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

Ph.D. Dissertation

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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., Vecsei 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. Csillik 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.**

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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.
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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.

Abbreviations

AEA	N-arachidonoylethanolamide /anandamide
AUC	area under the curve
BAL	bronchoalveolar lavage
cAMP	cyclic adenosine monophosphate
CB1	cannabinoid receptor type 1
CB2	cannabinoid receptor type 2
CGRP	calcitonin gene-related peptide
CI	confidence intervals
CNS	central nervous system
DMSO	Dimethylsulfoxide
DRG	dorsal root ganglion
ED50	50% effective dose
EM	endomorphin-1
EMT	endocannabinoid membrane transporter
FRAP	fluoride-resistant acid phosphatase
GABA	γ -aminobutyric acid
IKK	kappa B kinase complex
IL-1 β	interleukin-1 β
IT	intrathecal
I κ B	kappa B kinase inhibitor
Leu-enkephalin	leucine-enkephalin
L-Glu	L-glutamate
Met-enkephalin	methionine-enkephalin

MIP-2	macrophage inflammatory protein-2
mRNA	messenger RNA
NADA	N-arachidonoyl-dopamine
NF- κ B	nuclear factor kappa B
NGF	nerve growth factor
ORL-1	opioid-like receptor
P.B.S.	phosphate buffered saline
PDE1	phosphodiesterase 1
PMN	polymorphonuclear neutrophil
PWL	paw withdrawal latencies
RTX	resiniferatoxin
S.E.M.	standard error of the mean
SP	substance P
TDA	transganglionic degenerative atrophy
Δ 9-THC	Δ 9-tetrahydrocannabinol
TMP	thiamine monophosphatase
TNF- α	tumor necrosis factor alpha
TRP	transient receptor potential
TRPV1	transient receptor potential vanilloid type-1

1. Introduction

1.1 Basic mechanisms of pain

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 (Wall, 1995; Knyihár and Csillik, 2006). Damages or inflammation of sensory fibres or the central nervous system (CNS) itself can also cause pain sensation, the so called neuropathic pain. 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, e.g. the opioid morphine, codeine and thebaine, the cannabinoid Δ^9 -tetrahydrocannabinol (Δ^9 -THC) or the vanilloid receptor agonist capsaicin and resiniferatoxin (McCurdy CR and Scully SS, 2005). An exciting and rapidly developing field of pain research relates to the roles of different endogenous ligands. They may have potentially advantageous features: their synthesizing and breakdown enzymes or the mechanism of their excretion are available in the body. In general, they have short half-lives and they may have lower toxicity. On the other hand, most of the endogenous ligands exhibit lower specificity and affinity for their receptors as compared with synthetic drugs and/or they exert their effects at several types of receptors. Accordingly, their effectivity might be lower than that of synthetic drugs, suggesting that these ligands alone would not be ideal drugs for pain therapy (Csullog et al., 2011; Horváth et al., 1999a; Kekesi et al., 2002, 2004a,b). 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 (Fields et al., 1991; Sandkühler, 1996; Horváth and Kekesi, 2006). The investigation of natural products including endogenous ligands can lead not only

to new compounds with idealistic pharmacological profiles (adequate alleviation of pain without side effects and addictive potential), but may also contribute to our better understanding of the complex mechanisms of pain.

1.2. Important systems in the mechanisms of pain influenced by endogenous or plant derived agents

1.2.1. Opioids and their receptors

Opium, the alkaloid produced by Opium poppy is one of the oldest medications known to humans. Its efficacy in relieving pain and diarrhea has been known for thousands of years and opioids (derivatives of opium) have remained the most powerful drugs used for acute and chronic pain. During the nineteenth century, morphine was recognized as the principal alkaloid responsible for most of the beneficial effects of opium. It is also responsible for its undesirable side effects, the most important of which is the development of addiction on chronic use, but respiratory depression, mental clouding and constipation also limit the use of this drug in chronic pain conditions.

Three main classes of opioid receptors have been cloned, the μ -, δ - and κ -opioid receptors (Kieffer et al., 1992; Chen et al., 1993; Yasuda et al., 1993). Each of them belongs to the family of G-protein-coupled metabotropic receptors with opioids as ligands. Upon activation of exogenous or endogenous opioids, opioid receptors couple to inhibitory G-proteins and this leads to direct or (through decrease of cyclic adenosine monophosphate, cAMP) indirect suppression of Ca^{2+} or Na^{+} currents. All three receptors mediate pain inhibition and are found throughout the nervous system, in somatic and visceral sensory neurons, spinal cord projection- and interneurons, midbrain and cortex.

After the cloning of the three known opioid receptors, a fourth member of this family was identified, the opioid-like receptor (ORL-1), which was found not to bind any of the known natural or synthetic opioid ligands, but a 17-amino-acid peptide, named orphanin FQ or nociceptin, a derivative of pronociceptin (Reinscheid et al., 1995). Since the identification of the first endogenous ligands for δ -opioid receptors, methionine-enkephalin (Met-enkephalin) and leucine-enkephalin (Leu-enkephalin) (Hughes et al., 1975), other endogenous substances with opiate receptor affinity have been isolated including β -endorphin (a μ -agonist),

dynorphins (κ -agonists) and more recently a novel group of peptides named endomorphins (endomorphin-1 and endomorphin-2) (Zadina et al, 1997).

1.2.2. Cannabinoids and CB-Receptors

Cannabinoids (CB) are a group of substances that are structurally related to tetrahydrocannabinol (THC), a compound present in Cannabis (*Cannabis sativa* L) and bind to cannabinoid receptors. Cannabinoids can either be plant derived substances (phytocannabinoids), or synthetic drugs (synthetic cannabinoids) and finally endogenous compounds produced in the body of humans or animals (endocannabinoids).

Although new data suggest the existence of novel, yet uncloned cannabinoid receptors, there are two types of known cannabinoid receptors. The cannabinoid receptor type 1 (CB1 receptor) was first cloned from rat cerebral cortex (Matsuda et al., 1990) and can be found mainly in the nervous system: in the hippocampus, basal ganglia, cerebral cortex, amygdala and cerebellum (Herkenham et al., 1990; Glass and Felder, 1997; Tsou et al., 1998). Its localisation is dominantly presynaptical at central and peripheral nerve terminals where it mediates inhibition of transmitter release (Pertwee, 1997; Howlett et al., 2002). CB1 receptors have also been found peripherally among others in the spleen, tonsils, adrenal gland, prostate, uterus and ovary (Galiegue et al., 1995; Pertwee, 1996).

The CB2 receptor was cloned from human promyelocytic leukaemia cells (Munro et al., 1993) and is mainly restricted to the periphery. CB2 receptors have been found in the spleen (Munro et al., 1993., Schatz et al., 1997), in tonsils and on immune cells (B-cells, monocytes and T-cells) one of their roles being to modulate cytokine release (Munro et al., 1993; Galiegue et al., 1995; Schatz et al., 1997). Although often regarded as peripheral receptors, CB₂ receptors have been detected in the central nervous system, for example, on microglial cells (Howlett et al., 2002; Stella, 2010).

Both natural and synthetic cannabinoids potently reduce pain-related behavior in different pain models, and CBs are comparable with opiates in both potency and efficacy (Hohmann, 2002; Pertwee, 2001; Walker et al., 2002). The antinociceptive effects of exogenous and endogenous cannabinoids are exerted primarily via the CB1 receptor, which is coupled with G_i and G_o proteins (Matsuda et al., 1990; Munro et al., 1993). Activation of the cannabinoid CB1 receptor leads to inhibition of adenylyl cyclase activity (Felder et al., 1995; Howlett and Fleming 1984). In addition, CB1 receptor activation inhibits Ca²⁺ currents (Caulfield and Brown, 1992; Twitchell et al., 1997) and modulates various potassium currents (Deadwyler et

al., 1995; Mackie et al., 1995; Poling et al., 1996). The actions of CB₂ receptors are also signaled through the inhibitory G_i and G_o proteins, negatively to adenylate cyclase and positively to mitogen-activated protein kinase (Demuth and Mollemann, 2006). In general, cannabinoids inhibit glutamatergic transmission in the brain (Levenes et al., 1998; Shen et al., 1996; Szabo et al., 2000) and participate in the control of neuronal excitability and firing (McAllister et al., 1999; Mu et al., 1999; Pan et al., 1998; Poling et al., 1996; Schweitzer, 2000; Shen et al., 1996).

A major limitation for the potential use of CB agonists as therapeutic agents is the profile of side effects, which include dysphoria, dizziness, effects on motor coordination, memory and abuse potential (Carlini, 2004; Gardner, 2005). An alternative approach, which may avoid such side effects, is to influence the endogenous CB system. Most of the endocannabinoids are lipid derivatives and their physiological properties have been the target of several studies. Some of these agents, e.g. anandamide (AEA) and N-arachidonoyl-dopamine (NADA) bind not only to the cannabinoid receptors, but also possess agonist properties at the TRPV1 channel (Chu et al., 2003; De Petrocellis et al., 2004; De Petrocellis et al., 2000).

1.2.3. Capsaicin and TRPV1-Receptor

The transient receptor potential (TRP) vanilloid type-1 (TRPV1) receptor was identified due to its responsiveness to the plant derived pungent compound capsaicin isolated from hot chili peppers. TRPV1 also responds to other plant toxins, the most potent of which is resiniferatoxin (RTX) (Szallási & Blumberg, 1999), to temperatures at noxious range and acid, transducing mainly two of the principal types of pain stimuli: thermal and chemical (Caterina et al., 1997, Tominaga et al., 1998). TRPV1 can also be activated by inflammatory agents such as nerve growth factor, bradykinin and others (Tominaga & Caterina, 2004; De Petrocellis & Di Marzo, 2005).

It has been proved that capsaicin sensitive nociceptive neurons are located in the sensory ganglia and that the analgesic effects of capsaicin are accompanied to the degeneration of sensory ganglion cell axons and/or peripheral and central axon terminals (Jancsó et al., 1977; Jancsó, 1992; Simone et al., 1998; Dux et al., 1999; Nagy et al., 2004). It is a nonselective cation channel that belongs to the TRP family proteins and was the first to be cloned among the group of temperature-activated TRP ion channels (Caterina et al., 1997). The central role of TRPV1 receptor in the integration of various noxious stimuli and in the transmission of nociceptive information has also been well established (Nagy et al., 2004; Jancsó et al., 2008;

Holzer, 2008; Knotkova et al., 2008). The existence of endogenous ligands for TRPV1 receptor or „endovanilloids” was also suggested and N-arachidonoyl-ethanolamine (anandamide, AEA, known also to be a ligand of cannabinoid receptors) was first shown to act as endogenous activator for TRPV1 (Zygmunt et al., 1999). One year later another endogenous arachidonic acid derivative, N-arachidonoyl-dopamine was identified sharing endovanilloid and endocannabinoid biological activities (Bisogno et al., 2000).

1.3. Agents used in our experiments

1.3.1. Endomorphin-1

Endomorphins have been found unequally in the brain, they are stored in neurons and axon terminals with heterogeneous distribution, they are converted enzymatically by endopeptidases and they interact specifically and with high affinity with μ -opioid receptors (Horváth, 2000; Zadina et al., 1997). They differ from conventional endogenous opioid receptor ligands in their N-terminal sequence, peptide length and C-terminal amidation. It has been hypothesized that they possess partial rather than full agonist properties at μ -opioid receptors (Sim et al., 1998). It has also been suggested that they may produce antinociception through different subtypes of μ -opioid receptors, endomorphin-1 acting predominantly on the μ_2 -opioid receptor, while endomorphin-2 on the μ_1 -opioid receptor.

In studies using radioimmunoassay and immunocytochemistry endomorphin-1 was localized mostly in the thalamus, hypothalamus, cortex and striatum in the bovine brain and in the superficial dorsal horn of the spinal cord in rats (Zadina et al., 1997; Martin-Schild et al., 1999). Endomorphin-1 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 (Sbrenna et al., 1999).

In acute inflammatory heat-pain tests similar potencies of the endomorphins were found after intrathecal administration (Horváth et al., 1999), whereas Tseng et al. (2000) found a higher potency in the hot-plate test than in the tail-flick test. Intrathecal administration of endomorphin-1 (EM) dose-relatedly reduced both the C- and A β -fiber responses to electrically evoked noxious stimuli in the rat (Chapman et al., 1997). When intraplantarly injected, it produced a dose-dependent reduction of carrageenan-induced c-fos expression in the spinal cord, which could be prevented by the administration of naloxone (Jin et al., 1999).

In contrast to morphine, endomorphin-1 has short-lasting effects and there is also evidence suggesting a plateau effect and acute tolerance (Horváth et al., 1999; Horváth, 2000; Sanchez-Blazquez et al., 1999; Stone et al., 1997).

1.3.2. NADA

N-arachidonoyl-dopamine was identified as an endogenous ligand at both TRPV1 and cannabinoid CB1 receptors (Bisogno et al., 2000; Chu et al., 2003; De Petrocellis et al., 2004; Huang et al., 2002; Toth et al., 2003). 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) (Huang et al., 2002). It is the first endogenous compound identified in mammals that is almost equipotent to capsaicin at TRPV1 receptor (Huang et al., 2002). In addition, NADA also behaves as an agonist at CB1 receptor with an affinity similar to that of anandamide, the first identified endocannabinoid (Devane et al., 1992).

Some studies have shown its effects on pain threshold after different routes of administration (Bisogno et al., 2000; Huang et al., 2002; Huang and Walker, 2006; Pitcher et al., 2007; Price et al., 2004). NADA elicited analgesia following systemic administration (Bisogno et al., 2000). It caused nocifensive behavior when applied on the cornea, and it produced hyperalgesia when administered into the plantar skin of the hind paw (Huang et al., 2002; Price et al., 2004). As regards its effects after intrathecal (IT) administration, Pitcher et al. have found that NADA produced mechanical allodynia in mice (Pitcher et al., 2007). Our earlier data showed that TRPV1 receptor activation plays a substantial role in the antinociceptive effects of anandamide at spinal level (Horváth et al., 2008). Since NADA is more potent at TRPV1 than anandamide (De Petrocellis et al., 2000), it might be a more suitable endogenous ligand for the investigation of the role of TRPV1 receptors in the antinociception.

1.3.3. Vinpocetine

Vinpocetine (ethyl-apovincamate, Cavinton^R, Gedeon Richter, Budapest, Hungary) is a synthetic compound, but structurally related to the Vinca minor alkaloid vincamine, first synthesized by Lőrincz et al. (Lőrincz et al., 1976). Vinpocetine is a potent inhibitor of the voltage-dependent Na⁺ channels and the Ca²⁺/Calmodulin-dependent phosphodiesterase 1

(PDE1) (Jeon et al., 2010). Known for its minimal side effects (Balestreri et al., 1987; Szatmári et al., 2003) it is widely used in clinical pharmacotherapy of various cerebral circulatory diseases (Bonoczk et al., 2000), both *per os* and parenterally.

Although the molecular mechanism of its action has not yet been completely clarified, the blockage of voltage-dependent Na^+ channels appears to be a relevant and significant factor with regard to the neuroprotective effect of vinpocetine. Vinpocetine inhibited Na^+ currents in cortical neurons similar to the Na^+ -channel blocker phenytoin (Molnar and Erdő, 1995; Erdő et al., 1996). It was also able to reduce $[\text{Na}^+]_i$ rise induced by the neurotoxic agent veratridine in isolated cortical nerve terminals (Tretter and Adam-Vizi, 1998) proving that the protection of neurons against a Na^+ load may play an important role in the neuroprotective effect of vinpocetine. In addition to inhibiting Na^+ channels vinpocetine is capable of interacting with glutamate receptors (Kaneko et al., 1991; Kiss et al., 1991; Miyamoto et al., 1989), which also might have a special importance in the therapeutic effects of this drug.

It facilitates long-term potentiation (Molnár et al., 1994), improves spatial memory in animal models (Deshmukh et al., 2009; Filgueiras et al., 2010) and enhances performance on cognitive tests in humans (Hindmarch et al., 1991). Clinical and non-clinical investigations using positron emission tomography, near infrared spectroscopy and transcranial Doppler methods showed that intravenous or oral administration of vinpocetine leads to enhancement of regional brain glucose metabolism rate and cerebral blood flow in patients with chronic ischaemic post-stroke state. It also enhanced cerebral perfusion and parenchymal oxygen extraction in monkeys and humans (Vas et al., 2002; Gulyás et al.).

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- κ B), a key transcriptional factor (Jeon et al., 2010). NF- κ B is involved in the regulation of expression of proinflammatory mediators, including cytokines, chemokines and adhesion molecules (Rothwarf and Karin, 1999). *In vitro* vinpocetine inhibited tumor necrosis factor alpha (TNF- α)-induced NF- κ B-activation and the subsequent up-regulation of proinflammatory mediators in multiple cell types, including vascular smooth muscle cells, human umbilical vein endothelial cells, macrophages and lung epithelial cells. It also inhibited TNF- α -induced monocyte adhesion and chemotaxis. *In vivo*, intraperitoneally administered vinpocetine dose-dependently inhibited the induction of TNF- α , interleukin-1 β (IL-1 β), and macrophage inflammatory protein-2 (MIP-2) mRNA expression in the lungs and significantly inhibited polymorphonuclear neutrophil (PMN) infiltration in bronchoalveolar lavage (BAL) fluids in mice after intratracheal administration

of LPS or TNF- α . It was also shown that vinpocetine acts by the direct suppression of the inhibitor of kappa B kinase complex (IKK), preventing the degradation of the kappa B kinase inhibitor (IkB) and the consequent translocation of NF- κ B to the nucleus. Surprisingly, this mechanism was proved to be independent of vinpocetine action on PDE1 (Jeon et al., 2010; Medina, 2010).

2. Aims of our studies

In our studies we investigated the effects of plant-derived and endogenous compounds on the mechanisms of pain in the rat primarily at spinal level.

NADA

The first goal of this study was to characterize the antinociceptive potential of NADA on inflammatory thermal hyperalgesia in rats at spinal level.

The second goal was to characterize the role of CB1 and TRPV1 receptors in the effects of NADA.

There are a number of endogenous ligands that have antinociceptive effects in the central nervous system, thus, it is highly unlikely that they work alone, most likely they rather work in concert in the normal animals. The synergistic antinociceptive interactions between synthetic and plant-originated cannabinoids and opioids have been shown, but only few results are available yet about the interaction of endogenous ligands acting on these receptors (Cichewicz, 2004; Tuboly et al., 2009; Welch and Eads, 1999). Our recent study showed that anandamide potentiated the antinociceptive effects of endomorphin-1 (EM) at specific doses following IT administration (Tuboly et al., 2009). Both EM and NADA can influence the spinal dorsal horn neurons since their receptors are available pre- and/or postsynaptically (Coggeshall and Carlton, 1997; Horváth, 2000; Howlett, 2002; Huang et al., 2002).

Therefore, **the third aim** of the present study was to determine the interaction of NADA with EM at spinal level.

Vinpocetine

Central terminals of primary nociceptive neurons, terminating in lamina I and II of the dorsal horn in the form of scalloped terminals contain pain-related neuropeptides (substance P (SP)) and calcitonin gene-related peptide (CGRP)), and the marker enzymes fluoride-resistant acid phosphatase (FRAP) and thiamine monophosphatase or TMP (Knyihár, 1971; Knyihár-Csillik et al., 1986). In a series of publications (Knyihár and Csillik, 1976; Csillik et al., 1977,

Csillik et al., 1985 and Csillik et al., 2003; Knyihár-Csillik and Csillik, 1981; Csillik and Knyihár-Csillik, 1986), it has been shown that blockade of retrograde axoplasmic transport in a sensory nerve results in transganglionic degenerative atrophy (TDA) of central terminals of the affected primary nociceptive neurons. Blockade of axoplasmic transport was achieved formerly by perineural application of the microtubule inhibitors vincristin and vinblastin. TDA, causing an intermittent disruption of the nociceptive pathway, resulted in temporary loss of pain in the area supplied by the related sensory neurons. Based on these findings, transcutaneous iontophoresis of vincristin and vinblastin was successfully used in order to alleviate chronic pain deriving from postherpetic neuralgia, toxic polyneuropathies and terminal oncological states (Csillik et al., 1982; Knyihár-Csillik et al., 1982; Szűcs et al., 1984; Csillik and Knyihár-Csillik, 1986; Tajti et al., 1989; Rossano et al., 1989). However, this treatment has been discontinued for fear of unforeseen hepatotoxic side effects, that might arise from the usage of vincristine and vinblastine, known to be microtubule inhibitors. Accordingly, our interests were drawn to another vinca alkaloid, vinpocetine, which is proven to be devoid of any side effects (Kemény et al., 2005).

In our first study with vinpocetine, light- and electron microscopic effects of perineurally applied vinpocetine, upon the marker enzymes (FRAP and TMP), and the pain-related neuropeptide SP will be described in the ipsilateral, segmentally related upper dorsal horn of the spinal cord. The effect of vinpocetine on the retrograde axoplasmic transport of nerve growth factor (NGF) in a peripheral nerve will be reported on the basis of radiochemical studies.

In the second study with vinpocetine, the effect of perineural vinpocetine treatment in a nociceptive model will be reported on the basis of behavioral experiments and the expression of *c-fos* in the dorsal horn of the spinal cord, known to represent increased synaptic activity. On these premises, it is suggested that transcutaneous iontophoresis of vinpocetine might be promising to the pain clinician, aiming to alleviate chronic, neuropathic pain.

3. Materials and Methods

3.1. In vivo experiments with NADA

3.1.1. Intrathecal catheterization

The procedures involved in animal surgery and testing were approved by the Institutional Animal Care Committee of the University of Szeged, Faculty of Medicine. 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 (PE-10 tubing; Intramedic, Clay Adams; Becton Dickinson; Parsippany, NJ; I.D. 0.28mm; O.D. 0.61 mm) was inserted via the cisterna magna and passed 8.5 cm caudally into the subarachnoid space (Yaksh and Rudy, 1976), which served to place the catheter tip between Th12 and L2 vertebrae, corresponding to the spinal segments that innervate the hindpaws (Dobos et al., 2003). After surgery, the rats were housed individually and had free access to food and water. Animals exhibiting postoperative neurologic deficits (about 10%), and also those ones that did not show paralysis of one of the hindpaws (about 0.5%) after the administration of 100 μ g lidocaine were excluded (Dobos et al., 2003). After the surgery, animals were administered with antibiotic therapy (13 mg/kg gentamicin, subcutaneously) to prevent infection. 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.

3.1.2. Drugs

The following drugs were used: ketamine hydrochloride (Calypsol, Richter Gedeon RT, Budapest, Hungary), xylazine hydrochloride (Rompun, Bayer, Leverkusen, Germany), Gentamycin-Chinoin (Sanofi-Aventis, Budapest, Hungary), EM, NADA, AM 251 (CB1 receptor antagonist/inverse agonist) and AMG 9810 (TRPV1 receptor antagonist) (all were purchased from Sigma-Aldrich; Budapest, Hungary). EM was dissolved in saline, NADA was originally in ethanol solution, and it was diluted with saline, and the final concentration of ethanol was 10 %. AM 251 and AMG 9810 were dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich, Ltd., Budapest, Hungary) and ethanol and further diluted with distilled water. The final concentration of DMSO and ethanol was 15% and 9% respectively. IT administered

drugs were injected over 120 s in a volume of 10 μ l, followed by a 10 μ l flush of physiological saline. Our preliminary experiments did not show significant differences between the different vehicle-treated rats, therefore, our control group was formed by the 10% ethanol treated animals.

3.1.3. 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. A detailed description of this model has been published by Hargreaves et al. (Hargreaves et al., 1988). Briefly, rats were placed on a glass surface in a plastic chamber and were allowed to acclimatize to their environment for 15-30 min before testing. A heat stimulus was directed onto the plantar surface of each hindpaw, and the intensity of the thermal stimulus was adjusted to derive an average baseline latency (the time to withdrawal of the hind paw from the heat source, measured in seconds) 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) (Dobos et al., 2003). 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, AM251, AMG 9810, 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.

3.1.4. Experimental paradigm

The first series of experiments was performed to determine the dose response effect and time course for IT administered NADA (1.5, 5, 15 and 50 μ g) and EM (0.03, 0.1, 0.3, 1, 3 and 10 μ g). In the second series, 15 or 50 μ g NADA were co-administered with antagonist, either AM 251 (10 μ g) or AMG 9810 (0.3 μ g). The dose of antagonists was based on earlier results (Pitcher et al., 2007; Succar et al., 2007; Trang et al., 2006). The third series of experiments was performed with 1:15 and 1:50 ratios of EM and NADA in order to determine their interaction.

3.1.5. Statistical analysis

Data are presented as means $\pm$ SEM. The area under the curve (AUC) values were obtained by calculating the area between 5 and 90 min following IT-injection to construct dose response curves for different doses of NADA alone and/or with EM. AUC 828 (AUC_{max}) value would mean the complete relief of hyperalgesia (PWL: 9.74 s; the mean pre-carrageenan baseline value) during the whole period. We observed almost no effects as regards the AUC values after saline treatment (AUC_{min} = 281 ± 21.6). The mean AUC values were used for linear regression analysis (least square method) to determine the ED₅₀ values with 95% confidence intervals (CI). The 50% effective dose (ED₅₀) would mean the dose that yielded 50% increase in the PWD latency for the whole period ($[AUC_{max} + AUC_{min}]/2 = 554$). The AUC data sets were examined by one-way analysis of variance (ANOVA), and the time-course curves were analyzed by repeated measurement of ANOVA. *Post hoc* comparisons were carried out with the Bonferroni test.

To determine whether the interaction between the agents is additive or synergistic, isobolographic analyses were used according to earlier results (Tallarida et al. 1989; Ossipov et al., 1997). Tallarida et al. have provided a statistical interpretation of isobolographic data in which the experimental ED₅₀ of a mixture could be compared with a theoretic additive ED₅₀. A line of additive interaction was estimated by connecting the ED₅₀ for NADA with EM. For the ratios of drugs used, theoretic additive ED₅₀ values can be calculated with the confidence intervals (Tallarida, 1989). The theoretic additive points were then compared with the experimentally derived ED₅₀ for the mixture by means of a *t* test. A significant potency ratio with the experimental ED₅₀ significantly less than the theoretic additive ED₅₀ indicates a synergistic interaction.

A *p* value less than 0.05 was considered significant. Statistics were performed by STATISTICA (Statistica Inc., Tulsa, Oklahoma, USA) and GraphPad Prism (GraphPad software Inc. La Jolla, California, USA) softwares.

3.2. In vitro experiments with Vinpocetine

3.2.1. Perineural application of vinpocetine

Investigations were performed on 32 ($n = 5-7/\text{group}$) young adult male Wistar rats, 200–250 g body weight. Care of the animals complied with the guidelines of the Hungarian Ministry of Welfare; experiments were carried out in accordance with the European Communities Council Directive (November 24, 1986; 86/609/EEC) and the Guidelines for Ethics in Animal Experiments of the Szeged University, Albert Szent-Györgyi Medical School.

In experiments aiming to study the effect of vinpocetine on retrograde transport, a slab of Gelita tampon (Braun, Melsungen, Germany) measuring $5 \times 5 \times 20$ mm, was soaked in 10^{-6} , 10^{-7} or 10^{-8} M vinpocetine (Sigma, catalog number V 6383) 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, muscles were prepared with blunt forceps in order to avoid bleeding. The sciatic nerve was gently exposed; special care was taken not to crush or even to pinch the nerve trunk. The Gelita slab soaked in the solution to be tested was applied carefully 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, than the wounds were temporarily closed by hemostats. 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 as described below.

3.2.2. Histological techniques

For histochemical and immunohistochemical investigations, animals were anesthetized with 4% chloral hydrate intraperitoneally and subjected to transcardial perfusion with a formaldehyde solution containing picric acid (Zamboni and De Martino, 1965) to which glutaraldehyde was added. The spinal cord was excised and the distribution of FRAP and TMP was studied in $15 \mu\text{m}$ frozen cross-sections, according to the technique described earlier (Knyihár-Csillik et al., 1982 and Knyihár-Csillik et al., 1986; Csillik et al., 1986). SP immunoreactivity was visualized by means of the techniques described by Csillik et al. (2003).

3.2.3. Electron microscopic studies

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 Ultratome. Thin sections, silver interference color, were stained with lead citrate and studied with a Zeiss Opton 902 electron microscope (Oberkochen, Germany).

3.2.4. 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. NGF prepared from the murine submaxillary gland was obtained from Sigma (St. Louis, MO, USA); ^{125}I labeling was performed according to the conventional technique described by Rohrer and Barde (1981). 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.

3.2.5. 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 (FRAP, TMP) or immunocytochemistry (SP) with a SPOT RT Slider CCD camera (1600 $\times$ 1200 pixels, 8 bits) attached to a Nikon Eclipse E600 microscope using a 16 $\times$ front lens and a 10 $\times$ eyepiece. The captured images were analyzed by ImageProPlus v4.5 morphometric software (Media Cybernetics, Silver Spring, MD, USA). Areas of interest were rectangular areas measuring 200 $\times$ 200 μm . Fifteen rectangular fields were analyzed per animal; each field was chosen randomly in different sections in a blinded manner. Gray values of the selected areas were obtained using a ScionNIH image analysis software. The images were captured directly from the microscopic slides, using a 5 $\times$ objective lens by means of a

black and white camera Cohu CCD and displayed on a computer monitor. The software automatically assigned the average gray value of the screen pixels corresponding to the outlined area; a value of 0 indicated a white pixel and 225 indicated a black pixel.

3.3. In vivo experiments with Vinpocetine

3.3.1. Studies of the behavioral effects of vinpocetine

Investigations were performed on 15 young adult male albino Wistar strain rats ranging from 200 to 250 g in body weight. Care of the animals complied with the guidelines of the Hungarian Ministry of Welfare; experiments were carried out in accordance with the European Communities Council Directive (November 24, 1986; 86/609/EEC) and the Guidelines for Ethics in Animal Experiments of the Szeged University, Albert Szent-Györgyi Medical School. Vinpocetine (eburnamine-14-carboxylic acid ethyl ester) was obtained from Sigma-Aldrich (Saint Louis, MO, USA), catalog number V 6383.

The pain behavioral effect of subcutaneous formalin treatment was studied in eight rats by means of the formalin test of Dubuisson and Dennis (1977). A dilute formalin solution (10%, 10 μ L) was injected subcutaneously into the plantar surface of a hind paw using a 27-gauge syringe needle. This produced flinching, shaking and licking; the short-lived acute phase is comparable to acute nociception, while the second phase represents tonic pain. The number of flinches+shakes of the injected paw was counted each minute for a period of 60 min. The number of flinches+shakes was averaged into 3-min periods, according to a technique described recently by Butkevich et al. (2005).

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 μ L) was injected subcutaneously into the plantar surface of the hind paw using a 27-gauge syringe needle, and the number of flinches+shakes of the injected paw was counted as in the control experiments.

3.3.2. Immunohistochemistry

The immunohistochemical equivalent of perineural vinpocetine treatment was assessed on the basis of *c-fos* expression in the spinal cord of rats (Butkevich et al., 2006). One hour after the behavioral testing, rats were deeply anesthetized with 4% chloral hydrate and perfused transcardially with cold 4% paraformaldehyde buffered by 0.1 M phosphate saline. After perfusion, the lumbar spinal cord was excised and post-fixed in the same fixative overnight. After cryoprotection in an ascending series of sucrose, 15 μ m serial frozen sections were cut in a cryostat. Sections were pre-incubated in 0.5% hydrogen peroxide for 10 min, processed for immunohistochemical staining and incubated for 48 h at 4 °C in rabbit anti-Fos (Calbiochem, CA), diluted 1:2000 in PBS with Triton X. After rinsing, the sections were transferred into the second antibody for 1 h (goat anti-rabbit, Vector, Burlingame, CA) and processed according to the ABC kit protocol. Visibly labelled *c-fos* immunoreactive nuclei in the superficial dorsal horn (laminae I and II) were counted in a 0.1 mm² square and the mean number of *c-fos* expressing cells was calculated by averaging seven sections.

In evaluating the effects of perineural vinpocetine treatment, the Mann–Whitney test and Student's *t*-test were used; for repeated measures, ANOVA was employed. In the graphical representation of the data obtained the means $\pm$ S.E.M. are illustrated. A value of $p<0.05$ was considered statistically significant.

4. Results

4.1. The antinociceptive potential of NADA at spinal level

The basal thermal withdrawal latency was 9.74 ± 0.06 s. Carrageenan caused a significant decrease on PWL at the inflamed hind paw (2.97 ± 0.06 s), whereas the threshold contralaterally did not change significantly (9.67 ± 0.12 s). Higher doses of EM (≥ 1 μ g) alone and in combinations produced a short-lasting antinociception (between 5 and 10 min) contralaterally, but no other significant changes occurred (data are not shown), therefore, further analyses were performed ipsilaterally. Neither AM 251 nor AMG 9810 produced any effects compared to the control group (data are not shown).

NADA alone resulted in a dose-dependent effect (Fig 1A). ANOVA with repeated measurements showed significant effects of treatment ($F_{4,47}=16.8$, $p<0.001$), time ($F_{11,517}=34.5$, $p<0.001$), and interaction ($F_{44,517}=7.2$, $p<0.001$). The highest dose produced a prolonged antihyperalgesic effect - an increase of PWL back to pre-carrageenan baseline. The ED₅₀ value was 22.5 (CI: 15.1-30.5) μ g for the whole period. However, it should be mentioned that the highest dose (50 μ g) also caused temporary vocalization and excitation during injection, suggesting a pain-inducing potential of NADA.

4.2. The role of CB1 and TRPV1 receptors in the antinociceptive effects of NADA

With respect to interaction on the CB1 and TRPV1 receptors, AM 251 did not influence the antinociceptive effect of 15 μ g NADA, but significantly decreased the effect of 50 μ g NADA, while AMG 9810 significantly decreased the effects of both doses of NADA (ANOVA results for AUC with 15 μ g NADA: $F_{5,96}=16.08$, $p<0.0001$; with 50 μ g NADA: $F_{5,96}=17.79$, $p<0.0001$; Fig. 1B and 1D).

Figure 1. Time-course effects of NADA (A) and EM (C) and the AUC values of the effects of CB1 (AM 251, 10 μ g) and TRPV1 (AMG 9810, 0.3 μ g) receptor antagonists on the effects of NADA (15 and 50 μ g, B and D, respectively). Each point denotes the mean $\pm$ SEM of the results. Symbol * indicates a significant ($p < 0.05$) difference as compared with the vehicle treated group. + denotes a significant difference ($p < 0.05$) from NADA treated groups. Number of animals in the different groups is indicated in parentheses.

4.3. The interaction of NADA with EM at spinal level

As regards the effect of EM, ANOVA with repeated measurements showed significant effects of treatment ($F_{6,58}=31.5$, $p<0.001$), time ($F_{11,638}=164.5$, $p<0.001$), and interaction ($F_{66,517}=3.3$, $p<0.001$). The lower doses of EM alone caused dose-dependent short-lasting antihyperalgesia at 5th and 10th min, while the higher doses (3 and 10 μg) produced prolonged effects (Fig 1C). The ED₅₀ value was 4.2 (CI: 3.1-5.3) μg for the whole period.

Regarding the interaction of NADA and EM, in ratio 1:15 the lowest dose-combination (EM:NADA = 0.3:5 μg) was effective for almost all the investigated period, while the drugs alone did not produce a pronounced effect (Fig 2A). Surprisingly, the combination of 1 μg EM with 15 μg NADA produced shortened antinociception compared to NADA treatment (Fig 2B), while the highest dose-combination was more effective than the ligands alone only in two time points (Fig 2C). The combinations in 1:50 ratio produced significant antihyperalgesia in dose of 0.1:5 μg (EM:NADA) for 30 min (Fig 3B) and a higher dose-combination (EM:NADA = 0.3:15 μg) produced a temporary potentiation (Fig 3C). In the other two combinations the effects were similar to the NADA treatment alone (Fig 3A and D).

Figure 2. Time-course effects of different combinations of EM and NADA in 1:15 ratio. Each point denotes the mean $\pm$ SEM of the results. Symbol * indicates a significant ($p < 0.05$) difference as compared with the vehicle treated group. ° denotes significant difference from all the other groups. Number of animals in the different groups is indicated in parentheses.

Figure 3. Time-course effects of different combinations of EM and NADA in 1:50 ratio. Each point denotes the mean $\pm$ SEM of the results. Symbol * indicates a significant ($p<0.05$) difference as compared with the vehicle treated group. ^o denotes significant difference from all the other groups. Number of animals in the different groups is indicated in parentheses.

The dose-response curves and the isobolographic analysis of AUC values of EM-NADA combinations indicated that the experimentally derived ED₅₀ values did not differ significantly from the theoretical ED₅₀ values in both 1:15 and 1:50 ratios, indicating an additive interaction (Figures 4 and 5).

Figure 4. The magnitude of the dose-dependent effects of EM and NADA by themselves and their combinations (AUC values between 5-90 min) in 1:15 (A) and 1:50 (B) ratios.

Figure 5. ED₅₀ isobologram (additive line-) with confidence interval (---) for the interaction of EM and NADA. Points shown are the ED₅₀ values for EM and NADA alone (●), and the ED₅₀ values for combinations in ratios of 1:15 (*) and 1:50 (+).

4.4. Effect of perineurally applied vinpocetine on retrograde axoplasmic transport

Perineurally applied vinpocetine (Fig. 6), at a concentration of 10^{-6} M, caused disappearance of FRAP and TMP from the ipsilateral, segmentally related Rolando substance (lamina II) of the dorsal horn, within 7 days after application. At the same time, there was no alteration seen contralaterally (Figures 7 and 8). If applied at a concentration of 10^{-7} or 10^{-8} M, vinpocetine caused only a slight reduction in the histochemical enzyme reaction. At a concentration of 10^{-9} M, perineurally applied vinpocetine did not cause any alteration.

Figure 6. Structural formula of vinpocetine (a) and the Vinca alkaloids vincristin and vinblastin (b).

Figure 7. TMP activity of the Rolando substance at the spinal cord level L5 after unilateral perieneural vinpocetine treatment (apparent right). Note virtual absence of TMP reaction at the projection of the cuff treatment (arrow) as contrasted with the intense and uninterrupted enzyme reaction outlining lamina II contralaterally.

At the electron microscope level, thousands of scalloped axon terminals, displaying FRAP enzyme reactions at their surface membranes could be observed under normal conditions (Fig. 8a), which resulted in an enzyme active line in lamina II at the level of the light microscope. After vinpocetine treatment of the sciatic nerve, depletion of FRAP was accompanied by the formation of enzyme inactive labyrinthine structures (Fig. 8b).

Figure 8. Electron microscopic appearance of FRAP under normal conditions in a scalloped axon terminal (S) in the Rolando substance (a) and formation of an axonal labyrinth (arrow) devoid of any marker enzyme activity, 7 days after application of a 10^{-6} M containing Gelita tampon cuff around the sciatic nerve (b).

The effect of vinpocetine upon SP immunoreaction was evident but less conspicuous. These neuropeptides are known to be localized in lamina I and II but to a lesser degree also in lamina III of the dorsal horn. Perineural application of a Gelita tampon cuff containing 10^{-6} M vinpocetine induced a decrease in SP immunoreactivity in the ipsilateral, segmentally related upper dorsal horn (Fig. 9). The effects of 10^{-7} , 10^{-8} , and 10^{-9} M vinpocetine were equivocal.

Figure 9. Substance P immunoreactivity of the upper dorsal horn at the spinal cord level L5 after unilateral perineural vinpocetine treatment (10^{-6} M) of the sciatic nerve (apparent right). Note decreased substance P immunoreaction at the site of projection of the cuff treatment (arrow), as contrasted to the intense immunoreaction outlining lamina I and II contralaterally (arrow with asterisk).

Densitometric measurements of the histochemical and immunohistochemical reactions revealed that TMP was decreased highly ($p < 0.01$) significantly in the medial aspect of the ipsilateral spinal dorsal horn at levels corresponding to the representation of vinpocetine treatment of the sciatic nerve (Table 1.) Contralaterally, there could not be seen any alterations.

Table 1

Densitometric values of TMP in the Rolando substance. Areas of interest (AOI):Lateral (L): 200x200 μm ; Intermediate (I): 200x500 μm ; Medial (M): 200x200 μm .

Left (control)			Right (vinpocetine treated)		
Lateral	Intermediate	Medial	Medial	Intermediate	Lateral
80 ± 20	78 ± 20	80 ± 20	10 ± 9	41 ± 15	80 ± 20

n = 7;

Significances left versus right:

L: p = 1; I: p < 0,04; M: p < 0,01

Decrease of ipsilateral SP immunoreactivity at spinal cord levels corresponding to the representation of vinpocetine treated sciatic nerve (Table 2.) was also statistically significant (p<0.05).

Table 2

Densitometric values of Substance P in Lamina I-II-III of the spinal cord. Areas of interest (AOI):Lateral (L): 200x200 μm ; Intermediate (I): 200x500 μm ; Medial (M): 200x200 μm .

Left (control)			Right (vinpocetine treated)		
Lateral	Intermediate	Medial	Medial	Intermediate	Lateral
35 ± 22	25 ± 10	10 ± 9	11 ± 8	21 ± 12	10 ± 8

n = 7;

Significances left versus right:

L: p < 0,05; I: p = 1; M: p = 1

Radiochemical measurements of [^{125}I] NGF in 0.5 cm long portions of the sciatic nerve revealed that in control animals, where the sciatic nerve was surrounded by a Gelita tampon cuff soaked in isotonic saline, radioactivity of [^{125}I] NGF was more or less evenly distributed throughout the entire length of the sciatic nerve. Concentration of radioactivity was seen in the related dorsal root ganglia (Fig. 10).

Figure 10. Distribution of radioactivity in the sciatic nerve, in dorsal root ganglion L5 and in the related dorsal root, 15 h after injection of 600 ng (50 μ Ci) of 125 I-labelled NGF- β under the skin of the hind paw, 72 h after perineural application of physiological saline in a Gelita tampon cuff. #1 is the most distal portion, #13 is the dorsal root ganglion and #14 is the dorsal root. The isotonic saline cuff was applied at portions #7 and #8 (arrows).

After *perineural treatment* of the sciatic nerve with 10^{-6} M vinpocetine, retrograde transport of 125 I-labeled NGF was virtually blocked at the site of the cuff. A conspicuous accumulation of 125 I was observed just distal to the cuff and in the region of the cuff itself. In portions proximal to the cuff, as well as in dorsal root ganglia and dorsal roots, radioactivity was significantly lower than in controls (Fig. 11). After perineural treatment of the sciatic nerve with 10^{-7} or 10^{-9} M vinpocetine containing cuffs, the retrograde transport of 125 I-labeled NGF was virtually unchanged.

Figure 11. Distribution of radioactivity in the sciatic nerve, in dorsal root ganglion L5 and in the related dorsal root, 15 h after injection of 600 ng (50 μ Ci) of 125 I-labelled NGF- β under the skin of the hind paw, 72 h after perineural application of 10^{-6} M vinpocetine in a Gelita tampon cuff. #1 is the most distal portion; the vinpocetine cuff was applied at #7 and #8 (arrows); #13 is the dorsal root ganglion and #14 is the dorsal root. Asterisks indicate significant alterations (increase in #6; decrease in #13) as compared to control experiments.

4.5. The effect of perineural vinpocetine treatment in a nociceptive model

Subcutaneous injection of formalin into the plantar surface of a hind paw induced redness and swelling in the ipsilateral paw, followed by numerous flinchings, shakings and licking (Figure 12, filled circles). Perineural application of a Gelita tampon sponge soaked in 10^{-6} M vinpocetine, 72 h prior to formalin injection, did not affect redness and swelling of the paw, but reduced the number of flinchings, shakings and licking (Figure 12, empty circles). Immunohistochemical investigations demonstrated significant differences in *c-fos* expression between control and vinpocetine-treated animals.

Figure 12. Numbers of flinchings, shakings and licking in a unilateral formalin test (filled circles) and 3 days after perineural application of vinpocetine (empty circles). Number of animals tested was $n=7$ in both cases. The difference between the two curves is significant ($p<0.05$).

As shown in Figure 13 and Figure 14, the number of nerve cell nuclei displaying *c-fos* immunoreactivity in the superficial dorsal horn was significantly increased after formalin treatment, as contrasted to normal controls. The number of *c-fos*-expressing neurons in the segmentally related upper dorsal horn was 32 ± 6 on the formalin-injected side and 5 ± 1 on the control side.

Figure 13. Distribution of *c-fos* immunoreactive nuclei in sgment L5 of the spinal cord, 1 h after unilateral application of the formalin test (apparent right). Number of *c-fos* immunoreactive cells was determined in the rectangular area. Note that the number of *c-fos* immunoreactive cells increased tremendously as a consequence of the application of the formalin test.

Figure 14. Number of *c-fos* immunoreactive cells in the superficial dorsal horn 1 h after unilateral application of the formalin test (black column) as contrasted to the contralateral superficial dorsal horn (white column).

Figure 16. Number of *c-fos* immunoreactive cells in the superficial dorsal horn, 3 days after perineural application of vinpocetine and 1 h after unilateral application of the formalin test (black column) and in the contralateral superficial dorsal horn (white column).

In contrast, perineural application of a vinpocetine cuff (10^{-6} M) 3 days prior to formalin treatment prevented increased expression of *c-fos* in the ipsilateral, segmentally related superficial dorsal horn. Three days after application of a vinpocetine cuff (10^{-6} M), the number of *c-fos*-expressing cells increased only to 6 ± 3 on the formalin-injected side and to 5 ± 2 on the control side (Figure 15 and Figure 16).

Figure 15. Distribution of *c-fos* immunoreactive nuclei in segment L5 of the spinal cord, 3 days after perineural application of vinpocetine (10^{-6} M) on the sciatic nerve, and 1 h after unilateral formalin testing (apparent right). Number of *c-fos* immunoreactive cells was determined in the rectangular area outlined in Figure 13.

5. Discussion

5.1. The antinociceptive potential of NADA at spinal level

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. The co-administration of EM produced short-lasting potentiation of NADA antinociception, however, the isobolographic analysis of AUC values for the whole investigated period showed additive interaction.

The dual effects of NADA at TRPV1 and CB1 receptors have been confirmed in some earlier studies. Thus in vitro application of NADA to DRG neurons and insulinoma β -cells causes membrane depolarization and a significant increase in intracellular calcium, which are blocked by both TRPV1 and CB1-receptor antagonists (De Petrocellis et al., 2007; Sagar et al., 2004). NADA leads to the release of substance P and calcitonin gene related peptide (CGRP) from DRG, trigeminal ganglion neurons via activation of TRPV1 receptors (Huang et al., 2002; Price et al., 2004). Furthermore, NADA increases or decreases glutamatergic synaptic transmission to dopaminergic neurons via TRPV1 and CB1 receptor, respectively (Marinelli et al., 2007). It has also been established that NADA needs to be taken up by cells via the endocannabinoid membrane transporter (EMT) to interact with the TRPV1 receptors. After inhibition of its reuptake, NADA acts as a selective CB1 agonist suggesting that EMT plays a key role in modulating the stimulation of TRPV1 or CB1 receptors (Marinelli et al., 2007). It is well known that CB1 receptors are negatively coupled to adenylyl cyclase enzyme through G_i proteins and positively coupled to mitogen-activated protein kinases (Howlett, 2002). The further investigations of these intracellular mechanisms can help in ascertaining the exact role of CB1 receptors in the antinociceptive effects of NADA. NADA also inhibits the activity of fatty acid hydrolase enzyme, and this effect might increase the level of other endogenous cannabinoids (e.g. anandamide) (Bisogno et al., 2000).

As regards the in vivo results, intravenous administration of NADA to rats (1-, 4- and 10 mg/kg) induces dose-dependent decreases in mean arterial blood pressure via TRPV1 receptor in rats fed with normal or high sodium diet (Wang and Wang, 2007), while it (10 mg/kg intraperitoneally) causes hypothermia, hypolocomotion and immobility through the activation of CB1 receptor in mice (Bisogno et al., 2000). Furthermore, its antiemetic effect (1-2 mg/kg

intraperitoneally) in ferrets is inhibited by both receptor antagonists (Sharkey et al., 2007). Some data have shown that NADA may play a role in pain modulation, and these effects can also be mediated by CB1 or/and TRPV1-receptors. Thus, intraplantar administration of NADA (0.1-10 μ g) causes thermal hyperalgesia in rats and increases the spontaneous discharge of spinal nociceptive neurons (0.5-5.0 μ g) by activation of TRPV1-receptors (Huang et al., 2002; Huang and Walker, 2006). In contrast, Sagar et al. (2004) have investigated the effect of intraplantar NADA on mechanically evoked responses of dorsal horn neurons in anesthetized rats. NADA (5 μ g) significantly inhibited the innocuous evoked responses, and this effect was blocked by a CB1 antagonist, while the inhibitory effect of NADA on noxious evoked responses was inhibited by a TRPV1 (but not CB1) antagonist. In the eye-wipe-assay, topical NADA (0.1 %) has resulted in a nocifensive behavior in rats (Price et al., 2004). As regards the results after its systemic administration, NADA (10 mg/kg, intraperitoneally) has induced analgesia in the hot plate test in mice (Bisogno et al., 2000). Spinal administration of NADA (10 nM/4.4 μ g) caused mechanical allodynia in mice to hindpaw stimulation with von Frey hairs via TRPV1 receptor (Pitcher et al., 2007), whereas our results showed that NADA decreased the thermal hyperalgesia by the activation of both TRPV1 and CB1 receptors. It is possible that the differences in this study are related to differences in the applied models (acute pain vs. carrageenan-induced inflammation) and/or in the stimuli (mechanical vs. thermal). Thermal antihyperalgesia caused by 50 μ g NADA was blocked by both CB1 and TRPV1 receptor antagonists, whereas the effect of 15 μ g NADA was inhibited only by the application of TRPV1 antagonist. We suppose that in lower doses, NADA activates primarily the TRPV1 receptors, but in higher doses the greater degree of CB1 receptor activation might also be involved in its antihyperalgesic effect. The role of TRPV1 receptor in different pain syndromes both peripherally and spinally is well known, however, activation of TRPV1 receptors not only causes nociception and release of proinflammatory neuropeptides (e.g. SP, CGRP) but can also induce the release of endogenous antinociceptive ligands such as beta-endorphin, somatostatin, glycine or GABA leading to analgesic effects (Bach and Yaksh, 1995; Ferrini et al., 2007; Jancsó et al., 1985; Jancsó and Lawson, 1990; Kanai et al., 2006; Kanai et al., 2007; Pitcher et al., 2007; Szolcsányi et al., 1998; Yu et al., 2008). The long-lasting effects of NADA suggest that activation of TRPV1 receptors leads to prolonged release of endogenous antinociceptive ligands. However, it can not be excluded that NADA, similarly to anandamide, can induce acute desensitization of TRPV1 receptors (Lizanecz et al., 2006). Increasing evidence confirms that cannabinoid and vanilloid systems intensely cooperate in different systems,

therefore, their interaction should also be considered after NADA administration. At supraspinal level TRPV1 receptor activation has attenuated the anxiolytic effects of phyto- and endocannabinoids by increasing glutamate release in vivo in rats (Campos and Guimarpes, 2009), whereas capsaicin-evoked release of substance P has been increased by CB1 receptor antagonist in spinal cord slices (Lever and Malcangio, 2002). CB1 is found primarily on the primary sensory neurons and on both excitatory and inhibitory interneurons in the superficial spinal cord (Ahluwalia et al., 2000; Farquhar-Smith et al., 2000; Hegyi et al., 2009; Pernia-Andrade et al., 2009), and their activation inhibits these neurons and reduces the releases of several transmitters, however, the activation of TRPV1 receptors increases the transmitter releases, as was discussed above (Helyes et al., 2003; Lever and Malcangio, 2002; Richardson et al., 1998). Since NADA represents a “chimeric” ligand acting on both cannabinoid and TRPV1 receptors, and CBs and TRPV1 receptors show coexpression (Binzen et al., 2006), their coactivations can lead to an interaction between them (Mahmud et al., 2009), 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. Surprisingly, co-administration of 1 µg EM + 15 µg NADA produced a shortened antinociception. We also determined the effects of 1 µg EM with 5 µg NADA (ratio: 1:5), but this combination also did not induce significant potentiation (data are not shown). 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 (Farquhar-Smith et al., 2000; Hegyi et al., 2009; Hohmann, 2002; Szállási and Blumberg, 1999), the complex interactions of the endogenous ligands on different receptors and/or different cells may also result in these effects. The interaction between cannabinoid and opioid receptors is well-known, but results of the TRPV1-opioid receptor crosstalk are controversial. In vitro and in

vivo studies have shown a negative interaction between TRPV1 and μ -opioid receptor (Endres-Becker et al., 2007; Vetter et al., 2006; Welch and Eads, 1999). We can speculate that at spinal level, the activation of presynaptic TRPV1 and μ -opioid receptors on primary sensory neurons might interact by modifying the release of both excitatory and inhibitory transmitters. Furthermore, the interaction might occur as a consequence of the stimulation of TRPV1 channels and μ -opioid receptors located on distant (inhibitory and excitatory, respectively) neurons in the dorsal horn. Therefore, the net effects on transmitter releases might be dose-dependent. We suppose that in the case of combination of 1 μ g EM with 15 μ g NADA, the decreased antinociception might be due to the inhibitory effects of EM which might have antagonized the NADA at TRPV1 receptors therefore the release of inhibitory ligands might have decreased. Regarding the intracellular mechanisms, a recent study showed that opioid agonists decrease the translocation of TRPV1 channels into the plasma membrane (Vetter et al., 2008). Therefore, another possibility is that EM decreased the number of TRPV1 channels and this effect led to the decreased release of inhibitory transmitters and the surprisingly decreased effect. However, further in vitro investigations are necessary to reveal the possible intracellular changes. We did not measure the level of the different ligands in the spinal cord; thus, we could not disclose the role of the pharmacokinetic interaction too. Therefore, additional experiments in future are required for a further clarification of the pharmacokinetic interaction of these ligands.

In conclusion, the results show that both TRPV1 and CB1 receptor activation played a substantial role in the antinociceptive effects of NADA at spinal level. This study supports a role for the endogenous cannabinoid, opioid and TRPV1 receptor-related signalling systems in modulating nociceptive transmission in the spinal cord. 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. The complexity of the pro- and antinociceptive mechanisms remains to be cleared but the co-administration of endocannabinoids and endogenous opioids may be beneficial in special dose ranges, however, further experiments with other models are required in human, and with other endogenous ligands in animals.

5.2. The antinociceptive effects of perineural vinpocetine treatment

According to the enzyme histochemical, immunocytochemical and neurochemical studies described above, perineurally applied vinpocetine induces blockade of retrograde axoplasmic transport which consequently results in TDA in the ipsilateral, segmentally related upper dorsal horn. Vinpocetine, a derivate of vincamine, is proved to be devoid of any unwanted side effects. It is widely used in clinical pharmacotherapy of various cerebral circulatory diseases such as memory problems, motion disorders, climacterial complaints, vertigo and headache (Bonoczki et al., 2000). Vinpocetine is a drug very different from vincristine: principally, its effect is blockade of sodium channels (Sitges et al., 2005; Zhou et al., 2003) and of Ca^{2+} channels (Sitges and Nekrassov, 1999) as well as of glutamate receptors (Kiss et al., 1991). The chemical structure of vinpocetine, a derivate of *Vinca minor* L., shows little resemblance, if any, to that of vincristine and vinblastine, obtained from *Vinca major* L.

The effects of vinpocetine upon retrograde axoplasmic transport are very similar to those exerted by vincristine and vinblastine, two semi-synthetic derivatives of *Vinca major* L. (Knyihár-Csillik and Csillik, 1981; Csillik and Knyihár-Csillik, 1982). TDA caused by vinpocetine is followed by the appearance of labyrinthine structures similar to those found earlier in the upper dorsal horn after perineural application of microtubule inhibitors vincristine and vinblastine (Knyihár and Csillik, 1976; Csillik et al., 1977).

FRAP has been suspected to be a genuine marker enzyme of nociception as early as more than three decades ago (Knyihár, 1971); yet this idea was supported by experimental facts only considerably later (Schoenen et al., 1985; Glykys et al., 2003). TMP, an isoenzyme of FRAP (O'Brien et al., 1989) undergoes alterations in the course of TDA similar to those of FRAP. SP is regarded as a pain-related neuropeptide (Snyder and McMahon, 1998). Disappearance of FRAP and TMP, and diminishment of SP from the upper dorsal horn, can thus be suspected to be signs of a decreased nociception.

Vinpocetine was first synthesized by Lőrincz et al. (1976) from the alkaloid vincamine, obtained from the leaves of *Vinca minor* L. (Bonoczki et al., 2000). According to the studies of the Marburg school of pharmacologists, vinpocetine prevents ischemic cell damage in the hippocampus (Sauer et al., 1988); it enhances cerebral blood flow and glucose utilization after forebrain ischemia (Rischke and Kriegstein, 1990). Vinpocetine was reported to protect against excitotoxic cell death of cerebrocortical neurons (Erdő et al., 1990); it increases the neuroprotective effect of adenosine in cell cultures (Kriegstein and Rischke, 1991). According to Bonoczki et al. (2000) positron emission tomography proves that vinpocetine is

able to redistribute regional cerebral blood flow and enhance glucose supply of brain tissue in ischemic post-stroke patients. Lendvai et al. (2003) have shown that vinpocetine enhances morphological dynamics of dendritic spines of pyramidal cells in the neocortex.

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 (Csillik et al., 1985). Since transcutaneous iontophoresis of vincristin and vinblastin alleviates chronic pain resulting from postherpetic, trigeminal and other neuralgias (Csillik et al., 1982), the possibility arises that vinpocetine, applied by transcutaneous iontophoresis, can also be used for alleviation of pain.

According to Kemény et al. (2005), vinpocetine, widely used in clinical pharmacotherapy, is devoid of any side effects. The fact that perineural application of vinpocetine induces TDA by blockade of retrograde axoplasmic transport in a peripheral sensory nerve has been discussed in our previous study. The question regarding the mechanism by which retrograde axoplasmic transport was inhibited by vinpocetine, remained, however, enigmatic.

Intracellular transport is known to be mediated by microtubules (Porter and Tilney, 1965). In their pioneering studies, Schwab et al. (1979) and Thoenen et al. (1979) have shown that retrograde axoplasmic transport of NGF is due to microtubular function; therefore, blockade of retrograde axoplasmic transport of NGF by microtubule inhibitors vincristin and vinblastin is reasonable. Vinpocetine, however, is not a microtubule inhibitor; therefore, the question arises as to how it can block retrograde axoplasmic transport of NGF. Recently, however, new aspects of retrograde axoplasmic transport have become known. Therefore, this apparent controversy might be resolved by the studies suggesting that retrograde propagation of signalling complexes is due to the formation of a unique organelle called “signalling endosome” (Zweifel et al., 2005) and to the membrane trafficking protein called “pincher” that mediates endocytosis (Shao et al., 2002; Valdez et al., 2005). We assume that interactions of vinpocetine with these recently discovered substances might underlie the mechanism of the blockade of retrograde axoplasmic transport of NGF.

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 (Morgan and Curran, 1986; Hunt et al., 1987; Tassorelli et al., 2005; Abe et al., 2005; Knyihár-Csillik et al., 2007b). Recently, Butkevich et al (2006) demonstrated increased *c-fos* immunoreactivity in the superficial dorsal spinal cord of rats subjected to the painful formalin test. 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.

The antinociceptive effects of systemically administered vinpocetine in visceral pain have also been recently described (Abdel S. OM. 2006; 2007). On the basis of the observations of Musaev et al. (1998) that prove that vinpocetine can be administered successfully by iontophoresis, it can be assumed that iontophoretically applied vinpocetine might be an effective measure in alleviating chronic neuropathic pain without any side effects. Recent studies of Hua et al. (2004) 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.

Even though our experiments are related only to the lower extremities, they can be rationally extrapolated to the entire organism: a challenge for pain clinicians.

6. General 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 required 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.

7. References

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