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RESEARCH ARTICLE

Mitigation of nociception *via* transganglionic degenerative atrophy: Possible mechanism of vinpocetine-induced blockade of retrograde axoplasmic transport ☆

Bertalan Csillik[a,*], András Mihály[a], Beata Krisztin-Péva[a], Ibolya Farkas[a], Elizabeth Knyihár-Csillik[b]

[a]*Department of Anatomy, Albert Szent-Györgyi University Medical School, Szeged, Hungary*
[b]*Department of Neurology, Albert Szent-Györgyi University Medical School, Szeged, Hungary*

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Summary

Vinpocetine, a derivative of vincamine, widely used in the clinical pharmacotherapy of cerebral circulatory diseases, inhibits retrograde axoplasmic transport of nerve growth factor (NGF) in the peripheral nerve, resulting in transganglionic degenerative atrophy (TDA) in the related ipsilateral superficial spinal dorsal horn, as shown in our previous publications. TDA induced by vinpocetine has been demonstrated to be followed by depletion of the marker enzyme fluoride-resistant acid phosphatase (FRAP) and its isoenzyme thiamine monophosphatase (TMP), and by the decrease in the pain-related neuropeptide substance P from laminae I–II–(III) from the segmentally related, ipsilateral substance of Rolando of the spinal cord. In the present paper, we report on the behavioral effects of perineurally administered vinpocetine. Nociception, induced by intraplantar injection of formalin, was mitigated by vinpocetine; increased expression of *c-fos* in the ipsilateral, segmentally related upper dorsal horn was also prevented. Since vinpocetine is not a microtubule inhibitor, and its chemical structure differs from that of vincristin and vinblastin (used formerly by us in the therapy of intractable, chronic neuropathic pain), its mode of action is enigmatic. We assume that the effect of vinpocetine in blocking retrograde axoplasmic transport of NGF might be related to its interaction with membrane trafficking proteins, such as signalling endosomes and the endocytosis-mediating "pincher" protein. Temporary, locally restricted decrease of nociception, induced by vinpocetine, might be useful in the clinical

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*Corresponding author. Tel.: +36 62 544 918/+36 62 545 665; fax: +36 62 545 707.
E-mail address: csillik@anatomy.szote.u-szeged.hu (B. Csillik).

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treatment of intractable, chronic neuropathic pain, since vinpocetine can successfully be applied by transcutaneous iontophoresis.

Introduction

According to Wall (1995), pain is the equivalent of nociception transmitted to and perceived by the cerebral cortex. At the level of the spinal cord, central terminals of primary nociceptive neurons terminate mostly in laminae I and II of the dorsal horn, mainly in the form of scallopped terminals, which contain the pain-related neuropeptide substance P (SP) and the marker enzyme fluoride-resistant acid phosphatase (FRAP) and its isoenzyme thiamine monophosphatase (TMP; Snyder and McMahon, 1998).

It has been shown (Csillik, 1984; Csillik et al., 1985) 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 (see references in Knyihár-Csillik et al., 2007a).

Since the transcutaneous application of vincristin and vinblastin was not approved by health authorities for fear of unforeseen side effects of microtubule inhibitors, our interests were drawn to another vinca alkaloid, vinpocetine, which is proven to be devoid of any side effects (Kemény et al., 2005). Vinpocetine, a derivative of vincamine, is widely used in clinical pharmacotherapy of various cerebral circulatory diseases (Bonoczk et al., 2000), both *per os* and parenterally. In spite of the fact that vinpocetine is not a microtubule inhibitor and its chemical structure shows no resemblance to that of vincristin, we found that, at adequate concentration, vinpocetine inhibits retrograde axoplasmic transport of nerve growth factor (NGF), as shown by radiochemical studies (Knyihár-Csillik et al., 2007a).

In this paper, 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*, known to represent increased synaptic activity. In addition, the mechanism of the blockade of retrograde axoplasmic *transport* of NGF induced by vinpocetine will be discussed in the light of recent neurochemical investigations. On these premises, the possibility of transcutaneous iontophoresis of vinpocetine will be discussed for the alleviation of chronic neuropathic pain.

Experimental procedures

Investigations were performed on 15 young adult male albino Wistar strain rats (*Rattus norvegicus albus*) 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 μL) 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 the technique described by Butkevich et al. (2005). 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 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 μm 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 nuclei in the superficial dorsal horn (laminae I and II) were counted in a 0.1 mm^2 square and the mean number of *c-fos* expressing cells was calculated by averaging seven sections.

In seven further rats, the sciatic nerve was first enclosed in a Gelaspon cuff containing 10^{-6} M vinpocetine; 72 h later a dilute formalin solution (10%, 10 μL) 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. The number of *c-fos* immunoreactive nuclei was determined in a blinded manner and averaged as in controls. 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 ± S.E.M. are illustrated. A value of $p < 0.05$ was considered statistically significant.

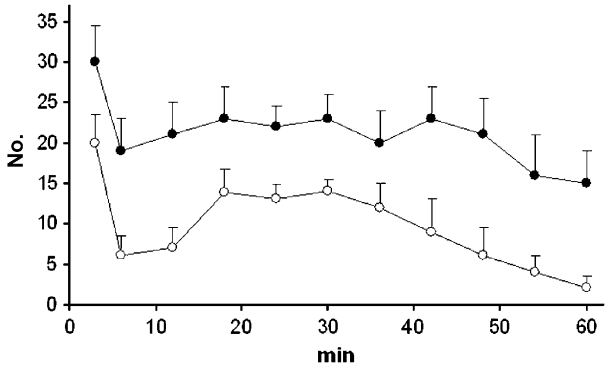

Figure 1. Numbers of flinchings, shakings and licking in a unilateral formalin test (filled circles) and 3 days after perineural application of vinpocetine (empty circles). Number of animals tested was $n = 7$ in both cases. The difference between the two curves is significant ($p < 0.05$).

Results

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 1, 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 1, empty circles). Immunohistochemical investigations demonstrated significant differences in *c-fos* expression between control and vinpocetine-treated animals. As shown in Figures 2 and 3, 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. 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 (Figures 4 and 5).

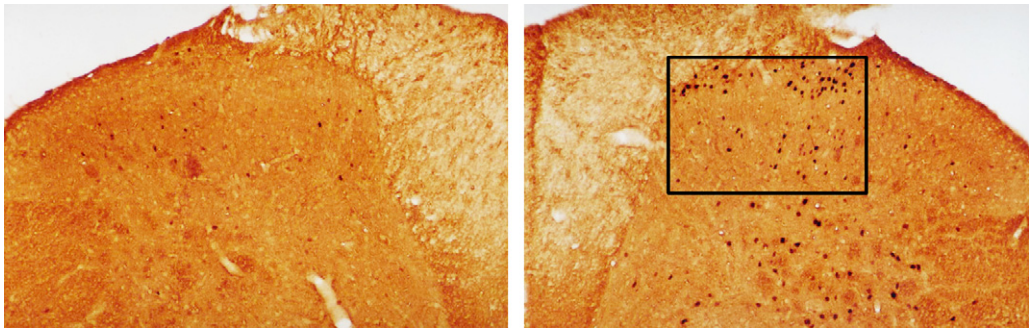

Figure 2. Distribution of *c-fos* immunoreactive nuclei in segment L5 of the spinal cord, 1 h after unilateral application of the formalin test (apparent right). Number of *c-fos* immunoreactive cells was determined in the rectangular area. Note that the number of *c-fos* immunoreactive cells increased tremendously as a consequence of the application of the formalin test.

Discussion

In our previous studies (Knyihár-Csillik et al., 2007a), we have shown that perineurally applied vinpocetine, a derivative of *Vinca minor*, the chemical structure of which bears no resemblance either to vinblastin or to vincristin, is able to block retrograde axoplasmic transport of NGF in peripheral nerves, inducing TDA of primary sensory axon terminals in the segmentally related, ipsilateral upper dorsal horn, which results in the depletion of FRAP (and TMP), and in the partial depletion of SP from the same area. Similar effects were observed decades earlier after perineural application of vinblastin and vincristin, two semi-synthetic derivatives of *Vinca major* L. (Csillik et al., 1977, 1978). Furthermore, the blockade of retrograde axoplasmic transport of ^{125}I after perineural application of vinpocetine is similar to that observed by us after perineural application of vinblastin and vincristin (Csillik, 1984; Csillik et al., 1985). Since transcutaneous iontophoresis of vincristin and vinblastin alleviates chronic pain in postherpetic, trigeminal and other neuralgias (Csillik and Knyihár-Csillik, 1986), the possibility arises that vinpocetine, if applied by transcutaneous iontophoresis, could also be used in the alleviation of pain.

Vinpocetine, synthetized by Lőrincz et al. (1976) from vincamine, is a Ca^{2+}/calmodulin-dependent phosphodiesterase 1 inhibitor (Han et al., 1999). 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 publication (Knyihár-Csillik et al., 2007a). The question regarding the mechanism by which retrograde axoplasmatic 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

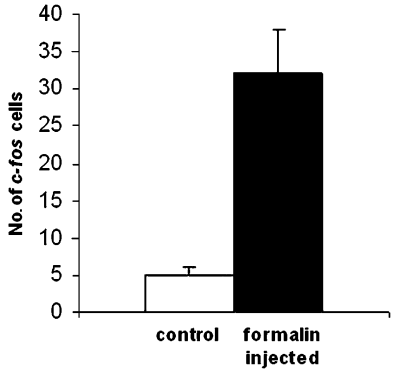

Figure 3. Number of *c-fos* immunoreactive cells in the superficial dorsal horn 1 h after unilateral application of the formalin test (black column) as contrasted to the contralateral superficial dorsal horn (white column).

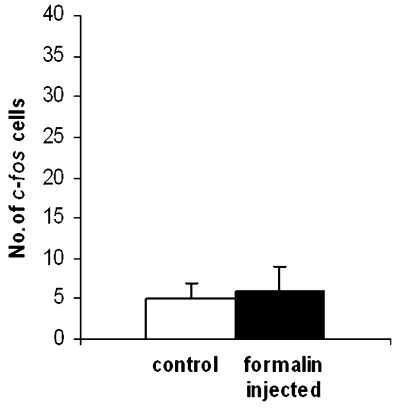

Figure 5. Number of *c-fos* immunoreactive cells in the superficial dorsal horn, 3 days after perineural application of vinpocetine and 1 h after unilateral application of the formalin test (black column) and in the contralateral superficial dorsal horn (white column).

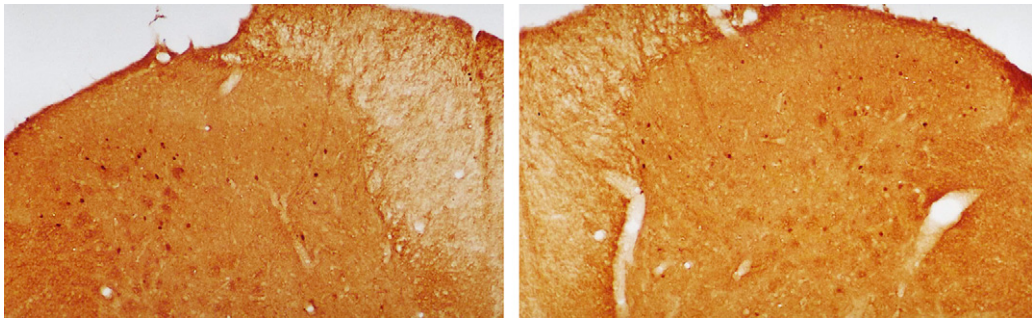

Figure 4. Distribution of *c-fos* immunoreactive nuclei in segment L5 of the spinal cord, 3 days after perineural application of vinpocetine (10^{-6} M) on the sciatic nerve, and 1 h after unilateral formalin testing (apparent right). Number of *c-fos* immunoreactive cells was determined in the rectangular area outlined in Figure 2.

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. 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.

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. 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.

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