Summary of Ph.D. Thesis

The effect of plant derived and endogenous substances on the spinal mechanisms of pain in rats

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2011

Szeged

1. Introduction

Pain is known to be the central representation of nociception transmitted to and perceived by the cerebral cortex. Nociceptive pain is a complex biological phenomenon that results from integrated mechanisms from the activation of peripheral primary sensory nociceptors by noxious chemical, mechanical or thermal stimuli via the dorsal horn of the spinal cord to higher structures of the nervous system.

Although acute pain has a warning role and can be mostly appropriately eased by drugs available, the treatment of chronic pain has not been solved yet. The reason for that is to some extent the limited efficacy of analgesic drugs, on the other hand chronic analgesic treatment often leads to undesirable side-effects. Therefore there has always been a need for investigations to find new medications and alternative approaches to pain treatment.

Many of the analgesic substances have been isolated from plants or other natural products including endogenous ligands. As regards the plant-originated agents, a huge amount have already been discovered and some of them applied in the therapy of pain. An exciting and rapidly developing field of pain research relates to the roles of different endogenous ligands. Various aspects of endogenous antinociceptive systems have been reviewed, some of them focusing on the results relating to antinociceptive interactions after the coadministration of different endogenous ligands.

The investigation of natural products including endogenous ligands can lead not only to new compounds with idealistic pharmacological profiles, but may also contribute to our better understanding of the complex mechanisms of pain.

Endomorphins have been found unequally in the brain, they are stored in neurons and axon terminals with heterogenous distribution, they are converted enzymatically by endopeptidases and they interact specifically and with high affinity with μ-opioid receptors. Endomorphin-1 produces antinociception acting predominantly on the μ_2-oipoid receptor. It has been shown to inhibit both the

spontaneous and K^+-evoked L-glutamate (L-Glu) efflux from superfused rat cerebrocortical synaptosomes and significantly inhibits the K^+-evoked γ-aminobutyric acid (GABA) efflux.

N-arachidonoyl-dopamine was identified as an endogenous ligand at both TRPV1 and cannabinoid CB1 receptors. This arachidonic acid derivative was found in the highest concentrations in the striatum and hippocampus, and was also detected in the cerebellum, thalamus and dorsal root ganglion (DRG). It is the first endogenous compound identified in mammals that is almost equipotent to capsaicin at TRPV1 receptor. In addition, NADA also behaves as an agonist at CB1 receptor with an affinity similar to that of anandamide, the first identified endocannabinoid.

Vinpocetine (ethyl-apovincaminate, Cavinton^R, Gedeon Richter, Budapest, Hungary) is a synthetic compound, but structurally related to the Vinca minor alkaloid vincamine. Vinpocetine is a potent inhibitor of the voltage-dependent Na^+ channels and the Ca^{2+}/Calmodulin-dependent phosphodiesterase 1 (PDE1). Known for its minimal side effects it is widely used in clinical pharmacotherapy of various cerebral circulatory diseases, both *per os* and parenterally. Recently, it was shown that vinpocetine acts as a potent antiinflammatory agent in vitro and in vivo by inhibiting the nuclear factor kappa B (NF-κB), a key transcriptional factor. Surprisingly, this mechanism was proved to be independent of vinpocetine action on PDE1.

2. Aims of our studies

1. Characterization of the antinociceptive potential of NADA on inflammatory thermal hyperalgesia in rats at spinal level.

2. Investigation of the role of CB1 and TRPV1 receptors in the antinociceptive effects of NADA.

3. Determination of the interaction of NADA with EM at spinal level.

4. Investigation of the effect of perineurally applied vinpocetine on the retrograde axoplasmic transport of nerve growth factor (NGF) in the periferal nerve.

5. Study of the antinociceptive effect of perineural vinpocetine treatment in a behavioral model.

3. Materials and Methods

Intrathecal catheterization: Male Wistar rats (239 ± 1.2 g) were anesthetized with a mixture of ketamine hydrochloride and xylazine (72 and 8 mg/kg intraperitoneally, respectively). An IT catheter was inserted via the cisterna magna and passed 8.5 cm caudally into the subarachnoid space, which served to place the catheter tip between Th12 and L2 vertebrae, corresponding to the spinal segments that innervate the hindpaws. The rats were allowed to recover for at least four days before testing, and were assigned randomly to the treatment groups (7-12 rats/group). The observer was blinded to the treatment administered.

Nociceptive testing: The hind paw response latency to a noxious heat stimulus was measured to assess the antinociceptive effects of the substances in rats with a hind paw carrageenan-induced

inflammation. Rats were placed on a glass surface in a plastic chamber and a heat stimulus was directed onto the plantar surface of each hindpaw. The intensity of the thermal stimulus was adjusted to derive an average baseline latency of approximately 10.0 s. The cut-off time was set at 20 s to avoid tissue damage. The baseline hindpaw withdrawal latencies (PWL; pre-carrageenan baseline values at -180 min) were then obtained. Unilateral inflammation was induced by intraplantar injection of 2 mg carrageenan in 0.1 ml physiological saline into one of the hindpaws (on the paralyzed side during the lidocaine effect). Carrageenan-induced thermal hyperalgesia peaked at 3-4 h after the injection. PWLs were obtained again 3 h after carrageenan injection (post-carrageenan baseline values at 0 min). NADA, AM 251 (CB1 receptor antagonist/inverse agonist) and AMG 9810 (TRPV1 receptor antagonist), EM or the combinations were injected after the determination of the post-carrageenan baseline value. PWLs were registered 5 min after the intrathecal injection and then every 10 min until 90 min.

Perineural application of vinpocetine: Investigations were performed on 32 (n=5-7/group) young adult male Wistar rats, 200–250 g body weight. In experiments aiming to study the effect of vinpocetine on retrograde transport, a slab of Gelita tampon was soaked in 10^{-6}, 10^{-7} or 10^{-8} M vinpocetine dissolved in physiological saline. The sciatic nerve was set free under 4% chloral hydrate anesthesia**.** After a 2 cm incision on the dorsal surface of the thigh, the sciatic nerve was gently exposed. The Gelita slab soaked in the solution to be tested was applied around the nerve in the form of a loose cuff. Contralaterally, the sciatic nerve was surrounded by a similar Gelita tampon cuff, soaked in physiological saline. Thirty minutes later the cuffs were removed and the wounds were closed with silk suture. Seven days after the application of the cuffs, the animals were killed with an overdose of 4% chloral hydrate, and subjected to transcardial fixation.

Histological techniques: For histochemical and immuno-histochemical investigations, animals were anesthesized with 4% chloral hydrate intraperitoneally and subjected to transcardial perfusion with a formaldehyde solution containing picric acid to

which glutaraldehyde was added. The spinal cord was excised and the distribution of FRAP and TMP was studied in 15 μm frozen cross-sections, SP immunoreactivity was visualized according to the technique described earlier. For the electron microscopic localization of FRAP, the enzyme has been visualized in vibratome sections of the spinal cord (50 μm) as described above; however, the last step of the reaction, i.e. sulfide treatment, was omitted. The samples were embedded in Durcupan ACM and sectioned on a Reichert Ultrotome. Thin sections, silver interference color, were stained with lead citrate and studied with a Zeiss Opton 902 electron microscope (Oberkochen, Germany).

Study of retrograde axoplasmic transport of nerve growth factor (NGF): The effect of vinpocetine upon retrograde axoplasmic transport of NGF was studied after injection of 600 ng (50 μCi) ^{125}I-labelled NGF-β under the skin of the hind paw. Radioactivity of 0.5 cm long portions of the sciatic nerve, the corresponding dorsal root ganglia and dorsal roots was determined 15 h after the injection of ^{125}I-labelled NGF-β in a Berthold 8F Gammascint apparatus. The left sciatic nerves of the rats were surrounded by Gelita tampon cuffs, soaked in 10^{-6}, 10^{-7} or 10^{-8} M vinpocetine, dissolved in physiological saline 72 h before the radioactivity measurement. The contralateral sciatic nerve was surrounded by a Gelita tampon cuff, soaked in physiological saline.

Densitometry: The *intensity* of the histochemical staining and that of the immunoreaction was measured by densitometry of the slides, performed by digitalizing microscopic pictures obtained by histochemistry or immunocytochemistry with a SPOT RT Slider CCD camera (1600×1200 pixels, 8 bits) attached to a Nikon Eclipse E600 microscope using a ×16 front lens and a ×10 eyepiece. The captured images were analyzed by ImageProPlus v4.5 morphometric software (Media Cybernetics, Silver Sring, MD, USA).

Studies of the behavioral effects of vinpocetine: Investigations were performed on 15 young adult male albino Wistar rats (200 - 250 g). The pain behavioral effect of subcutaneous formalin treatment was studied by means of the formalin test. A dilute

formalin solution (10%, 10 μL) was injected subcutaneously into the plantar surface of a hind paw (n=8). This produced flinching, shaking and licking. The number of flinches+shakes of the injected paw was counted each minute for a period of 60 min and then it was averaged into 3-min periods. In seven further rats, the sciatic nerve was first enclosed in a Gelaspon cuff containing 10^{-6} M vinpocetine as described above by the in vitro experiments. Seventy-two hours later a dilute formalin solution (10%, 10 μL) was injected subcutaneously into the plantar surface of the hind paw, and the number of flinches+shakes of the injected paw was counted as in the control experiments.The **immunohistochemical** equivalent of perineural vinpocetine treatment was assessed on the basis of *c-fos* expression in the spinal cord of rats.

4. Results

1. NADA alone resulted in a significant, dose-dependent antinociceptive effect. The ED_{50} value was 22.5 (CI: 15.1-30.5) µg for the whole period

2. AM 251 did not influence the antinociceptive effect of 15 µg NADA, but significantly decreased the effect of 50 µg NADA, while **AMG 9810 significantly decreased the effects of** both doses of **NADA.**

3. Regarding the interaction of NADA and EM, in ratio 1:15 (EM:NADA = 0.3:5 µg) the combination was effective for almost all the investigated period. The combinations in 1:50 ratio produced significant antihyperalgesia in dose of 0.1:5 µg (EM:NADA) for 30 min and a higher dose-combination (EM:NADA = 0.3:15 µg) produced a temporary potentiation.

4. Perineurally applied vinpocetine at a concentration of 10^{-6} M caused **disappearance of FRAP and TMP** and induced a **decrease in SP** immunoreactivity from the ipsilateral, segmentally related Rolando substance of the dorsal horn. It also **blocked retrograde transport** of ^{125}I-labeled NGF in the sciatic nerve.

5. Perineural application of a vinpocetine cuff (10^{-6} M) 3 days prior to formalin treatment **reduced the number of flinchings, shakings and licking** of the animals in the behavioral model and **prevented increased expression of *c-fos*** in the ipsilateral, segmentally related superficial dorsal horn of the spinal cord.

5. Discussion

The results of our study show that IT administration of NADA caused dose-dependent antihyperalgesic effect in the inflammatory pain model. Both CB1 and TRPV1 receptor antagonists decreased the antihyperalgesic effect of NADA, indicating that these receptors are involved at spinal level. CB1 is found primarily on the primary sensory neurons and on both excitatory and inhibitory interneurons in the superficial spinal cord, and their activation inhibits these neurons and reduces the releases of several transmitters, however, the activation of TRPV1 receptors increases the transmitter releases. Since NADA represents a "chimeric" ligand acting on both cannabinoid and TRPV1 receptors, and CBs and TRPV1 receptors show coexpression, their coactivations can lead to an interaction between them, thus NADA can influence both the antinociceptive and pronociceptive processes. Furthermore, the exogenously administered ligands can also interact with the endogenously released substances during inflammation. Therefore, the complex changes at the level of different inhibitory and excitatory ligands and their effects on several neurons/receptors in the spinal cord might lead to the prolonged antihyperalgesia.

Our results showed that in contrast to the well known synergistic antinociceptive interaction of exogenous cannabinoids and opioids, the co-administration of NADA and EM did not produce synergistic interaction in the applied ratios. Several combinations produced effective short-lasting antihyperalgesia, but the long-lasting potentiation was observed only in one combination. As regards the action mechanism of their interaction, EM exerts its effect on μ-opioid receptor, while the action mechanism of NADA is more complex, therefore, at least three receptor-mechanisms (μ-opioid, CB1 and TRPV1) should be calculated. The additive interaction might be the result of the simple summation of the effects of the ligands. Since TRPV1, CB1 and μ-opioid receptors are expressed in the spinal dorsal horn, the complex interactions of the endogenous ligands on different receptors and/or different cells may also result in these effects. Therefore, additional experiments in future are required

for a further clarification of the pharmacokinetic interaction of these ligands.

Our studies reported here prove that perineurally applied vinpocetine is able to block retrograde axoplasmic transport of NGF in a peripheral nerve that induces TDA of primary sensory axon terminals in the segmentally related, ipsilateral upper dorsal horn, resulting in the depletion of FRAP and TMP, and partial depletion of SP from the same area. Similar effects were observed earlier after perineural application of vinblastine and vincristine; the blockade of retrograde axoplasmic transport of ^{125}I NGF after perineural application of vinpocetine is similar to that observed by us after perineural application of vinblastine and vincristine. Since transcutaneous iontophoresis of vincristin and vinblastin alleviates chronic pain resulting from postherpetic, trigeminal and other neuralgias, the possibility arises that vinpocetine, applied by transcutaneous iontophoresis, can also be used for alleviation of pain. According to our behavioral studies, perineural vinpocetine pre-treatment diminished the number of flinchings, shakings and licking, and prevented increased *c-fos* immunoreactivity in the ipsilateral, segmentally related superficial dorsal horn of the spinal cord. Increased expression of *c-fos* is a widely accepted marker for increased neuronal activity, therefore, decrease in the number of flinchings, shakings and licking, and decreased amount of *c-fos* immunoreactive cells in the ipsilateral superficial dorsal horn are incontestable signs of decreased nociception and pain. Recent studies revealed that an optimized microemulsion of vinpocetine represents a nonirritant transdermal delivery system. Accordingly, transdermal or transcutaneous administration of vinpocetine seems to be a promising possibility in the clinical treatment of neuropathic pain.

6. Conclusions

1. Intrathecal administration of the endogenous ligand **NADA caused dose-dependent thermal antihyperalgesia** in rats in the inflammatory pain model proving the antinociceptive effect of NADA at spinal level.

2. Both CB1 and TRPV1 receptor antagonists decreased the antihyperalgesic effect of intrathecally administered NADA, indicating that both receptors play a substantial role in its antinociceptive effects at spinal level. Thus, NADA may be involved in both antinociceptive and pronociceptive processes and its exact mechanism of action remains to be cleared.

3. We found **additive interaction between NADA and EM**, which in itself does not reveal a functional cross-talk in vivo of these systems within the framework of spinal nociceptive transmission. Further investigations are requiered with other models and other endogenous ligands to verify the effect of coadministration of endocannabinoids and endogenous opioids which may be beneficial in the treatment of painful conditions.

4. As regards the investigations with the plant-derived substance vinpocetine, our studies showed, that after perineural application **vinpocetine induced blockade of retrograde axoplasmic transport** which consequently resulted in TDA in the ipsilateral, segmentally related upper dorsal horn of the spinal cord. Disappearance of pain related neuropeptides from the upper dorsal horn can thus be suspected to be a sign of a decreased nociception showing the antinociceptive effect of vinpocetine.

5. According to our behavioral studies with vinpocetine, after perineural application, it caused a decrease in the number of flinchings, shakings and licking, and prevented increased *c-fos* immunoreactivity in the ipsilateral superficial dorsal horn of the spinal cord in rats. These results confirm the **decreased nociception after perineurally applied vinpocetine** assuming that vinpocetine might be effective in alleviating chronic neuropathic pain.

Publications

Full papers related to the Thesis

I. **Farkas I**., Tuboly G., Benedek G., Horváth G. The antinociceptive potency of N-arachidonoyl-dopamine (NADA) and its interaction with endomorphin-1 at the spinal level. Pharmacol Biochem Behav 2011 May. Epub ahead of print. **Impact factor: 2,967**

II. Knyihár-Csillik E., Vécsei L., Mihály A., Fenyő R., **Farkas I**., Krisztin-Péva B., Csillik B. Effect of vinpocetine on retrograde axoplasmic transport. Ann Anat. 2007; 189:39-45. **Impact factor: 0,817**

III. Csillk B., Mihály A., Krisztin-Péva B., **Farkas I.,** Knyihár-Csillik E. Mitigation of nociception via transganglionic degenerative atrophy: possible mechanism of vinpocetine-induced blockade of retrograde axoplasmic transport. Ann Anat. 2008;190:140-145. **Impact factor: 0,932**.

Abstracts related to the Thesis

1. **Farkas I**., Tuboly G., Benedek G., Horváth G. Az endogén N- arachidonoyl-dopamin antinociceptív hatása gerincvelői szinten. *MÉT LXXII. Vándorgyűlése*, Debrecen, 2008, June 4-6.

2. Horváth G., **Farkas I.,** Tuboly G., Benedek G. The antinociceptive effects of intrathecal injection of N-arachidonoyl-dopamine (NADA) are mediated by cannabinoid receptors. *IBRO International Workshop*, Debrecen, 2008, January 24-26.

3. Tuboly G., **Farkas I.**, Benedek G., Horváth G. Antinociceptive potency of N-arachidonoyl-dopamine at spinal level. *European Opioid Conference*, Ferrara, Italy, 2008, April 9-11.

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5. **Farkas I.**, Tubly G., Benedek G., Horváth G. Az endogén N-arachidonoyl-dopamin antinociceptív hatása gerincvelői szinten. *MAT Kongresszus*, Budapest, 2009, June 11-13.

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Acknowledgements

I would like to express my special thanks to **Prof. Dr. György Benedek** for providing the opportunity for me to work at the Department of Physiology many years ago as a medical student, and now again, as a Ph.D. student. I am grateful to him for raising my attention and introducing me into science.

I greatly acknowledge and give special thanks to **Prof. Dr. Gyöngyi Horváth** for allowing me to join her research group, for scientific guidance and support during my Ph.D. course.

I would like to express my deep and sincere gratitude to **Prof. Dr. Bertalan Csillik and Dr. Erzsébet Knyihár** for teaching me and for their continuous encouragement and support throughout my studies. I would like to thank **Prof. Dr. László Vécsei** for giving the opportunity to perform these experiments.

I am much obliged to **Prof. Dr. Gábor Jancsó** for making it possible to work in the neuroscience program as a Ph.D. student and for supporting me in my work at the Department of Physiology. I am grateful to the co-workers at the Department of Physiology, to **Péter Liszli**, **Gabriella Kékesi** and **Gabriella Joó**.

I would like to give special thanks to **Prof. Dr. András Mihály** for his continuous help and to all of the co-workers at the Department of Anatomy, especially to **Beáta Krisztin-Péva**, **Róbert Fenyő**, **Mihály Dezső, Zoltán Imre** and **Andrea Kobolák.**

This work would not have been possible to accomplish without the assistance of **Ágnes Szelepcsényiné Tandari**, **Zita Petrovszki and Valéria Széll.**

I have to say thanks to my current chief **Prof. Dr. Lajos Kemény** for his help and encouragement.

My deepest gratitude goes to **my family** for their love and support throughout my life.
I dedicate this thesis to them.