Accepted Manuscript

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

Authors: Gábor Nagy-Grócz, Zsuzsanna Bohár, Annamária Fejes-Szabó, Klaudia Flóra Laborc, Eleonóra Spekker, Lilla Tar, László Vécsei, Árpád Párdutz

PII: S0891-0618(17)30034-0
DOI: http://dx.doi.org/doi:10.1016/j.jchemneu.2017.06.002
Reference: CHENEU 1496

To appear in:

Received date: 24-2-2017
Revised date: 2-6-2017
Accepted date: 14-6-2017

Please cite this article as: Nagy-Grócz, Gábor, Bohár, Zsuzsanna, Fejes-Szabó, Annamária, Laborc, Klaudia Flóra, Spekker, Eleonóra, Tar, Lilla, Vécsei, László, Párdutz, Árpád, Nitroglycerin increases serotonin transporter expression in rat spinal cord but anandamide modulated this effect. Journal of Chemical Neuroanatomy http://dx.doi.org/10.1016/j.jchemneu.2017.06.002

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Nitroglycerin increases serotonin transporter expression in rat spinal cord but anandamide modulated this effect

Gábor Nagy-Grócz M.Sc. a,b, Zsuzsanna Bohár M.Sc., Ph.D. a,c, Annamária Fejes-Szabó M.Sc., Ph.D. a, Klaudia Flóra Laborc M.D. c, Eleonóra Spekker M.Sc. c, Lilla Tar M.D. d, László Vécsei M.D., Ph.D., D.Sc. a,c*, Árpád Párdutz M.D., Ph.D. c

aMTA-SZTE Neuroscience Research Group, University of Szeged, Szeged, Hungary
bFaculty of Health Sciences and Social Studies, University of Szeged, Szeged, Hungary
cDepartment of Neurology, Faculty of Medicine, Albert Szent-Györgyi Clinical Center, University of Szeged, Szeged, Hungary
dDepartment of Neurology, University of Ulm, Oberer Eselsberg 45, 89081, Ulm, Germany

*Corresponding author:
László Vécsei M.D., Ph.D., D.Sc.
Department of Neurology, Faculty of Medicine, Albert Szent-Györgyi Clinical Centre, University of Szeged, Szeged, Hungary
Semmelweis utca 6. H-6725 Szeged, Hungary
Tel: +36-62-545-351, Fax: +36-62-545-597
Email: vecsei.laszlo@med.u-szeged.hu

Highlights
- Systemic administration of nitroglycerin and anandamide are able to increase the expression of serotonin transporter at the level of caudal trigeminal nucleus
- The combined nitroglycerin and anandamide treatment attenuated this effect
- A complex interaction might be present between the serotoninergic, nitrergic and the endocannabinoid system

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 has 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 (10mg/kg). Rats in the third and fourth groups received i.p. AEA (2x5mg/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.

**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, intraperitoneal-i.p., nNOS-neuronal nitric oxide synthase, NO-nitric oxide, NTG-nitroglycerin, PBS-phosphate-buffered saline, TBST-Tris-buffered saline containing Tween 20

**Key words:** migraine, trigeminal system, nitroglycerin, anandamide, serotonin, serotonin transporter

1. **Introduction**

Migraine is a chronic neurological disorder characterized by recurrent headaches lasting for 4-72 hours 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 and colleagues have 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 and co-workers 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 presynaptic terminals, mitigating the effect of 5-HT. In patients with familial hemiplegic migraine, Horvath and colleagues have found low 5-HT levels in the platelets, reduced 5-HT transport 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 (Annimalai 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: ∆1-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., 2015). 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 grams 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 hour dark-12 hour 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 (2x5 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% H2O2 in or PBS for 30 minutes. 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% igepeal, 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 minutes 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 minutes. 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: 1:2000, incubation: overnight at 4 °C) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Cell Signaling Technologies, 8884, dilution: 1:1000, incubation: overnight at room temperature). Next day, membranes were incubated in TBST containing 1% non fat dry milk and horseradish peroxidase-conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology; sc-2030) for two hours at room temperature. Protein bands were visualized after incubation of membranes with the SuperSignal West Pico Chemiluminescent Substrate using Carestream Kodak BioMax Light film.

2.5. Data evaluation:
All evaluations were implemented by an observer blind to the experimental groups. The detailed methods were described previously (Nagy-Grocz et al., 2015).

Immunohistochemistry:
The photomicrographs of the stained sections of C1-C2 were taken using Zeiss AxioImager microscope supplied with an AxioCam MRc Rev.3 camera (Carl Zeiss Microscopy, Jena, Germany). The density of the 5-HTT-immunoreactive fibres was analyzed by Image Pro Plus 6.2® software (Media Cybernetics). After capturing the images, the borders of laminae I, II and III of the dorsal horn were defined manually as areas of interest (AOI). The background level of the staining intensity was first determined in the recorded gray scale images, which was used as a threshold to segment pixels in the AOI with grey levels above the background, as described in details earlier (Nagy-Grocz et al., 2015). The area innervated by the immunoreactive fibres was expressed as the cumulative number of pixels with densities above the threshold. Finally, the relative area covered by immunoreactive fibres and in the different laminae was calculated as area fractions (%) of the appropriate AOI. To exclude possible errors originating from different staining efficiency in separate experiments, background intensities in indifferent areas on the sections with non-specific staining were measured and used as inter-experimental controls.

Higher magnification photos were also taken to determine the sizes of immunoreactive varicosities by the same digital system using 40x objective. For the determination of the cross-sectional areas we selected processes which were in focus and were recognized and measured by the program as single objects. Measurements were carried out as above.

Western blot analysis:
For densitometric analyses, films were scanned and quantified using Java ImageJ 1.47v analysis software (National Institutes of Health). GAPDH served as a control to ensure
loading of equivalent amounts of sample proteins and measured protein levels of 5-HTT were normalized to GAPDH.

2.6. Statistical analysis:

Statistical analysis of measurements were performed in SPSS Statistics software (Version 20.0 for Windows, SPSS Inc.) by one-way analysis of variance followed by the Fishers Least Significant Difference post hoc test, with p<0.05 taken as statistically significant. Normality was tested with Shapiro-Wilk test. Group values are reported as means ± S.E.M.

3. Results

NTG and AEA raise 5-HTT expression in the C1-C2, but the combined treatment inhibits this effect-Immunohistochemistry data

On microscopic investigation (20x objective) of transverse sections of the C1-C2 segments, there were abundant 5-HTT-positive fibres in the superficial layers (I-III) of the dorsal horn. In animals sacrificed four hours after i.p. NTG-injection the 5-HTT-immunoreactive area was significantly higher in each layers compared to the placebo-treated group (p<0.01). This effect has been experienced also in the AEA-treated animals (p<0.01; p<0.001). It is interesting that, in the combined NTG+AEA treated group the 5-HTT area fractions were significantly decreased (p<0.01) as compared either with the NTG or the AEA treated groups (lamina I: F(3,20)=26.556; p=2x10^{-6}; lamina II: F(3,20)=92.104; p=2.62x10^{-10}; lamina III: F(3,20)=45.300; p=4.81x10^{-8}). (Fig.1)

NTG and AEA also enhance the sizes of the 5-HTT varicose fibres in the C1-C2, but the combined treatment reduces this effect-Immunohistochemistry data

On higher magnification (40x objective), the microscopic examination of the sections revealed numerous immunoreactive processes. The average size of the fiber varicosity was significantly higher in the NTG or AEA treated animals compared to the placebo-treated group (p<0.05). In the combined NTG+AEA treated group, the sizes was reduced (p<0.05) as compared to the NTG or the AEA treated groups (F(3,20)=12.071; p=9.9x10^{-5}) (Fig.2)

NTG and AEA increase 5-HTT expression in the C1-C2, but the combined administration attenuates this effect-Western blot data
Western blot analysis of the C1-C2 region confirmed the results obtained by 5-HTT immunohistochemistry. The characteristic band of 5-HTT was visualized at 64 kDa. Densitometric analysis showed that, in the NTG and AEA treated groups 5-HTT bands were significantly increased (p<0.01) as compared with the placebo-treated group. After the combined NTG+AEA treatment the bands were significantly decreased (p<0.05; p<0.01) as compared either with the NTG or the AEA treated groups (F(3,16)=8.088; p=2x10⁻³). (Fig.3)

4. Discussion

Despite the fact that the role of 5-HT in the migraine pathogenesis is generally accepted, the exact molecular mechanism of its effect is far from being clearly understood. One of the key molecules of 5-HT signaling mechanisms is its membrane transporter 5-HTT, which is responsible for the cellular internalization of the released transmitter, thereby terminating its effect.

In our present study, we found an increase in 5-HTT and processes expression after the NTG administration in rat C1-C2 segments, where most of the trigeminal nociceptive afferents terminate. Our western blot data demonstrate that the endocannabinoid AEA has similar effect; its administration increases the 5-HTT expression.

From functional point of view the observed changes, i.e. the increased transport of 5-HT may indicate a decrease in the serotonergic activity in these brain areas. These observations are in line with previous neuroimaging and molecular biology studies. By using neuroimaging methods (SPECT-images coregistered with MRI-scans) (Schuh-Hofer et al., 2007) it was shown that there is an increased availability of 5-HTT in brainstem in migraine patients during the interictal period. The recent PET-study also confirms that the 5-HTT availability is crucial in the pathomechanisms of migraine (Park et al., 2016). The limitation of the complete comparison of our results with the clinical studies is that the human observations are examining migraine during the interictal period opposite the NTG model of migraine, which is the ictal model of migraine. On the other hand, clinical and experimental data from several laboratories clearly indicate that the 5-HTT gene is one of the genetically contributing factors of migraine (Murphy et al., 2001). In migraine patients with and without aura it has been shown an altered allelic division of the 5-HTT (Ogilvie et al., 1998).

Our data give more emphasis on the role of the transporter in the regulation of 5-HT levels and provide further evidences for the crosstalk existing between serotonergic and NO systems (Miller and Hoffman, 1994; Zhu et al., 2004). By using proteomic methods Chanrion et al. showed that the 5-HTT interacts with the nNOS and they concluded that this physical
association may make reciprocal modulation possible (Chanrion et al., 2007). It was also shown that the application of L-NAME (a nonspecific nNOS inhibitor) reduces the vascular abnormalities which are induced by the CSD-triggered 5-HT depletion, suggesting that NO production has a pivotal role in the CSD caused 5-HT depletion (Saengjaroentham et al., 2015). In addition, Harkin and co-workers showed that inhibition of NO synthase could be used as a strategy to raise the clinical efficacy of serotonergic antidepressants, enhancing the activity of the drug (Harkin et al., 2004).

A previous study found an increase in the 5-HT-IR fibres in C1-C2 of rats in the NTG-model, which may indicate a reduced release of 5-HT from the terminals (Pardutz et al., 2002). Our present data suggests that NTG may increase 5-HT reuptake and may reduced its levels in the synaptic cleft. In addition, the increased 5-HTT varicosity may suggest a raised 5-HT turnover, as well. This data is in accordance with the HPLC results of Tassorelli and her group, which was shown that NTG can decrease the levels of 5-HT in rats medullary segments (Tassorelli et al., 2002). It has been observed, that the depletion of tryptophan (precursor of 5-HT) is positively correlated with the severity of the headache in migraineurs (Drummond, 2006), and for that reason the decreased level of 5-HT is a crucial factor in the trigeminal activation and the pathomechanism of migraine.

According to our data the endocannabinoid AEA had an effect on the 5-HTT too, it increased its expression. Literature data show that AEA regulates the expression of several genes and some of its effect is receptor (CB1) dependent, but receptor independent actions have also been reported (Correa et al., 2008; Mestre et al., 2011; Sancho et al., 2003). One can not exclude the possibility of some indirect action, because it is known that endocannabinoids are able to raise NO production by upregulating the nNOS activity (Carney et al., 2009). It is therefore possible that the increase of 5-HTT expression is due to the increase of NO after the administration of AEA. The possible role of the endocannabinoid system in the descending modulation of the trigeminal complex was characterized by Akerman and his group. They showed that CB1 receptor activation is able to inhibit the trigeminal nociceptive pathway and AEA is involved in the modulation of the descending trigeminovascular nociception (Akerman et al., 2013; Akerman et al., 2004). In our previous study, we investigated the effect of AEA on the markers of sensitization and inflammation in the NTG model (Nagy-Grocz et al., 2015). Taken together, AEA was able to inhibit the NTG induced changes, suggesting that AEA would have beneficial effect of migraine pathophysiology, while the interaction with the serotonergic axis might be more complex.
It was quite unexpected that in animals treated with NTG+AEA we could not detect changes in 5-HTT expression. The present data do not permit us to explain all aspects of this phenomenon, but we can not exclude the possibility of a negative feed-back mechanism, since both NTG and AEA increase cGMP and NO levels (Carney et al., 2009). It is also worth considering, that administration of NTG increases the activity of enzymes, which break down endogenous endocannabinoids in the midbrain of rats (Greco et al., 2010b), suggesting, that NTG is able to influence the endocannabinoid pathway.

On the other hand, we do not exclude that the combined treatment may act at gene expression level, and the accelerated induction of 5-HTT synthesis and the rapid reversability of expression may result in a recovery to the control level by 4 hours.

Further experiments are needed to reveal the molecular background of this interesting observation.

5. Conclusion
The present study has demonstrated that NTG and AEA alone, and in a combined treatment are able to modulate 5-HTT expression. These finding suggest a complex interaction between the serotonergic and endocannabinoid system on the NTG-induced trigeminal activation and sensitization phenomenon, which are important during migraine attack.

Authors contributions
GNN: participated in the design and implementation of experiments, statistical analysis, interpreted data and wrote the manuscript, ZSB, AFSZ, KFL, ES, LT: participated in the implementation of the experiments, statistical analysis LV: participated in the design of the experiments, in the final approval of the version to be published, AP: participated in the conception and design of the experiments, the interpretation of the data and writing, all authors: critical revision of the manuscript

Conflict of interest
We declare that we have not conflict of interest.

Acknowledgement
This work was supported by the Hungarian Brain Research Program [Grant No. KTIA_13_NAP-A-III/9]; the EUROHEADPAIN [FP7-Health 2013-Innovation; Grant No. 602633] and GINOP-2.3.2-15-2016-00034. Gábor Nagy-Grócz was supported through the ÚNKP-ÚNKP-16-3 New National Excellence Program of the Ministry of Human Capacities. Dr. Árpád Párdutz was supported by the Bolyai Scholarship Programme of the Hungarian Academy of Sciences. We are indebted to Mrs. Valéria Vékony for histotechnical assistance.
References


Saengjaroentham, C., Supornsilpchai, W., Ji-Au, W., Srikiatkhachorn, A., Maneesri-le Grand, S., 2015. Serotonin depletion can enhance the cerebrovascular responses induced by cortical spreading depression via the nitric oxide pathway. Int J Neurosci 125, 130-139.


Figure 1. Summary of immunohistochemistry results. A. Representative photomicrographs of the 5-HTT expression in the C1-C2 segments from the treatment groups. The expression of 5-HTT is not equal between the measured laminae. Laminae I and III contain higher density of fibres compared to lamina II. B. Changes in area fractions of 5-HTT-immunoreactive fibres in superficial laminae I, II and III of the C1-C2 segments. In the NTG and AEA group, the area covered by 5-HTT was significantly higher than in the placebo group. NTG+AEA treatment abolished this effect. Bars present means ± SEM. ##p<0.01; §§p<0.01; **p<0.01; ***p<0.001 Scale bar: 100 µm
Figure 2. Summary of immunohistochemistry results II.

A. Representative photomicrographs of the 5-HTT varicose fibres expression in laminae I-III of C1-C2 segments from the treatment groups.

B. Changes in varicosity sizes of 5-HTT in superficial laminae I-III of the C1-C2 segments. In the NTG and AEA group, the varicosity size of 5-HTT was significantly higher than in the placebo group. NTG+AEA treatment reduced this effect. Figure shows means ± SEM. *p<0.05; Scale bar: 100 µm
Figure 3. Summary of Western blot data. A. Western blots of 5-HTT and GAPDH expression in the C1-C2. B. The quantitative analysis shows that in the NTG and AEA groups, the relative optical density of 5-HTT specific bands was significantly higher than in the placebo group. NTG+AEA treatment abolished this effect. \#p<0.05; $$$p<0.01; **p<0.01