PRESENCE AND ROLES OF NEUROPEPTIDES (PACAP, VIP) IN THE CHICKEN CENTRAL NERVOUS SYSTEM

PhD thesis

Tibor Hollósy MD.

University of Pecs, Medical Shool, Department of Anatomy

Supervisors
Dóra Reglődi, Rita Józsa

Pécs, 2013.

Contents

1. Introduction

Pituitary adenylate cyclase activating polypeptide PACAP)

PACAP was isolated in 1989 from ovine hypthalamus on the basis of its adenylate cyclase activating activity. It exists in two forms: PACAP27 and PACAP38. In vertebrate species, PACAP38 is the dominant form. Shorter fragments have antagonistic effects, the most commonly used antagonist is PACAP6-38. PACAP belongs to the vasoactive intestinal polypeptide (VIP)/secretin/glucagon peptide family, and shows closest homology to VIP, but its adenylate cyclase activating effect is 1000-10000 times stronger than that of VIP. PACAP can also be found in protochordate species and in non-mammalian vertebrates. The primary structure of PACAP is highly conserved, implying its important physiological functions. Since its discovery, widespread occurrence of the peptide has been described and its functions have been revealed to go beyond pituitary effects. According to previous studies, PACAP can be found not only in the central and peripheral nervous system, but in several other tissues, including endocrine glands and in the gastrointestinal tract. In mammals, PACAP has been found in highest level in the hypothalamic area. In the peripheral nervous system PACAP is expressed in spinal ganglia and pre- and postganglionic neurons.

PACAP plays a role in brain development, differentation and proliferation of neuroblasts, and the development of the neural tube. Mice deficient of PACAP or its receptor show severe developmental deficits, among others, they have memory insufficiency.

Distribution of PACAP in birds

Presence of PACAP in the central nervous system of birds shows a pattern similar to that in mammals. In the telencephalon strong PACAP expression can be found within the hippocampus, neostriatum intermedium and nucleus basalis. Strong labeling has been described in the olfactory bulbs and tract. In the diencephalon, cells and fibers expressing PACAP occur in the preoptic area. At the level of the anterior commissure, the bed nucleus is also rich in PACAP, similarly to the dorsal hypothalamus. Within the pretectal area, distinct populations of heavily labeled neurons are found in the nucleus spiriformis medialis and

nucleus pretectalis medialis. The distribution of PACAP-containing neurons were restricted mainly to tectum opticum. Within the brainstem, a dense cluster of PACAP-expressing neurons can be found near the nucleus lemnisci lateralis, nucleus parabrachialis, nucleus subceruleus ventralis and locus ceruleus. In the cerebellum, Purkinje cells are also labeled.

Distribution of PACAP receptors in birds

The effects of PACAP are mediated by specific G-protein coupled receptors. PAC1 receptors exhibit a high binding affinity for PACAP27 and 38, but not for VIP. VPAC1 and VPAC2 receptors bind VIP and PACAP with equal affinity. PAC1 receptors in birds are distributed homogenously throughout the brain. High expression can be found in the dorsal telencephalon, neostriatum, olfactory bulb, nucleus accumbens and ventral paleostriatum. Moderate labeling is present in the preoptic region and supraoptic and paraventricular areas. Numerous cells expressing PAC1 receptor can be found in the nucleus infundibularis, and nucleus mamillaris. Highest level of receptor gene expression is found in the thalamus. The granular layer of the cerebellum and the optic tectum show moderate-high receptor gene expression.

Physiological effects of PACAP

PACAP has several physiological effects. The first described effect was the influence of the hormone production of both the anterior and posterior lobes of the pituitary gland. Subsequently, other endocrine effects have also been described: it influences the thyroid hormone production, and steroidogenesis in the gonads as well as it plays a role in spermatogenesis and ovarian follicular development. PACAP also stimulates chatecholamine synthesis in the adrenal gland and insulin production in the pancreas.

PACAP has also been shown to play a central role in the regulation of circadian rhythm. Our research team has also investigated the effects of PACAP on melatonin production of the pineal gland. We have shown that PACAP stimulates release ot melatonin without influencing its circadian pattern. This effect can already be observed in embryonic life. Recent results have shown that PACAP also influences the migratory behavior of birds. PACAP has numerous other functions: it is involved in regulation of sleep-wake cycle, it has thermoregulatory effects and it influences the chemoreception in the carotid glomus. PACAP stimulates memory functions, which has also been confirmed by the memory deficits observed in PACAP knockout mice. PACAP has other behavioral effects, like in reproductive behavior of mice and rats, it increases locomotor activity in rodents, it is involved in stress adaptation mechanisms and has antidepressant effects. PACAP is released from sensory nerve endings, where it plays a role in pain suppression.

The neurotrophic and neuroprotective effects of PACAP have been shown in several in vitro and in vivo models. PACAP and its receptors appear early in the nervous system during development and they play a role in neurogenesis, neuronal differentiation, gliogenesis and neuronal patterning. The upregulation of PACAP after neuronal injuries has been described in several reports. The endogenous neuroprotective effect of PACAP has also been confirmed in studies using PACAP deficient mice or using PACAP antagonist with PACAP6-38. Our research team provided evidence for its protective effects in several neuronal disorders, such as in Parkinson`s disease, Huntington chorea, traumatic brain injury and cerebral ischemia.

Distribution of VIP in mammals and chicken

VIP and PACAP are well conserved throughout phylogenesis: the amino acid sequence is the same in almost all mammalian species. The structure of VIP is also known in a few non-mammalian vertebrates. The distribution of VIP-containing nervous structures has been described in several species, early studies focused on the distribution of the peptide in the intestinal system. VIP receptors have also been characterized in several mammalian and other vertebrate species. In birds, the amino acid sequence of VIP differs from mammalian VIP by only four amino acids, but significant differences in biological activity between mammalian and chicken VIP have also been reported. VIP can been found in the spinal cord, sympathetic ganglia, submucous plexus in the nervous system of the chicken embryo. However detailed distribution in the brain of chicken and the its role in chicken embryos are less known.

2. Aims

Examination of the distribution of PACAP- and VIP-containing structures in the chicken brain.

Examination of the distribution and daily rhythm of PACAP in the pineal body.

Examination of the effects of pinealectomy and starvation on PACAP levels and the daily rhythm in the nervous system and gastrointestinal tract.

Examination of effects of in ovo PACAP treatment on the motor and social behavior.

Examination of the role of PACAP on olfactory memory formation.

3. Methods

Animals

Fertilized eggs of domestic chicken were obtained from a local hatchery (Hubbard Flex egg). All procedures were performed in accordance with the ethical guidelines approved by the University of Pecs.

Radioimmunoassay

Different brain areas were removed and tissues were homogenized. The supernatant were used for the RIA analysis. 88111-3 antiserum was raised against a conjugate of PACAP24-28 and bovine thyreoglobulin coupled by carbodiimide in rabbit. Ovine PACAP24-28 C-terminal fragment was iodinated and the reaction mixtures were separated on a reverse-phase HPLC column. For VIP RIA analysis 85/24 antiserum was raised against a conjugate of porcine VIP and bovine thyreoglobulin coupled by glutaraldehyde in rabbit.

Daily rhythm

Animals were distributed into four groups: ***LD*** (light/dark) was maintained under 14 light hours and 10 dark hours. ***DL*** (dark/light) group was kept under reversed conditions. The animals in the ***LL*** (light/light) group were illuminated continuously in a room with no natural light. The ***DD*** (dark/dark) group was kept in complete darkness. The animals were decapitated every three hours brains and retinas were removed. The following tissues were collected: whole diencephalon, brainstem (pons and medulla oblongata), tectum, pineal gland, hypophysis, anterior part of telencephalon, cerebellum and retina. Cosinor analysis was used to assess circadian variation of PACAP levels in the different brain areas.

Immunohistochemistry

Chicken pineal gland sections wer used for immunohistochemistry. After two days fixation period, sections (15-50 µm) were cut in cryostat and processed for immunohistochemistry. PACAP-immunoreactive structures were identified in the section with a standard ABC method.

Pinealectomy

Under pentobarbital anaesthesia a short incision was made on top of the head in a craniocaudal direction to expose the surface of the skull. The bone was removed to expose the dura mater over the superior saggital sinus. The dura was slit, exposing the underlying pineal gland. The gland was gently grasped and carefully removed, observing the stalk as it emerged. The same procedure was followed for sham-operated birds, expect the the pineal body was not removed. Based on observations that chicken need at least one week of recovery after pinealectomy, the animals were decapitated and brains removed after one week of survival, and we observed the following parts of the brain: brainstem (pons and medulla oblongata), diencephalon and the frontal part of the telencephalon.

Starvation

Food deprivation of chickens was started at 8 pm, and animals were sacrificed 12, 36, 48 hours after the beginning of starvation, when their brains were removed. Control animals were sacrificed at each time point. Hypothalamus, brainstem and telencephalon were removed, weighed and further processed for RIA analysis of PACAP and VIP content. Statistical comparisons were made using the analysis of variance (ANOVA) test.

In ovo treatments

The fertilized eggs were kept in an incubator. The treatment of chicken embryos was made in the first and second embryonic periods. A small window was made on the eggshell by a sterile needle. Treatment solutions were given beneath the chorioallantois membrane with a sterile Hamilton-needle. Embryos were administrated 20 µg PACAP6-38 dissolved in 25 µl physiological saline. The given dose of PACAP6-38 was calculated based on earlier descriptions. Control animals were treated only with saline in the same volume.

Examination of the olfactory memory

Treatments were started on day 15 and ended on day 20. The stimulus used was undiluted strawberry flavoring solution, a commercially available food additive. Eggs were gently removed individually from the incubator, and a 3x2 cm piece of cottonwool was attached to the eggshells. The strawberry solution (300 ml) was injected daily into the cottonwool piece, at the same time of the day. Preceding the first exposure, eggs were treated with PACAP6-38 (20 µg) or physiological saline. Soon after the hatching, chicks of either sex were selected, and were placed in cages supplied with two bottles. One of them contained

strawberry-flavored water the other one had normal tap water. The amount of scented and unscented water was measured daily, for 6 days after hatching.

Behavioral testing

Open field test

General exploratory and locomotor activity were tested in an open-field at two days and two weeks after hatching. Activity was videorecorded for five minutes and the following behavioral parameters were evaluated: latency to walk and to vocalize, the number of stapes, areas entered, burst activity, escape attemps, peckings, preenings, wing moves, forward-backward and lateral head moves, fecal boluses, wall runs, diagonal runs and jumps. Parametric data in the test were compared by Student's t-test, while nonparametric data were compared using Man-Whitney test.

General behavior test

An instantaneous scan sampling method was used to record the number of birds within their homecage performing defined behaviors at 1 min intervals for 15 minutes each time. Behaviors recorded were ingestion standing, drinking, moving, sitting, aggressive pecking, preening, other pecking and gentle feather. Data are expressed as mean percentage of animals. Values of control and treated animals are compared with Student's t-test.

Runway test

A runway test was used to measure social behavior. The runway consisted of a start and a goal box at the end of a two meters long runway path. The test subject was acclimatized in the start box for two minutes, during which it could see the other birds. The door of the start box was then raised, and the time to enter the goalzone (the 20 cm zone next to the goal box) as well as the total time spent in the goalzone during the ten minutes test were measured. The runway test was done at 4 and 14 days. Results were compared using Student's t-test.

4. Results

Presence of PACAP and VIP in the chikcen brain

The results of the RIA measurements show that both PACAP and VIP are present in high levels during the second half of the embryonic developmental period. PACAP levels were significantly higher than those of VIP in all examined brain areas. Highest PACAP levels were measured in the brainstem, followed by the hypothalamus, cerebellum, while telencephalon showed the lowest PACAP levels. Levels of PACAP showed a tendency to decrease during the second half of embryonic development in almost every observed brain area, reaching statistical significance in the brainstem and hypothalamus between 15. and 20. days. Compared with PACAP, VIP levels were significantly lower in all examined brain areas, which might suggest that the regulatory mechanisms mediated by PACAP are more dominant than those of VIP during the second half of embryonic development.

Daily variations of PACAP

Brainstem:

The daily variation of PACAP values was similar in the *LD*, *LL* and *DD* groups. During subjective day hours, no significant difference was found between the groups, values varied between 4-10 ng/mg protein. During subjective night hours, PACAP levels increased to 12-19 ng/mg protein. Average PACAP levels of all subjective night hours vere significantly higher in all three groups than those of subjective day hours. In the *LD* group, a single peak was observed at 24 hour, which was significant compared to all other time points. In the *LL* group, the peak was observed at 21 hour, which levels significantly different from all previous points. In the *DD* group, the peak occured also at 21 hour, being significantly different from the lowest levels at 15, 18 and 03 hours. A different pattern was observed in the *DL* group: levels of PACAP were highest between 15 and 21 hours, while they were significantly different from preceding and succeeding time points. Cosinor analysis revealed significant daily rhythms for *LD* and *DL* groups.

Diencephalon:

In the *LD*, *LL* and *DD* groups, PACAP levels varied in the range of 6-10 ng/mg protein during the subjective day. In the subjective nighttime, PACAP levels increased in all three groups (11-14 ng/mg protein), with highest levels at 21 hour in *LL* and *DD* groups, and at 24 hour in the *LD* group. Although no significant difference could be shown comparing values at different time points, averages of levels of all subjective night hours were significantly higher in all three groups than those of all subjective day hours, regardless of the light conditions. The pattern observed in the *DL* group was similar to that of the brainstem: values were highest at the end of dark and beginnig of light hours (15-21 hours). Cosinor analysis revealed significant circadian variations in all four groups.

Telencephalon:

The *LD*, *LL* and *DD* groups showed a nearly similar daily variation of PACAP. PACAP levels showed a decreasing pattern during subjective daytime, reaching low levels between 12-21 hours, with lowest values at 21, 12-15 or at 18 hour. The difference between the low and high levels was significant. In *DL* group, two peaks were observed, at 18 and 03 hours (at the beginning and the end of light hours). Using Cosinor analysis, significant rhythm was obtained in groups, except in the *DL* group.

Retina:

All four groups showed a similar pattern: values started decreasing after 6 hours, with lowest levels reached at 12-15 hours. Levels gradually returned to the original levels during the subjective night hours independent of the lighting conditions. The difference between the lowest and highest levels was significant in all four groups. In the *LD* group, high values were measured at 03, 06, 21 and 24 hours, low levels at 9, 12 and 15 hours. Similar pattern could be observed in the *LL* group, although all values of PACAP were lower than in the *LD* group. In the *DL* group, high levels were measured at 06 and 24 hours. In the *DD* group, highest levels were observed at 03 and 06 hours. Significant difference was found in the *LD* and *LL* groups between average values. Cosinor test also revealed daily variation in all groups.

Tectum, cerebellum, hypohysis, pineal body:

No circadian rhythm of PACAP levels was observed in these areas using either statistical test. Levels of PACAP varied in the tectum and cerebellum in the range of 4-4.5 and 2-3.3 ng/mg protein. However, a slight but not significant increase in average PACAP

levels could be seen in *DL* group, during subjective day hours in the tectum and during subjective night in the cerebellum. In the pineal body, significant increase was observed only in the *DL* group during subjective nigth hours. The hypophysis showed a slight but not significant increase only in the *LD* and *DD* groups during subjective night hours.

Examination of changes of levels of PACAP after pinealectomy and starvation

Pinealectomy:

In the present study, we investigated the possible functional role of the pinealectomy in influencing PACAP and cAMP levels in the brainstem and diencephalon of he chickens. In our previous study highest levels and most pronounced daily variations of PACAP levels were found in the brainstem and diencephalon. In these areas, lowest level of PACAP was measured between 15 and 18 hours and highest level during nighttime, between 21 and 3 hours. PACAP levels were significantly higher in both the brainstem and diencephalon of pinealectomized animals than in sham-operated chickens. In pinealectomized birds, 41 and 68% elevations of PACAP levels were found in the brainstem at 15 and 24 hours. In the diencephalon of pinealectomized animals, PACAP levels increased by 21 and 28%, at 15 and 24 hours. These elevations in PACAP levels resuleted to be statistically significant in each group.

When comparing values measured at 15 and 24 hours in the sham-operated groups, a nighttime elevation of PACAP levels was found both in the diencephalon and brainstem. In pinealectomized animals, PACAP levels showed a significant nighttime elevation in the brainstem, but not in the diencephalon.

Starvation:

A significant elevation was observed 36 hours after food deprivation in the hypothalamus, brainstem and telencephalon. At later time points, levels returned to those measured in normal control animals.

VIP levels in the chicken showed an opposite pattern to that found with PACAP: levels gradually decreased, and it was significant 36 hours after food deprivation. By 84 hours, levels returned to normal, except in the telencephalon.

Data obtained in chicken were compared with those of rats. A significant elevation of PACAP was observed after 12 hours of starvation in the rat hypothalamus, brainstem and telencephalon. VIP levels showed a gradual increase in the hypothalamus and telencephalon, then levels returned to nearly ad libitum fed state. In the brainstem, a significant decrease was observed after 84 hours of starvation in rats.

Effects of in ovo treatment on motor and social behavior

In the open-field, similary to the behavior observed in their home cage, chicken treated with PACAP6-38 at E8 were more active than control animals. PACAP6-38-treated animals spent significantly less time inactive, crossed more areas and made more steps as well as more escape attempts. Only a few animals displayed pecking, turning, wing moves or preening activities at this age. By P14, most differences between the same groups treated at E8 dissappeared. Results of the runway test showed that chicken treated with PACAP6-38 at E8 exhibited reduced social behavior. The latency to leave the startbox was not different between the control and treated groups. All chicken left the startbox, but 50% of the treated chicken and 25% of the control animals did not reach the goalzone at all at P4. At P14, most chicken

reached the goalzone, but again more of the treated animals did not (18%). PACAP6-38-treated chicken reached the goalzone in a longer time. Although the time was longer at both P4 and P14, significant difference was only observed at P14. In addition, animals required less time to reach the goalzone at P14 than at P4. The time spent in the proximity of their penmates was significantly shorter in the treated group at both examined time points. There was no difference between control and treated at P16.

The role of the PACAP in olfactory memory formation

Normal control animals – not exposed to strawberry scent – showed a preference for plain water throughout the 6 days observed. The consumption of strawberry-scented water was less than half of that of plain water. The differences were significant throughout the observation period without significant differences between the individual days observed. Chickens exposed to strawberry scent and injected only with saline showed no preference after hatching: the difference between unscented and scented water consumption was not significant.

Animals exposed to strawberry scent during embryonic life and injected with PACAP6-38 consumed unscented and scented water in a similar proprotion to that observed in normal, untreated chicken: the consumption of strawberry-scented water less than half of that of plain water. This could be observed throughout the 6 days, with no significant differences between the individual days.

5. Discussion

Presence of PACAP and VIP in the chicken brain

The results of the RIA measurements show that both PACAP and VIP are present at high levels during the second half of the embryonic developmental period. Compared with PACAP, VIP levels were significantly lower in all examined brain areas, which might suggest that regulatory mechanisms mediated by PACAP are more dominant that those of VIP during the second half of embryonic development. And lower levels of VIP during the second half of embryonic development may indicate important region- and stage-specific roles of these peptides in brain development, the discussion of which is beyond the scope of the present thesis.

Daily variation of PACAP

Our result shows that highest levels of PACAP were found in the diencephalon and brainstem followed by the cerebral cortex, tectum and cerebellum. Lowest levels were found in the hypophysis, pineal body and retina. We found that the changes in levels of PACAP showed similar daily pattern in the diencephalon and brainstem. In the *LD*, *DD* and *LL* groups, the levels of PACAP increased during night. Elevated levels of PACAP observed in *LD* are in accordance with other studies demonstrating higher levels of PACAP, VIP and other peptides in rat hypothalamic nuclei during night hours. We found that under reversed lighting condition (*DL*), the circadian variation did not follow the pattern observed in the

other three groups. The daily variation of PACAP could be seen in the telencephalon in *LD*, *DD* and *LL* groups. Levels of PACAP and cAMP were increased after pinealectomy, suggesting that the pineal gland, via melatonin, may play an important role in the daily rhythm of PACAP secretion of neurons. According to our data melatonin modulates PACAP secretion. We found higher levels of PACAP during night hours, and lower levels during day hours. After pinealectomy, significantly higher levels of PACAP were found in some brain areas. Elevations were more pronounced during night hours, especially in the brainstem. We also found a difference in changes of daily PACAP levels in the brainstem and diencephalon between young and aging chickens.

Influence of pinealectomy and starvation on levels of PACAP

In the present study we showed that levels of PACAP and cAMP increased in the brainstem and diencephalon after pinealectomy. These data suggest that the pineal gland has an inhibitory impact on the formation of PACAP and also on PACAP-induced effects. Inhibitory effects have been documented for VIP, which is the closest structural relative peptide to PACAP. These studies indicate that the diencephalon (hypothalamus) in one site for functional interaction between PACAP and pineal hormone melatonin. Our results provide evidence that the pineal gland suppresses PACAP and cAMP formation in the chicken brain and diencephalon, and due to the absence of this inhibitory effect, levels of PACAP and cAMP are increased one week after pinealectomy. Our findings show that in control animals, PACAP- and cAMP-levels are significantly higher at nighttime than at daytime. After pinealectomy, significant nighttime elevation of PACAP was found only in the brainstem, but not in the diencephalon. In the diencephalon, PACAP levels in pinealectomized animals increased at 15 hour compared to the controls, but no further elevation was detected. These results indicate the significance of the pineal gland in the regulation of PACAP levels in the diencephalon and the presence of additional regulatory mechanisms resulting in the nighttime elevation of cAMP-levels in these brain areas after pinealectomy. Our present results indicate that the pineal gland is not the primary regulatory factor of the daily variations in PACAP-levels, and rhythmical changes of hormonal and neuropeptide levels are not controlled exclusively by the pineal gland.

According to results of starvation study our results show that PACAP-levels are increased in both species, 12 hours after food deprivation in rats, and 24 hours later in chickens. VIP-levels show a more complex pattern: a gradual increase in the hypothalamus and telencephalon, and a significant decrease in the brainstem in rats. In chickens, a significant decrease was observed in every brain area after 36 hours of starvation.

Effect of in ovo treatment on motor and social behavior

A single injection of the PACAP6-38 during the first half of embryonic life caused subtle transient changes in general and motor behaviors, when compared to saline-treated control animals. Most of these behavioral differences disappeared by P14. Treatment during the second half of embryonic life, however, resulted in no modified behavioral pattern. Measurement of PACAP content in selected brain areas from animals treated at E8 showed, that PACAP-levels significantly decreased during development, but there was no difference in PACAP content between control and treated animals. Observing the daily general behavior of the animals revealed that PACAP6-38-treated chicken were more active in all observed behaviors in their familiar home-cage, but significant difference was observed in running,

preening and pecking. These differences disappeared by two weeks, but two weeks old animals exhibited less anxiety, as shown by the less time spent near the wall of the open-field. The transient nature of locomotor differences observed in our study may be due to the single injection during the sensitive period of embryonic development. The effect of PACAP6-38 on social behavior was more striking than on general exploratory and motor behaviors. Chicken treated with PACAP6-38 during the first half of embryonic life approached the goalzone of their penmates, significantly more slowly and spent significantly less time in the proximity of the familiar birds. This behavioral difference was present also at P14, in contrast to other behavioral differences, which almost disappeared by two weeks. The finding that treated chickens reached the goalzone later, than control animals is of special interest, since treated animals moved more in the open-field test. Our results also showed that PACAP content significantly decreased during development in all examined brain areas. These findings further underline the significance of PACAP during development. PACAP6-38 treatment did not influence the PACAP content, no significant difference was found at either time-point. A single injection of PACAP6-38 during a sensitive period may induce slight beavioral differences.

The role of the PACAP in the olfactory memory formation

Animals exposed to strawberry scent in ovo showed no preference after hatching for unscented or scented water, in contrast to unexposed chickens. When chickens were exposed to strawberry but were also injected with PACAP6-38, there was a clear preference for plain water, similarly to that in unexposed chickens. These results indicate that endogenous PACAP plays a role in embryonic chemosensory learning in the chicken. The exact mechanism how PACAP can exert these effects in the chicken is not known, but the presence and distribution of PACAP and its receptors are well known. Knockout studies show that PACAP or PAC1 deficient mice exhibit deficits in learning or show alterations in learning-associated factors. Also VIP, which shares structural similarity with PACAP, has been shown to be involved in memory processes. Our present study provides further data for the involvement of PACAP in embryonic development of the nervous system, and points to the possibility of PACAP being important in embryonic memory formation.

7. Publications related to the thesis

1. **Hollósy T.**, Józsa R., Jakab B., Németh J., Lengvári I., Reglődi D.: Effects of in ovo treatment with PACAP antagonist on general activity, motor and social behavior of chickens. Regul. Pept. 2004; 123:99-106. *(IF: 2,531)*

2. Jakab B., Reglődi D., Józsa R., **Hollósy T.**, Tamás A., Lubics A., Lengvári I., Oroszi G., Szilvássy Z., Szolcsányi J., Németh J.: Distribution of PACAP-38 in the central nervous system of various species determined by a novel radioimmunoassay. J. Biochem. Biophys. Meth. 2004; 61:189-198. *(IF: 1,302)*

3. Józsa R., Somogyvári-Vigh A., Reglődi D., **Hollósy T.**, Arimura A.: Distribution and daily variations of PACAP in the chicken brain. Peptides 2001; 22:1371-1377. *(IF: 2,137)*

4. Józsa R., **Hollósy T.**, Tamás A., Tóth G., Lengvári I., Reglődi D.: PACAP plays a role in olfactory memory formation in chicken. Peptides 2005; 26:2344-2350. *(IF: 2,231)*

5. Józsa R., **Hollósy T.**, Németh J., Tamás A., Lubics A., Jakab B., Olah A., Arimura A., Reglődi D.: Presence of PACAP and VIP in embrionic chicken brain. Ann. NY. Acad. Sci. 2006; 1070: 348-353. *(IF: 1,9)*

6. Józsa R., Németh J., Tamás A., **Hollósy T.**, Lubics A., Jakab B., Oláh A., Lengvári I., Arimura A., Reglődi D.: Short-term fasting differentially alters PACAP and VIP levels in the brain of rat and chicken. Ann. NY. Acad. Sci. 2006; 1070:354-358. *(IF: 1,93)*

7. Németh J., Jakab B., Reglődi D., Lubics A., Józsa R., **Hollósy T.**, Tamás A., Lengvári I., Görcs T., Szolcsány J.: Comperative distriburion of VIP in the central nervous system of various species measured by a new radioimmunoassay. Regul. Pept., 2002; 109:3-7. *(IF: 3,205)*

8. Somogyvári-Vígh A., Józsa R., Reglődi D., **Hollósy T.**, Meggyesi R., Lengvári I., Arimura A.: Influence of pinealectomy on levels of PACAP and cAMP in the chicken brain. Regul. Peptides 2002; 109:9-13. *(IF: 3,205)*

9. Németh J., Jakab B., Józsa R., **Hollósy T.**, Tamás A., Lubics A., Lengvári I., Kiss P., Oberritter Zs., Horváth B., Szilvássy Z., Reglődi D.: PACAP-27 radioimmunoassay: Description and application of a novel method. J. Radioanal. Nucl. Chem., 2007; 273:327-332. *(IF: 0,499)*

10. Csernus V., Józsa R., Reglődi D., **Hollósy T.**, Somogyvári-Vigh A., Arimura A.: The effect of PACAP on rhythmic melatonin release of avian pineals. Gen. Comp. Endocrinol. 2004; 135:62-69. *(IF: 1,751)*

Impact factor of all publications: 20,757.