GALANIN AFFECTS VASOPRESSIN AND OXYTOCIN RELEASE FROM THE HYPOTHALAMO-NEUROHYPOPHYSIAL SYSTEM IN HAEMORRHAGED RATS*

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The effect of centrally administered galanin (Gal; 100 pM i.c.v.) on the hypothalamo-neurohypophysial storage as well as blood plasma level of vasopressin and oxytocin was estimated in haemorrhaged (1 ml per 100 g b.w.) male Wistar rats. Gal i.c.v. treatment did not alter vasopressin and oxytocin content both in the hypothalamus and neurohypophysis as well as their concentration in blood plasma of not haemorrhaged rats. Haemorrhage decreased the hypothalamic and neurohypophysial vasopressin and oxytocin storage but increased the neurohormones plasma level in animals injected with vehicle solution. During the haemorrhage, the increase in plasma vasopressin and oxytocin was inhibited in rats previously treated i.c.v. with galanin. The hypothalamic and neurohypophysial vasopressin as well as oxytocin content significantly increased in animals treated with galanin and subsequently haemorrhaged. These results suggest that galanin may have a regulatory role in the hypothalamo-neurohypophysial function especially under condition of hypovolemia.

Key words: galanin, vasopressin, oxytocin, haemorrhage

INTRODUCTION

The neuropeptide galanin (Gal), of 29 amino acids in length, is widely distributed in the rat central nervous system and peripheral tissues (1, 2). This peptide is implicated in the regulation of processes such as food intake (3), memory, neuroendocrine release (4), gut secretion and motility (5, 6). The

highest densities of galanin-like immunoreactivity (Gal-LI) are found in brain structures such as the amygdaloid complex, the hypothalamus and the brain stem as well as posterior pituitary (7, 8). In the hypothalamus Gal is present especially in median eminence, supraoptic nucleus (SON), paraventricular nucleus (PVN) and medial preoptic area (9, 10). Moreover, SON and PVN appear a high density of Gal binding sites (11).

There are some reports regarding the possible interactions between galanin and the hypothalamo-neurohypophysial system function. It has been shown earlier that the states of dehydratation or hyperosmolality reduce Gal content in the magnocellular hypothalamic neurons as well as in posterior pituitary; however, Gal mRNA level increases in these conditions (12, 13). Furthermore, centrally administered galanin inhibits osmotically stimulated arginine vasopressin (AVP) release in the rat (14). Gal is also shown to inhibit oxytocin (OT) release (15).

The aim of this study was to evaluate the influence of galanin on the hypothalamic and neurohypophysial AVP and OT content and their release in haemorrhaged rats.

MATERIALS AND METHODS

Animals. The experiments were carried out on 40 adult male Wistar rats weighing 350 ± 36 g (±S.D.). All the experiments were performed with the acceptance of the Ethical Committee of Medical University of Lodz. All animals were kept at a temperature of about +20°C and in regulated light-dark conditions (light phase: from 6.00 to 18.00; night phase: from 18.00 to 6.00); there were four animals per a cage. They had free access to standard pelleted food, as well as tap water ad libitum.

Surgical preparations. The implantation of intracerebroventricular cannula for chronic injections was performed under light hexobarbital anaesthesia (intraperitoneal (i.p.) injection of 7% Hexobarbital Natrium solution: 0.3 ml/100 g b.w.). The animals were immobilized in a simple stereotaxic apparatus as recommended by Noble et al. (16); a small hole was drilled in the skull (1.5 - 2.0 mm laterally and 1.5 - 2.0 posteriorly to the crossing of the sagittal and coronal sutures). A simple stainless steel cannula was inserted into the left lateral cerebral ventricle; its tip was 4.0 mm below the dorsal skull surface and its inner diameter was 0.5 mm. The cannula was fixed, permanently, to the skull with dental cement. After surgery, the animals were left to recover for up to 7-10 days before starting the experimental protocol. The intracerebroventricular (i.c.v.) injections were made only once, on the day of experiment, to anaesthetized rats; a 50 µl Hamilton syringe (Hamilton Comp., Reno, NV) with plunger pushed by a microscrew was used. The duration of the infusion was about 15 sec. The effectiveness of i.c.v. infusions was verified by injecting 10 µl of 0.25% trypan blue solution to similarly operated separate control animals (one rat injected with trypan blue solution for every ten animals applied i.c.v. with Gal or aCSF) and was found to be satisfactory, i.e., the dye was distributed in an uniform manner within all cerebral ventricles.

Experimental design. The rats were devided into two groups: A - animals injected i.c.v. with 5 µl of artificial cerebrospinal fluid (aCSF) (control intact group); B - animals applied with galanin (Gal; Sigma Chemical Co., lot 124H09511, St. Louis, USA) administered i.c.v. at a dose of 100 pM dissolved in 5 µl of aCSF. The solution of artificial cerebrospinal fluid was composed as follows:
The brain with intact pituitary was quickly (i.e., not later than 2 min after decapitation) removed. The infundibular stalk was cut up and the neurointermediate lobe was separated. From the brain, hardened for a few minutes at -70°C, the hypothalamic block was dissected as follows; rostral limit – frontal plane situated about 2.0 mm more rostral to the anterior margin of the optic chiasma; caudal limit – frontal plane just behind the mamillary bodies; lateral limits – sagittal planes passing on both sides, just through the hypothalamic fissures. The depth was about 2.0 mm from the base of the brain. The wet weight of such a block of the brain (containing the hypothalamus and a part of the thalamus) was 36.6 ± 1.4 mg (mean ± S.D.).

Experimental procedure. The animals were sacrificed by decapitation 20 minutes following haemorrhage (i.e., between 9.00 and 11.00 a.m.). Immediately thereafter the blood from the trunk, drawn into heparinized tubes, was centrifuged for 20 min (+4°C; rpm = 2500-3000) and the plasma was preserved. For the radioimmunoassay of vasopressin and oxytocin, the samples of plasma were extracted using C18 "Sep-Pak" microcolumns (Sep-Pak®, C18, Lot No W9224G1, Waters Corp., Milford, Massachusetts, USA) as described by Forsling (17). The recoveries of hormones during extraction procedure were > 85% and, therefore, the values were not corrected for procedural losses. The final extracts were preserved in frozen (temperature about -70°C) sealed glass vials until radioimmunoassay.

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The neurointermediate lobe (respectively, the hypothalamus) was homogenized, in 2 ml of 0.25\% (resp. 0.5\%) acetic acid in 0.15 M sodium chloride solution, by the use of ultrasonic disruptor (Microson\textsuperscript{TM} Ultrasonic Homogenizer, LABCAIRE, UK). The samples were heated for 5 minutes on a boiling water bath (in order to inactivate the proteolytic enzymes present in the homogenized tissue) and centrifuged for 30 min in a temperature +4°C (rpm = 4000), the supernatant was removed and collected. The final extracts were stored at -70°C until radioimmunoassay for AVP and OT.

Radioimmunoassay of vasopressin and oxytocin

Characteristic of antisera. Anti-AVP (serum No 1228/1987-08-24) and anti-OT (serum No 1232/1988-02-03) antibodies were raised in rabbits in Department of Physiology, Institute of Physiology and Biochemistry, Medical University of Lodz, Poland. The antibody titer to be used in the radioimmunoassay was 1: 40 000 for anti-AVP and 1: 80 000 for anti-OT (both final dilutions). Cross reactivity with oxytocin for anti-AVP antibodies was 0.016\%, with lysine vasopressin (LVP) - 2.7\%, with luliberin (LH-RH), leucine enkephalin (Leu-Enk), angiotensin II (Ang II), substance P (SP), hexapeptide (Tyr\textsuperscript{6}Pyr\textsuperscript{8}Glu\textsuperscript{6-11}SP), and hexadecapeptide (Tyr\textsuperscript{8}Tyrglu\textsuperscript{6}Pyr\textsuperscript{11}SP\textsuperscript{6-11}) it was < 0.002\%. Cross reactivity with AVP for anti-OT antibodies was 1.12\%; with LH-RH, Leu-Enk and Ang II it was < 0.002\%. The sensitivity of anti-AVP and anti-OT antisera was 0.78 pg/100 \(\mu\)l. Intra- and extra-assay coefficients of variation (c\(\sigma\)v) for the vasopressin assay were 2.5\% and 6.3\%, respectively; for the oxytocin assay c\(\sigma\)v were 3.3\% and 8.3\%, respectively.

Iodination of arginine vasopressin and oxytocin. Arginine vasopressin (Arg\textsuperscript{8})-Vasopressin, (Bachem AG, lot 511731) and oxytocin (Peninsula Lab. Ltd., lot 027179) were iodinated with \(^{125}\text{I}\) using the chloramine-T method (18). Unreacted iodide was removed by mixing the reaction mixture with Amberlite (Aldrich Chemical Company, USA). Further purification was carried out on a column of Sephadex G-25 (Aldrich Chemical Company, USA) fine pre-equilibrated and eluated with 0.05 M/l acetic acid. Labelled vasopressin and oxytocin were identified in the third peak by their ability to bind to the corresponding antibodies (19). The effectiveness of the iodination procedure was 80\% - 90\%. The top or the 1th descending portion of this peak was used as the tracer in RIA. Labelled hormones retained their antibody bindability for up to four weeks. All the specimens from particular experiments were measured in duplicate in the same assay. For a single estimation 100 \(\mu\)l of the respective extracts were used.

Statistical evaluation of the results. The vasopressin or oxytocin content was finally expressed in nanograms per milligram of the hypothalamic tissue, in nanograms for the whole neurointermediate lobe and in picograms per millilitre of plasma. All findings are reported as mean ± standard error of the mean (±S.E.M.). Data were calculated by the analysis of variance (ANOVA); if ANOVA revealed significant effects, post hoc analyses were done using U Mann-Whitney test (p≤ 0.05 was considered to be statistically significant). For statistical assessment of the data the programme "STATISTICA" (Version 5.0), copyright StatSoft Inc., licensed to Department of Pathophysiology, Medical University of Lodz, was used.

RESULTS

The results are summarized on Figs 1-6.

The effect of Gal on the vasopressin and oxytocin content in the hypothalamo-neurohypophysial system (see Fig.1, 2, 4, 5). Haemorrhage was followed by a significant decrease in hypothalamic vasopressin and oxytocin content in animals injected i.c.v. with aCSF (Fig.1 and 4: subgroup A-II versus A-I). Injection of a
single i.c.v. dose of Gal to otherwise not treated animals resulted in no significant change of the hypothalamic vasopressin and oxytocin content (Fig.1 and 4: subgroup B-I). In haemorrhaged rats, previous injection of Gal resulted in a considerable increase in both neurohormones content in the hypothalamus (Fig.1 and 4: subgroup B-II versus B-I).
Haemorrhage decreased neurohypophysial AVP and OT storage in animals applied i.c.v. with aCSF (Fig. 2 and 5: subgroup A-II versus A-I). In not haemorrhaged rats previous injection of Gal resulted in no significant changes of the neurohypophysial AVP and OT content (Fig. 2 and 5: subgroup B-I). In rats bleeded and simultaneously treated i.c.v. with Gal neurohypophysial AVP and OT content were distinctly higher when compared to rats similarly bleeded but injected with aCSF (Fig. 2 and 5: subgroup B-II versus A-II).
The effect of Gal on the vasopressin and oxytocin concentration in blood plasma (see Fig. 3 and 6). In rats haemorrhaged and injected with aCSF a distinct rise in plasma vasopressin and oxytocin levels has been observed (Fig. 3 and 6: subgroup A-II versus A-I). Gal administered i.c.v. to haemorrhaged rats resulted in a significant decrease in both neurohormones plasma concentration (Fig. 3 and 6: subgroup B-II versus A-II).
DISCUSSION

**Haemorrhage and vasopressin and oxytocin release.** Haemorrhage as the state inducing the hypovolemia and the hypotension is known to be a major stimulus (behind the dehydration) affecting the release of vasopressin and oxytocin from the hypothalamo-neurohypophysial system. Haemorrhage is also known to influence the electrical activity of vasopressinergic and oxytocinergic neurons of magnocellular hypothalamic nuclei (20). Moreover, these both states (i.e., bleeding and deprivation of water) induce the expression of the immediate early gene c-fos in specific brain regions involved in osmotic mechanisms regulation [i.e., supraoptic nucleus, paraventricular nucleus, organum vasculosum of the lamina terminalis (OVLT), median preoptic nucleus (MnPO) and subfornical organ (SFO)] (21, 22). In these conditions, the decrease of AVP and OT content in the neurohypophysis as well as the elevation of their plasma concentration was reported in rats, dogs and humans (23, 24, 25, 26). The neurohypophysial AVP and OT contents associated with their plasma levels reflect the actual degree of neurohormones release into the circulation. On the other hand, the rate of AVP and OT biosynthesis as well as their transport towards the neurohypophysis points out the level of both hypothalamic neurohormones storage. It has been shown that all these processes are influenced by the states of water deprivation or haemorrhage (27, 28, 29). The present results (i.e., increased secretion of AVP and OT into the blood accompanied by the diminution of both neurohormones content in the neurohypophysis as well as in the hypothalamus) are entirely consistent with these earlier findings.

The reflex mechanisms involved in the release of AVP and OT are, however, not identical for hypertonic dehydration and haemorrhage. Deprivation of water is followed by an increased osmolality of body fluids as well as by secondary hypovolemic hypotension. The respective mechanisms involved in AVP and OT release (brought about by acute hypovolemia due to bleeding) are thought to be related rather to atrial (low pressure receptors) than to sinoaortic receptors (high pressure receptors). The reduction in blood volume (as caused by haemorrhage) is followed by the decrease of low pressure receptors activity and by increased AVP release into the blood (24, 26, 30). However, paradoxically, it has been recently noted that centrally applied AVP intensifies hypotension and bradycardia after haemorrhage in spontaneously hypertensive rats (31).

Accordingly, in this experiment the amount of the blood taken away by haemorrhage was set as 1 ml/100 g b.w., i.e., about 20% of total blood volume [in the rat, the blood volume has been estimated to be about 8.0 ml/100 g b.w. (32)]. In the similar experiments the haemorrhage was generated by withdrawal of 3-4 ml of the blood from adult rats (33, 34).

The simultaneous release of both AVP and OT as brought about by bleeding continues to be discussed. In present study, a distinct increase of both neurohormones concentration in blood plasma following the haemorrhage was
noted. Simultaneously, both hypothalamic and neurohypophysial AVP and OT storages meaningly decreased in these bleeded rats. These findings seem to be consistent with previous data of Brizzee et al. (35) and Kasting (23) as well as Ciosek and Orłowska-Majdak (26). On the other hand, increased plasma level of AVP - but not that of OT - has been reported earlier following haemorrhage in the dog (36) and goat (37).

**Galanin as a neuromodulator of vasopressin and oxytocin release.** Galanin has been demonstrated to have potent effects on the neuroendocrine responses of the hypothalamo-pituitary system (38, 39). It has been showed in the humans and in the rats that Gal stimulates among other things the secretion of growth hormone and prolactin but inhibits dopamine release from the median eminence (38, 40, 41, 42). The hypothalamus is thought to be one of the major sites of galanin action in the central nervous system. The high affinity binding receptors for galanin (GAL R1, Gal R2, Gal R3) have been detected in the brain, especially in the medial preoptic area, paraventricular and supraoptic nuclei (39, 43).

In the hypothalamo-pituitary system galanin-like neurons are found in the magnocellular hypothalamo-neurohypophysial system as well as in the parvocellular hypothalamo-pituitary - anterior pituitary system (7). Cell bodies of galanin-immunoreactive neurons were seen mainly in the supraoptic and paraventricular nuclei with axons terminating in the posterior lobe of the pituitary (7, 8). Immunohistochemical data as well as the studies in vitro have shown that galanin coexists with vasopressin in the magnocellular and/or parvocellular neurons in the supraoptic nucleus and paraventricular nucleus of the human and rat hypothalamus (12, 13, 44, 45, 46, 47, 48). Some amounts of Gal appear also in oxytocinergic neurons after experimental manipulations such as colchicin treatment (44, 49) or hypophysectomy (12, 50).

It has been speculated on the basis of some findings that galanin may be important neuromodulator for water-electrolyte balance regulation. Some earlier reports have been showed that Gal mRNA content raised in the supraoptic and paraventricular nuclei of rats deprived of tap water as well as Brattleboro rats with hereditary diabetes insipidus; Gal-like immunoreactivity in the neurohypophysis was then reduced (12, 13, 45).

Some data suggest a modulatory role for Gal in the release of neurohypophysial hormones (14, 15, 51). Kondo et al. (14) investigated the effect of centrally administered galanin on plasma vasopressin level in hypertonic saline-treated rats. These authors demonstrated that Gal inhibits osmotically stimulated vasopressin release. Landry et al. (51) showed in dehydrated animals that osmotically stimulated AVP mRNA content in the PVN and SON decreases after i.c.v. injection of galanin. No evidence has been found for an action of galanin on OT mRNA content after stimulation by dehydration. On the other hand, Björkstrand et al. (15) reported a significant decrease of OT plasma level in anesthetized rats 20 min after i.c.v. injection of 0.1 or 1 µg of galanin. In the study of Gayman and Falke (52) galanin had no effect on oxytocin release from rat
neurosecretory endings. What is more, Skofitsch et al. (45) observed that Gal injected intravenously resulted in mild diuresis, however, had no effect on AVP-induced anti-diuresis process. Similarly, Balment and al Barazanji (53) observed a transitory diuresis after central galanin infusion.

Our present results seem to be consistent with these reports. We demonstrate for the first time the consequences of central injection of Gal on vasopressin and oxytocin release in the rats in the state of hypovolemia induced by haemorrhage. Therefore, we have showed that galanin distinctly suppressed vasopressin as well as oxytocin release (intensified in the state of hypovolemia) from the neurohypophysis into the blood. Accordingly, hypothalamic and neurohypophysial stores of AVP and OT significantly raised in these animals. Gal administered i.c.v. to not haemorrhaged animals did not affect these neurohormones secretion.

The question that still remains is to know the mechanisms that may be used by galanin to regulate the activity of vasopressinergic and oxytocinergic neurons. It has been assumed that exogenous galanin injected intracerebroventricularly may modify the neurohypophysial hormones release by direct action on vasopressinergic and oxytocinergic neurons sending their axons from the hypothalamus mainly towards the posterior pituitary. This influence Gal exerts through the binding to the respective galanin receptors at the level of the hypothalamo-neurohypophysial system. However, the type of galanin receptor engaged in this mechanism remains unclear. Galanin, injected i.c.v., probably modulates vasopressin and oxytocin biosynthesis, infundibular transport as well as their release into the blood. Landry et al. (51) has been observed a distinct decrease in AVP mRNA content in SON and PVN, after injection of Gal in dehydrated rats, that supposed the inhibiting effect of Gal on the AVP biosynthesis. In our experiments AVP and OT content in the hypothalamus of bleeded rats raised remarkably following galanin injection. This observation need not be inconsistent with the results achieved by Landry et al. (51). On the basis of our experiments and accessible data we suggest that galanin may have an inhibitory influence not only on the biosynthesis of AVP and OT but it can also inhibit axonal transport and the secretion of these neurohormones into the circulation. Restrained axonal transport of AVP and OT under Gal treatment could be the reason of accumulation and the increase of the neurohormones content in the hypothalamus. Therefore, the fall of blood plasma AVP and OT concentrations with simultaneous increase of their hypothalamic and neurohypophyseal content completely confirms this conclusion.

Moreover, it cannot be excluded the indirect influence of Gal on the mechanisms of AVP and OT release via participation of specific type of innervation of vasopressinergic and oxytocinergic neurons. It has been shown that galanin may be involved in the modulation of activity of monoamine system with widespread projections in the central nervous system (54). For example, galanin-positive nerve endings could be seen in the synaptic contact with dendrites and
soma of noradrenergic neurons in the locus coeruleus (55, 56). What is more, galanin-immunoreactive neurons from the locus coeruleus project to the paraventricular nuclei and are mostly confined to the anterior and periventricular parts of the PVN (55). Galanin was found to coexist with dopamine-beta hydroxylase immunoreactivity in the neurons of the A_1 and A_6 (locus coeruleus) catecholamine cell groups. Electron microscope studies of Landry et al. (58) demonstrated synaptic contacts between galanin-containing fibers and magnocellular neurons. Apart from this, the adrenergic afferentation of supraoptic and paraventricular neurons modify the neurohormones biosynthesis and release (59, 60, 61). It could be therefore possible that functional interactions between galaninergic and monoaminergic neurons could modify magnocellular neurons activity under exogenous galanin influence.

It seems to be supposed that galanin is one of most important endogenous factors inhibiting vasopressin and oxytocin release especially in the state of haemorrhagic hypovolemia.

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