Electronic Journal of Polish Agricultural Universities is the very first Polish scientific journal published exclusively on the Internet, founded on January 1, 1998 by the following agricultural universities and higher schools of agriculture: University of Technology and Agriculture of Bydgoszcz, Agricultural University of Cracow, Agricultural University of Lublin, Agricultural University of Poznań, Higher School of Agriculture and Teacher Training Siedlce, Agricultural University of Szczecin, and Agricultural University of Wrocław.

ELECTRONIC
JOURNAL
OF POLISH
AGRICULTURAL
UNIVERSITIES

1999
Volume 2
Issue 2
Series
FISHERIES

POPEK W., EPLER P. 1999. EFFECTS OF INTRAVENTRICULAR MELATONIN MICROINJECTIONS ON HYPOTHALAMIC CATECHOLAMINE ACTIVITY IN CARP FEMALES DURING A YEAR **Electronic Journal of Polish Agricultural Universities**, Fisheries, Volume 2, Issue 2.
Available Online http://www.ejpau.media.pl

EFFECTS OF INTRAVENTRICULAR MELATONIN MICROINJECTIONS ON HYPOTHALAMIC CATECHOLAMINE ACTIVITY IN CARP FEMALES DURING A YEAR

Włodzimierz Popek, Piotr Epler
Department of Ichthyobiology and Fisheries, Agricultural University of Krakow, Poland

ABSTRACT

Assays, made on 64 mature carp females aged 5 years, were performed during the spawning season (summer) and in mid-winter. Some fish were subject to intraventricular melatonin microinjections, while other had their pineal gland excised. Intensity of fluorescence in the hypothalamic aminergic nuclei was determined with the fluorescence histochemical method. The lowest fluorescence intensity was revealed in those individuals lacking the pineal gland, the highest intensity being typical of the fish subject to intraventricular melatonin

microinjections. In the winter series, all the fish showed a similar fluorescence intensity in the hypothalamic region studied. The results demonstrate a relationship between the pineal gland, melatonin, and the hypothalamic aminergic system, present in carp during the spawning period.

Key words: pineal gland, melatonin, hypothalamic catecholamines, hormonal control of maturity, carp.

INTRODUCTION

In many animal species reproducing seasonally, photoperiod – in addition to temperature – is the key environmental factor controlling the endocrine activity. The photoperiod-related information is transmitted, via the pineal gland and its major hormone, melatonin, to the hypothalamus-hypophysis-gonad pathway [14, 15, 34]. Most probably, the hypothalamic provides the functional coupling between melatonin and the endocrine system, the site of the interaction being, however, unknown [18, 19, 20].

The fish pineal gland and melatonin have also been shown to be involved in photoperiod-related mediation of maturation [11, 28, 33, 38]. Earlier studies on carp [25, 27] demonstrated the pineal gland– via melatonin – to stimulate the final stages of gonad maturation and to enhance post-spawning vitellogenesis. A hypothesis has been advanced that the hypothalamic, and in all likelihood its aminergic system, is the melatonin target. This is possible as, in numerous fish species (cyprinids and silurids in particular) the hypothalamic dopamine functions as a hypothalamic gonadotropin release inhibiting factor from the hypophysis [6]. On the other hand, noradrenalin stimulates secretion of the hypothalamic GnRH and gonadotropin (GtH2) [7, 22].

The fish catecholamines originate in two large aggregations of neurons: the *nucleus recessus lateralis* (NRL) and the *nucleus recessus posterioris* (NRP). The nuclei were found in the hypothalamic of numerous species, e.g., the gold fish [3], eel [16], and roach [9]. In addition, the fluorescence intensity in NRL and NRP of carp was shown to vary not only throughout a day (circadian rhythm), but also throughout a year, and to depend on fish maturity [31].

The research described in this paper was aimed at determining effects of the pineal gland and melatonin on the hypothalamic aminergic system activity in mature carp females during a year. If detected, changes in the system's activity, related to the absence of the pineal gland or resulting from intraventricular melatonin microinjections, could be interpreted as supporting the hypothesis on the role of CA in the mechanism of melatonin involvement in carp sexual maturation.

MATERIALS AND METHODS

The assays were performed on 64 mature carp (*Cyprinus carpio* L.) females aged 5 years, obtained from the culture run by the Fisheries Experimental Station of the Agricultural University in Cracow. The assays were made twice during a year. In the summer, a total of 32 individuals were retrieved and transferred to four 2 m^3 flow-through concrete tanks, each housing 8 individuals. The water in the tanks was aerated in order to maintain the dissolved oxygen level above 4 mg O_2/l. Water temperature (22°C) and illumination (L:D = 16:8; light, of intensity >1500 lux, switched on at 4.00 hrs) were electronically controlled and identical with actual conditions in the original fish ponds. All the females in Group I (Px) had their pineal gland removed, as described by Popek et al. [33]. In Groups 2 (ivM) and 3 (ivC), injection needle conduits leading to the third cerebral ventricle were implanted as described by Popek [25]. Group 4 (C) constituted the control. Four days later, at the mid-point of the

dark phase, the Group 2 fish were subject to intraventricular melatonin microinjection, 1 ug/ml/kg body weight doses being used. Prior to injection, melatonin (Sigma Chemical Co.) was dissolved first in 5 ul 96% ethanol and then in the physiological fluid. The Group 3 females were subject to intraventricular microinjections of the physiological fluid with 5 ul 96% ethanol. Five minutes after microinjection the fish were sacrificed by decapitation. All the surgical and manipulation procedures were carried out on fish anaesthetised with 1% monophenyl ether-ethylene glycol (Merck Schuchardt Co.). The hypothalamic was excised and analysed with the Falck-Hillarp fluorescence histochemical method [4, 10]. The slices were viewed under the fluorescence microscope equipped with a HBO-200 high pressure mercury lamp and 2BG-12 and GG-9 filters which ensured wavelength ranges optimal for the following catecholamines (CA): adrenalin (A), noardrenalin (NA), and dopamine (DA). Fluorescence intensity in the two hypothalamic aminergic nuclei: the lateral recess nucleus (*nucleus recessus lateralis*, NRL) and the posterior recess nucleus (*nucleus recessus posterioris*, NRP) was estimated (on coded slides) with a 5-score scale in which 1 was equivalent to very weak and 5 to very strong fluorescence. Such a scale had already been used in earlier studies on catecholamine metabolism [23, 24, 26]. To check the specificity of the fluorescence observed in the brain tissue, a test with 0.01 sodium borohydride ($NaHBO_4$) was run [8].

The winter series (4°C water temperature; L:D 8:16 light regime; light, of intensity >1500 lux, switched on at 8.00 hrs) involved 32 mature carp females as well. The handling and assay procedures were identical as those used in the summer series.

RESULTS

In view of the identical changes in fluorescence activity observed in the aminergic nuclei (NRL and NRP) in the hypothalamic, they were treated jointly as a mean aminergic intensity of the hypothalamic.

In the summer series, the lowest fluorescence activity was recorded in Group 1 (Px) (mean estimate equal to 2). A total of 75% of slides in that group scored 2 (weak fluorescence). The highest fluorescence (mean estimate equal to 3.5) was recorded in the hypothalamic of those fish which had received intraventricular melatonin microinjections (Group 2, ivM). A total of 50% of slides in that group showed intermediate and the remaining 50% strong fluorescence. In the control groups 3 (ivC) and 4 (C), the average fluorescence was weak (the respective mean estimates equalled 2.4 and 2.5) (Table 1).

Table 1. Changes in carp hypothalamic fluorescence activity in summer. Each column shows number of individuals with a given fluorescence activity. On the average, each individual yielded 10-15 estimates

Group	Summer series					
	fluorescence intensity					
	1	2	3	4	5	mean estimate
Group 1 (Px)	1	6	1	-	-	2.0
Group 2	-	-	4	4	-	3.5

(ivM)						
Group 3 (ivC)	1	3	4	-	-	2.4
Group 4 (C)	1	3	3	1	-	2.5

In the winter series, fluorescence intensity in the hypothalamic of all the groups was estimated as strong and very strong (most slides scored 5). Mean estimates in different groups were equal to 4.2; 4.7; 4.4; and 4.4 (Table 2).

Table 2. Changes in carp hypothalamic fluorescence activity in winter. Each column shows number of individuals with a given fluorescence activity. On the average, each individual yielded 10-15 estimates

Group	Winter series					
	fluorescence intensity					
	1	2	3	4	5	mean estimate
Group 1 (Px)	-	1	-	3	4	4.2
Group 2 (ivM)	-	-	-	2	6	4.7
Group 3 (ivC)	-	-	1	3	4	4.4
Group 4 (C)	-	1	-	2	5	4.4

DISCUSSION

In spite of an ample body of research, no information is thus far available on the precise mechanism of melatonin involvement in both the seasonal reproductive function [1] and the circadian rhythm control [17].

In fish, melatonin has been shown to indirectly affect the gonadotropin level [32, 33] and the steroidogenesis [30]; no direct effects of the hormone on ovaries was demonstrated [29].

The world literature lacks evidence on specific binding sites, hence specific receptors, of melatonin in fish brain. It was as late as in 1989 that a high affinity to ^{125}I-melatonin was autoradiographically detected in brain tissue of higher vertebrates: reptiles [35] and birds [36]. Similar assays performed in mammals demonstrated the mammalian brain to have three such sites only: the suprachiasmatic nucleus (*nucleus suprachiasmaticus*, SCN), the median eminence (*eminentia mediana*, ME), and the dorsoposterior part of the fourth cerebral ventricle [37]. It should be added that melatonin receptors occur with the highest density in ME (near the arquate nucleus, *nucleus arquatus*) [39], i.e., in areas housing the terminal parts of axons originating from LH-RH producing cells and the DA-containing tubero-infundibular

neurons. By virtue of its inhibiting LH-RH release to hypophysis portal capillaries, DA controls LH and FSH secretion from the hypophysis [13]. The presence of melatonin in just those areas clearly demonstrates its mediating role in reproduction control in mammals. Moreover, Zisapel and Laudon [41] and Zisapel et al. [40] showed melatonin to be capable of inhibiting DA release from dissected hypothalamic cells of rats.

Since, as already mentioned in the Introduction, NA, too, is active in control of the release of both the hypothalamic GnRH and GtH2 [7, 22], a question can be raised whether, as is the case in mammals, melatonin is capable of affecting DA and NA activity via the aminergic system. The results obtained in the work described here demonstrate the mechanism to be possible in fish as well. The increased hypothalamic aminergic nuclei fluorescence intensity in summer (i.e., during the carp's spawning season), resulting from intraventricular melatonin microinjections (Group 2), as well as the weak fluorescence intensity in the fish after pinealectomy (Group 1) provide a clear evidence that melatonin inhibits CA release in the carp hypothalamic. The high fluorescence intensity is related to a low activity of the aminergic system, as confirmed by the results of assays on rat females at different stages of their reproductive cycle [12]. The actual CA content can be, with a high probability, inferred from changes in fluorescence intensity, particularly in view of the fact that other studies demonstrated a linear relationship between the actual catecholamine level as measured microspectrofluorometrically and biochemically and the observed fluorescence intensity in tissues under study [2].

The time of the day was important in the study presented, as the pilot experiment failed to demonstrate changes in the hypothalamic fluorescence intensity in carp individuals decapitated at the beginning of the light phase. The effect was most likely a result of a varying amount of active melatonin receptors in the carp hypothalamic. The hypothesis is supported by the results of experiments on mammals, which showed the melatonin efficiency to have been dependent on melatonin receptor concentration in the nervous tissue, higher at night than during the morning hours [21].

The seasonally varying sensitivity of the hypothalamic CA to melatonin is most probably related to the amount of active melatonin receptors as well. That was demonstrated by the winter series of assays (L:D = 8:16; 4°C water temperature) when no between-groups differences in fluorescence intensity could be found. Fluorescence was equally strong in all the groups (most estimates yielded scores of 4 and 5), thus evidencing a low activity of the aminergic system (Table 2).

Another proof of the pineal gland effect on the hypothalamic CA level in carp is the interaction between endogenous melatonin and CA rhythms. As demonstrated by Popek et al. [32], the maximum and low melatonin levels in the fish pineal gland and peripheral blood system occur at night and during the day, respectively. Consequently, the highest and the lowest levels of the hypothalamic biogenic amines should occur in the reverse order. This inference is supported by results of studies on the circadian and seasonal rhythms in the carp hypothalamic CA contents [31]: the highest and the lowest CA levels in the carp hypothalamic occur at night and during the day, respectively, the circadian rhythmicity being present in the sexually mature fish only.

Although the Falck-Hillarp fluorescence histochemical method used in the experiments described does not allow to qualitatively discriminate between adrenalin, noradrenalin, and dopamine, studies carried out by Brass [5] on eel and by Popek on carp (unpubl. data) show

dopamine to contribute almost 70% to the fish cerebral CA. Thus it can be inferred with a high probability that, similarly to mammals, the pineal gland melatonin can inhibit DA release from the hypothalamic aminergic nuclei cells in the sexually mature carp during summer. This is particularly important in view of the fact, mentioned in the Introduction, that the hypothalamic DA functions as a hypothalamic gonadotropin release inhibiting factor in cyprinids, whereby not only the basal, but also the spontaneously released GtH2 levels are reduced.

The results obtained in the study described here unequivocally demonstrate a coupling between the pineal gland and melatonin and the hypothalamic CA activity in the sexually mature carp during the spawning season. They also point to a possible target site of melatonin effects on the hypothalamic-hypophysis-gonads axis. For this reason, further studies in which quantitative relations between the hypothalamic DA and NA on the one hand and melatonin on the other would provide a basis from which to study mechanisms of melatonin effects in development, maturation, and reproduction in cyprinids are necessary.

CONCLUSIONS

1. The pineal gland and melatonin mediate the hypothalamic aminergic system activity in carp during the spawning season.
2. The results obtained demonstrate that the two hypothalamic aminergic nuclei: NRL and NRP are most probably the sites of melatonin action in the hypothalamic-hypophysis maturation control.

ACKNOWLEDGEMENT

The research described in this paper was supported by the State Committee for Scientific Research Grant No. 5 PO6D 019 12.

REFERENCES

1. Arendt J., 1986: Role of the pineal gland in seasonal reproductive function in mammals. Oxford Rev. Reprod. Biol. 8, 266-320.
2. Bacopoulos N.G., Bhatnagar R.K., Schute W.J., van Orden III L.S., 1975: On the use of the fluorescence histochemical method to estimate catecholamine content in brain. Neuropharmacol. 14, 291-299.
3. Baumgarten H., Braak H., 1967: Catecholamine im hypothalamic vom goldfish (*Carassius auratus)* Z. Zellforsch. 80, 246-263.
4. Bjorklund A., Falck B., Owman Ch., 1972: Fluorescence microscopic and mocrospectrofluorometric techniques for the cellular localization and characterization of biogenic amines. In: “Methods of Investigative and Diagnostic”. Endocrinology 1, 318-368.
5. Brass Y.M., 1979: Circadian rhythm in brain catecholamine concentrations in the teleost *Anguilla anguilla* L. Comp. Biochem. Physiol. 62, 115-117.
6. Chang J.P., Peter R.E., 1983: Effects of dopamine on gonadotropin release in goldfish, *Carassius auratus*. Gen. comp. Endocrinol. 49, 22-31.
7. Chang J.P., Van Goof F., Acharya S., 1991: Influences of norepinephrine, and adrenergic agonist and antagonist on gonadotropin secretion from dispersed pituitary cells of goldfish, *Carassius auratus*. Neuroendocrinol. 54, 202-210.
8. Corrodi H., Hillarp N.A., Jonsson G., 1964: Fluorescence methods for the histochemical demonstration of monoamines. 3: Sodium borohydride reduction of the fluorescent compounds as a specificity test. J. Histochem. Cytochem. 12, 582-582.
9. Ekengreen B., 1975: Aminergic nuclei in the hypothalamic of the roach (*Leuciscus rutilus*). Cell. Tiss. Res. 159, 493-502.

10. Falck B., Hillarp N.A., Thieme G., Torp A., 1962: Fluorescence of catecholamines and related compounds condensed with formaldehyde. J. Histochem. Cytochem. 10, 348-354.
11. Falcon J., Meissl H., 1981: The photosensory function of the pineal organ of the pike (*Esox lucius* L.). Correlation between structure and function. J. comp. Physiol. A. 144, 127-137.
12. Fuxe K., Hokfelt T., Nilsson O., 1972: Effect of constant light and androgen-sterylization on the amine turnover of the tubero-infundibular dopamine neurons: blockade of cyclic activity and induction of a persistent high dopamine turnover in the median eminence. Acta Endocrinol. 69, 625-639.
13. Gallo R.V., 1981: Further studies on dopamine- induced suppresion of pulsatile LH release in ovariectomized rats. Neuroendocrinol. 32, 187-192.
14. Karsch F.J., Bittman E.L., Robinson J.E., Yellon S.M., Wayne N.L., Olster D.H., Kaynard A.H., 1986: Melatonin and photorefractoriness: Loss of response to the melatonin signal leads to seasonal reproductive transitions in the ewe. Biol. Reprod. 34, 265-274.
15. Kennaway D.J., Sanford L.M., Godfrey B., Friesen H.G., 1983 : Patterns of progesterone, melatonin and prolactinsecretion in ewes maintained in four different photoperiods. J. Endocrinol. 97, 229-242.
16. Lefranc G., L'Kermite A., Tysques J., 1970: Etude topographique et cytologique des differentes noyaux monoaminergiques de l'encephale *d'Anguilla vulgaris*. C. R. Soc. Biol. 164, 1629-1634.
17. Lewy A.J., Ahmed S., Lathan Jackson J.M., Sack R., 1992: Melatonin shifts human circadian rhythm according to a phase-response curve. Chronobiol. Int. 9, 380-392.
18. Lincoln G.A., Short R.V., 1980: Seasonal breeding: nature's contraceptive. Rec. Prog. Horm. Res. 36, 1-52.
19. Malpaux B., Daveau A., Maurice F., Locatelli A.,: Thierry, J.C., 1994: Evidence that melatonin binding sites in the pars tuberalis do not mediate the photoperiodic actions of melatonin on LH and prolactin secretion in ewes. J. Reprod. Fertil. 101, 625-632.
20. Maywood E.S., Hastings M.H., 1995: Lesions of the iodomelatonin-binding sites of the mediobasal hypothalamic spare the lactotropic, but block the gonadotropic response of male Syrian hamsters to short photoperiod and to melatonin. Endocrinology 136, 144-153.
21. Peschke E., Peschke D., Peil J., Ruzsas C., Mess B., 1988: Changes in the daily rhythm of serum testosterone levels following superior cervical sympathetic ganglionectomy in the cold-exposed rat: The role of the pineal. J. Pineal Res. 5, 179-189.
22. Peter R.E., Trudeau V.L., Sloley B.D., Peng C., Nahorniak C.S., 1991: Actions of catecholamines, peptides and sex steroids in regulation of gonadotropin-II in the goldfish. In: Reproductive Physiology of Fish. Pp. 30-34. A.P. Scott, J.P. Sumpter, D.E. Kime and M.S. Rolfe Eds. FishSymp 91, Sheffield.
23. Popek W., 1983: Seasonal variations in circadian rhythm of hypothalamic catecholamine content in the eel (*Anguilla anguilla* L.). Comp. Biochem. Physiol., 75C, 1, 193-198.
24. Popek W., 1988: Effects of hormonal stimulation of sexual maturation on the hypothalamic catecholamine content in European eel (*Anguilla anguilla* L.). Pol. Arch. Hydrobiol., 35(1), 119-126.
25. Popek W., 1991: Role of pineal gland in development and gonad maturation in common carp (*Cyprinus carpio* L) Ph. D. Thesis No 157: 1-103, Academy of Agriculture, Krakow.
26. Popek W., Sokolowska M., Bieniarz K., 1981: Effect of stress on the circadian rhythmicity of hypothalamic catecholamine content in the eel (*Anguilla anguilla* L.). Comp. Biochem. Physiol., 70C, 135-138.
27. Popek W., Bieniarz K., Epler P., 1991: Role of pineal gland in sexual cycle in common carp. In: "Chronobiology and Chronomedicine" (J. Surowiak and M.H. Lewandowski Eds.). pp. 99-102. Verlag Peter Lang, Frankfurt.
28. Popek W., Bieniarz K., Epler P., 1992: Participation of the pineal gland in sexual maturation of female rainbow trout (*Oncorhyncus mykiss* Walbaum). J. Pineal. Res. 13, 97- 100.
29. Popek W., Epler P., Sokolowska-Mikolajczyk M., 1996: Melatonin does not affect steroidogenesis or maturation of carp oocytes *in vitro* during the prespawning period. Pol. Arch. Hydrobiol. 43(3), 379-385.
30. Popek W., Galas J., Epler P., 1997: The role of pineal gland in seasonal changes of blood estradiol level in immature and mature carp females. Arch. Pol. Fish. 5(2), 259-265.
31. Popek W., Natanek H, Bieniarz K. Epler P., 1994: Seasonal changes in the catecholamine levels of the hypothalamic in mature and immature carp (*Cyprinus carpio* L.). Pol. Arch. Hydrobiol. 41(2), 227-236.
32. Popek W., Epler P., Bieniarz K., Sokolowska-Mikolajczyk M., 1997: Contribution of factors regulating melatonin release from pineal gland of carp (*Cyprinus carpio* L.) in normal and in polluted environments. Arch. Pol. Fish. 5(1), 59-75.
33. Popek W., Breton B., Piotrowski W., Bieniarz K., Epler P., 1994: The role of the pineal gland in the control of a circadian pituitary gonadotropin release rhythmicity in mature female carp. Neuroendocrinol. Lett. 16(3), 183-193.

34. Reiter R.J., 1995: Functional pleiotropy of the neurohormone melatonin: antioxidant protection and neuroendocrine regulation. Frontiers in Neuroendocrinology 16, 383-415.
35. Rivkees S.A., Carlson L.L., Reppert S.M., 1989: Guanine nucleotide- binding protein regulation of melatonin receptors in lizard brain. Proc. Natl. Acad. Sci. USA. 86, 3. 882.
36. Rivkees S.A., Cassone V.M., Weaver D.R., Reppert S.M., 1989: Melatonin receptors in avian brain: characterization and localization. Endocrinology, 125, 363. .
37. Vanecek J., 1988: Melatonin binding sites. J. Neurochem. 51, 1436-1440.
38. Vivien-Roels B., Pevet P., Dubois M.P., Arendt J., Brown G.M., 1981: Immunohistochemical evidence for the presence of melatonin in the pineal gland, the retine and the Harderian gland. Cell. Tissue Res. 217, 105-115.
39. Weaver D.R., Rivkees S.A., Reppert S.M., 1989: Localization and characterization of melatonin receptors in rodent brain by *in vitro* autoradiography. J. Neurosci. 9, 2581.
40. Zisapel N., Egozi Y., Laudon M., 1985: Circadian variations in the inhibition of dopamine release from adult and newborn rat hypothalamic by melatonin. Neuroendocrinol. 40, 102-108.
41. Zisapel N., Laudon M., 1983: Inhibition by melatonine of dopamine release from rat hypothalamic: regulation of calcium entry. Brain Res. 272, 378-381.

Submited:

Włodzimierz Popek, Piotr Epler
Department of Ichthyobiology and Fisheries
Agricultural University of Krakow
Poland