synthetic drugs is lower, their continuous administration is relevant. As the main points of developmental pharmacology of pain are the inhibition of the excitatory pathways and the exaggeration of the inhibitory pathways, using NMDA receptor antagonists, opioid and adrenergic receptor agonist in pain therapy is relevant. Therefore the aims of the thesis were:

1. to determine whether the agmatine pre- or posttreatment influences carrageenan-induced thermal hyperalgesia,
2. to investigate whether agmatine influences or not the antinociceptive properties of the cumulatively administered morphine,
3. to investigate the antinociceptive properties of the μ -opioid receptor agonist endomorphin-1, the α_2 -adrenoceptor-agonist agmatine, and the NMDA-receptor antagonist kynurenic acid on carrageenan-induced inflammation and
4. to analyse the possible interaction of the continuously administered mixture of these endogenous ligands at the spinal level.

Methods

Intrathecal catheterization

In order to determine the antinociceptive effects of endogenous ligands in awake rats at the spinal level, we implanted an intrathecal catheter into the subarachnoid space previously.

Male Wistar rats were anaesthetized with a mixture of ketamine hydrochloride and xylazine (72 and 8 mg/kg intraperitoneally, respectively). An intrathecal catheter (PE-10 tubing) was inserted via the cisterna magna and passed 8.5 cm caudally into the subarachnoid space, which serves to place the catheter tip between vertebrae Th12 and L2 corresponding to the spinal segments, which innervate the hindpaws. Rats exhibiting postoperative neurologic deficits (about 10%) were excluded. The rats were allowed to recover for at least 4 days before testing, and assigned to the treatment groups (n=4-12 rats/group) randomly.

Inflammatory pain test

We used the paw withdrawal (PWD) test to measure the antinociceptive effects of the applied substances on carrageenan-induced inflammation. The baseline hindpaw withdrawal latencies (pre-carrageenan baseline values at -180 min) were measured. Unilateral inflammation was induced by intraplantar injection of 1.5 mg/0.1 ml lambda carrageenan into one of the hindpaws, that induces a period of hyperalgesia to peripheral thermal and mechanical stimulation peaking at 2-3 h after injection. The PWD latencies were obtained again 3 h after carrageenan injection (post-carrageenan baseline values at 0 min), and at 10-min intervals subsequently for 130 min during the continuous intrathecal infusion. In case of

the agmatine pre- and posttreatment the latencies were obtained 10 and 30 min after the injections for 90 min. The values obtained at 10 and 30 min after each dose were averaged for the value for that dose.

Materials

- Agmatine sulphate (Sigma-Aldrich Kft., Budapest, Hungary),
- endomorphin-1 (Sigma-Aldrich Kft., Budapest, Hungary),
- kynurenic acid (Sigma-Aldrich Kft., Budapest, Hungary),
- morphine chloride (Alkaloida, Tiszavasári, Hungary).

The solutions were prepared freshly on the day of the experiments by dissolving them in sterile physiological saline. Saline was used as control in all series.

In case of single drug administration the drugs were administered in a volume of 5 μ l a flushed with additional 10 μ l of physiological saline during 30 s.

We used microinjection pump in case of continuous intrathecal infusion in a volume of 60 μ l, with a flow rate of 1 μ l/min. The drugs were flushed with additional 10 μ l of physiological saline.

Morphine sulphate was administered cumulatively similarly to the single drug studies.

Statistical analysis

Data are presented as means \pm S.E.M. Analysis of variance (ANOVA) of data for repeated measures was used for overall effects. The area under the curve (AUC) values were obtained by calculating the area during (10-70 min) and after (100-130 min) drug administration. These data sets were examined by one-way ANOVA. The significance of differences between experimental and control values was calculated by using the Neuman-Keuls test for *post hoc* comparison. A probability level of 0.05 ($P < 0.05$) was considered significant.

Experimental paradigm

Single drug studies

In the first series we investigated the antinociceptive effects of endogenous ligands by themselves using continuous intrathecal administration.

- Agmatine: 0.3, 1 and 3 μ g/min and 1, 10, 50, and 100 μ g/ 5 μ l in pre- or posttreatment,
- endomorphin-1: 0.1, 1 and 3 μ g/min,
- kynurenic acid: 0.1, 0.3, 1 and 4 μ g/min,
- morphine: 1-4-5 μ g/5 μ l cumulatively.

Drug combination studies

In the second series we investigated the antinociceptive properties of different combinations of endogenous ligands and the type of their interaction on nociception.

- Agmatine + endomorphin-1: in a fixed dose ratio 3:1
0.3, 1, 3 μ g/min agmatine + 0.1, 0.3, 1 μ g/min endomorphin-1,
- endomorphin-1 + kynurenic acid : in a fixed dose ratio 10:1
0.1, 0.3, 1 μ g/min endomorphin-1 + 0.01, 0.03, 0.1 μ g/min kynurenic acid
- agmatine + morphine: 50, 100 μ g/5 μ l agmatine pre- or posttreatment + 1, 4 and 5 μ g/5 μ l morphine cumulatively.

Results and conclusions

There was no significant difference in PWD responses to noxious thermal stimuli between the right and left hindpaws prior to intraplantar injection of carrageenan. The overall mean PWD latencies for the ipsilateral and contralateral paws were 9.3 ± 0.16 and 9.5 ± 0.15 s in the first; 9.94 ± 0.15 and 9.92 ± 0.16 s in the second; and 9.9 ± 0.13 and 9.8 ± 0.14 s in the third experimental series. Carrageenan injection induced acute inflammation of the injected hindpaw, as evidenced by oedema and erythema. The PWD latencies of the carrageenan-injected paw were significantly reduced to 2.9 ± 0.14 ; 2.95 ± 0.001 and 3.3 ± 0.09 s ($P < 0.001$) in non-pretreated animals. Thus, thermal hyperalgesia was consistently produced in rats with carrageenan-induced inflammation.

The following observations have been made:

1. Intrathecal pre- or posttreatment of AGM did not influence significantly the PWD latencies on the normal paws. Both treatment procedure of AGM decreased the carrageenan-induced hyperalgesia, but did not relieve it. The higher doses (more than 200 μ g) resulted in side-effects: vocalization and fighting lasted for several hours in all animals and accompanied by a decrease in PWD latency of the normal paw.
2. Both pre- and posttreatment increased the MO-induced analgesia, but the pretreatment had higher efficacy.
3. Intrathecally administered EM-1 did not alter the PWD latency significantly on the non-inflamed paw. The higher doses on the inflamed side, as compared to the control group, resulted in significant increases in PWD latency.
4. The continuous agmatine infusion did not influence the pain threshold on the non-inflamed paw. On the inflamed paw it resulted in significant increase in the PWD latency during and after the infusion. Significantly reduced but did not reveal thermal hyperalgesia. The highest dose (3 μ g/min) caused excitation in 50% of the animals.
5. The continuous intrathecal infusion of KYNA resulted in a dose-dependent increase on PWD latency on the normal and inflamed paws too. Administered alone induced a dose-dependent, reversible motor impairment as a severe side effect.
6. On the inflamed side, AGM significantly potentiated the antihyperalgesic effect of EM-1 during the infusion. It should also be mentioned that the highest dose caused a temporary excitation in 30% of the animals.
7. In low doses, KYNA potentiated the antihyperalgesic effect of EM-1 in the inflammatory pain model without causing side-effects. Termination of the infusion was followed by a gradual decrease of the effects, suggesting that this method provides well-controlled antinociception.

Summary

The aims of our study were to induce effective antinociception without side-effects. Thus we decided to use endogenous substances in combinations using a continuous, intrathecal drug administration protocol.

Our results demonstrated a well-controlled antinociception and reinforced the advantages of combination treatment despite monotherapy.

These results suggest an important direction for the development of new strategies for pain therapy that focus on the coadministration of different endogenous ligands into the subarachnoid space.

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Full papers and abstracts related to the thesis

- I. Horvath, G., **Kekesi, G.**, Dobos, I., Szikszay, M., Klimscha, W. and Benedek, G. Effect of intrathecal agmatine on inflammation-induced thermal hyperalgesia in rats. *Eur. J. Pharmacol.* 368:197-204, 1999.
- II. **Kekesi G.**, Joo G., Csullog E., Dobos I., Klimscha W., Toth K., Benedek G., Horvath, G.. The antinociceptive effect of intrathecal kynurenic acid and its interaction with endomorphin-1 in rats. *Eur. J. Pharmacol.* 445:93-96, 2002.
- III. **Kekesi G.**, Horvath Gy. A kinurénsav fájdalomcsillapító hatása. / The antinociceptive effect of kynurenic acid. *Ideggy Szle / Clin Neurosci.* 55(9-10): 313-322, 2002.
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Full papers and abstracts not related to the thesis

- I. Joo, G., Horvath, G., Klimscha, W., **Kekesi, G.**, Dobos, I., Szikszay, M., and Benedek, G. The effects of ketamine and its enantiomers on the morphine- or dexmedetomidine-induced antinociception after intrathecal administration in rats. *Anesthesiology* 93:231-241, 2000.
- II. **Kekesi G.**, Dobos I., Benedek G., Horvath G. Antinociceptive activity of *Sempervivum tectorum* L. extract in rats. *Phytother. Res.* In press

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