Interaction between Kisspetin and Dopamine in the Regulation of in vitro LH Release in Prussian carp (Carassius gibelio Bloch, 1782) Females at the Time of Gonad Recrudescence and Spawning Period

Miroslawa Sokołowska-Mikołajczyk1,*, Grzegorz Gosiewski1, Jarosław Chyb1, Magdalena Socha1

1Department of Ichthyobiology and Fisheries, University of Agriculture, ul. Prof. T. Spiczakowa 6, 30-199 Krakow-Mydlniki, Poland.

Abstract

The aim of the present study was to demonstrate the in vitro response (LH release) of Prussian carp pituitary cells to mammalian kisspeptin in static incubations. Pituitary glands were collected at two periods of the season (at the time of gonad recrudescence or spawning period) from untreated fish or those with dopaminergic system blocked in vivo by injection of pimozide - a dopamine receptor antagonist. LH measurement (by ELISA method) have shown that there was no significant impact of kisspeptin on the level of this gonadotropin at both investigated seasons of the year if pituitary glands for the study were collected from untreated females. In case of fish pre-treated with pimozide there was a significant stimulation of LH release in both investigated seasons. The results demonstrate the direct effect of kisspeptin on LH release from the Prussian carp gonadotropic cells, dependent on the strength of dopamine inhibition on LH secretion.

Introduction

Presently a great number of neuropeptides and neurotransmitters of brain origin have been shown to affect pituitary gonadotropic activity in fish. Some of them have a proven functional significance, mainly gonadoliberin (GnRH) and dopamine (DA). The use of superactive GnRH analogues to synchronize or to stimulate the last phases of gonad maturation - ovulation and spermiation - is a common practice in aquaculture of many economically important species (Mylonas, Fostier, & Zanuy, 2010). The analogues of GnRH combined with dopamine antagonists (pimozide, domperidone, metoclopramide) significantly improve the effectiveness of hormonal treatment (Yaron, 1995; Brzuska, 2001; Mikołajczyk et al., 2003; Arabaci & Sari, 2004). In some species such hormonal treatment is less effective, because the inhibitory strength of dopamine at the time of final maturation is less pronounced, like in salmonid fish (Vacher, Mañanos, Breton, Marmignon, & Saligaut, 2000; Vacher, Ferrière, Marmignon, Pellegrini, & Saligaut, 2002; Levavi-Sivan, Bogerd, Mañanós, Gómez, & Lareyre, 2010) or does not exist, like in Atlantic croaker Micropogonias undulatus (Copeland & Thomas, 1989) or gilthead sea bream, Sparus aurata (Zohar & Mylonas, 2001).

In some species there are other key hormones involved in gonadotropin release control, like GABA, NPY (Kah et al., 1989b, 1992; Peng, Gallin, Peter, Blomqvist, & Larhammar, 1994) and in such cases the application of hormones other than GnRH agonist together with dopamine antagonist could be more effective. The good candidate which could improve recently applied methods of hormonal control of reproduction are the naturally occurring substances like kisspeptins (kisspeptin1 and kisspeptin2) or its...
analogues, which seem to be the most important regulator of maturation and reproduction of vertebrates. Having quite conservative structure among vertebrates, kisspeptins could serve as this universal hormonal preparation controlling maturation of different fish species.

There are several fish species investigated to date and the knowledge of kisspeptin system includes the details on the anatomy, expression of kisspeptin genes and receptors, localization or distribution: tilapia, Oreochromis niloticus (Parhar, Ogawa, & Sakuma, 2004), grey mullet, Mugil cephalus (Nocillado, Levavi-Sivan, Carrick, & Elizur, 2007), cobia, Rachycentron canadum and Senegalese sole, Solea senegalensis (Mohammed, Benninghoff, Holt, & Khan, 2007; Mechaly, Viñas, & Pferrer, 2009). In medaka (Oryzias latipes) and zebrafish (Danio rerio) a novel gene Kiss2 was identified (Kitahashi, Ogawa, & Parhar, 2009), which can play a role in reproductive as well as nonreproductive processes. Two different kisspeptin genes (Kiss1 and Kiss2) have been characterized in the zebrafish, Danio rerio (Shahjahan, Kitahashi, Ogawa & Parhar, 2012), goldfish, Carassius auratus (Li et al., 2009) sea bass, Dicentrarchus labrax (Felip et al., 2009) and chub mackerel, Scomber japonicus (Selvaraj et al., 2010, 2012).

It was experimentally proven that administration of kisspeptin increases LH secretion in the following fish species: goldfish, Carassius auratus (Li et al., 2009), European sea bass, Dicentrarchus labrax (Felip et al., 2009), striped bass, Morone saxatilis (Zmora et al., 2012) and affects gonad maturity: accelerates gonadal development in basses of genus Morone (Beck, Fulle, Peatman McEntire, Darwish, & Freeman, 2012), accelerates spermatogenesis in prepubertal male chub mackerel, Scomber japonicus (Selvaraj et al., 2013), stimulates gonadal development in pre-pubertal male yellowtail kingfish Seriola lalandi during the breeding and non-breeding season (Nocillado, Zohar, Biran, Levavi-Sivan & Elizur, 2013). Before the trials with kisspeptins in commercial fish farms, the necessary basic research should be conducted in order to find the effective doses, timing, way of administration, delivery systems, time of the season for the specific fish species, the possible effects on the fertilisation process, hatching and the offspring. There are already interesting conclusions from the paper of Selvaraj et al. (2013) who found that subcutaneous application of kisspeptin-1 induces spermatiation in sexually immature chub mackerel and that this treatment is more effective than GnRH analogue application. They suggest to use this peptide for other farmed fish.

Kissepeptin acting directly on GnRH neurons (Zohar, Munoz-Cueto, Elizur, & Kah, 2010) and on gonadotropic cells in the pituitary (Chang, Mar, Wlachuk, & Wong, 2012) may also coordinate the information coming from other brain systems involved in the control of gonadotropin secretion, namely from dopaminergic system. The putative link between kisspeptin and dopamine is not unfounded, as in mammals it was already demonstrated that dopaminergic system is one of the target places for kisspeptin (Clarkson & Herbison, 2011; Goodman et al. 2012; Ozawa, Sawai, Iwata, Takumi & Iijima, 2012). According to our knowledge there is no data showing the direct connections between kisspeptin and dopamine systems in fish brain.

Fish are seasonally reproducing animals which mature and spawn in the most advantageous, for the offspring survival, season of the year. Dopamine inhibiting GnRH secretion at the brain level or inhibiting the action of GnRH at the level of pituitary gland (Yu & Peter, 1992) controls the release of gonadotropins, thus onset of puberty and the final maturity of gonads. At the brain level dopamine may also affect other than GnRH neurons and the kisspeptin neurons are very likely to be one of its target. This hypothesis on the possible interaction of dopamine, kisspeptin and GnRH system cannot be yet verified, but the experiments described in this paper were carried out to demonstrate the in vitro response (LH release) of Prussian carp pituitary cells to mammalian kisspeptin. Pituitary glands for the experiments were collected at two periods of the season (at the time of gonad recrudescence or spawning period) from untreated fish or those with dopaminergic system blocked in vivo by injection of pimozide (dopamine antagonist).

Materials and Methods

Fish

Fish, Prussian carp (Carassius gibelio Bloch, 1782) were purchased from Experimental Fish Farm in Zator of Inland Fisheries Institute in Olsztyn and were kept in the outdoor ponds of the Fisheries Research Station of the Department of Ichthyobiology and Fisheries, University of Agriculture in Krakow, Poland. The experiments were approved by the First Local Ethical Committee on Animal Testing in Krakow (decisions: 90/2013, 91/2013).

In two consecutive years experiments were performed at the time of gonad recrudescence (February) or at the spawning season (June). Every season fish were collected from outdoor ponds and transferred to 300 liters volume flow-through glass basins, in which they were kept for two days for acclimatization. At the time of gonad recrudescence water temperature was kept at 12±1°C and simulated natural photoperiod Light:Dark (L:D) was 8:16 and at spawning season water temperature was kept at 20±1°C and simulated natural photoperiod L:D was 16:8.

Pituitary glands for the in vitro incubation of cells were collected from sexually mature, two-year-old females of Prussian carp (Carassius gibelio, Bloch 1782). Average body weight of fish was 105.88±30.78 g. Gonad maturity was specified as a percentage of body weight (gonadosomatic index - GSI): at the time of gonad...
recrudescence and spawning season 4.30 ± 2.06 % and 15.96 ± 3.47 %, respectively.

For the whole experiment 60 pituitary glands were used (30 in one year and another 30 in the consecutive year, as a repetition). Each year half of the fish were used at the time of gonad recrudescence and another half at the spawning season. In each season glands were obtained from 7 fish injected with saline or from 8 fish treated with pimozide - a dopamine receptor antagonist (Sigma Aldrich Co., USA), at 3 hours before decapitation. Pimozide was injected intraperitoneally at a dose of 5 mg kg⁻¹ body weight.

**Culture Technique**

On the day of the experiment fish were anaesthetised with 2-phenoxo-ethanol (Merck, Germany) at 0.3 mL L⁻¹ of water, killed by decapitation and the pituitary glands were collected and placed in sterile ice-cold medium (MEM-Eagle, Sigma-Aldrich, USA) buffered with 15 mM Heps (Sigma-Aldrich, USA) and 9 mM sodium bicarbonate (P.O.Ch., Poland).

The enzymatic dispersion of the pituitary glands and the technique of cell culture were described in detail elsewhere (Mikolajczyk, Weil, Epler, & Breton, 1990; Sokolowska-Mikolajczyk, Socha, Szczcerbik, & Epler, 2009). In brief, collected glands were chopped into small pieces and subjected to dispersion for 6–8 h at 20°C in the medium containing 0.1% (w/v) collagenase H (Boehringer Mannheim, Germany) and 1% BSA (Sigma-Aldrich, USA). The cells were harvested by 10-min centrifugation (200 m s⁻²) at 20°C and washed twice with pre-incubation medium containing 2% (v/v) serum substitute (Ultroser SF, Sepracor S.A., France) and 1% (v/v) antibiotic-antimycotic (Sigma-Aldrich, USA). Cell viability test (trypan-blue test) and cell counting were performed with a Thoma haemocytometer and it was routinely better than 95%. Cells were resuspended in the pre-incubation medium and transferred into four 96-well microplates (Nunc A/S Denmark) coated with Poly-L-lysine (Sigma-Aldrich, USA). Each well contained approximately 5 × 10⁴ cells in 250 μL of medium. Then the plates were sealed and incubated for 48 h at 22°C. On the third day of culture the pre-incubation medium was replaced with medium containing kisspeptin (Metastin 45–54 amide human, Sigma Aldrich Co., USA) at a concentrations of 10⁻⁸, 10⁻⁹, 10⁻⁷ or 10⁻⁶ M. Control wells were filled up with medium without any supplementation. Each treatment group consisted of five wells containing cells from the pool of pituitaries. For each variant in each experiment n=5. At the end of the incubation period (24 hours) the plates were centrifuged (200 m s⁻²) for 10 min at 20°C and the media were collected and frozen at −20°C until LH determination by ELISA (Kah, Pontet, Nurroz Rodriguez, Calas, & Breton, 1989a). Sensitivity of the performed ELISA was in the range of 0.6–100 ng·mL⁻¹ with the intra- and inter-assay coefficients of variance at 5% and 9%, respectively.

**Statistical Analysis**

LH concentrations were analysed using GraphPad Prism statistical software (version 5 GraphPad software, USA). LH levels in the experimental groups were normalized as a percentage of the control group values (without drug treatment) and then data were analysed using nonparametric two-tailed Mann–Whitney U-test with Bonferroni correction. The differences between the means were considered as significant for P < 0.05. Data presented on the graphs (mean percentage of LH levels ± SEM) come from two independent experiments.

**Results**

A. The effects of different concentrations of kisspeptin on LH levels in the incubation medium of pituitary cells at the time of gonad recrudescence:

a) pituitary cells of untreated fish (Figure 1A)

Changes in LH levels in the media of pituitary cells incubated in the presence of kisspeptin at the concentration range from 10⁻⁸ to 10⁻⁶ M were not significant in relation to the basal LH concentration (control media).

b) pituitary cells of fish treated with pimozide (Figure 1B)

Kisspeptin at the concentrations of 10⁻⁸ and 10⁻⁷ M increased LH levels in the media by 45 and 40 percent, respectively. Other investigated concentrations of kisspeptin (10⁻⁸ and 10⁻⁶ M) did not cause any statistically significant changes.

B. The effects of different concentrations of kisspeptin on LH levels in the incubation medium of pituitary cells at the spawning season:

a) pituitary cells of untreated fish (Figure 2A)

Changes in LH levels observed in the incubation media containing the tested concentrations of kisspeptin were not statistically significant when compared with control incubations.

b) pituitary cells of fish treated with pimozide (Figure 2B)

All concentrations of kisspeptin (from 10⁻⁸ to 10⁻⁶ M) evoked almost the same, statistically significant increase of LH levels in the media (by 45, 42, 36 or 37 percent, respectively).

**Discussion**

Although there is no doubt about the role of kisspeptin in the control of the reproductive axis in vertebrates, there are still some discrepancies, if the site of its action is considered (Richard, Corvaisier, Camacho, & Kottler, 2009). According to Gutierrez-Pascual et al. (2007), Yang, Jiang, Chan, Ko, & Wong (2010) and Chang et al. (2012) kisspeptin acts both at the hypothalamic and pituitary levels. Li et al. (2009) did not observe a direct influence of both kisspeptins (Kiss1 and Kiss2) on LH secretion from goldfish pituitary cells. Yang et al.
The first who demonstrated a direct effects of kisspeptin on pituitary hormone (LH, GH) secretion in goldfish were Chang et al. (2012) and Espigares, Zanuy, and Gomez (2015).

In the present experiments we found that the changes in the levels of LH in the incubation media of pituitary cells (derived from untreated fish), exposed to four different concentrations of human kisspeptin: $10^{-9}$, $10^{-8}$, $10^{-7}$ or $10^{-6}$ M (Figs 1A and 1B) were not statistically significant in comparison to the levels observed in the control incubations (without kisspeptin). Such insignificant changes occurred in both investigated seasons, i.e. at the time of gonad recrudescence (Fig 1A) and the period of natural spawning (Fig. 2A). Based on these results we could conclude that in the Prussian carp human kisspeptin is not acting directly at the level of pituitary cells. Considering the results of in vivo work by Gosiewski, Sokolowska-Mikolajczyk, Chyb, and Socha (2015), who have already demonstrated the effects of human kisspeptin on LH release in Prussian carp, we could reason that the place of kisspeptin action would have to be the brain (central action at the level of hypothalamus). However, looking for the potential interaction of kisspeptin and dopamine in the process of gonadotropin release control we repeated the above mentioned in vitro experiment according the same schedule with one difference: pituitary cells for the static incubation with kisspeptin were obtained from fish pre-treated with pimozide - a dopamine receptor antagonist 3 hours before cell harvesting. The results were different - both, at the time of gonad recrudescence (Fig. 1B) as well as in the period of natural spawning (Fig. 2B), kisspeptin significantly increased LH concentrations if compared with control incubations. In case of recrudescence period only two concentrations of kisspeptin were stimulatory: $10^{-9}$ and $10^{-7}$ M (Fig. 1B), but at the spawning period all of them were equally potent in the stimulation of LH release to the medium (Fig. 2B). These results demonstrate that the removal or the reduction of dopamine inhibitory tone in vivo by pimozide, greatly facilitates the direct action of kisspeptin on pituitary gonadotrophs resulting in increase of LH levels secreted by these cells to the incubation medium. It is possible that temporary

**Figure 1.** Percentage of control LH secretion from Prussian carp female pituitary cells harvested at the time of gonad recrudescence and incubated for 24 h with human kisspeptin at $10^{-9}$, $10^{-8}$, $10^{-7}$ or $10^{-6}$ M. (A) pituitary cells of control fish, (B) pituitary cells of fish pretreated with pimozide - a dopamine receptor antagonist 3 hours before cell harvesting. Bars represent means ± SEM. Different letters of the alphabet identify significant differences between groups ($P < 0.05$; Mann-Whitney U-test with Bonferroni correction). Data presented on the graphs (mean percentage of LH levels ± SEM) come from two independent experiments.
reduced inhibitory tone of dopamine up-regulates the number of kisspeptin receptors on gonadotropic cells in the pituitary. This is rather a speculative theory, because we did not measure the kisspeptin receptor number, however such a phenomenon may take place, as KISS1r mRNA expression have been reported in the pituitary of the rat (Richard, Galmiche, Corvaisier, Caraty, & Kottler, 2008) and human (Ohtaki et al., 2001). Also Yang et al. (2010) presented evidence for KISS1r expression in goldfish somatotrophs, lactotrophs and gonadotrophs and direct action at the pituitary level to induce pituitary hormone secretion and gene expression in goldfish. If kisspeptin receptors are present in fish pituitary, the process of their up- or down-regulation is also possible and may depend on the strength of dopamine action exerted on LH release, changing with the season (external factors) and the state of gonad maturity.

In already mentioned in vivo experiments on Prussian carp (Gosiewski et al. 2015) pimozide alone significantly increased LH release in fish at the time of gonad recrudescence as well as at the spawning period and the combined treatment with pimozide and human kisspeptin caused significantly higher LH release in recrudescent fish than did pimozide alone, demonstrating the potentiating effects of kisspeptin on pimozide action. These results fit well with the in vitro data of the present paper and let us suggest that both, dopamine and kisspeptin, participate in the process of gonadoliberin and gonadotropin release in Prussian carp integrating hormonal and environmental signals. Dopamine seems to dominate kisspeptin, which stronger or weaker stimulates gonadolibin or gonadotropin secretion depending on the dopamine inhibitory tone associated with current environmental conditions (suitable or not for reproduction). Seasonality of kisspeptin system activity in fish is confirmed by Migaud, Ismail, Cowan, & Davie, (2012) and Alvarado, Carrillo, & Felip, (2013) who presented sex-dependent differences in the dynamic of kisspeptin and kisspeptin receptor gene expression in the hypothalamus of European sea bass during gonad maturation cycle.

Kisspeptin influence on GnRH/LH release in fish (measured by blood LH levels) is present throughout the

Figure 2. Percentage of control LH secretion from Prussian carp female pituitary cells harvested at the spawning season and incubated for 24 h with human kisspeptin at 10−9, 10−8, 10−7 or 10−6 M. (A) pituitary cells of control fish, (B) pituitary cells of fish pretreated with pimozide - a dopamine receptor antagonist 3 hours before cell harvesting. Bars represent means ± SEM. Different letters of the alphabet identify significant differences between groups (P < 0.05; Mann-Whitney U-test with Bonferroni correction). Data presented on the graphs (mean percentage of LH levels ± SEM) come from two independent experiments.
sexual cycle, but it is the more pronounced when the inhibitory dopamine effect gets weaker. This conclusion is important for understanding the whole complexity of stimulating and inhibiting factors influencing LH release in fish. This is especially visible in the light of the results showing that in fish, besides dopamine, acting as a gonadotropin release inhibiting factor the presence of another hormone - the gonadotropin inhibitory hormone (GnIH), negatively regulating LH release in teleost is confirmed (Sawada et al., 2002; Zhang et al., 2010; Ogawa & Parhar, 2014). Only consideration of the multifactorial regulation of sexual maturation and reproduction in fish (stimulatory and inhibitory), may provide an important tool for controlling these processes in aquaculture.

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