

GONADOTROPIN RELEASING HORMONE AND SUBSTANCE P RELEASED INTO THE CEREBRAL VENTRICULAR FLUID IN INTACT, ORCHIDECTOMIZED AND SEX STEROID TREATED MALE RATS

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Objective. To evaluate whether orchidectomy (ORCX) and sex steroid hormones can modify the release of gonadotropin releasing hormone (Gn-RH) and substance P (SP) are released into the cerebral ventricular fluid (CSF).

Methods. The perfusions of lateral ventriculo-cerebello-medullary cistern were performed in anesthetised (urethane with chloralose) rats, three 30 min samples of perfusion fluid being collected in each animal. Four weeks prior to this experiment adult male rats (except INTACT group) were bilaterally orchidectomized and implanted s.c. either with empty silastic capsules (ORCX) or with these containing 17 β -estradiol (ORCX+E₂) or testosterone propionate (ORCX+T). This procedure was performed in two series of experiments: first one for Gn-RH and second one for SP estimation by RIA. In some animals the unconjugated fraction of E₂ and T was estimated in blood samples collected at the end of experiment.

Results. In all groups Gn-RH and SP were continuously released into the perfusion fluid. The mean Gn-RH concentration was higher than that of SP in the collected samples. A long-term modification of sex steroid hormone level in blood resulted in significant decrease of Gn-RH concentration only in ORCX as compared to INTACT rats, while Gn-RH and SP levels in the perfusion fluid were found relatively unchanged by a high concentration of 17 β -estradiol or testosterone in the peripheral circulation.

Key words: Gonadotropin releasing hormone (Gn-RH) – Substance P – Cerebrospinal Fluid - Sex steroids - Orchidectomy

The analysis of cerebrospinal fluid (CSF) in clinical (BLACK 1982) and experimental (BEGLEY and CHAIN 1992) studies has become increasingly important. Using a radioimmunoassay procedure, most neuropeptides can now be assayed in the human cerebrospinal fluid (BLACK 1982).

The hypothalamic releasing hormones or inhibitory hormones, gonadoliberin (JOSEPH et al. 1975; PAU et al. 1991; VAN VUGHT et al. 1985), somatostatin (NEMEROFF et al. 1985; STEPIEŃ et al. 1986; JOST et al. 1991), tyreoliberin (PEKARY et al. 1991) and corticoliberin (KLING et al. 1993) are present in CSF, although their functions are still not definitely elucidated. It is suggested that the neuropeptides reach CSF by direct re-

lease from the peptidergic nerve terminals bathed in CSF and play an important role in neuroendocrine regulation. A lot of evidence suggests that the specific system for neuropeptide transport is the tanycyte system – specialized ependymal cells in the floor of the 3rd ventricle (KNOWLES 1974; KIZER et al. 1976). The tanycyte system may provide with two-way transport of the substances between the CSF of the 3rd ventricle and the pituitary gland (BLACK 1982). The autoradiographic findings indicate that Gn-RH can pass from the CSF into the median eminence (ME) (GOLDGEFTER 1976). Also the infusion of labelled Gn-RH into the 3rd ventricle leads to the penetration of the peptide into the blood of pituitary portal vessels and

to the anterior pituitary lobe in rats as well as to a significant and long-lasting increase of the LH level in the peripheral circulation (BEN-JONATHAN et al. 1974). On the contrary, radioactive Gn-RH injected into the third ventricle of ewes caused extremely low levels of radioactivity being detected in hypophysial portal blood (SKINNER et al. 1998). In rabbits (PAU et al. 1991) and in ovariectomized monkeys (VAN VUGT et al. 1985) and ewes (SKINNER et al. 1995; SKINNER et al. 1997) pulsatile changes of Gn-RH levels in CSF were observed. In monkeys they were synchronized with pulsatile LH secretion from the pituitary into the blood (VAN VUGT et al. 1985). Similar pulsatile Gn-RH changes had been previously demonstrated at the medial basal hypothalamus (MBH) in rats (LEVINE AND RAMIREZ 1982) and median eminence (ME) in sheep (LEVINE et al. 1982) using the push-pull method. If Gn-RH in CSF was indeed fulfilling such a physiological role and acting as a humoral signal, there are a number of places at which it could act to influence gonadotropin secretion. Gn-RH in CSF can act via an ultrashort-loop feedback system to regulate its own secretion or Gn-RH may pass from the ventricles to the hypophysial portal system via the tanycytes and act directly at the pituitary gland to stimulate gonadotropin release or synthesis (VAN VUGT et al. 1985). However, the latest study strongly suggests that Gn-RH in CSF does not modulate LH secretion from the pituitary gland because the infusion of Gn-RH into the third ventricle had no effect on the mean inter-pulse interval, nadir pulse amplitude or circulating level of systemic LH (SKINNER et al. 1998).

The infusion of substance P into the cerebral ventricles proved effective in the modification of prolactin (KATO et al. 1976; ECKSTEIN et al. 1980; TRACZYK et al. 1992) and LH release (VIJAYAN and McCANN 1979; TRACZYK et al. 1992) as well as in the modification of the estrus cycle and ovulation (POTARGOWICZ and JAKUBOWSKA-NAZIEMBLO 1987) in rats.

SP injected into the 3rd cerebral ventricle caused a decrease of Gn-RH content in MBH of OVX rats treated with 17β -estradiol (WALCZEWSKA et al. 1996) and an increase of Gn-RH in pituitary portal blood after injection of SP into the internal carotid artery only in intact rats i.e. with an optimal steroidal milieu (WALCZEWSKA et al. 1998). It was suggested that SP may be involved in the release of Gn-RH from Gn-RH-ergic neurones located in the medial basal

hypothalamus. It is possible that SP may pass from CSF in the cerebral ventricles to this area and act directly on Gn-RH neurones or it may pass from the ventricles via tanyctic transport to the hypophysial portal system and act at the hypophyseal cells releasing the gonadotropin. As repeatedly observed, the influence of SP on Gn-RH release is dependent on the sex steroid hormones levels.

The release of Gn-RH from neuron terminals in the medial basal hypothalamus/median eminence is under the neural control of classic transmitters, neuropeptides and circulating gonadal steroid hormones (FINK et al. 1991). Also sex steroid hormones are known to affect the content of substance P in the same structure of the brain (KREAM et al. 1987) It is interesting whether gonadal hormones can alter Gn-RH and SP concentration in the cerebrospinal fluid, in a similar way as it was observed in brain tissue.

The present study was designed to determine effects of orchidectomy and long-term sex steroid hormone replacement on the levels of both SP and Gn-RH basal release from the brain tissue into the artificial cerebrospinal fluid (CSF) perfusing the rat cerebral ventricles.

Materials and Methods

The experiments were performed on the first (F1) generation of Wistar female and Buffalo male rats from the animal farm of the Institute of Oncology in Gliwice which were cross-bred in the Department of Physiology. The animals were kept in standard conditions of temperature (20-22 °C) and artificial light between 06.00-20.00 h. They received standard rat pellets and water ad libitum.

Orchidectomy and hormones administration:

Four weeks prior to the experiment all adult male rats except the intact group were orchidectomized bilaterally via midline ventral incision under i.p. hexobarbital (80 mg/kg b.w.) anaesthesia. At the same time the animals were implanted (subcutaneously into the neck area) either with empty silastic capsules or with these containing 1 mg testosterone propionate or 1 mg 17β -estradiol.

The 20 mm capsules (I.D.-1.4 mm and O.D.-2.5 mm) were made from silastic medical grade tubing produced by Dow Corning Corp. The capsules were made by packing testosterone propionate (Serva, N

3585) or 17 β -estradiol (Serva, N 31100) into silastic tubing, both ends of which were sealed with silastic cement and kept in 10 % ethanol in 0.9 % NaCl solution for 5-7 days and next in 0.9 % NaCl four days before the implantation (KREAM et al. 1987).

Lateral ventricles cerebello-medullary cistern perfusion: Lateral ventricles cerebello-medullary cistern perfusion was performed by i.p. administration of urethane (60 mg/100 g, Fluka AG 231080 985) and chloralose (6 mg/100 g b.w., Carl Roth KG, 086469). Using a simple stereotaxic instrument for rats, the following points were marked on the cranial bones: 5 mm anterior to the frontal zero plane and 3 mm lateral from the sagittal zero plane (KOENIG and KLIPPEL 1963). The holes in the cranial bones were made with a dental drill and stainless steel cannulae (external diameter 0.6 mm) were inserted into both lateral cerebral ventricles to a depth of 4 mm from the surface of the skull. The cannulae were connected to a vessel containing perfusion fluid (PF) consisting of Krebs-Ringer solution: mM per liter (123 NaCl, 4.0 KCl, 0.8 CaCl₂, 2H₂O, 2 MgSO₄ in 800 ml of bidistilled water, to which 120 ml of 0.1 M sodium phosphate buffer was then added). The solution was stirred and the flask was topped to 1000 ml with bidistilled water (pH 7.4). In all animals three 30-min samples (mean 500 μ l) of the PF were collected in polyethylene tubes by an outflow cannula inserted into the cerebello-medullary cistern according to the method described elsewhere (ŁUCZYŃSKA et al. 1980). The polyethylene tubes containing 10 μ l of glacial acetic acid were kept in a bath at 0 °C. 30-minute portions of collected PF were centrifuged at 4 °C. Then the volumes were measured, the collected fluids were lyophilized and kept at -20 °C until analysed. This procedure was performed in two series of experiments: first Gn-RH, and second SP radioimmunoassays in the obtained fluid perfusing the cerebral ventricles. The numbers of animals used for a final evaluation are presented in the legends to figures 1 and 2.

In some animals blood samples (0.5 ml) were collected from the femoral vein after the end of perfusion procedure. All blood samples were immediately centrifuged and plasma was stored at -20 °C until radioimmunoassay for 17 β -estradiol and testosterone.

Radioimmunoassays: Before the radioimmunoassay for Gn-RH and SP, 100 μ l of bidistilled water

was added to each lyophilized sample of perfusate. In the first series of experiments, Gn-RH was determined by a double antibody radioimmunoassay.

Gn-RH (Serva no 52345) was used as standard and for radioiodination by the Na¹²⁵I (Polatom, Otwock-Swierk) in the presence of chloramine-T (Serva) method (GREENWOOD et al. 1963) followed by the purification on Sephadex (Sephadex G-25 Fine. Pharmacia lot 2198) column. The Gn-RH antiserum was supplied by Prof. B. Kerdelhué (Institute National de la Recherche Agronomique, Jouy-en-Josass, France) and was used at final dilution 1:20.000 when the binding to the radioligand was 25-40 %. Intra- and interassay coefficients of variation were 2.5 % and 22.5 %, respectively. The limit of RIA sensitivity (90 %-10 % displacement range of the standard curve) was 6 pg to 792 pg Gn-RH per tube.

In the second series of experiments, the assay for SP concentration in perfusate samples was carried out using ¹²⁵I RIA Kit (Peninsula Laboratories, INC lot 032340 and 032746). The detection limit was 1 pg to 64 pg SP per tube.

Testosterone ¹²⁵I RIA Kit (ICN Biomedicals, INC, Lot TK 9404 L) and 17 β -estradiol ¹²⁵I RIA Kit (ICN Biomedicals, INC., Lot EJK 9405) were used for the measurement of unconjugated form of this steroid in peripheral blood. Gn-RH and SP concentration in the samples of the PF or testosterone and 17 β -estradiol in the peripheral blood were measured in duplicate samples.

Statistical evaluation: The levels of Gn-RH and SP in the samples collected during the perfusion of the cerebral ventricular system were expressed as pg/30 min of the perfusion. The concentrations of Gn-RH (Fig. 1) and SP (Fig. 2) were estimated as the mean \pm SEM in three 30-min samples for each group and the mean concentration of these peptides was calculated from all the samples obtained during the 90 min of the experiment (total) in each group. The significance of the differences between the mean values was determined by nonparametric Wilcoxon's matched pairs test and Mann-Whitney U-test.

Results

Gn-RH and SP concentration in artificial CSF perfusing the cerebral ventricular system. The course of Gn-RH and SP release into the perfusion

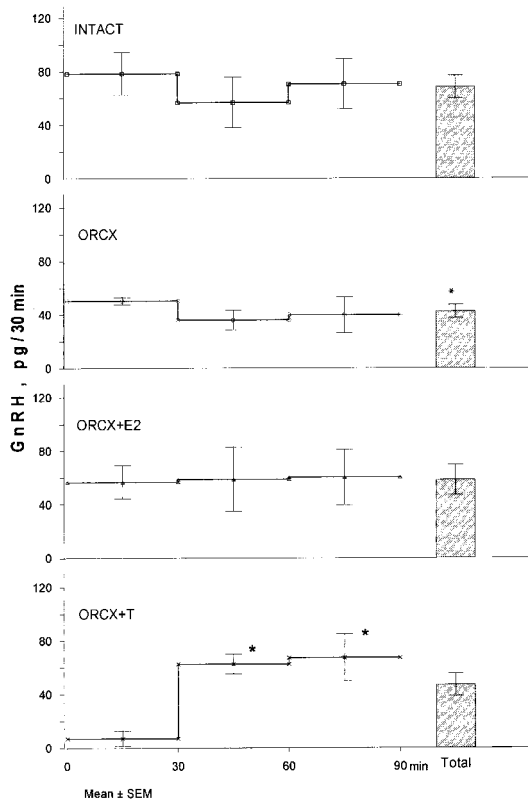


Fig. 1

Gn-RH concentration in three consecutive 30-minute samples of perfusate and mean Gn-RH concentration calculated from all samples obtained during 90 min of the perfusion (total) in male rats: INTACT (n=8), ORCX (n=8), ORCX+E₂ (n=8) and ORCX+T (n=9). Mean \pm SEM, $P < 0.01$.

fluid passing through the cerebral ventricular system in 90-min testing time for the four groups is shown in Fig. 1 and Fig. 2, respectively. No significant changes of the mean Gn-RH concentration among the subsequent 30-min samples of PF occurred in INTACT, ORCX and ORCX+E₂ groups during 90 min perfusion. However, in ORCX+T group a significant increase of the mean Gn-RH concentration was observed, e.g. from 7.00 ± 5.7 pg/30 min in the first sample to 62.5 ± 7.45 pg/30 min in the second sample and 67.4 ± 17.66 pg/30 min in the third sample. The differences between Gn-RH concentration in the first and the two subsequent samples were significant ($P < 0.01$). In addition, the total mean level of this peptide in PF was the lowest in ORCX group (42.4 ± 4.90 pg/30 min) which was significantly lower ($P < 0.01$) only as compared

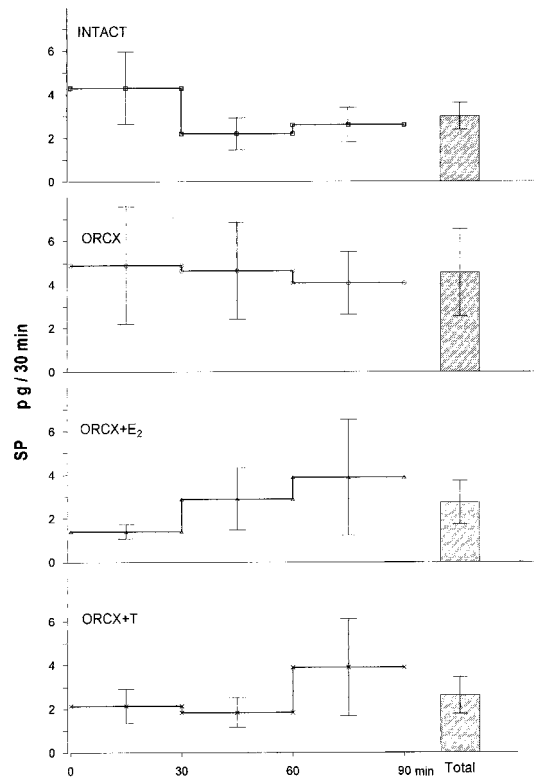


Fig. 2

SP concentration in three consecutive 30-minute samples of perfusate and mean SP concentration calculated from all samples obtained during 90 min of the perfusion (total) in male rats INTACT (n=7), ORCX (n=10), ORCX +E₂ (n=11) and ORCX+T (n=10). Mean \pm SEM .

to that found in INTACT group (68.3 ± 8.40 pg/30 min) but not different from the two remaining groups: ORCX+E₂ (58.40 ± 11.26 pg/30 min) and ORCX+T (47.10 ± 8.30 pg/30 min).

In the case of SP no significant changes of the mean SP concentration among the subsequent 30-min samples of PF were found in all four groups. Although the differences in the mean SP concentration between the four groups were not significant, it should be noted that the mean total SP level in the perfusion fluid was the highest in ORCX rats (4.55 ± 2.00 pg/30 min) as compared to that in INTACT (2.99 ± 0.61 pg/30 min), ORCX + E₂ (2.73 ± 1.00 pg/30 min.) and ORCX+T (2.62 ± 0.84 pg/30 min) rats.

As is shown, orchidectomy only induced Gn-RH decrease, but did not change SP level in the perfusates significantly. Gn-RH and SP levels were found

relatively unchanged by a high concentration of 17β -estradiol or testosterone.

17β -estradiol (E₂) and testosterone (T) blood levels. The sex hormone blood levels in INTACT animals as well as in those implanted with E₂ and T capsules were evaluated. E₂ concentration in the INTACT group was 0.024 ± 0.011 ng/ml and that in ORCX+E₂ group it was considerably higher (4.11 ± 0.09 ng/ml). In the INTACT group T level was 1.50 ± 0.39 ng/ml, while in ORCX+T group it was 11.54 ± 2.61 ng/ml.

Discussion

The method of lateral ventricles cerebello-medullary cistern perfusion applied to follow the basal release of Gn-RH and SP into the lumen of the cerebral ventricles was selected as it allows the evaluation of the time course of these neuropeptides release. The spontaneous outflow of endogenous CSF from the cerebello-medullary cistern in rats allows obtaining only a small volume of CSF (CANNON et al. 1980).

As far as some neuropeptides present in CSF are concerned, it has been demonstrated that there is a gradient of their concentration at various levels of the cerebral ventricular system (CRAMER et al. 1991). This method depends on the perfusion of the ventricular system with artificial CSF, which flows through the lateral ventricles, the 3rd ventricle, the aqueduct, the 4th ventricle, and then leaves the system through the cerebello-medullary cistern. The perfusion eliminates the possibility of obtaining site-related differences in the concentrations of the investigated peptides dependent on the site of CSF collection. The method was used in the investigation of effects of sciatic nerve stimulation on the release of SP (CANNON et al. 1980) and vasopressin (AVP) (ORLOWSKA-MAJDAK et al. 1994).

The results obtained in these experiments demonstrated that in intact rats, orchidectomized ones and those with subcutaneously implanted 17β -estradiol and testosterone capsules, Gn-RH and SP are continually released into the cerebral ventricles.

The mean levels of SP in the perfusing fluid were considerably lower than the mean levels of Gn-RH in all of the experimental animals groups. The levels of Gn-RH and SP in the perfusion fluid determined in the course of the presented experiments should

not be compared with other literary data because of considerable methodological differences with respect to the technique and site of CSF samples collection as well as the species of animals. In the CSF outflowing freely from the 3rd ventricle of sheep, no Gn-RH activity was detected (COPPINGS 1977), while SKINNER et al. (1995, 1997, 1998) demonstrated the presence of Gn-RH in CSF of ewe. Little or no Gn-RH in CSF of women (MIYAKE 1980) was found, whereas in rats (MORRIS et al. 1975 and JOSEPH et al. 1975) considerably high concentrations of this peptide in the CSF were reported. MATSUBARA et al. (1988) observed a significant increase of Gn-RH concentrations by comparison to control values in the CSF collected by lumbar puncture in humans with severe brain damage.

In rats, CANNON et al. (1980) determined SP levels in pure CSF as remaining within the range 0.7-5 ng/ml, but in CSF obtained by lumbar puncture from humans with various CNS disorders (NUTT et al. 1980; RIMON et al. 1984; MEYERSON et al. 1985; HIGA et al. 1989; JOST et al. 1991; CRAMER et al. 1991) or directly from the 3rd ventricle, SP levels were within the range from a few to several fmol/l (JOST et al. 1991).

In rat median eminence, the Gn-RH content is higher than that of SP (PARNET et al. 1990). However, a reverse relation between SP and Gn-RH levels was also observed. Thus, SP levels were 10-50 fold higher than these of Gn-RH in the anterior hypothalamus and medial basal hypothalamus/median eminence of hamsters (KREAM et al. 1987). The diversity of these data may be species-related. The comparison of Gn-RH and SP level in the median eminence and perfusing fluid has been supported by the fact that this structure is regarded as an area of good contact of the CSF with the tissue adjacent to the 3rd ventricle and with the pituitary portal vessels system. The abundance of Gn-RH axon terminals in the ME region is well documented (KOZLOWSKI et al. 1985). The hypothalamus contains also large numbers of SP-positive cell bodies, many of which appear to have terminal fields in the ME (MAKARA et al. 1986). The difference between the mean values of Gn-RH and SP in the perfusates may be caused either by the fact that Gn-RH-ergic neurons show more intensive secretory activity than SP-ergic neurons or it results from a different contact area range of those neurons in hypothalamus with the lumen of the cerebral ventricles.

It cannot be discounted that the pulsatile pattern of Gn-RH release to the CSF occurs as in pituitary portal vessels. Thus, in ovariectomized awake monkeys the Gn-RH levels in CSF aspirated from the 3rd ventricle at 15-min intervals ranged from 8 to 800 pg/ml (VAN VUGT et al. 1985). In this author's and others authors' (SKINNER et al. 1995, 1997) opinion Gn-RH release to the CSF has a pulsatile pattern. Specification of Gn-RH pulses in CSF is of longer duration than portal-Gn-RH pulses, and the appearance of the peak occurs after peripheral LH pulses (SKINNER et al. 1995). It was also found that Gn-RH pulses in the hypophysial portal blood peak takes place before Gn-RH pulses in CSF counterparts (SKINNER et al. 1997). The pulsatile basal Gn-RH release from the MBH freely moving rats during stages of the estrus cycle and after ovariectomy was observed (LEVINE and RAMIREZ 1982). In our experiments it was difficult to prove because of the insufficient number of samples and frequency of samples collection. No significant changes of the mean Gn-RH concentration among the subsequent 30-min samples of perfusates occurred in INTACT, ORCX, and ORCX+E₂ groups during 90 min of the perfusion.

Numerous studies have demonstrated the influence of gonadal steroid application on Gn-RH levels in the MBH and ME. This influence was expressed as a considerable decrease in Gn-RH levels after orchidectomy or ovariectomy (OLSON and BLAKE 1991) and their increase after the administration of exogenous gonadal steroids in rats (GROSS 1980; KOBAYASHI et al. 1978; KALRA and KALRA 1980, 1982). On the contrary, in CSF of ovariectomized rats an increase of Gn-RH concentration (JOSEPH et al. 1975) and a decrease after i.v. estradiol injection in monkeys (XIA et al. 1992) were observed. But no quantitative differences have been found in women's CSF content of Gn-RH in the follicular and luteal phases of the menstrual cycle, or during pregnancy and menopause (MIYAKE et al. 1980).

As a matter of controversy, in our experiment it was found that orchidectomy significantly decreased the Gn-RH concentration in the perfusate as compared to intact animals. But orchidectomy induced changes were not reversible to Gn-RH concentration in the INTACT rats by chronic 17 β -estradiol or testosterone applied. Recently, in the same way implanted capsules containing 17 β -estradiol caused a significantly increased Gn-RH content in medial basal hypothalamus in OVX rats (WALCZEWSKA et al.

1996). As shown, a high level of T and E₂ in the peripheral circulation cannot change Gn-RH concentration in the perfusates as well as in medial basal hypothalamus. It can be the negative feedback actions on Gn-RH neurones releasing this peptide into the cerebral ventricular system. This can explain a different effect of sex steroid hormone on Gn-RH content in the medial basal hypothalamus recently observed and in the perfusates obtained from cerebroventricular system during their perfusing. The most likely explanation for the increase in Gn-RH content in MBH/ME after estrogen replacement is a suppression of Gn-RH release from neuron terminals associated with the hypophysial portal system (GOODMAN and KNOBIL 1981). The statistical analysis of mean SP level values in PF points to the lack of significant differences of the mean SP concentrations in subsequent 30-minute samples of perfusates among the four experimental animal groups. But we observed no significantly higher total SP concentration in the perfusates higher in ORCX group than in following groups. The cyclic SP level changes in median eminence of hamsters (DEBELJUK et al. 1992) and of rats (JAKUBOWSKA-NAZIEMBLO et al. 1985) or medial preoptic nucleus and arcuate nucleus (PARNET et al. 1990) indicate a correlation between the blood level of gonadal steroid and SP concentration in some structures of the brain (KREAM et al. 1987). The injection of estradiol caused a decrease of SP in the anterior pituitary lobe of rats (COSLOVSKY et al. 1984), Siberian hamsters (DEBELJUK and BARTKE 1994) as well as monkeys (KERDELHUÉ et al. 1993). In our experiments we also observed a lower (but not significant) total SP concentration in perfusates of the animal groups after implantation of capsules containing testosterone or 17 β -estradiol than in orchidectomized groups. It can suggest a weak modifying gonadal steroid implantation influence on the release of this peptide into the cerebral ventricles. It should be noted that the lack of significant difference may result from the high value of mean standard error, especially in the ORCX group.

Summarizing the results obtained, it should be stated that the mean Gn-RH and SP concentrations in the subsequent samples of perfusates were generally stable during 90 minutes of the perfusion. The mean total concentration of SP was lower than that of Gn-RH in fluid outflowing from the cerebral ventricular system. A long-

term modification of gonadal steroids blood level due to 28-day orchidectomy, or orchidectomy combined with chronic subcutaneous implantation of capsules with high testosterone or 17 β -estradiol, generally, did not lead to statistically significant differences of Gn-RH and SP values in the perfusing fluid, except a decrease of Gn-RH after orchidectomy.

The problem of the effect of gonadal steroids on Gn-RH and SP release to the cerebral ventricle system requires further investigation because some tendency to modification of its concentration in the perfusates was observed similarly to a change in medial basal hypothalamus and median eminence.

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