

**The modulatory effect of anandamide on nitroglycerin-induced sensitization in the trigeminal system of the rat**

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The modulatory effect of anandamide on nitroglycerin-induced sensitization in the  
trigeminal system of the rat

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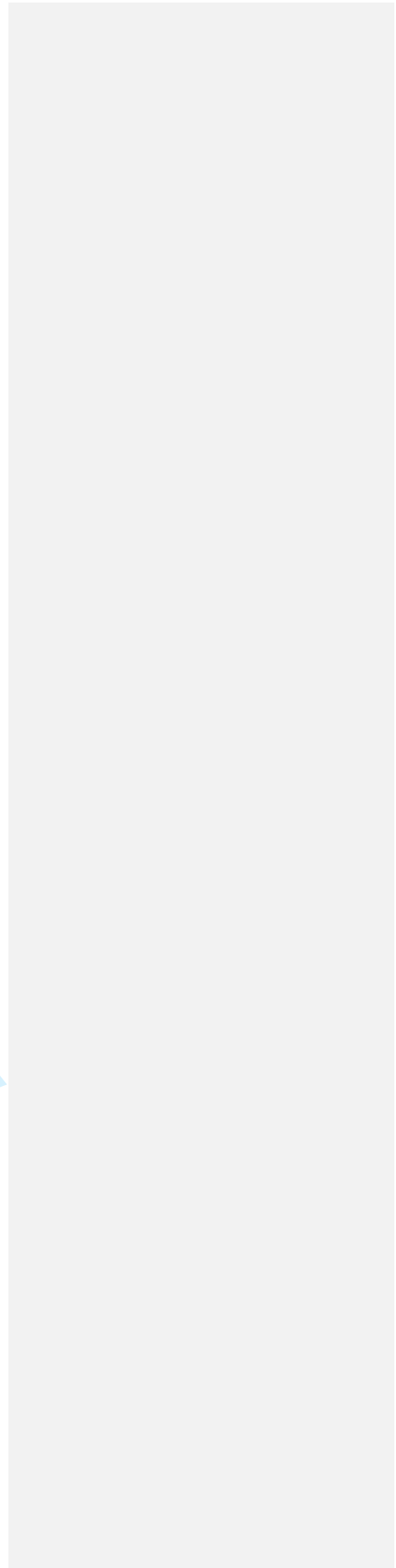
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## Abstract

**Background:** One of the human and animal models of migraine is the systemic administration of the nitric oxide donor (NO) nitroglycerin (NTG). NO can provoke migraine like attacks in migraineurs and initiates a self-amplifying process in the trigeminal system, probably leading to central sensitization. Recent studies suggest that the endocannabinoid system is involved in nociceptive signal processing and cannabinoid receptor (CB) agonists are able to attenuate nociception in animal models of pain.

**Aim:** The purpose of the present study was to investigate the modulatory effects of a CB agonist anandamide (AEA) on the NTG-induced expression of transient receptor potential vanilloid type 1 (TRPV1), neuronal nitric oxide synthase (nNOS), nuclear factor kappa B (NF- $\kappa$ B), cyclooxygenase-2 (COX-2) and kynurenine aminotransferase-II (KAT-II) in the upper cervical spinal cord (C1-C2) of the rat, where most of the trigeminal nociceptive afferents convey.

**Methods:** Half hour before and one hour after NTG (10 mg/kg) or placebo injection, adult male Sprague-Dawley rats (n=44) were treated with AEA (2x5 mg/kg). Four hours after placebo/NTG injection, the animals were perfused and the cervical spinal cords were removed for immunohistochemistry and Western blotting.

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11 **Results and conclusion:** Our results show that NTG is able to increase TRPV1, nNOS,  
12 NF- $\kappa$ B, COX-2 and decrease KAT-II expression in the C1-C2 segments. On the other  
13 hand, we have found that AEA modulates the NTG-induced changes, thus it influences  
14 the activation and central sensitization process in the trigeminal system, probably via  
15 CBs.  
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23 **Key words:** migraine; trigeminal system; nitroglycerin; anandamide; TRPV1; nNOS;  
24 NF- $\kappa$ B; COX-2; KAT-II  
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## Introduction

Migraine belongs to the group of primary headaches, and it is one of the most abundant neurological syndromes, which affects 15% of the European population (1). Despite intensive research, the exact pathomechanism of the disorder is not fully understood, but it is well-known that activation and sensitization of the trigeminal system is essential during the attack (2). Continuous activation of peripheral trigeminal afferents leads firstly to peripheral (first order) sensitization. Sustained inputs can lead to second order, and ultimately to third order central sensitization (3). Previous data have shown, that after the onset of central sensitization during the migraine attack, the acute treatment becomes less effective (4). Although, this theory has been contested recently, stating that the severity of headache might be a better indicator, than the symptoms of sensitization (5, 6), it is generally accepted that the latter plays an essential role in the genesis of migraine (7).

One of the models of migraine is the systemic administration of nitroglycerin (NTG), generating a short lasting headache in humans *via* the rapid vasodilatatory effect of nitric oxide (NO) followed by a typical migraine without aura in migraine patients (8).

In rats, NTG is able to increase c-Fos-immunoreactivity in the caudal trigeminal nucleus (TNC) suggesting the activation of the second order trigeminal neurons there (9). NO is also involved in the central sensitization process in the trigeminal system (10), probably

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11 acting *via* the activation of trigeminal A $\delta$  and C fibres, since the destruction of the latter  
12 with capsaicin abolishes the effect of NTG (11).  
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15 Cannabinoid receptors (CB) are G-protein-coupled receptors, which have two subtypes:  
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17 CB1 and CB2. CB1 is present in the central nervous system, liver, lung and CB2 is  
18 expressed primarily in the immune system (12).  
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21 Animal models of pain have shown that fluctuations in the endocannabinoid levels in  
22 the nervous system are related to pain processing and antinociception (13). CB1 is  
23 present in the trigeminal ganglion (TG) and on the axon terminals of primary sensory  
24 neurons in the nociceptive areas in the spinal cord in rats (14). CB1 is able to inhibit the  
25 responses of trigeminal neurons with A- and C-fiber inputs from the dura mater (15),  
26 pointing to the importance of the endocannabinoid system in pain processing (16).  
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30 Although the psychoactive properties of cannabinoids (17), restrict their therapeutic  
31 application, the interactions between the endocannabinoid system and pain mediation is  
32 intensively studied in several laboratories.  
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38 N-Arachidonylethanolamide or anandamide (AEA) is the first discovered  
39 endocannabinoid, and is an agonist of CBs and transient receptor potential vanilloid  
40 type 1 (TRPV1), which is a nonselective cation channel activated by numerous stimuli,  
41 such as heat and vanilloids (18, 19). AEA has vasodilatory actions (20), which is not  
42 mediated by CBs (21). It is well known that AEA is able to reduce NTG-induced  
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11 hyperalgesia and c-Fos expression in the TNC in rats (22), which means that AEA is  
12 capable of modulating the activation of the trigeminal system.

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14 TRPV1 is present in the spinal cord and is considered as a molecular integrator of  
15 chemical and physical stimuli that elicit pain (23). In addition, NO donors can activate  
16 TRPV1 resulting in an increase of intracellular calcium concentration in different cell  
17 types (24, 25), which suggests that TRPV1 may be modulated by NO.

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19 NO is synthesized from arginine by nitric oxide synthase, neuronal isoform of which  
20 (nNOS) has an utmost importance in nociception and sensitization and it is present in  
21 the trigeminal system (26). NO donors may trigger a self-amplifying process at the level  
22 of central projection site of the trigeminal system by increasing endogenous NO  
23 synthesis, which might be relevant in central sensitization phenomenon (7).

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25 Nuclear factor kappa B (NF- $\kappa$ B) has a crucial role in inflammation process by  
26 controlling many genes including cytokynes. Several studies have shown that  
27 proinflammatory cytokynes contribute to the development of pain and hyperalgesia  
28 (27). Cyclooxygenase-2 (COX-2) is present in the dorsal horn of spinal cord too and it  
29 has a substantial role in the processing of pain (28). Gao and Duan have found that  
30 COX-2 expression increased in the TNC after orofacial nociception (29).

31  
32 Kynurenine aminotransferase-II (KAT-II) is a key enzyme in the kynurenine pathway,  
33 which converts L-kynurenine (L-KYN) to kynurenic acid (KYNA), a known ionotropic  
34 glutamate receptor antagonist molecule (30), which can also block  $\alpha$ -7-nicotinic  
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11 acetylcholine receptors (31). In NTG-induced animal model of migraine L-KYN,  
12 KYNA and KYNA analogues inhibited the trigeminal activation and sensitization (32-  
13 34).  
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16 Thus modulatory effects of cannabinoids can be suggested on trigeminal activation both  
17 on peripheral and central levels. In the present paper we studied the effect of NTG  
18 injection on the TRPV1, nNOS, NF- $\kappa$ B, COX-2 and KAT-II expression levels in the  
19 superficial laminae of C1-C2 and its modulation by CB agonist AEA.  
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## Materials and methods

### *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). 44 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 hour dark-12 hour light cycle.

### *Drug administration*

The animals were divided into four groups (n=6 per group for immunohistochemistry, n=5 per group for Western blot analysis). The animals in the first group, called placebo group, received only the vehicle solution (physiological saline) as treatment. In the second group, the rats were treated with an intraperitoneal injection of NTG (10 mg/kg bodyweight, Pohl Boskamp). In the third and fourth group, animals received intraperitoneal AEA (2x5 mg/kg bodyweight, Simga Aldrich) half hour before and one hour after the placebo or NTG treatment. Greco and colleagues have demonstrated that single 20 mg/kg doses of AEA before the NTG administration is able to reduce NTG-

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11 induced c-Fos expression in the caudal part of spinal trigeminal nucleus (22). Since in  
12 our other running experiments (not reported here) we obtained results showing the  
13 efficacy of AEA in lower dosages we used 2x5 mg/kg of AEA in this context using two  
14 injections due to the short half-life of the drug (35). AEA was dissolved in physiological  
15 saline. In the case of the first and second groups, animals were treated with  
16 physiological saline instead of AEA.  
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#### 24 ***Immunohistochemistry***

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26 Four hours after the placebo/NTG injection, the rats were perfused transcardially with  
27 100 mL phosphate-buffered saline (PBS, 0.1 M, pH 7.4), followed by 500 mL 4%  
28 paraformaldehyde in phosphate-buffer under chloral hydrate (0.4 g/kg bodyweight)  
29 anesthesia. The C1-C2 segments of the cervical spinal cord between -5 and -11 mm  
30 from the obex were removed and postfixed overnight for immunohistochemistry in the  
31 same fixative. After cryoprotection, 30 µm cryostat sections were cut and serially  
32 collected in wells containing cold PBS. The free-floating sections were rinsed in PBS  
33 and immersed in 0.3% H<sub>2</sub>O<sub>2</sub> in methanol or PBS for 30 minutes. After several rinses in  
34 PBS containing 1% Triton X-100, sections of C1-C2 were kept for overnight at room  
35 temperature in anti-TRPV1 antibody (Santa Cruz, s.c.28759) at a dilution of 1:500, or  
36 for two nights at 4 °C in anti-nNOS antibody (EuroProxima, 2263B220-1) at a dilution  
37 of 1:5000, or for two nights at 4 °C in anti- NF-κB antibody (Abcam, ab97726) at a  
38 dilution of 1:100. The immunocytochemical reaction was visualized by the Vectastain  
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11 avidin-biotin kit of Vector Laboratories (PK6101), and nickel ammonium  
12 sulphate-intensified 3,3'-diaminobenzidine. The specificity of the immune reaction was  
13 controlled by omitting the primary antisera.  
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### 16 17 18 *Western blot analysis*

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20 Four hours after the placebo/NTG injection, the animals were perfused transcardially  
21 with 100 mL PBS and the dorsal horns of C1-C2 segments were extracted. Until the  
22 measurements, the samples were stored at -80 °C. The samples were sonicated in ice  
23 cold lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.1% igePAL, 0.1% cholic  
24 acid, 2 µg/mL leupeptin, 2 mM phenylmethylsulphonyl fluoride, 1 µg/mL pepstatin, 2  
25 mM EDTA and 0.1% sodium dodecyl sulphate. The lysates were centrifuged at 12 000  
26 RPM for 10 minutes at 4 °C and supernatants were aliquoted and stored at -20 °C until  
27 use. Protein concentration was defined with BCA Protein Assay Kit using bovine serum  
28 albumin as a standard. Previous to loading, each sample was mixed with sample buffer,  
29 and denaturated by boiling for 3 minutes. Equal amounts of protein samples (20  
30 µg/lane) were separated by standard SDS polyacrylamide gel electrophoresis on 10%  
31 Tris-Glycine gel and electrotransferred onto Amersham Hybond-ECL nitrocellulose  
32 membrane (0.45 µm pore size). We used the Page Ruler Prestained Protein Ladder (10-  
33 170 kDa) to define approximate molecular weights. Following the transfer, membranes  
34 were blocked for one hour at room temperature in Tris-buffered saline containing  
35 Tween 20 (TBST) and 5% non fat dry milk powder. Then, they were incubated in TBST  
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11 containing 1% non fat dry milk and nNOS antibody (BD Biosciences, 610308, dilution:  
12 1:2000, incubation: overnight at room temperature), or COX-2 antibody (Proteintech,  
13 12375-1-AP, dilution: 1:1000, incubation: overnight at room temperature), or KAT-II  
14 antibody (Santa Cruz, sc-67376, dilution: 1:10 000, incubation: overnight at room  
15 temperature) or  $\beta$ -actin antibody (Calbiochem, CP01, dilution: 1:100 000, incubation:  
16 overnight at room temperature). Next day, membranes were incubated in TBST  
17 containing 1% non fat dry milk and horseradish peroxidase-conjugated anti-rabbit or  
18 anti-mouse secondary antibody (Santa Cruz Biotechnology, sc-2030, sc-2031) for two  
19 hours at room temperature. Protein bands were visualized after incubation of  
20 membranes with the SuperSignal West Pico Chemiluminescent Substrate using  
21 Carestream Kodak BioMax Light film.  
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### 33 ***Data evaluation***

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35 All evaluations were implemented by an observer blind to the experimental groups. The  
36 detailed methods were described previously ([34-36](#), [34, 36, 37](#)).  
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### 40 ***Immunohistochemistry (TRPV1, nNOS, NF- $\kappa$ B)***

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42 The photomicrographs of the stained sections of C1-C2 were taken using Zeiss  
43 AxioImager microscope supplied with an AxioCam MRc Rev.3 camera (Carl Zeiss  
44 Microscopy, Jena, Germany).  
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11 The area covered by TRPV1-immunoreactive fibres and nNOS-immunoreactive cells  
12 was determined by Image Pro Plus 6.2® image analysis software (Media Cybernetics).  
13 After image acquisition, the laminae I-II in dorsal horn were defined manually as area  
14 of interest and a threshold grey level was validated with the image analysis software, as  
15 described in an earlier study (32, ~~3536~~). The program calculated the area innervated by  
16 the immunoreactive fibres and cells as the number of pixels with densities above the  
17 threshold, the data were expressed as area fractions (%) of the corresponding  
18 immunolabelled structures.  
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21 We used the unbiased optical disector method to calculate the volume densities of the  
22 NF-κB-immunoreactive cells (~~3637~~).  
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### 27 ***Western blot analysis (nNOS, COX-2, KAT-II)***

28 For densitometric analyses, films were scanned and quantified using Java ImageJ 1.47v  
29 analysis software (National Institutes of Health). The results were normalized to β-actin.  
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### 32 ***Statistical analysis***

33 Statistical analysis of measurements were performed in SPSS Statistics software  
34 (Version 20.0 for Windows, SPSS Inc.) using one-way analysis of variance (ANOVA)  
35 followed by the Tukey or Tamhane post hoc test depending on variances of data, with  
36 P<0.05 taken as statistically significant. Group values are reported as means ± SEM.  
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## Results

### *NTG induced an increase in TRPV1 expression in the C1-C2 - AEA inhibited this phenomenon*

On transverse sections of the C1-C2 segments, there were abundant TRPV1-positive fibres in the superficial layers of the dorsal horn. The TRPV1-immunoreactive area in the NTG-treated group was significantly higher compared to the placebo-treated group ( $P<0.05$ ). The NTG-induced increase was attenuated by treatment with AEA ( $P<0.05$ ). (Fig 1.)

[Insert Figure 1.]

### *NTG increased nNOS expression in the C1-C2 and AEA attenuated this effect*

On transverse sections of the C1-C2 segments, nNOS-immunoreactive neurons and processes with cytoplasmic staining can be observed in the superficial layers of the dorsal horn. In the NTG group, the area fraction of nNOS-immunoreactive structures was significantly higher than in the placebo-treated group ( $P<0.01$ ). AEA treatment resulted in a decrease of nNOS-immunopositive structures ( $P<0.001$ ). (Fig 2.)

[Insert Figure 2.]

Western blot analysis of the C1-C2 region confirmed the results obtained by nNOS immunohistochemistry. A band characteristic of the nNOS protein was identified at 155

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11 kDa. Densitometric analyses confirmed that the nNOS bands were significantly  
12 enhanced ( $P<0.01$ ) in C1-C2 after NTG administration as compared with the placebo-  
13 treated animals. This effect was blocked by the treatment with AEA ( $P<0.05$ ). (Fig 3.)  
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17 [Insert Figure 3.]  
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20 ***NTG treatment enhanced NF- $\kappa$ B expression and AEA mitigated this phenomenon***  
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22 In transverse sections of the C1-C2 region high number of NF- $\kappa$ B-positive cells can be  
23 seen in the superficial layers of the dorsal horn. In the NTG treated animals the volume  
24 density of NF- $\kappa$ B-positive cells was significantly higher than in the placebo group  
25 ( $P<0.05$ ). This value decreased in the AEA injected group ( $P<0.05$ ). (Fig 4.)  
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30 [Insert Figure 4.]  
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33 ***NTG enhanced expression of COX-2 enzyme and AEA inhibited this action***  
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35 A band characteristic of the COX-2 protein was identified at 68 kDa in Western blot  
36 assay. Densitometric analyses showed that the COX-2 bands were significantly  
37 enhanced ( $P<0.01$ ) in segments C1-C2 after NTG administration as compared with the  
38 placebo-treated animals. The effect of NTG was decreased by the AEA treatment  
39 ( $P<0.01$ ). (Fig 5.)  
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11 ***NTG decreased KAT-II expression, which was alleviated by AEA***

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13 A band characteristic of the KAT-II protein was referred at 60 kDa in Western blot  
14 assay. Densitometric analyses confirmed that the KAT-II bands were significantly  
15 weaker ( $P<0.05$ ) in segments C1-C2 after NTG administration as compared with the  
16 placebo-treated animals. This effect was reversed by treatment with AEA ( $P<0.05$ ). (Fig  
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23 [Insert Figure 6.]  
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## Discussion

The present data indicate that NTG treatment activates the trigeminal system and the observed changes can be interpreted as a central sensitization phenomenon. The NTG induced alterations are reversed by the administration of AEA suggesting the involvement of CBs in this process.

It is generally accepted that the trigeminal pain processing and sensitization is an extremely complex phenomena involving numerous cellular and molecular components but the associated pathways are not fully known. Figure 7. shows a schematic and simplified flow diagram, which indicates that the connections are sometimes indirect and the signaling molecules may modulate the complex response at different target points.

[Insert Figure 7.]

### *TRPV1*

TRPV1 is present in the terminals of primary sensory neurons in the dorsal part of spinal cord (3738) and co-expressed with CB1 (3839). Experimental data indicate that TRPV1 contributes to the peripheral sensitization, allodynia and hyperalgesia (3940), central blockade of this receptor is able to attenuate central terminal sensitization (4041). Our results show that NTG significantly increases TRPV1 expression in the C1-C2 segments of the rat, thus it may be the indicator of sensitization phenomena in the trigeminal system in our experimental setting.

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11 It is well evidenced that NO donors can activate TRPV1 in several cell types (24, ~~4442~~)  
12 and in inflammatory pain models increased receptor expression was also reported (~~4243~~,  
13 ~~4344~~). This indicates that the NTG effect on TRPV1 expression is indirect - it may  
14 ~~4344~~. This indicates that the NTG effect on TRPV1 expression is indirect - it may  
15 cause neurogenic inflammation (~~4445~~), and the inflammatory mediators (~~like~~ serotonin  
16 (~~5-HT~~), bradykinin (~~BK~~), etc.) - can activate TRPV1 by stimulating trigeminal  
17 nociceptive neurons (~~4546~~). ~~In the animal model of inflammation, Complete Freud's~~  
18 ~~adjuvant increases TRPV1 mRNA expression in the dorsal root ganglion. Pro-~~  
19 ~~inflammatory mediators, such as tumor necrosis factor  $\alpha$ , interleukin 1 (IL-1),~~  
20 ~~interleukin 6 (IL-6) and BK enhance TRPV1 (~~4647~~);. In the animal model of~~  
21 ~~inflammation, Complete Freud's adjuvant increases TRPV1 mRNA expression in the~~  
22 ~~dorsal root ganglion (48), this also supports the idea that inflammation upregulates~~  
23 TRPV1 expression. ~~To summarize, we may assume that NTG is able to activate TRPV1~~  
24 ~~mainly via inflammatory mediators. On the other hand, there are research data~~  
25 ~~indicating that systemic administration of calcitonin gene-related peptide (CGRP)~~  
26 ~~increases the expression of TRPV1 in the TG of rats (49), which might also play a role~~  
27 ~~in this process.~~

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42 Concerning the possible mechanisms we have to consider that intravenous injection of  
43 calcitonin gene related peptide (CGRP) increases the expression of TRPV1 in TG of  
44 rats (47). As NTG treatment results in an increased CGRP release from the primary  
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~~terminals of the caudal trigeminal nucleus (48), we may assume that NTG is able to activate TRPV1 via CGRP release or/and by inflammatory mediators.~~

Our recent data also show that AEA, a CB receptor and TRPV1 agonist attenuates the effect of NTG on TRPV1 changes. Activation of ionotropic CBs can result in inhibition of nociceptors and antihyperalgesia and antinociception in certain pain models (4950).

Intrathecal administration of AEA decreases thermal pain sensitivity and its effect can be altered with the TRPV1 antagonist capsazepine (5051). AEA may also cause a desensitization of TRPV1 in skeletal muscle arterioles (5152), suggesting that AEA is

able to mitigate TRPV1 activity. On the other hand, AEA can inhibit neurogenic, CGRP- and NO-induced dural vasodilatation, and this involves pre- and postsynaptic mechanisms (5253). Recent report shows that the AEA level changes are able to

modulate CGRP mRNA-expression after NTG-treatment in human peripheral blood mononuclear cells (5354). We do not know exactly the role of TRPV1 in the AEA modulated sensitization process, but based on the available literature data (15, 5455),

we hypothesize that the role of CB1 is more pronounced than TRPV1 in this context.

### *nNOS*

It is known that nNOS is a key player in nociception (5556) and its role in the sensitization cascade is intensively studied. In our present experiment administration of NTG increased the expression of nNOS in the C1-C2, which is in line with earlier results (10). The most probable explanation for this phenomenon is, that NO activates

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11 small caliber fibres in the trigeminal system and the increase of nNOS expression in the  
12 second order trigeminal neurons induces a self-amplifying mechanism (10, 11). The  
13 present results indicate that AEA is able to inhibit this effect. Several studies have  
14 shown that there is an interaction between NO and cannabinoid system e.g. nNOS and  
15 CB1 are co-localized in neurons in lamina II of the spinal cord (~~5657~~), NTG-induced  
16 hyperalgesia is associated with a fluctuation of endocannabinoid system in various brain  
17 areas of rats (22). Our data are in line with Hillard and co-workers findings, who  
18 reported that CB1 agonists inhibit KCl-induced activation of nNOS in cultured  
19 cerebellar granule cells (~~5758~~). In addition, Carney and co-workers have detected that  
20 cannabinoid agonist downregulated nNOS protein and mRNA in neuronal cells (~~5859~~).  
21 These data suggest that NTG is able to generate sensitization process and AEA inhibits  
22 this effect by blocking nNOS.  
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#### 41 *NF-κB*

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43 In our experiment NTG increased NF-κB expression in the superficial layers of the  
44 dorsal horn in the C1-C2 segments. Similar effect was reported by Reuter et al., who  
45 demonstrated that NTG infusion is able to trigger the activation of NF-κB in dura mater  
46 (~~5960~~). It is not clear how NTG can activate NF-κB-pathway, it might be related to a  
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11 | direct neuronal effect of NTG, or indirect effect *via* dural inflammation (~~6061~~).

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12 | Concerning the cellular mechanisms it is important to note that both TRPV1 and nNOS

13 | might play a role in this effect. It was shown that Ca<sup>2+</sup> influx through TRPV1 may

14 | modulate the nuclear translocation and increased the activity of NF-κB (~~6162~~), on the

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15 | other hand the increase in nNOS expression is accompanied by increased NF-κB

16 | expression and activation (~~6263~~). Furthermore, Sancho and colleagues have noticed that

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17 | AEA inhibits tumor necrosis factor-α-induced activation, by inhibition of a cytokine-

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18 | induced cascade (~~6364~~). In addition, Nakajima and co-workers have found that AEA

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19 | also blocked lipopolysaccharide-induced activation, suggesting that AEA inhibits

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20 | proinflammatory mediators by blocking NF-κB activation (~~6465~~). Tassorelli and her

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21 | group reported that parthenolide (inhibitor of NF-κB) attenuated NTG-induced c-Fos

22 | activation in TNC (64), which indicates that NF-κB may be important in the NTG-

23 | induced trigeminal activation and its inhibition is able to modulate the nociceptive

24 | process. Our data reconfirm, that NTG is able to activate NF-κB, thus can trigger

25 | neurogenic inflammation, which has a key role in sensitization phenomena.

26 | Furthermore, we detected that AEA is able to reduce this effect. Endocannabinoids

27 | might operate a negative feedback control over the proinflammatory process by

28 | suppressing the activation of transcription factors involved in the inflammatory action

29 | (~~6566~~).

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## COX-2

NO may also cause neurogenic inflammation by increasing NF- $\kappa$ B levels, which may lead to the upregulation of COX-2 in inflammatory pain (6667). It is well-known, that NSAIDs, which exert their effects through the inhibition of COX-enzymes (6768), are effective in the treatment of migraine and tension-type headache (6869). In animal studies, it has been shown that COX-2 is involved in the NTG-induced activation and sensitization process of the trigeminal system. Pre-treatment with indomethacin (non-selective COX inhibitor) and NS398 (selective COX-2 inhibitor) reduced the NTG-induced c-Fos (11), nNOS and calmodulin-dependent protein kinase II alpha expression-changes in the TNC (6970, 7071). Tassorelli and colleagues have demonstrated that COX-2 expression is increased in the hypothalamus and caudal brain stem after NTG injection (7172), thus COX is one of the mediators of NTG-induced neuronal activation. Furthermore, NO is able to activate COX-enzymes in fibroblasts, probably by an interaction with the iron-hem center of the enzyme (7273). It is important to note, that AEA is one of the substrates of COX-2 producing prostaglandin and ethanolamids (7374). In our study, we have found that AEA is able to inhibit the NTG-induced COX-2 increase. The cellular and molecular background of this effect remains to be determined. Our assumption is that it may be associated with a negative feedback mechanism, but it is possible that after the cleavage of AEA some metabolites may downregulate COX-2 expression. Since AEA is able to reduce cytokine-induced

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11 cascade and proinflammatory mediators, it is also possible, that the reduction of the  
12 inflammatory process is able to downregulate the COX-2 expression.  
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### 14 *KAT-II*

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16 In our experiments NTG decreased KAT-II expression in the C1-C2 segments, which  
17 may indicate that fluctuation of KAT is involved in the NTG-triggered trigeminal  
18 activation. Recent studies have shown, that migraine is associated with mitochondrial  
19 dysfunction (7475). It has also been demonstrated that an NO donor is able to  
20 downregulate the respiratory chain complex and cause release of the mitochondrial  
21 cytochrome C, thus causing a mitochondrial dysfunction (7576). The decrease in KAT-  
22 II activity might be associated with mitochondrial damage as shown in the striatum of  
23 patients suffering in Huntington's disease (7677). This is in line with our earlier data  
24 showing, that administration of mitochondrial complex II inhibitor 3-nitropropionic acid  
25 is able to reduce KAT-II activity in rats (7778). On the other hand, NTG may cause  
26 neurogenic inflammation and the released pro-inflammatory cytokines can modulate the  
27 kynurenine pathway (7879), which may also contribute to the change in KAT-II  
28 expression. Nevertheless, in another model of trigeminal activation (electrical  
29 stimulation of the TG), decreased KAT-immunoreactivity in dural macrophages,  
30 Schwann cells and mastocytes was found in rats (7980), suggesting a prominent role of  
31 this enzyme in the process of trigeminal activation.  
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11 In the present experiment, we have detected that AEA is able to mitigate the NTG-  
12 induced KAT-II expression decrease. AEA and the other endocannabinoid molecule, 2-  
13 arachidonoylglycerol causes reduction of calcium-induced cytochrome C release from  
14 mitochondria, protect mitochondria from the cytochrome mediated damage, which leads  
15 to DNA fragmentation and apoptosis (8081, 8182). Taken together it is possible, that  
16 NTG may cause mitochondrial dysfunction, and AEA is able to inhibit this effect  
17 probably by decreasing cytochrome C release.  
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### 27 *Conclusions*

28 In conclusion, our results show that AEA has modulating effect on central sensitization  
29 markers in our experimental setting. These data suggest that the endocannabinoid  
30 system plays a significant role in the cellular mechanism of trigeminal sensitization and  
31 thus it may modulate the pathomechanism of migraine.  
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**Conflict of interest**

The authors declare that they have no conflict of interest.

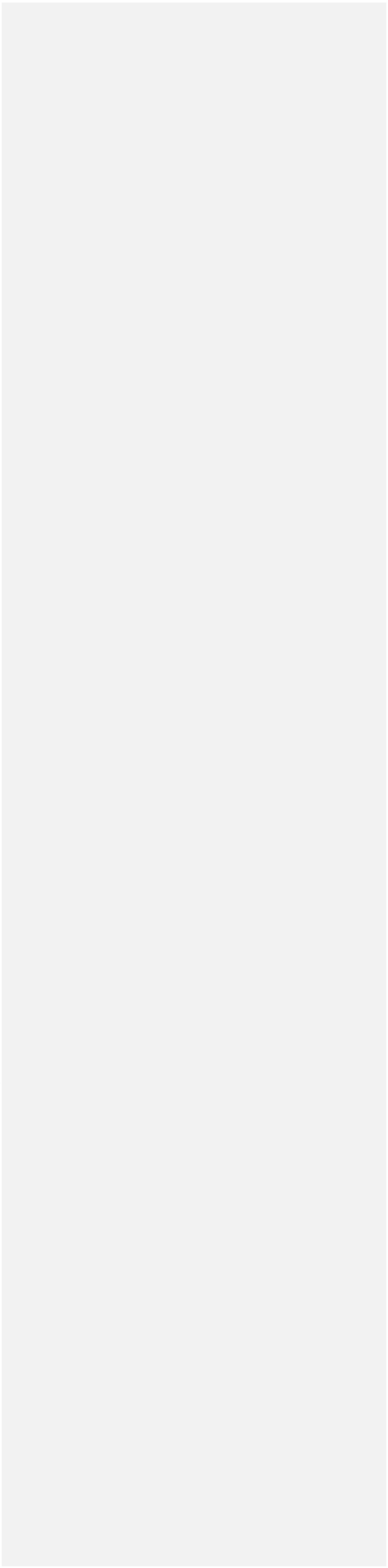
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**Article highlights**

- Systemic administration of nitroglycerin is able to activate and sensitize the trigeminal system in rats
- This phenomenon is reflected by alteration of the expression of biological markers (TRPV1, nNOS, NF- $\kappa$ B, COX-2 and KAT-II) of this process
- Anandamide, a cannabinoid receptor agonist is able to modulate these changes caused by nitroglycerin
- Cannabinoid receptors are involved in the activation and sensitization of the trigeminal system

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**-Figure captions**

Figure 1. A. Representative photomicrographs of the TRPV1 expression in the C1-C2 segments. B. Changes in area fractions of TRPV1-immunoreactive fibers in superficial laminae I and II of the C1-C2 segments. In the NTG group, the area covered by TRPV1 was significantly higher than in the placebo group. AEA seemed to block this effect. Bargraph data presented here and in succeeding figures are means  $\pm$  SEM. Scale bar: 100  $\mu$ m, \*P<0.05

Figure 2. A. Representative photomicrographs of nNOS expression in the C1-C2 segments. B. Changes in the nNOS-immunoreactive area fractions. In the NTG group, the area fraction of nNOS-IR structures was increased compared to the placebo-treated group. AEA was able to attenuate this effect. Scale bar: 100  $\mu$ m, \*\*P<0.01; \*\*\*P<0.001

Figure 3. A. Western blot of nNOS and  $\beta$ -actin (~~loading control~~) expression in the C1-C2. B. The quantitative analysis shows that in the NTG group, the relative optical density of nNOS specific band was significantly higher than in the placebo group. AEA treatment weakened this effect. \*P<0.05; \*\*P<0.001

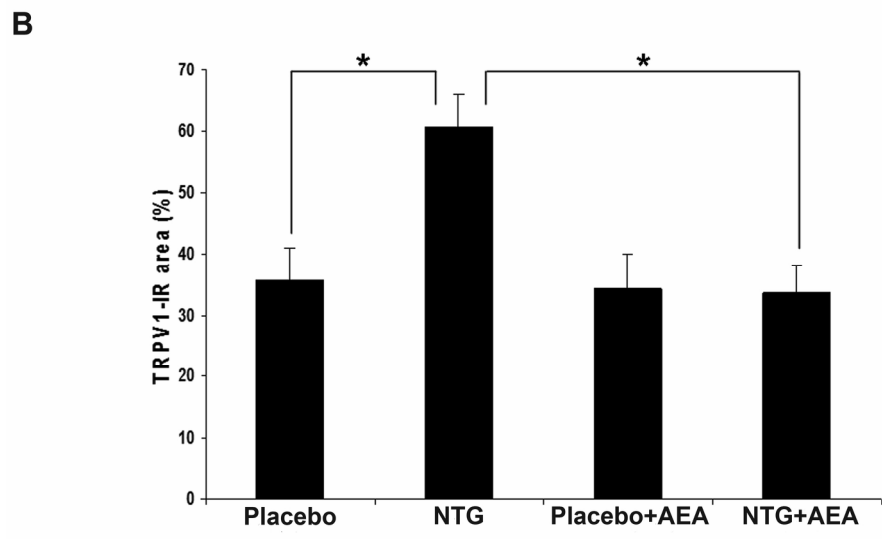
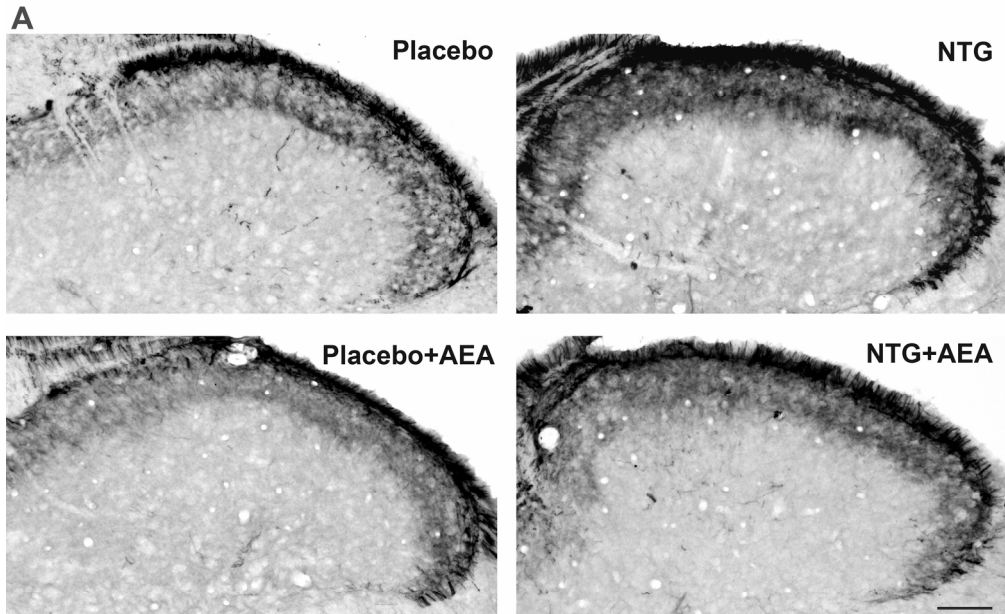
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11 Figure 4. A. Photomicrographs of the NF- $\kappa$ B expression in the C1-C2 segment. B.  
12 Diagram shows that NTG treatment resulted in a significant increase in the volume  
13 density of NF- $\kappa$ B-immunoreactive cells. This effect was not observed in AEA-injected  
14 animals. Scale bar: 50  $\mu$ m, \*P<0.05  
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21 Figure 5. A. Western blot of COX-2 and  $\beta$ -actin expression in the C1-C2. B.  
22 Densitometry of the individual bands indicated that in the NTG-treated animals the  
23 expression of COX-2 was significantly higher than in the placebo group. No such effect  
24 was seen in the AEA-injected group. \*\*P<0.01  
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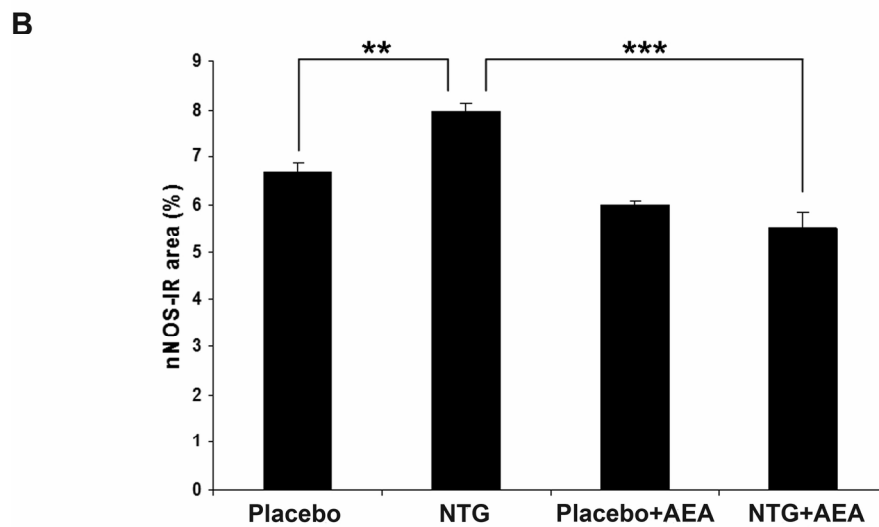
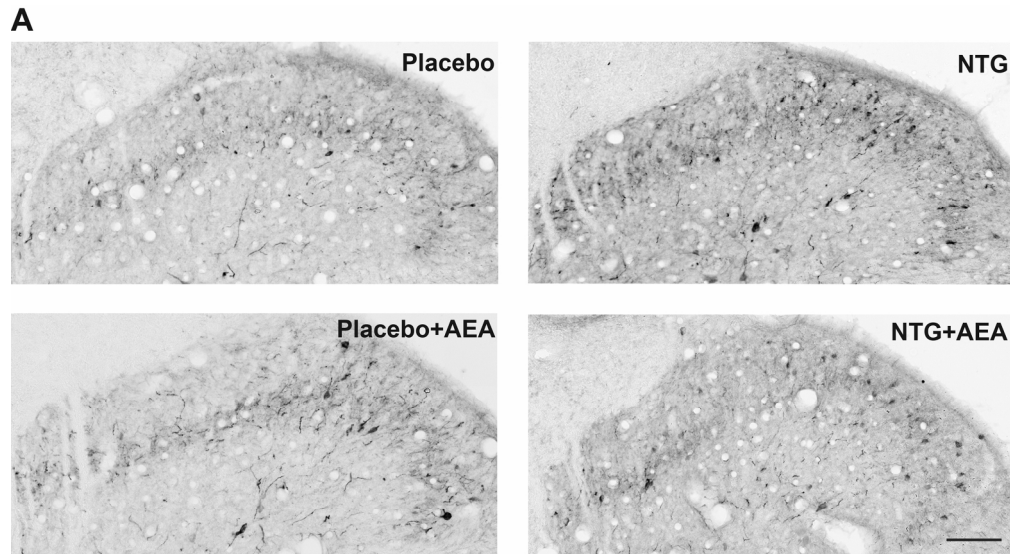
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31 Figure 6. A. Western blot of KAT-II and  $\beta$ -actin expression in the C1-C2. B.  
32 Quantitative data demonstrate that in the NTG group, the relative optical density of  
33 KAT-II is significantly lower than in the placebo group. AEA reduced this effect.  
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36 \*P<0.05  
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41 Figure 7. Schematic illustration of the interactions among the NO-influenced molecules  
42 involved in the trigeminal pain processing and sensitization showing the possible sites  
43 of action for cannabinoids.  
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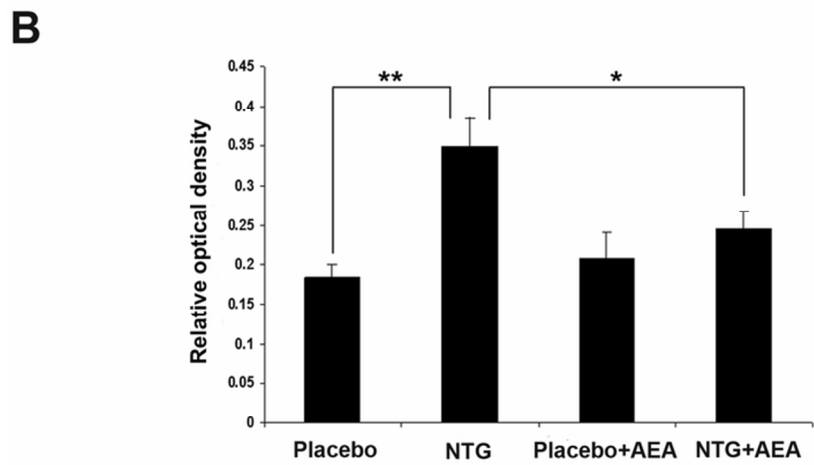
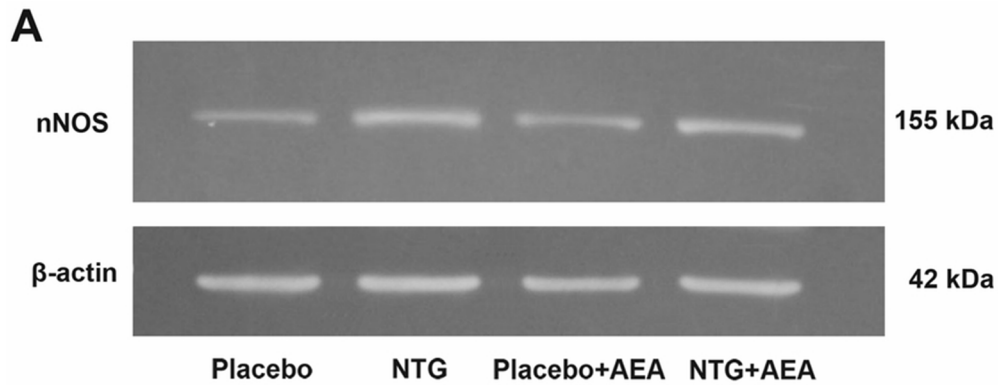


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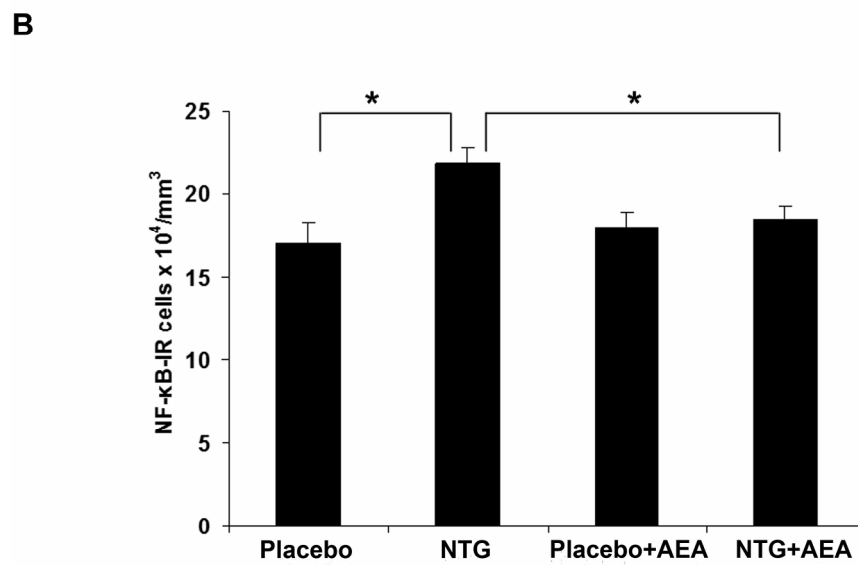
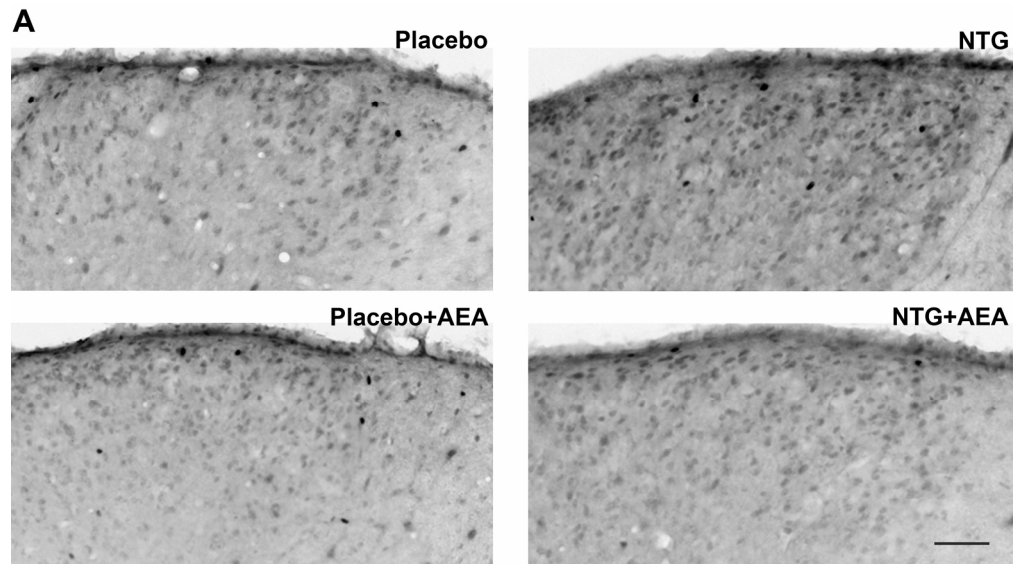
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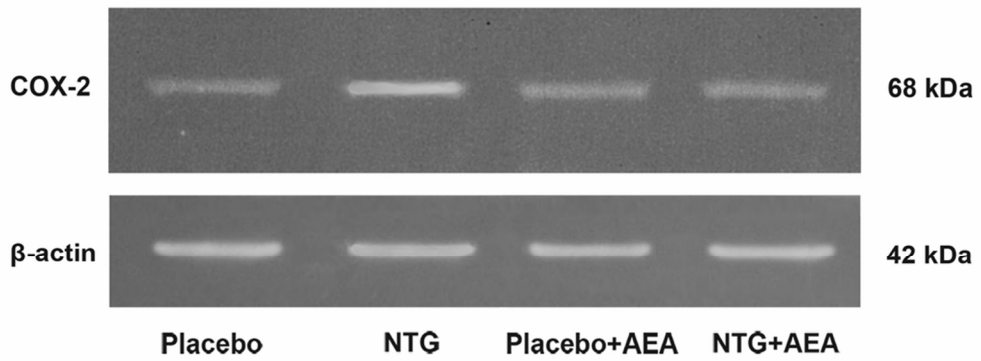




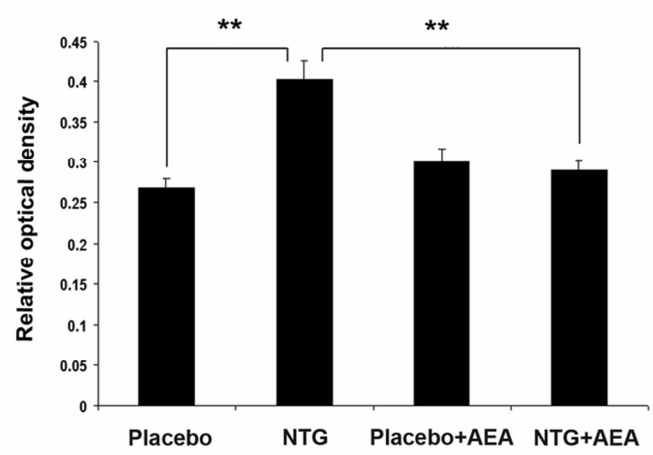
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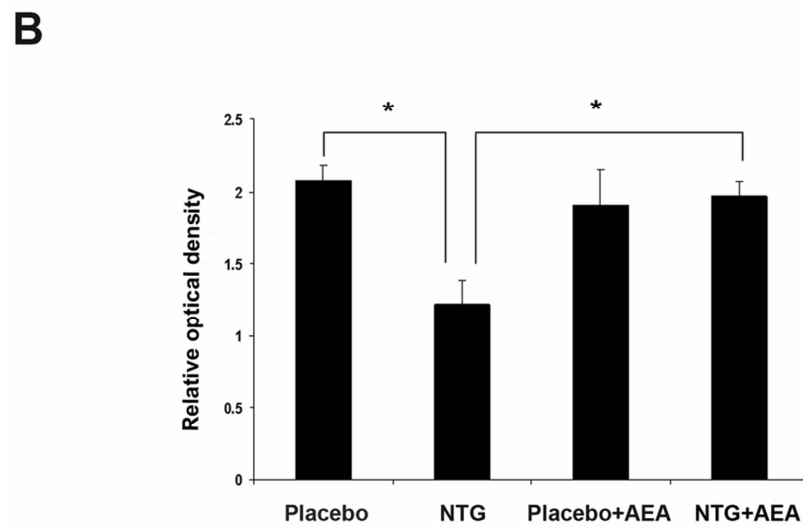
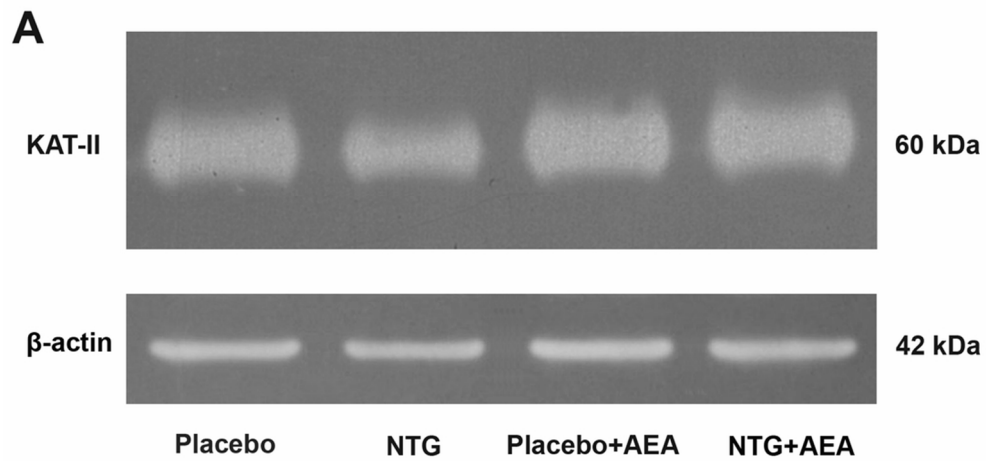
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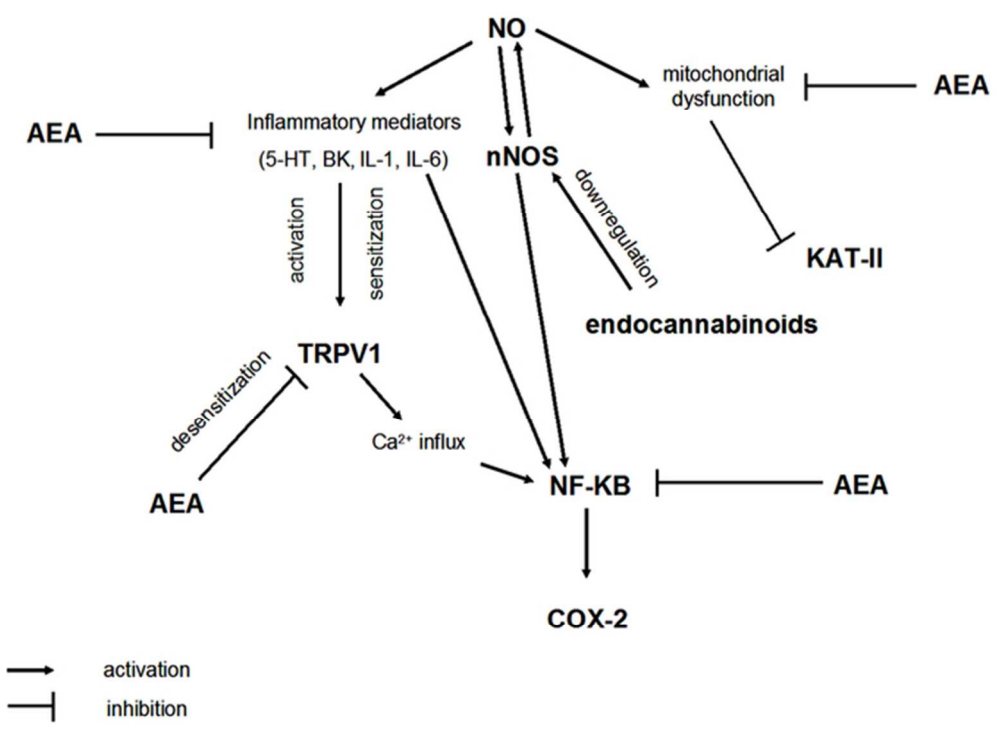


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# The ARRIVE Guidelines Checklist

## Animal Research: Reporting In Vivo Experiments

Carol Kilkenny<sup>1</sup>, William J Browne<sup>2</sup>, Innes C Cuthill<sup>3</sup>, Michael Emerson<sup>4</sup> and Douglas G Altman<sup>5</sup>

<sup>1</sup>The National Centre for the Replacement, Refinement and Reduction of Animals in Research, London, UK, <sup>2</sup>School of Veterinary Science, University of Bristol, Bristol, UK, <sup>3</sup>School of Biological Sciences, University of Bristol, Bristol, UK, <sup>4</sup>National Heart and Lung Institute, Imperial College London, UK, <sup>5</sup>Centre for Statistics in Medicine, University of Oxford, Oxford, UK.

	ITEM	RECOMMENDATION	Section/ Paragraph
Title	1	Provide as accurate and concise a description of the content of the article as possible.	Title
Abstract	2	Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.	Abstract
<b>INTRODUCTION</b>			
Background	3	a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale. b. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology.	Introduction
Objectives	4	Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested.	Introduction-last paragraph
<b>METHODS</b>			
Ethical statement	5	Indicate the nature of the ethical review permissions, relevant licences (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research.	Materials and methods-animals paragraph
Study design	6	For each experiment, give brief details of the study design including: a. The number of experimental and control groups. b. Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when). c. The experimental unit (e.g. a single animal, group or cage of animals). A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.	Materials and methods-drug administration paragraph
Experimental procedures	7	For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example: a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s). b. When (e.g. time of day). c. Where (e.g. home cage, laboratory, water maze). d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).	Materials and methods-animals and drug administration paragraph
Experimental animals	8	a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range). b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc.	Materials and methods-animals and drug administration paragraph

The ARRIVE guidelines. Originally published in *PLoS Biology*, June 2010<sup>1</sup>

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For Peer Review

Housing and husbandry	9	<p>Provide details of:</p> <p>a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish).</p> <p>b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment).</p> <p>c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.</p>	Materials and methods-animals paragraph
Sample size	10	<p>a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group.</p> <p>b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used.</p> <p>c. Indicate the number of independent replications of each experiment, if relevant.</p>	Materials and methods-animals and drug administration paragraph
Allocating animals to experimental groups	11	<p>a. Give full details of how animals were allocated to experimental groups, including randomisation or matching if done.</p> <p>b. Describe the order in which the animals in the different experimental groups were treated and assessed.</p>	Materials and methods-animals and drug administration paragraph
Experimental outcomes	12	Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).	Materials and methods-Data evaluation
Statistical methods	13	<p>a. Provide details of the statistical methods used for each analysis.</p> <p>b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron).</p> <p>c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.</p>	Materials and methods-Statistical analyses paragraph
<b>RESULTS</b>			
Baseline data	14	For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naïve) prior to treatment or testing. (This information can often be tabulated).	Results
Numbers analysed	15	<p>a. Report the number of animals in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50%<sup>2</sup>).</p> <p>b. If any animals or data were not included in the analysis, explain why.</p>	Results
Outcomes and estimation	16	Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).	Results
Adverse events	17	<p>a. Give details of all important adverse events in each experimental group.</p> <p>b. Describe any modifications to the experimental protocols made to reduce adverse events.</p>	Results
<b>DISCUSSION</b>			
Interpretation/scientific implications	18	<p>a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature.</p> <p>b. Comment on the study limitations including any potential sources of bias, any limitations of the animal model, and the imprecision associated with the results<sup>2</sup>.</p> <p>c. Describe any implications of your experimental methods or findings for the replacement, refinement or reduction (the 3Rs) of the use of animals in research.</p>	Discussion
Generalisability/translation	19	Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.	Conclusions

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Funding	20	List all funding sources (including grant number) and the role of the funder(s) in the study.	Funding
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References:

1. Kilkenney C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS Bio* 8(6): e1000412. doi:10.1371/journal.pbio.1000412
2. Schulz KF, Altman DG, Moher D, the CONSORT Group (2010) CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 340:c332.

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