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Nitroglycerin increases serotonin transporter expression in rat spinal cord but anandamide modulated this effect

Gábor Nagy-Grócz[a,b], Zsuzsanna Bohár[a,c], Annamária Fejes-Szabó[a], Klaudia Flóra Laborc[c], Eleonóra Spekker[c], Lilla Tar[d], László Vécsei[a,c,*], Árpád Párdutz[c]

[a] MTA-SZTE Neuroscience Research Group, University of Szeged, Szeged, Hungary
[b] Faculty of Health Sciences and Social Studies, University of Szeged, Szeged, Hungary
[c] Department of Neurology, Faculty of Medicine, Albert Szent-Györgyi Clinical Center, University of Szeged, Szeged, Hungary
[d] Department of Neurology, University of Ulm, Oberer Eselsberg 45, 89081, Ulm, Germany

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ABSTRACT

Migraine is one of the most prevalent neurological diseases, which affects 16% of the total population. The exact pathomechanism of this disorder is still not well understood, but it seems that serotonin and its transporter have a crucial role in the pathogenesis.

One of the animal models of migraine is the systemic administration of nitroglycerin (NTG), a nitric oxide (NO) donor. NO can initiate a central sensitization process in the trigeminal system, which is also present in migraineurs.

Recent studies showed that the endocannabinoid system has a modulatory role on the trigeminal activation and sensitization.

Our aim was to investigate the effect of an endogenous cannabinoid, anandamide (AEA) on the NTG-induced changes on serotonin transporter (5-HTT) expression in the upper cervical spinal cord (C1–C2) of the rat, where most of the trigeminal nociceptive afferents convey.

The animals were divided into four groups. Rats in the first group, called placebo, received only the vehicle solution as treatment. In the second group, they were treated with an intraperitoneal (i.p.) injection of NTG (10 mg/kg). Rats in the third and fourth groups received i.p. AEA (2 × 5 mg/kg) half hour before and one hour after the placebo or NTG treatment. Four hours after placebo/NTG injection, the animals were perfused and the cervical spinal cords were removed for immunohistochemistry and Western blotting.

Our results show that both NTG and AEA alone are able to increase 5-HTT expression in the C1–C2 segments. Combination of NTG and AEA has an opposing effect on this marker, nullifying the effects of non-combined administration, probably by negative feedback mechanisms.

1. Introduction

Migraine is a chronic neurological disorder characterized by recurrent headaches lasting for 4–72 h and commonly accompanied by nausea, photophobia and phonophobia. This syndrome affects 16% of the total population (Smitherman et al., 2013). The exact pathomechanism of the disease is not fully known, but it has been suggested that serotonin or 5-hydroxytryptamine (5-HT) has an important role in the migraine attack (Ferrari and Saxena, 1993). In 1961, Sicuteri has shown that the excretion in the urine of 5-hydroxyindoleacetic acid, the principal catabolite of 5-HT, was increased during some attacks of migraine headache (Sicuteri, 1961) and these findings were verified by Curran et al. in 1965 (Curran et al., 1965). Despite these data, the exact role of 5-HT in the pathogenesis of migraine is not fully clear.

Serotonin transporter (5-HTT) removes 5-HT from the synaptic cleft back into the pre-synaptic terminals, mitigating the effect of 5-HT. In patients with familial hemiplegic migraine, Horvath et al. have found low 5-HT levels in the platelets, reduced 5-HT transport

Abbreviations: 5-HT, serotonin; 5-HTT, serotonin transporter; AEA, anandamide; C1–C2, upper cervical spinal cord; CSD, cortical spreading depression; GAPDH, glyceraldehyde 3, phosphate dehydrogenase; i.p., intraperitoneal; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NTG, nitroglycerin; PBS, phosphate-buffered saline; TBST, Tris-buffered saline containing Tween 20.

* Corresponding author at: Department of Neurology, Faculty of Medicine, Albert Szent-Györgyi Clinical Centre, University of Szeged, Szeged, Semmelweis utca 6, H-6725 Szeged, Hungary.
E-mail address: vecsei.laszlo@med.u-szeged.hu (L. Vécsei).

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capacity and low metabolite levels in the cerebrospinal fluid (Horvath et al., 2011). In a neuroimaging study increased 5-HTT availability in the mesopontine brainstem of migraineurs has been detected (Schuh-Hofer et al., 2007). Data show that the vast majority of 5-HTT is localized on the axolemma, in the vicinity of the synapses, and along the axons as well. This distribution suggests that the transporter may play a role not only in the termination of synaptic transmission, but in the general extracellular 5-HT regulation. Intracellularly, it has been demonstrated in low amount in the soma and dendrites (Tao-Cheng and Zhou, 1999; Zhou et al., 1998). Depending on the stimulus, 5-HT uptake and 5-HTT trafficking may be differentially affected, but are often linked with altered 5-HTT basal phosphorylation by Ser/Thr protein kinases (Annamalai et al., 2012; Ramamoorthy et al., 1998).

Nitroglycerin (NTG)-administration is a model of migraine, being able to generate migraine attacks in migraineurs (Sicuteri et al., 1987), and trigger activation and sensitization in the trigeminal system (Di Clemente et al., 2009). It is also well-known, that NTG-a nitric oxide donor (NO)-can initiate trigeminal activation and sensitization in animals as well (Pardutz et al., 2000; Tassorelli and Joseph, 1995). In rats, it has been shown that NTG produced an increase in the area covered by 5-HT-immunoreactive fibres (Pardutz et al., 2002), which suggests that NO influences the 5-HT system.

Cannabis has been sporadically used to reduce nausea and vomiting during chemotherapy and to treat pain, migraine and muscle spasticity (Borgelt et al., 2013). The interactions between the endocannabinoid system and pain perception are intensively studied in several laboratories, but the psychoactive properties of cannabinoids (Crawley et al., 1993) restrict their therapeutic application. On the other hand, a recent retrospective study shows, that medical marijuana is able to decrease the frequency of migraine attacks (Rhyne et al., 2016). The alteration of platelet 5-HT homeostasis was considered to be connected with the pathogenesis of migraine headache (Danese et al., 2014). Research data show a strong interaction between the cannabinoid and 5-HT system in platelets: Δ1-tetrahydrocannabinol blocked 5-HT release from the thrombocytes (Volfe et al., 1985), whereas platelet 5-HT uptake was inhibited by various cannabinoids (Velenovska and Fisar, 2007; Volfe et al., 1985). The interaction of cannabinoid and 5-HT systems at the periphery is well documented, but for the better understanding of migraine pathophysiology experimental data are needed about such possible mechanism in the CNS.

Anandamide (AEA) is an extensively studied endocannabinoid, which is effective in the inhibition of trigeminal activation and central sensitization in animals (Greco et al., 2010a; Nagy-Grocz et al., 2016). AEA is an agonist of both cannabinoid 1 and 2 receptors and the transient receptor potential vanilloid type 1 receptor.

The goal of the present study was to investigate the effect of NTG and AEA on the 5-HTT expression levels, in one of the central nervous system structures relevant in migraine: the superficial laminae of the upper cervical spinal cord (C1–C2) with immunohistochemistry and Western blotting.

2. Materials and methods

2.1. Animals

The procedures utilized in this study followed the guidelines for the Use of Animals in Research of the International Association for the Study of Pain and the directive of the European Economic Community (86/609/ECC). They were permitted by the Committee of the Animal Research of University of Szeged (I-74-12/2012) and the Scientific Ethics Committee for Animal Research of the Protection of Animals Advisory Board (XI./352/2012). Forty-four adult male Sprague-Dawley rats weighing 200–250 g were used. The animals were raised and maintained under standard laboratory conditions, with tap water and regular rat chow available ad libitum on a 12 h dark–12 h light cycle.

2.2. Drug administration

The animals were divided into four groups (n = 6 per group in the immunohistochemistry, n = 5 in the Western blot). The animals in the first (placebo) group, received only the vehicle solution as pretreatment. In the second group, the rats were pretreated with an intraperitoneal (i.p.) injection of NTG (10 mg/kg bodyweight, Pohl Boskamp). In the third and fourth groups, rats were given AEA (2×5 mg/kg) injection half hour before and one hour after the placebo or NTG treatment. AEA was dissolved in physiological saline. In the first and second groups, animals were treated with physiological saline instead of AEA.

2.3. Immunohistochemistry

Four hours after the placebo/NTG injection, the rats were perfused transcardially with 100 ml phosphate-buffered saline (PBS, 0.1 M, pH 7.4), followed by 500 ml 4% paraformaldehyde in phosphate-buffer in chloral hydrate (0.4 g/kg bodyweight) anesthesia. The C1–C2 segments of the cervical spinal cord between −5 and −11 mm from the obex, which receive important nociceptive information from the head (Strassman et al., 1993) were removed and postfixed overnight for immunohistochemistry in the same fixative. After cryoprotection, 30 μm cryostat sections were cut and serially collected from C1–C2 in wells containing cold PBS. The free-floating sections were rinsed in PBS and immersed in 0.3% H_2O_2 in or PBS for 30 min. After several rinses in PBS containing 1% Triton X-100, sections of C1–C2 were kept for two nights at 4 °C in anti-5-HTT antibody (Merck Millipore, ab9726) at a dilution of 1:100 000. The immunocytochemical reaction was visualized by the avidin-biotin kit of Vectastain (PK6101), and nickel ammonium sulphate –intensified 3,3′-diaminobenzidine. The specificity of the immune reaction was controlled by omitting the primary antiserum.

2.4. Western blot analysis

Four hours after the placebo/NTG injection, the animals were perfused transcardially with 100 ml PBS then the dorsal horns of C1–C2 segments were extracted. Until the measurements, the samples were stored −80 °C. The specimens were sonicated in ice cold lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.1% igepal, 0.1% cholic acid, 2 μg/ml leupeptin, 2 mM phenylmethylsulphonyl fluoride, 1 μg/ml pepstatin, 2 mM EDTA and 0.1% sodium dodecyl sulphate. The lysates were centrifuged at 12,000 RPM for 10 min at 4 °C and supernatants were aliquoted and stored at −20 °C until use. Protein concentration was defined with BCA Protein Assay Kit using bovine serum albumin as a standard. Previous to loading, each sample was mixed with sample buffer, and denaturated by boiling for 3 min. Equal amounts of protein samples (20 μg/lane) were separated by standard SDS polyacrylamide gel electrophoresis on 10% Tris–Glycine gel and electrotransferred onto Amersham Hybond-ECL nitrocellulose membrane (0.45 μm pore size, GE Healthcare). We used The Page Ruler Prestained Protein Ladder (Thermo Scientific, 10–170 kDa) to define approximate molecular weights. Following the transfer, membranes were blocked for one hour at room temperature in Tris-buffered saline containing Tween 20 (TBST) and 5% non fat dry milk. Then they were incubated in TBST containing 1% non fat dry milk and 5-HTT antibody (Merck Millipore, ab9726, dilution: