Interactions of Galanin with Endomorphin-2, Vasopressin and Oxytocin in Nociceptive Modulation of the Trigemino-Hypoglossal Reflex in Rats

M. ZUBRZYCKA¹, A. JANECKA²

¹Department of Experimental Physiology and ²Laboratory of Biomolecular Chemistry, Medical University, Lodz, Poland

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Summary
Galanin (GAL) is suggested to be a neuropeptide involved in pain transmission. In this study we tried to determine, whether the increase of GAL concentration in brain cells affects impulse transmission between the motor centers localized in the vicinity of the third and fourth cerebral ventricles. The experiments were carried out on rats under chloralose anesthesia. The study objectives were realized using the method allowing to record the amplitude of evoked tongue jerks (ETJ) in response to noxious tooth pulp stimulation during the perfusion of the cerebral ventricles with solutions containing tested compounds. Perfusion of the cerebral ventricles with GAL concentration-dependently inhibited the ETJ amplitude. The antinociceptive effect of GAL was blocked by a galanin receptor antagonist, galantide (GLT) and by opioid antagonists: non-selective naloxone (Nal) and μ-selective β-funaltrexamine (β-FNA). In contrast, a δ-opioid receptor antagonist, naltirindole (NTI) or the κ-opioid receptor antagonist, nor-binaltrophimine (nor-BNI) did not inhibit the effect of GAL. The antinociceptive effect of GAL was more pronounced when GAL was perfused in combination with other neuropeptides/neurohormones, such as endomorphin-2 (EM-2), vasopressin (AVP) and oxytocin (OT). The present results demonstrate that in the orofacial area analgesic activity is modulated by GAL, OT and AVP and that EM-2-induced antinociception involves GAL.

Key words
Antinociception • Galanin • Vasopressin • Oxytocin • Galanin antagonist • Opioid antagonists • Perfusion of cerebral ventricles

Introduction
Galanin (GAL) is a 29/30-amino-acid neuropeptide (Tatemoto et al. 1983) present in the central nervous system (CNS) together with other neuropeptides. GAL exerts its biological effect by interacting with three high affinity cell surface receptors GALR1-3, which all belong to the family of G-protein coupled receptors (Branchez et al. 1998, 2000, Lismaa and Shine 1999, Waters and Krause 2000) GAL has been shown to be involved in a variety of physiological processes (Gundlach 2002, Rajendren 2002, Wiesenfeld-Hallin and Xu 2001). A substantial body of work suggests that GAL plays an important role as a modulator in pain perception (Jimenez-Andrade et al. 2005, Liu and Hökfelt 2002, Malkmus et al. 2005, Sun and Yu 2005, Wiesenfeld-Hallin and Xu 2001, Xu et al. 2000a). It has been proposed that GAL produces a biphasic, dose-dependent effect on nociception through activation of antinociceptive (inhibitory) GALR1 or nociceptive (excitatory) GALR2 receptors (Liu and Hökfelt 2002, Xu et al. 2000b). The complex effect of GAL on nociception is assumed to be a result of differential activation of the GAL receptor subtypes (Liu and Hökfelt 2002, Xu et al. 2000b). The complex effect of GAL on nociception is assumed to be a result of differential activation of the GAL receptor subtypes (Liu and Hökfelt 2002, Xu et al. 2000b). The complex effect of GAL on nociception is assumed to be a result of differential activation of the GAL receptor subtypes (Liu and Hökfelt 2002, Xu et al. 2000b). There are also multiple lines of evidence suggesting that under normal conditions, the effect of exogenous GAL is predominantly inhibitory (Hua et al. 2004, Xu et al. 2000b) due to blocking the excitatory effect of substance P and calcitonin gene-related peptide (Hua et al. 2005, Xu et al. 1990).

In many rat and human CNS structures, GAL is co-expressed with other neuropeptides or neuromediators. Most neurons of the hypothalamic
paraventricular nuclei (PVN) and supraoptic nuclei (SON) contain GAL, co-localized with vasopressin (AVP) (Gai et al. 1990, Jimenez-Andrade 2005, 2003, Landry and Hökfelt, 1998, Melnikowa et al. 2006), oxytocin (OT) (Gai et al. 1990, Melnikowa et al. 2006) and opioids (Sanchez et al. 2001). The hypothalamus is known to be one of the key structures involved in pain modulation and transmission (Dafny et al. 1996). The hypothalamic fibers containing opioid neurons, terminate in periaqueductal central gray (PAG) (Pilcher et al. 1988), which plays an important role in ascending antinociceptive pathways (Sandküklicher 1996, Wang et al. 1999, 2000). Descending vasopressinergic and oxytocinergic pathways extend from the hypothalamus to the brain stem. Hypothalamic fibers containing AVP and OT, modulating afferent noxious stimuli, project to the thalamus, medulla oblongata (including the trigeminal nerve nuclei) and the substantia gelatinosa of the dorsal horn of the spinal cord (Sawchenko and Swanson 1982).

The antinociceptive role of GAL at the spinal level has been extensively studied (Blakeman et al. 2003, Hua et al. 2005, Liu et al. 2001, Liu and Hökfelt 2002, Wiesenfeld-Hallin et al. 2005), whereas the role of GAL in nociceptive transmission in the orofacial area remains to be elucidated. The co-existence of GAL with AVP, OT and opioids in the hypothalamus prompted us to study the interactions between these neuropeptides/neurohormones in pain processing, i.e. in the trigemino-hypoglossal reflex induced by tooth pulp stimulation in rats.

**Methods**

**Experimental animals and anesthesia**

The experimental protocol in the present study was approved by the Local Ethical Committee for Animal Research and it complies with the European Community guidelines for the use of experimental animals. Male Long-Evans rats weighing 330-360 g were used for the experiments. The animals were kept under standard conditions: temperature 22 °C, a 12 h light-dark cycle, and allowed tap water and rodent chow ad libitum. The rats were anesthetized with a single intraperitoneal injection of chloralose solution in a dose of 150 mg/kg body weight. Chloralose does not eliminate completely transmission in the ascending reticular formation, which constitutes a non-specific pathway for sensory impulsion and therefore does not interfere with synaptic transmission between sensory neurons and allows recording ETJ amplitude. For each experiment ten animals were used.

**Chemicals**

The artificial cerebrospinal fluid (aCSF) was prepared according to Daniel and Lederis (1967) and contained: 120 mM NaCl, 4.8 mM KCl, 2.8 mM CaCl₂, 1.2 mM KH₂PO₄, 1.3 mM MgSO₄, 26 mM NaHCO₃, 10 mM glucose, 1.0 g/l bovine serum albumin and 0.1 g/l ascorbic acid (pH=7.4-7.5). The solution was placed in a water bath at 37 °C and constantly gassed with carbogen (a mixture of 95 % O₂, and 5 % CO₂). Solutions for intracerebroventricular (i.c.v.) perfusions were prepared in aCSF: 100 and 200 nM galanin (GAL) (Tocris, Bristol, UK), 100 nM galantide (GLT) (Bachem, Switzerland), 100 nM naloxone hydrochloride (Nal) (Sigma, St. Louis, MO, USA), 100 nM β-funaltrexamine hydrochloride (β-FNA) (Tocris, Bristol, UK), 100 nM nor-binaltorphimine dihydrochloride (nor-BNI) (Tocris, Bristol, UK), 100 nM oxytocin (OT) and vasopressin (AVP) (Peninsula Laboratories Inc., San Carlos, CA, USA) and 100 nM endomorphin-2 (EM-2), which was synthesized in our laboratory, as described elsewhere (Janecka et al. 2004).

**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 (Zubrzycka et al. 1997). 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 co-ordinates given by De Groot’s stereotaxic atlas (1963), 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. The system of cerebral ventricles was perfused by inserting stainless steel cannulae into both lateral ventricles and to the cerebellomedullary cistern. The container with perfusion fluid was positioned 20 cm above the animal’s head. 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 during perfusion was 0.5-0.7 ml/10 min. After control perfusion with aCSF the cerebral ventricles were perfused with peptide solutions. There were 5-min intervals between solutions.
Tooth pulp stimulation

After placing the animal’s head in a stereotaxic instrument, the tips of both lower incisors were cut off with a dental separator and stainless steel wire electrodes were inserted into the pulp and fixed with dental cement (Duracil, Spofa). The pulp bipolar stimulation was delivered 6 times per minute, with a train of four electrical impulses, of 200 Hz frequency, 3 ms single impulse duration with 2 ms intervals and 4-6 V amplitude, using a programmed stimulator. Trains of 4 impulses were delivered to the pulp at 10 s intervals. A Grass stimulator, model S4K, connected with a gating circuit, was used. 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 on a paper using a Line Recorder TZ-4620 (Laboratorní přístroje, Prague, Czech Republic). For each animal during the first 10 min of perfusion or with 10 min after injection the amplitude of tongue jerks evoked by tooth pulp stimulation was recorded. The mean amplitude of tongue jerks by tooth pulp stimulation was regarded as an indicator of magnitude of the trigemino-hypoglossal reflex. Mean amplitudes of ETJ induced by tooth pulp stimulation during perfusion the control solution (aCSF) and with peptide solutions, were compared separately.

Statistical analysis

Statistical analyses were performed using Prism 4.0 (GraphPad Software Inc.). The data are expressed as means ± SEM. Differences between groups were assessed by one-way analysis of variance (ANOVA) followed by a post-hoc multiple comparison Student Newman-Keuls test. Antagonist effects in the combination experiments were analyzed using two-way analysis of variance (ANOVA) and a post-hoc multiple comparison Student Newman-Keuls test was used for multiple comparisons between groups. A probability level of p<0.05 or lower was considered as statistically significant.

Results

Antinociceptive activity of galanin in the trigemino-hypoglossal reflex in rats

Cerebral ventricles were perfused with GAL in 50, 100 and 200 nM concentration. To obtain an effect of a peptide perfused i.c.v. it is necessary to get an appropriate concentration of this peptide in the extracellular fluid in the sensory and motor centers for trigemino-hypoglossal reflex. These centers are located in the vicinity of the fourth cerebral ventricle wall, so the perfused peptide is first diluted in the CSF and then it diffuses through the whole cerebroventricular and cerebromedullary cistern wall area to the extracellular fluid of the brain. With extracellular fluid, it reaches the regions surrounding structures responsible for trigemino-hypoglossal reflex. Thus, it can be presumed that the concentrations of the peptide, effective in the specific structures, are much lower than their initial concentrations. For this reason in our experiments concentration of GAL used for i.c.v. perfusion was
several times higher than in other studies where GAL was administered directly to brain nuclei (Zhang et al. 2000b, Sun et al. 2003).

Perfusion of cerebral ventricles with GAL caused a significant reduction of the amplitude of ETJ compared to control perfusion with aCSF (Fig. 1). The analgesic activity of GAL, expressed as the amplitude of the evoked retractor movement of the tongue after electrical tooth pulp stimulation (ETJ), was concentration-dependent and the values for the perfusion with 50, 100 and 200 nM solutions were 82.8±6.2, 59.8±6.2 and 51.4±7.1 %, respectively, as compared with control regarded as a 100 % (Fig. 2).

Influence of co-administration of galanin with galantide or opioid antagonists on galanin-induced analgesia in trigemino-hypoglossal reflex in rats

The effect of different antagonists on GAL-induced analgesia was studied. Antagonists selected for the experiment were GAL antagonist, GLT and four opioid antagonists: non-selective Nal, μ-selective β-FNA, δ-selective NTI, and κ-selective nor-BNI. GAL and antagonists were all in 100 nM concentration and GAL administration was always preceded by the perfusion with an antagonist. As expected, GLT decreased the antinociceptive effect produced by GAL. The amplitude of the ETJ increased from 56.2±5.4 to 91.2±8.1 % (Fig. 3A). Similar effect was observed when cerebral ventricles were perfused with Nal or β-FNA. The obtained ETJ values were 95.2±7.8 and 93.1±8.4 %, respectively. The δ antagonist, NTI and κ antagonist, nor-BNI did not suppress GAL-induced analgesia (Fig. 3B).

Influence of galantide on the endomorphin-2-induced analgesia in trigemino-hypoglossal reflex in rats

The endogenous μ-opioid receptor selective ligand, EM-2, was shown to produce a strong analgesic effect in the same in vivo test (Zubrzycka et al. 2005). Here the administration of EM-2 was preceded by the perfusion of the cerebral ventricles with GAL antagonist, GLT. The amplitude of the ETJ when EM-2 was perfused alone was 30.0±4.6 and increased to...
84.3±5.8 %, when GLT was used (Fig. 4) which indicated that GLT strongly antagonized the antinociceptive action of the μ-opioid agonist, EM-2.

Antinociceptive activity of galanin co-administered with μ-opioid agonist endomorphin-2 and neurohormones oxytocin and vasopressin in trigemino-hypoglossal reflex in rats

The antinociceptive activity of GAL was significantly increased when GAL was perfused through the cerebral ventricles in combination with EM-2, OT, or AVP. The ETJ amplitude decreased from 55.8±5.7 to 21.1±2.8 % for EM-2 (Fig. 5A) and to 27.3±3.2 %, and 35.4±2.9 %, when cerebral ventricles were perfused with OT and AVP, respectively (Fig. 5B, C).

Discussion

The reflexes used for over ten years in the studies of the transmission of pain within the brainstem are trigemino-digastric jaw-opening reflex (JOR) (Aletaret et al. 1997) and trigemino-hypoglossal evoked tongue jerk reflex (ETJ) (Zubrzycka et al. 1997).

In our studies we have used trigemino-hypoglossal reflex to test the effects of various neuropeptides present in the cerebrospinal fluid (Zubrzycka et al. 1997, 2002). The excitation or inhibition of this reflex may be evaluated according to the mean amplitude of the recorded tongue jerks induced by
incisor pulp stimulation during perfusion of the cerebral ventricles with the solution of tested compounds.

Our studies indicate that GAL exerts its antinociceptive action on the ETJ center located in the vicinity of the third and fourth cerebral ventricles, as well as on nerve V sensory nuclei. The afferent impulses induced by stimulation reached, via the inferior alveolar nerve fibers and the trigeminal ganglion, the nucleus sensorius principalis n.V. From the sensory nuclei, impulsion was transmitted to motoneurons located under the fundus of the fourth ventricle, concentrated in the hypoglossal motor nucleus and then via hypoglossal nerve fibers to the muscles of the tongue, causing tongue jerks.

For several years we have been studying the role of different neuropeptides (Met-enkephalin, EM-2, OT and AVP) on nociception in trigemino-hypoglossal reflex. In the present study we have demonstrated the antinociceptive effect of GAL, perfused through the cerebral ventricles alone or in combination with other neuropeptides/neurohormones, on trigemino-hypoglossal reflex in rats. GAL was shown to produce a concentration-dependent analgesic effect, which was blocked by a GAL antagonist, GLT. The antinociceptive effect of GAL was also reversed by opioid receptor antagonists, non-selective naloxone and µ-selective β-FNA, but δ- and κ-selective opioid antagonists had no effect on GAL-induced antinociception. These results indicate that GAL exerts its antinociceptive effects in the orofacial area through the µ-opioid receptor. Attenuation of antinociception by opioid receptor antagonists is consistent with other studies showing that analgesic effect of GAL in the spinal cord (Zhang et al. 2000a), periaqueductal central gray (PAG) (Wang et al. 1999, 2000) or arcuate nucleus (Sun and Yu 2005) was attenuated by naloxone. Similar findings were reported at the spinal level (Sun and Yu 2005, Zhang et al. 2000b), where the analgesic effect of GAL was attenuated by the µ- but not δ- and κ-opioid receptor antagonists. These results suggest that GAL can modulate the release of the µ-selective opioids in the CNS. Furthermore, the present results showed that GLT can antagonize the antinociceptive effect of the µ-opioid agonist, EM-2. Thus, GLT abolishes the inhibitory effect of both GAL and EM-2 on ETJ, which further indicates that the interactive function between the opioid and galaninergic systems is mediated by the µ-opioid receptors.

Earlier we have shown that OT and AVP (Zubrzycka et al. 2005, Zubrzycka and Janecka 2005) produced a significant antinociceptive effect after i.c.v. administration, as assayed in the same experimental setting (Zubrzycka et al. 2005). Inhibition of this effect was mediated by opioid receptors, indicating that there is a synergy between oxytocin, vasopressin and opioid systems in transmitting and modulating pain stimuli. Here we showed that GAL also influences the analgesic activity in the trigemino-hypoglossal reflex arc and enhances the antinociceptive effect of EM-2, OT and AVP. These data support earlier results of others, showing that GAL potentiated the effect of morphine at the spinal level (Wiesenfeld-Hallin et al. 1990, Hua et al. 2004).

In conclusion, our results demonstrate that in the orofacial area analgesic activity exerted by opioids is modulated by GAL, OT and AVP.

**Conflict of Interest**

There is no conflict of interest.

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**References**


