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THE EFFECT OF NALOXONE ON TRIGEMINO-HYPOGLOSSAL REFLEX INHIBITED BY PERIAQUEDUCTAL CENTRAL GRAY STIMULATION IN RATS

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The aim of the study was to determine whether opioid receptor antagonist naloxone abolishes the influence of periaqueductal central gray (PAG) on nociceptive evoked tongue jerks (ETJ) — a trigemino-hypoglossal reflex induced by tooth pulp stimulation. In rats under chloralose anesthesia three subsequent series of perfusions of lateral ventricles — cerebellomedullary cistern with Mc Ilwain-Rodnight’s solution, Met-enkaphalin (Enk-Met) and naloxone were carried out. The amplitudes of tongue jerks induced by tooth pulp stimulation were recorded during subsequent 10 min perfusions. Mean amplitude of tongue movements induced by tooth pulp stimulation was regarded as the indicator of the magnitude of trigemino-hypoglossal reflex. We observed that perfusion of the cerebral ventricles with Enk-Met (100 nmol/mL) inhibited the trigemino-hypoglossal reflex by 46%, whereas naloxone (100 nmol/mL), added to the solution perfusing the cerebral ventricles system, increased the reflex by 42%. The amplitude of ETJ was significantly reduced during PAG stimulation with a train of electrical impulses. After obtaining a significant — 93% — inhibition of ETJ, naloxone (100 nmol/mL) was added to the perfusion fluid. This led to a significant increase of the reflex by 68%. The above results suggest that the inhibition of ETJ due to PAG stimulation is partially reversed by naloxone and mediated via interactions with endogenous opioid systems involved in modulation of nociception.

Keywords: periaqueductal central gray, tongue jerks, cerebral ventricle, nociceptive and antinociceptive effects

INTRODUCTION

In the midbrain periaqueductal gray matter (PAG) is a region which has been identified as important in pain modulation, particularly in the generation of analgesia. This activity can be elicited by electrical stimulation (1—10) and by intraventricular or direct injection of opiates (11—14) and non-opioid peptides (15—17). Akil et al. (18) and Oliveras et al. (5) confirmed that there is
evidence for the role of endogenous opioid peptides in analgesia induced by 
stimulation (SPA), demonstrating, that analgesia induced by PAG stimulation 
in rats and cats was partially blocked by a specific opioid receptor antagonist — naloxone.

The role of endogenous opioid peptides in nociceptive mediation from 
PAG is controversial and depends on the pathway, injection site and dose. 
Some authors did not observe any interactions of naloxone with SPA (12,19,20) 
and even if naloxone was effective, it caused a slight reduction of SPA (21), 
which resulted from the simultaneous excitation of internal nerve terminals 
containing opiates and efferent PAG neurons, dependent on stimulation 
parameters (22).

The aim of our study was to determine whether naloxone, perfused through 
the cerebroventricular system, abolishes the effect of PAG on nociceptive 
evoked tongue jerks induced by tooth pulp stimulation and what is the role of 
endogenous opioid peptides present in the cerebrospinal fluid passing through 
the cerebral aqueduct in the effect of PAG surrounding the aqueduct and the 
nervous centres located in the vicinity of the cerebral ventricles on transmission 
of neural impulses.

MATERIALS AND METHODS

Experimental animals and anesthesia

The animal protocols used in these experiments were approved by the Institutional Animal 
Care and Use Committee of Department of Physiology, Institute of Physiology and Biochemistry, 
Medical University of Lodz.

The experiments were carried out on male Long-Evans rats of body weight 470—500 g, aged 
approx. 5 months, bred in the Department of Physiology, Medical University of Lodz. The animals 
were kept under standard conditions: temperature 22°C, alternating 14 h periods of light and 
10 h of darkness. The rats were fed with granulated LSM rodent chow and received water ad 
libitum.

The rats were anesthetized with a single intraperitoneal injection of chloralose solution in 
a dose of 150 mg/kg body weight. The experimental animals were divided into 3 groups, 10 
animals in each group. In group 1 subsequent 10 min perfusions of the cerebral ventricles 
with McIlwain-Rodnight’s solution and Enk-Met (100 nmol/mL) were carried out. In group 
2 subsequent 10 min perfusions of the cerebral ventricles with McIlwain-Rodnight’s solution 
and naloxone (100 nmol/mL) were carried out. In group 3—10 min perfusion of the cerebral 
ventricles with McIlwain-Rodnight’s solution with simultaneous tooth pulp stimulation, and 
then PAG stimulation and perfusion of the cerebral ventricles with naloxone (100 nmol/mL) 
were carried out.

Perfusion of cerebral ventricles in rats

The rat’s head was immobilized by introduction of ear bars into the external auditory 
meati and fixing the maxilla with jaw clamps in a stereotaxic instrument specially adapted
for perfusion of the cerebral ventricles. The skin of the animal’s head, anesthetized with 2% polocaine solution, was incised in the midline and the skull bones were exposed. On the basis of modified coordinates given by De Grott’s stereotaxic atlas (23), the sites for drilling holes in the skull bones were determined: to the lateral ventricles — 9 mm anterior to the frontal interaural zero plane and 3 mm lateral to the sagittal zero plane, and to the central midbrain PAG — 3 mm anterior to the frontal interaural zero plane and 1 cm lateral to the sagittal zero plane.

The system of cerebral ventricles was perfused by inserting stainless steel cannulae into both lateral ventricles and to the cerebellomedullary cistern, according to the method described earlier (24, 25). The container with perfusion fluid was positioned 20 cm above the animal’s head. Mc Ilwain-Rodnight’s solution, prepared according to Daniel and Lederis (26) was used for perfusion. The outflow cannula inserted into the cerebellomedullary cistern was connected to a polyethylene tube ca 100 cm long which provided the outflow for the perfusion fluid. The flow rate at the end of the tubing in the course of perfusion was 0.7 mL/10 min.

After control perfusion with Mc Ilwain-Rodnight’s solution, the cerebral ventricles were perfused with met-enkephalin (Enk-Met) (Peninsula Lab.) added to Mc Ilwain-Rodnight’s solution to obtain 100 nmol/mL concentration and naloxone (Sigma) to obtain 100 nmol/mL concentration.

At the end of each experiment the cerebral ventricles were perfused with 1% trypan blue solution till the stain appeared in the outflow tubing leading out of the cerebellomedullary cistern.

**Midbrain periaqueductal gray stimulation**

The stimulating electrodes consisted of stainless steel guiding tubes 35 mm long and 0.5 mm in diameter with inserted Insect Pint 00 pins with 0.3 mm diameter. The electrodes were covered with five coats of RDB — 9 transformer lacquer. After each coating the tubes with pins were dried in a dryer at 170°C for 30 min, and checked with an ohmmeter whether the coat was continuous. The Insect Pint 00 pins were inserted into the tubes so that they projected 7 mm below the ends of the tubes.

The PAG was stimulated with electrical current trains using a pair of electrodes which insulated tips were positioned on either side of the cerebral aqueduct. Each time a 10 min train of square electrical impulses of 100 Hz frequency, 1 ms impulse duration and constant 0.5—1.0 mA intensity were delivered using a Disa Multistim stimulator.

**Tooth pulp stimulation**

After placing the animal’s head in a stereotaxic instrument, the tips of incisors were cut off with a dental separator and stainless steel wire electrodes were inserted into the pulp and fixed with dental cement. Bipolar stimulation was delivered 6 times per minute, with a train of 4 impulses of 200 Hz frequency, 3 ms single impulse duration and 4—5 V amplitude, using a programmed stimulator.

The amplitudes of electrical impulses stimulating the incisor pulp were adjusted individually for each animal. At the beginning of each experiment the intensity of stimulus inducing maximum tongue jerks was determined. Then, the amplitude of impulses was reduced to obtain the amplitude of tongue jerks equal to half of the maximum values. The amplitude of stimulating impulses adjusted in this way, as well as their other parameters, remained unchanged till the end of the experiment.
Recording tongue jerks

The tip of the animal's tongue was attached with a silk thread to an isotonic rotating tensometric transducer. The amplitude of tongue jerks was recorded by a Line Recorder TZ-4620 (Laboratori Pristroje Praha, Czech Republic). The tongue was stretched with the same force, ca. 5.8 G throughout the experiment, the amplification of the recorder also remained unchanged.

In each animal during the first 10 min of perfusion and during PAG stimulation, the amplitude of tongue jerks evoked by tooth pulp stimulation was recorded. The mean amplitude of tongue jerks evoked by tooth pulp stimulation was regarded as an indicator of magnitude of the trigemino-hypoglossal reflex. Mean amplitudes of ETJ induced by pulp stimulation during perfusion with McIlwain-Rodnight's solution, investigated solutions and PAG stimulation were compared separately.

At the end of each experiment, constant electric current was passed through the electrodes positioned in the central midbrain PAG. For that purpose, a reference electrode (cathode) connected to the negative pole was placed in the rectum, then the electrode delivering PAG stimulation was connected to an anode (positive pole) and 10 mA constant current was delivered for 5 sec. Then the animal's thoracic cavity was opened and the left cardiac ventricle was perfused with 250 mL solution of the following composition: potassium ferricyanide (15 g), acetic acid (20 mL), ethanol (740 mL), 40% formalin (150 mL), and water (1350 mL).

The rats were beheaded and the heads were placed in 10% formalin solution. After a week the brains were removed from the skulls and the staining of the lateral ventricles, 3rd and 4th ventricles, as well as correct positioning of cannulae in the lateral ventricles was checked. 60 um thick sections were cut parallel to the frontal plane from the midbrains frozen with solid CO₂, and the electrode insertion sites in the PAG and the midbrain were verified histologically.

Statistical analysis

The amplitude of evoked tongue jerks recorded on a tape was measured in millimeters, and the arithmetical mean was calculated from 60 ETJ obtained in the course of perfusion with the investigated solution. Statistical comparison between groups were performed by Anova analysis of variance. A value of p<0.01 was considered to be statistically significant. Values were presented as means ± standard error of the mean SEM.

RESULTS

Effect of Enk-Met, naloxone and PAG stimulation on trigemino-hypoglossal reflex

The experiments were carried out on 3 groups of rats, 10 animals in each group. In group 1 perfusion of the cerebral ventricles with Enk-Met (100 nmol/mL) inhibited the trigemino-hypoglossal reflex by 46% p<0.01 (Fig. 1 and Fig. 4).

In group 2 the cerebral ventricles were perfused with McIlwain-Rodnight's solution followed by naloxone (100 nmol/mL). It was demonstrated that the blockade of the opioid receptors by naloxone led to a significant, 66% increase of the amplitude of evoked tongue jerks induced only by tooth pulp stimulation (Fig. 2 and Fig. 5).
Fig. 1. Original recordings of evoked tongue jerks (ETJ) induced by incisor pulp stimulation in rat (one of experimental group 1) during perfusion of cerebral ventricles with McIlwain-Rodnight’s solution. (A) 1—10 min control perfusion and 60 ETJ records (1—10 min), mean amplitude 2.6 cm, (B) 10 min perfusion with 100 nmol/mL Enk-Met, 60 ETJ records (1—10 min), mean amplitude 1.2 cm.

Fig. 2. Original recordings of evoked tongue jerks (ETJ) induced by incisor pulp stimulation in rat (one of experimental group 2) during perfusion of cerebral ventricles with McIlwain-Rodnight’s solution: (A) 1—10 min control perfusion and 60 ETJ records (1—10 min), mean amplitude 2.5 cm, (B) 10 min perfusion with 100 nmol/mL of naloxone, 60 ETJ records (1—10 min), mean amplitude 3.5 cm.
Fig. 3. Original recordings of evoked tongue jerks (ETJ) induced by incisor pulp stimulation in rat (one of group 3) during perfusion of cerebral ventricles with McIlwain-Rodnight’s solution: (A) 1—10 min control perfusion and 60 ETJ records (1—10 min), mean amplitude 2.6 cm; (B) 10 min perfusion of McIlwain-Rodnight’s solution and stimulation of PAG, 60 ETJ records (1—10 min), mean amplitude 0.2 cm; (C) 10 min perfusion with 100 nmol/mL naloxone and 60 ETJ records (1—10 min), mean amplitude 1.7 cm.

In group 3 cerebral ventricles were perfused first with McIlwain-Rodnight’s solution and then, after PAG stimulation, with naloxone (100 nmol/mL). It was demonstrated that during tooth pulp stimulation delivered 6 times per minute and simultaneous continuous PAG stimulation, the amplitude of evoked tongue jerks was reduced by approx. 93%. The inhibition of ETJ amplitude was statistically significant. After adding naloxone to the fluid perfusing the cerebral ventricles system, the reflex tongue movements were significantly increased — by 68%, as compared with the control (Fig. 3 and Fig. 6).
**Fig. 4.** Effect of perfusion of cerebral ventricles with 100 nmol/mL Enk-Met solution on evoked tongue jerks (ETJ) induced by tooth pulp stimulation in rats of group 1 (n = 10). Values illustrated in the graph represent arithmetical means ± standard error of the arithmetical mean (x ± SEM).

**Fig. 5.** Effect of perfusion of cerebral ventricles with 100 nmol/mL naloxone solution on evoked tongue jerks (ETJ) induced by tooth pulp stimulation in rats of group 2 (n = 10). Values illustrated in the graph represent arithmetical means ± standard error of the arithmetical mean (x ± SEM).

**Fig. 6.** Effect of perfusion of cerebral ventricles with 100 nmol/ml naloxone solution on evoked tongue jerks (ETJ) inhibited by PAG stimulation in rats of group 3 (n = 10). Values illustrated in the graph represent arithmetical means ± standard error of the arithmetical mean (x ± SEM).
Histological verification

Histological verification of frontal cross-section of rat midbrain was performed according to the atlas by J. König and R. Klippel (27). It was shown that the tips of bipolar electrodes and the area of the electric field induced by electrical impulses used to stimulate midbrain PAG were located in ventral PAG.

DISCUSSION

Numerous experimental data supporting the hypothesis that there is a multineuronal network in the brainstem which is responsible for the inhibition of nociceptive impulses at the level of spinal dorsal horns and nucleus tractus spinalis nervi trigemini have appeared in the literature in the recent years (22, 28). The main portion of spinal and trigeminal pathways to PAG originates from the neurons of lamina I (28).

The discovery made by Cannon et al. (21) that the midbrain PAG is not equally capable of inducing a certain degree of anesthesia throughout the whole structure confirms the hypothesis that within PAG itself there is more than one system mediating the inhibition of nociceptive reactions. These authors observed that the threshold of current intensity necessary to induce anesthesia from the ventral PAG is lower, whereas anesthesia obtained by stimulation of the dorsal part of PAG persisted for a longer time. Cannon et al. (21), as well as Thorn et al. (29) suggest that the dorsal PAG constitutes a part of a non-opioid system of inhibition of nociceptive impulsion, whereas the ventral part belongs to an opioid system. Our experiments involved stimulation of the ventral PAG.

On the basis of experiments performed in group 3, direct evidence was obtained that blocking with naloxone the opioid receptors in the nervous centres adjacent to the cerebral ventricles abolishes, in a considerable degree, the inhibition of nociceptive retractoratory tongue reflex induced by PAG stimulation. This inhibition could be effected both at the level of the nerve XII nucleus, and of the nucleus tractus spinalis nervi trigemini, because the presence of enkephalins has been detected in both these structures. The inhibition of the investigated reflex is reduced after naloxone administration, because it blocks opioid receptors (30). Thus, PAG neurons exert inhibitory influence on the central neurons of trigemino-hypoglossal reflex arc via opioids, probably endogenous Enk-Met. It has been confirmed by the experiments performed in group 1 and 2 because exogenous Enk-Met perfused through the cerebral ventricles penetrates the blood-brain barrier and significantly reduces the trigemino-hypoglossal reflex.
The results of experiments involving perfusion of neuropeptides through the
cerebral ventricles in rats, obtained so far, indicate that ETJ can serve as
a good model for testing the effects of various neuropeptides on brainstem
nervous centres. Tongue stretching does not affect the excitability of
motoneurons of the XII nerve nucleus, because no intrafusal spindles were
observed in the tongue muscles (31). Annulospiral nerve endings in the
intrafusal spindles modulate the excitability of motoneurons supplying other
skeletal muscles via the spinomuscular loop. The magnitude of the
trigemino-hypoglossal reflex and the reflex-modulating factors are also
dependent on the branch of the trigeminal nerve which is stimulated with
electrical impulses to evoke tongue jerks.

On the basis of the obtained results, it can be concluded that perfusion of
the cerebral ventricles with naloxone effectively abolishes the inhibition of
tongue jerks evoked by PAG stimulation, which proves that both, Enk-Met
and naloxone, penetrate through the lining of the cerebral ventricles and act on
the structures of the CNS, modulating the trigemino-hypoglossal reflex directly
— by exerting an influence on the neurons of the reflex arc, or indirectly — by
acting on the neurons of the PAG, spinal and medullary nuclei, and that opioid
receptors are involved in modulation of the magnitude of this reflex.

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