Steroid effects on the *in vitro* LH secretion from pituitary cells of sexually immature carp (*Cyprinus carpio* L.) under the influence of naltrexone and morphine

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ABSTRACT: Dispersed cells from sexually immature carp (footlings) pituitaries were exposed to estradiol (E_2) or testosterone (T) (both at 3×10^{-8} M) in the presence of opioid receptor antagonist naltrexone (10^{-8} or 10^{-6} M) and/or agonist – morphine (10^{-8} or 10^{-6} M). Naltrexone alone at 10^{-6} M increased the LH level as compared with the control. Morphine (10^{-8} or 10^{-6} M), T and E_2 had no influence on LH levels. The combination of T with naltrexone (10^{-8} M) stimulated LH release if compared with the control or with T alone. Morphine (both concentrations) with T caused significantly higher LH secretion than the control medium and T alone. Estradiol with naltrexone (10^{-8} and 10^{-6} M) had no influence on LH concentration. In media with E_2 and morphine (10^{-8} M) LH levels were higher than in the control and estradiol alone. The results show that in common carp sex steroids affect the response of pituitary cells to opioid agonist or antagonist giving an evidence on the role of steroids in LH release mediated by the opioid system.

Keywords: common carp; *in vitro* LH secretion; morphine; naltrexone; sex steroids

In mammals endogenous opioid systems inhibit the gonadotropin-releasing hormone (Gopalan et al., 1989; Yilmaz et al., 1996; Smith et al., 1998) by μ, κ or δ opioid receptor type (Thom et al., 1996; Lieberman et al., 1998). The reduction of this inhibition is necessary for the preovulatory surge of LH (Spijkstra et al., 1988; Walsh and Clarke, 1998). Gonadal steroids also exert an inhibitory effect on gonadotropin secretion (negative feedback action) and this effect is mediated (at least in part) by an increase in the opioidergic tone of the hypothalamus which is the cause of decreased GnRH secretion and a subsequent reduction of LH release (Ferin et al., 1984; Melis et al., 1984). Sex steroids influence the number of the brain opioid receptors (Casulari et al., 1987; Martini et al., 1989) and their binding characteristic (Piva et al., 1995; Thom et al., 1996). There is also a direct evidence on the involvement of steroid hormones in the modulation of the opioidergic tone (Morrell et al., 1985, 1992).

The involvement of endogenous opioid peptides in the control of gonadotropin secretion was also demonstrated in fish. Several forms of endogenous opioid peptides (Follenius and Dubois, 1979; Kawauchi et al., 1980; Salbert et al., 1992) as well as the specific opioid receptors (Bird et al., 1988; Rosenblum and Callard, 1988) were found in the fish brain and in the pituitary gland. By *in vivo* and *in vitro* investigations on goldfish and common carp (Rosenblum and Peter, 1989; Cheng, 1996; Sokolowska-Mikolajczyk et al., 2002a,b; Socha et al., 2003; Sokołowska-Mikołajczyk et al., 2005) the influence of opioid agonists (morphine, DAGO) or antagonists (naloxone, naltrexone) on LH secretion and on the release of immunoreactive GnRH was demonstrated. This influence is exerted

by the modulation of GnRH and dopamine release – the two most important hypothalamic factors (stimulatory and inhibitory) controlling LH release in teleosts.

In fish, like in mammals, gonadal steroids affect LH secretion acting at the level of the brain and/or pituitary. The steroid binding sites and oestrogen-immunoreactive cells in the brain of teleosts are localised in the close vicinity of GnRH neurons and may suggest that the effects of sex steroids on GnRH release in fish may be mediated by other neurotransmitters or neuropeptides (Trudeau et al., 1993) like endogenous opioid peptides. The mechanism of the steroid action on LH release in fish is not clear yet, as there are only a few papers dealing with the problem of opioid involvement in the sex steroid feedback effects on LH release (Rosenblum and Peter, 1989; Sokolowska-Mikolajczyk et al., 2002a,b). They demonstrated that the action of opioid agonists or antagonists depends on the sex of fish and the actual stage of their sexual cycle, suggesting the importance of steroids produced by the gonads in the mechanism of LH release control.

In the present investigations we used the pituitary cells from two years old, sexually immature carp (footlings), in which the endogenous levels of LH and sex steroids are relatively low: about 2.4 and 2 ng/ml of LH and testosterone, respectively (Billard et al., 1992; Chyb, personal communication). Cells were exposed to estradiol (E_2) or testosterone (T) in the presence of opioid receptor agonist and/or antagonist. As in mammals gonadotropin secretion is controlled mainly by μ or δ receptor type (Smith and Gallo, 1977a,b; Bondanelli et al., 1988; Lieberman et al., 1988; Thom et al., 1996; Kumru et al., 2001) and in the whole brain of regularly cycling female rats the number of μ receptors shows variation during the different phases of the oestrous cycle (Casulari et al., 1987; Martini et al., 1989; Piva et al., 1995), in the presented experiments we tested morphine and naltrexone (agonist of μ receptor type) and naltrexone (opioid receptor antagonist).

MATERIAL AND METHODS

An experiment was conducted in April on 15 carp (*Cyprinus carpio* L.) footlings netted from commercial carp ponds belonging to the Fisheries Research Station of the Department of Ichthyobiology and Fisheries, University of Agriculture, Krakow, Poland. Prior to the experiment fish were kept in flow-through basins for 3 days, exposed to a simulated, natural photoperiod (L:D = 13:11). Average body weight of fish was 206 ± 10 g. After the anaesthesia with 2-phenoxy-ethanol (Merck, Germany) – 0.3 ml/l of water, fish were killed by decapitation and the pituitary glands were collected and placed in a sterile ice-cold medium (MEM-Eagle, Sigma-Aldrich, USA) buffered with 15mM Hepes (Sigma-Aldrich, USA) and 9mM sodium bicarbonate (P.O.Ch. Poland). An osmotic pressure of the medium was 275 mOsm/kg and pH of 7.7 (the values characteristic of the common carp plasma).

The enzymatic dispersion of the glands was performed with collagenase H (Boehringer Mannheim, Germany) according to the method of Weil et al. (1986) for trout and adapted to carp by Mikolajczyk et al. (1990), as described elsewhere (Sokolowska-Mikolajczyk et al., 2005). After dispersion the cells

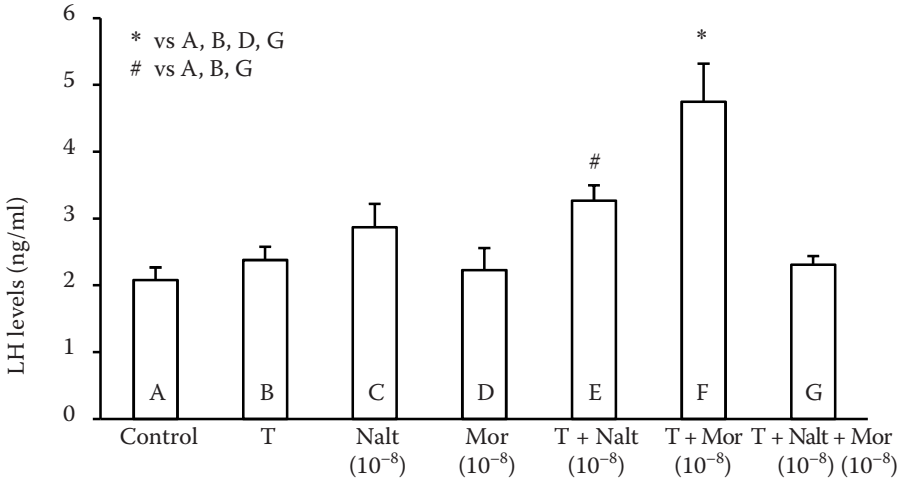

Figure 1. The effects of morphine (Mor) and/or naltrexone (Nalt) at the concentration of 10^{-8} M in combination with testosterone (T) (3×10^{-8} M) on LH secretion from the dispersed pituitary cells of sexually immature carp; bars represent mean LH levels ± SEM

were resuspended in a pre-incubation medium and transferred into two 96-well microplates (Nunc A/S Denmark), approximately 5 × 10^4 cells in each well. Then the plates were sealed and incubated for 48 hours at 22°C.

On the third day of culture the pre–incubation medium was replaced (after washing) with a medium containing the tested concentrations of naltrexone (10^{-8} or 10^{-6} M) (Sigma Aldrich USA), morphine sulphate (10^{-8} or 10^{-6} M) (Polfa, Poland), testosterone propionate (T) (3 × 10^{-8} M) (Calbiochem, Calif.) or beta-estradiol 17-propionate (E_2) (3 × 10^{-8} M) (Sigma Aldrich USA) and the combination of each concentration of naltrexone or morphine with testosterone or estradiol.

Each concentration of naltrexone, morphine, T or E_2 was tested in five wells. The plates were then incubated for another 24 hours at 22°C. At the end of this period the plates were centrifuged (200 g for 10 minutes at 20°C), the media were collected and frozen at –20°C until LH determination by the modified ELISA method (Kah et al., 1989).

LH levels measured in the medium samples were analysed statistically using the nonparametric two-tailed Mann-Whitney U-test. The differences between the means were determined as significant for $P < 0.05$.

RESULTS

(1) The effects of naltrexone on LH concentration in the incubation medium

Naltrexone alone at the concentration of 10^{-8} M did not change LH release (Figure 1), but the ten times higher concentration (10^{-6} M) caused a statistically significant increase ($P < 0.05$) in LH level as compared with the control (Figure 2).

(2) The effects of morphine on LH concentration in the incubation medium

Neither concentration of morphine (10^{-8} and 10^{-6} M) had an influence on LH levels in the culture medium in comparison with LH concentration in the control (Figures 1 and 2).

(3) Testosterone effects on LH levels under the influence of

(a) naltrexone

Testosterone alone had no effect on LH levels in comparison with the control medium (Figure 1). The combination of testosterone with a lower concentration of naltrexone (10^{-8} M) ensured significant stimulation of LH release if compared with the control ($P < 0.01$) or with testosterone alone ($P < 0.05$) (Figure 1). In the case of testosterone with a higher naltrexone concentration (10^{-6} M) the LH levels were not significantly different either from the control or from testosterone alone (Figure 2) and were lower ($P < 0.05$) than in wells with the combination of testosterone and naltrexone at 10^{-8} M.

(b) morphine

Both concentrations of morphine in combination with testosterone caused significantly higher ($P < 0.01$)

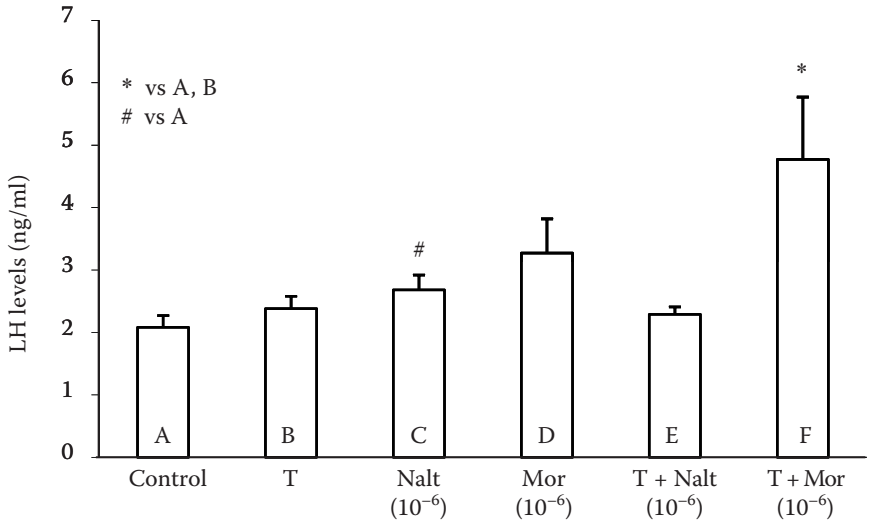

Figure 2. The effects of morphine (Mor) or naltrexone (Nalt) at the concentration of 10^{-6} M in combination with testosterone (T) (3 × 10^{-8} M) on LH secretion from the dispersed pituitary cells of sexually immature carp; bars represent mean LH levels ± SEM

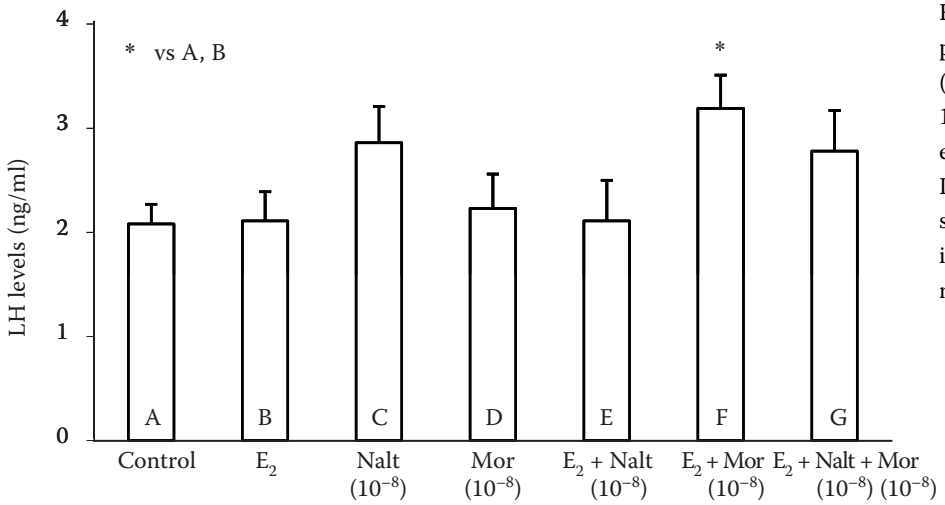

Figure 3. The effects of morphine (Mor) and/or naltrexone (Nalt) at the concentration of 10^{-8} M in combination with estradiol (E_2) (3×10^{-8} M) on LH secretion from the dispersed pituitary cells of sexually immature carp; bars represent mean LH levels ± SEM

secretion of LH from the cells than in the control medium and in the medium with testosterone alone ($P < 0.01$ and $P < 0.05$, respectively) (Figure 1).

(c) naltrexone and morphine

In the medium containing naltrexone (10^{-8} M), morphine (10^{-8} M) and testosterone LH levels were significantly lower ($P < 0.01$) than in the combination of testosterone with opioid antagonist or agonist (Figure 1).

(4) Estradiol effects on LH levels under the influence of

(a) naltrexone

Neither estradiol alone nor in combination with naltrexone (10^{-8} and 10^{-6} M) had an influence on LH concentration in the medium (Figures 3 and 4).

(b) morphine

In the medium containing estradiol and morphine (10^{-8} M) the LH levels were significantly higher than those in the control ($P < 0.01$) and in the medium with estradiol alone ($P < 0.05$) (Figure 3). In the ten times higher concentration of morphine no significant changes in LH concentration were observed (Figure 4).

(c) naltrexone and morphine

The combination of naltrexone (10^{-8} M) and morphine (10^{-8} M) in the presence of estradiol had no effect on LH levels if compared with estradiol and morphine alone (Figure 3). The same situation was observed if naltrexone was used at the higher concentration (10^{-6} M) (Figure 4).

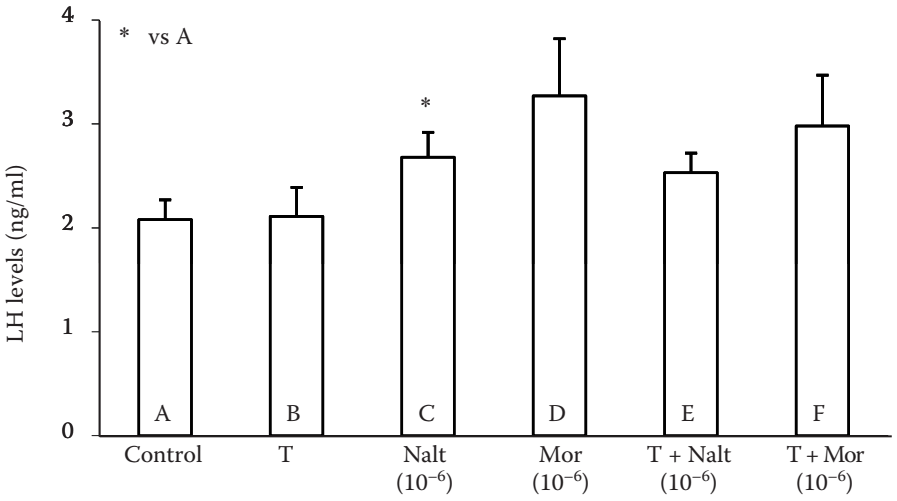

Figure 4. The effects of morphine (Mor) or naltrexone (Nalt) at the concentration of 10^{-6} M in combination with estradiol (E2) (3×10^{-8} M) on LH secretion from the dispersed pituitary cells of sexually immature carp; bars represent mean LH levels ± SEM

DISCUSSION

Despite the proofs of a sex steroid feedback effect on LH release in teleost fish (Goos, 1987; Peter et al., 1991), the mechanism and the site of negative or positive feedback on the brain-pituitary axis to influence LH secretion are not clear yet. Sex steroids may act directly at the level of the pituitary gland or indirectly *via* other neurons: GABAergic, serotoninergic, opioid, which in turn affect dopaminergic (inhibitory) and/or GnRH (stimulatory) systems controlling LH secretion and release (Somoza et al., 1988; Rosenblum and Peter, 1989; Kah et al., 1992). Data on the role of opioid peptides in the control of gonadotropin secretion in fish are not abundant (see Introduction) and the relation of opioids to other endocrine factors which ultimately determine blood LH levels is not clear yet. Rosenblum and Peter (1989) and Sokolowska-Mikolajczyk et al. (2002b, 2005) demonstrated in the *in vivo* and *in vitro* experiments on sexually mature goldfish and common carp that the putative (not measured) sex steroid levels connected with different state of gonad maturity or the sex of fish are responsible for the differential effects of opioid agonist (morphine) and antagonist (naltrexone) on LH release.

The results of the present paper demonstrate data obtained on sexually immature carp footlings. The use of fish at this stage of gonadal development was interesting for two reasons: firstly, because we tested the direct response of carp pituitary cells derived from immature fish in the milieu containing opioid agonist and antagonist with sex steroids and secondly, because for the first time opioids were tested on sexually immature fish in which the moment of the opioidergic system component development and its involvement in the control of gonadotropin secretion is unknown so far.

The analysis of LH concentration in the medium containing naltrexone alone showed that only the higher concentration of this antagonist (10^{-6} M) significantly increased LH levels in comparison with the control medium (Figure 2). The *in vitro* experiment on pituitary cells of mature male and female carp (Sokolowska-Mikolajczyk et al., 2005) demonstrated that naltrexone at identical concentrations had no influence on LH secretion.

On the other hand, morphine alone in mature fish caused an increase or a decrease in LH levels, depending on the sex of fish but in the present experiment both concentrations of this agonist were ineffective. This result shows that in immature fish endogenous opioids may have a certain (opposite than in mature fish) influence on gonadotropin secretion despite of the relatively low activity of the hypothalamo-pituitary-gonad axis.

Neither steroid (testosterone and estradiol) used in the present experiment affected LH secretion to the incubation medium (Figures 1 and 3). Similar results, but *in vivo* demonstrating that in goldfish sex steroids do not affect basal LH but they enhance GnRH induced LH secretion were reported by Trudeau et al. (1993). In the conditions of our *in vitro* experiment pituitary cells were not stimulated by GnRH in the presence of steroids, but they were exposed to naltrexone and/or morphine. Testosterone with naltrexone at 10^{-8} M (this dose was not effective in changing the spontaneous release of LH) significantly stimulated LH release into the culture medium (Figure 1). The higher dose of naltrexone (10^{-6} M), effective alone in the stimulation of LH secretion, had no influence on LH levels if present in the medium with testosterone (Figure 2). It is clear that the addition of testosterone to opioid antagonist changes the response of cells expressed as the actual level of LH secreted to the culture medium. This was not the case of estradiol, which was not effective either alone or in combination with naltrexone (Figures 3 and 4). Similarly to testosterone, estradiol with the lower dose of morphine significantly increased LH levels in the culture medium (Figure 3).

Testosterone also stimulates LH secretion when present in the medium together with morphine (both concentrations) (Figure 1). The opioid antagonist and agonist, together with testosterone, seem to induce the same type of reaction: they stimulate LH release. However, in the situation when testosterone was present in the medium simultaneously with both opioid agents (Figure 1), naltrexone antagonised the stimulatory action of morphine – LH levels in the medium with both naltrexone and morphine were significantly lower than in the case of morphine or naltrexone with testosterone. This result confirms that in fish testosterone acts on gonadotropin release through the typical opioid receptors and that μ receptors, among other types, may be involved in this process.

The results of this experiment show for the first time that sex steroids (mainly testosterone and estradiol to a lesser extent) present in the incubation medium affect the response of pituitary cells to opioid agonist or antagonist. This is direct evidence on the role of steroids in the process of LH

release mediated by the opioid system in fish, as the previous papers (Sokolowska-Mikolajczyk et al., 2002a,b, 2005) gave the indirect assumptions only, not supported by the measurements of steroid levels or the presence of steroids in the medium.

The results also show for the first time that in sexually immature fish the opioid system is active in the control of gonadotropin release. It is not surprising taking into account that in mammals the neuroendocrine control of reproduction starts very early in life and hypophyseal gonadotropin secretion is controlled by the hypothalamus at around mid-gestation in the foetal sheep and pig (Parvizi, 2000). In these species opioid receptors are functioning well before birth and the inhibitory action of morphine on LH release was demonstrated in male and female foetuses (Behrens-Herrler and Parvizi, 1992). According to our knowledge, there is no data showing the development of opioid system in fish before and after hatching.

The present paper gives some preliminary results showing that in fish like in mammals endogenous opioid peptides may be involved in the control of many functions, also those that are connected with early stages of sexual maturation of fish.

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