How does oestradiol influence the AVT/IT system in female round gobies during different reproductive phases?

Hanna Kalamarz-Kubiak*, Magdalena Gozdowska, Tatiana Guellard and Ewa Kulczykowska

ABSTRACT

In this in vitro gradient perfusion study, we determined whether there is a functional relationship between oestradiol and the arginine vasotocin/isotocin (AVT/IT) system in the female round goby (Neogobius melanostomus). Brain explants were perfused in medium supplemented with 17β-oestradiol (E2) at doses mimicking the plasma levels of this hormone in nature during the spawning-capable phase and regressing phase. We aimed to establish which pathway, genomic or non-genomic, is involved in this mechanism in different reproductive phases. For this purpose, brain explants were perfused in medium supplemented with Fulvestrant (ICI 182,780) or Actinomycin D (Act D) separately or in combination with E2. The contents of AVT and IT in the perfusion media were determined using high-performance liquid chromatography (HPLC) with fluorescence and UV detection. During the spawning-capable phase, the effect of E2 on AVT release is mediated through oestrogen receptors (ERs) via both genomic and non-genomic pathways, while IT release is mediated through ERs via a genomic pathway only. In the regressing phase, release of both nonapeptides is mediated through ERs via a genomic pathway. This is the first study to present a feasible mechanism of oestradiol action on the AVT/IT system in female fish during different phases of the reproductive cycle.

KEY WORDS: Oestradiol, AVT, IT, In vitro gradient perfusion, Genomic and non-genomic pathways, Female round goby

INTRODUCTION

Oestradiol, which is synthesized in gonads and in the brain, is crucial to the neuroendocrine control of reproduction and sexual behaviour in vertebrates (Knobil and Neill, 1994; Compagnone and Mellon, 2000). The functions of oestrogens include the regulation of oocyte growth within the gonads, secondary sex characteristics and behaviour (Nagahama et al., 1995). Oestrogens act at the level of the brain and pituitary, and influences the secretion of gonadotropin-releasing hormone and other neuropeptides that control the ventral telencephalon, diencephalon and various mesencephalic structures, and to the neurohypophysis (Holmqvist and Ekström, 1995; Saito et al., 2004). In Teleostei, AVT has been reported to influence sexual behaviour, including courtship (Salek et al., 2002; Grober and Bass, 2002), aggression (Semsar et al., 2001; Greenwood et al., 2008; Kleszczyńska et al., 2012), vocalization (Goodson and Bass, 2000a) and spawning reflex (Knapp et al., 1999). Less is known about the role of IT in the regulation of sexual behaviour in fish. The investigations on this subject are limited to the species Tautogolabrus adspersus (Porichthys notatus) (Goodson and Bass, 2000b), Lythrypnus dalli (Black et al., 2004) and three-spined stickleback (Gasterosteus aculeatus) (Kleszczyńska et al., 2012; Kulczykowska and Kleszczyńska, 2014). It has been established that IT is involved in controlling aggression, courtship, final oocyte maturation and/or egg deposition in the three-spined stickleback (Kleszczyńska et al., 2012; Kulczykowska and Kleszczyńska, 2014), and vocal-motor responses in plainmidshipman fish (Goodson and Bass, 2000b). Because AVT and IT immunoreactivity (IR) was found within multiple components of the ascending auditory pathway, the modulatory role of both neuropeptides was suggested in fish vocalization (Goodson et al., 2003). However, in female plainmidshipman fish, only local administration of IT inhibited vocal-motor responses elicited by anterior hypothalamic stimulation, and this effect is reversed by an OT antagonist. In tetrapods and fish, there are indications of possible interactions between oestrogens and AVP/AVT-ergic or OT/IT-ergic systems.

However, studies have focused on the location of these hormones or their receptors in the brain (Axelson and Leeuwen, 1990; Shughrue et al., 1996, 1997; Simonian and Herbison, 1997; Harbovszky et al., 1996, 1997; Simonian et al., 2000). As was mentioned earlier, the teleost brain is well known as the site of de novo synthesis of oestrogens, catalysed by a brain-specific aromatase enzyme. However, to the authors’ knowledge, only Lorenzi et al. (2012) has presented 17β-oestradiol (E2) concentration in the brain of Lythrypnus dalli. In female L. dalli, the gonadal E2 concentration was ~10 times higher than that in the brain. In the brain, there are three oestrogen receptor (ER) subtypes (ER-α, -β1, -β2) recognized in fish. ER-α protein and ER-α mRNA have been localized in the forebrain of rainbow trout (Oncorhynchus mykiss) (Kah et al., 1997; Menuet et al., 2003). In Atlantic croaker (Micropogonias undulatus), ER-α, -β1, -β2 mRNA expression has been found in the suprachiasmatic nucleus of the POA (Hawkins et al., 2000). In addition, the three subtypes of ERs are distributed in the anterior POA, the ventral hypothalamus and the posterior tuberculum of the zebrafish (Danio rerio) (Menuet et al., 2002). More information is currently available about the distribution of ERs in the plainmidshipman fish (Porichthys notatus) brain. The anatomical
localisation of ER-α was noticed in the telencephalon, POA, diencephalon, anterior hypothalamus and hindbrain; ER-β in the telencephalon, POA and hindbrain; ER-β2 in the telencephalon, POA, anterior hypothalamus, midbrain and hindbrain (Forlano et al., 2005; Fergus and Bass, 2013). In fish, ERs are located in brain regions of AVT and IT synthesis, similar to tetrapods, where ERs are located in AVP- and OT-synthesizing cells (Anglade et al., 1994; Holmqvist and Ekström, 1995; Hawkins et al., 2005). Moreover, ERs are distributed in the hypothalamic and extrahypothalamic areas related to the control of reproduction (e.g. mediobasal hypothalamus, ventral telencephalon), where both nonapeptides give projections (Anglade et al., 1994; Goodson and Bass, 2001; Goodson et al., 2003; Forlano et al., 2005; Thompson and Walton, 2009; Fergus and Bass, 2013).

According to current knowledge, oestradiol controls gene expression via ERs by activation of both genomic (nuclear) and non-genomic (extranuclear) pathways. Genomic pathways encompass the classical interactions between ligand-bound ER dimers and oestrogen-responsive elements in target gene promoters (Gronemeyer et al., 2004) and the non-classical pathway, where a ligand activates ER/specificity protein and ER/activating protein-1 complexes (Safe and Kim, 2008). The non-genomic effect is mediated through the membrane ER (mER) ‘family’, consisting of classical ER-α and ER-β or splice variants, ER-X, two G-protein-coupled oestrogen receptors, GPER (also known as GP30) and Gq-mER (Toran-Allerand et al., 2002; Qiu et al., 2003; Micevych and Kelly, 2012). Oestrogens may also exert non-genomic action without receptor involvement through a physicochemical interaction with plasma membrane at only micromolar concentrations (Falkenstein et al., 2000; Simoncini and Genazzani, 2003).

In fish, there is little information on the effects of oestradiol on the synthesis and release of AVT and IT. Available information is related only to seasonal changes in gene expression and immunoreactivity of the nonapeptides, which are probably linked with changes in steroid hormones. In the female grass puffer (Takifugu niphobles), brain AVT mRNA expression is augmented during the spawning period (Motohashi et al., 2008). Ota et al. (1999) have demonstrated that increases in AVT mRNA level and AVT-IR in the POA are accompanied by elevation of plasma oestriadiol levels in immature female masu salmon (Oncorhynchus masou) in November. In female medaka (Oryzias latipes) and halfspotted goby (Asterropteryx semipunctata), AVT-IR and IT-IR signals in the preoptic–hypothalamic regions in the pre-spawning and spawning phases were stronger than those in the post-spawning phase (Ohya and Hayashi, 2006; Maruska et al., 2007). Based on our studies with sticklebacks (Gozdowska et al., 2006; Kleszczyńska et al., 2012; Kulczykowska and Kleszczyńska, 2014) and round goby (Sokołowska et al., 2015), we presume that there is a functional link between oestriadiol and the AVT/IT system in fish during different phases of the reproductive cycle. In a socially controlled situation such as the masculinization process of females, a link between sex steroids and brain AVT and IT was shown in black molly (Poecilia sphenops) (Kulczykowska and Kleszczyńska, 2015). So far, to the authors’ knowledge, there is no evidence that a functional relationship between oestriadiol and the AVT/IT system exists in fish. In this study, we determine whether there is a functional relationship between circulating oestriadiol and AVT and IT in the female round goby (Neogobius melanostomus). We try to establish which pathway, genomic or non-genomic, is involved in this mechanism in different reproductive phases. The in vitro gradient perfusion technique is the method of choice (Kalamarz-Kubiak et al., 2011) because it monitors the dynamic hormone secretion and registers even small and shortened fluctuations in hormone secretion before and after treatment. The brain explants are perfused in medium supplemented with E2 at doses mimicking the plasma levels of this hormone in nature during different reproductive phases. In the perfusion of brain explants, we use E2 separately or in combination with Fulvestrant (ICI 182.780) or Actinomycin D (Act D). Fulvestrant is an ER antagonist (Robertson, 2001), which affects the reproduction processes in fish females (vitellogenesis, oocyte maturation) and males (steroidogenesis in testes) (Bouma et al., 2003; Pang and Thomas, 2009; Nagler et al., 2010). Actinomycin D is an effective inhibitor of ER processing because it directly blocks ER access to a specific region of DNA (Horwitz and McGuire, 1978).

RESULTS

Base levels of AVT and IT release into the perfusion media (control) and 17β-oestradiol in plasma during the spawning-capable phase and the regressing phase

Levels of AVT and IT release were significantly higher (P<0.001) during the spawning-capable phase than in the regressing phase (Table 1). Plasma E2 was significantly higher (P<0.001) during oocyte maturation in the spawning-capable phase than during the regressing phase (Table 1).

The influence of 17β-oestradiol on AVT and IT during the spawning-capable phase

During the spawning-capable phase, E2 (3.67×10⁻⁸ M) significantly increased AVT release (P<0.01; P<0.001) compared with the control (Fig. 1A). The AVT response to E2 occurred within the first 20 min and persisted for the next 100 min of perfusion. IT release was also stimulated by E2 (P<0.05; P<0.01), but the response appeared after 40 min of perfusion and persisted for the next 60 min (Fig. 1B).

The influence of Fulvestrant on AVT and IT during the spawning-capable phase

A higher dose of Fulvestrant (1×10⁻⁶ M) caused a 60% inhibition of AVT and a 50% inhibition of IT release, and therefore the dose was not used in further in vitro perfusion experiments (data not shown). A lower dose of Fulvestrant (1×10⁻⁷ M) did not influence AVT and IT release into perfusion media (Fig. 1A,B); this dose was used further. Fulvestrant (1×10⁻⁷ M) significantly inhibited the fast response of AVT (P<0.01; P<0.001) (Fig. 1A) and the slower response of IT (P<0.05; P<0.01) (Fig. 1B) to E2 stimulation (3.67×10⁻⁸ M) during the spawning-capable phase.

The influence of Actinomycin D on AVT and IT during the spawning-capable phase

A higher dose of Actinomycin D (1×10⁻⁶ M) caused a 45% inhibition of AVT release and a 35% inhibition of IT release, and therefore the dose was not used in further in vitro experiments.

| Table 1. Base levels of AVT and IT released into the perfusion media and E2 concentration in the plasma of female round gobies during the spawning-capable phase and regressing phase |
|-------------------------------|-----------------|-----------------|-----------------|
| Spawning-capable phase      | Regressing phase |
| AVT (pmol ml⁻¹ of perfusion medium) | 23.82±2.2*** | 6.61±1.54 |
| IT (pmol ml⁻¹ of perfusion medium) | 24.22±1.9*** | 13.15±1.56 |
| E2 (ng ml⁻¹ of plasma) | 10±1.2*** | 5±1.6 |

Values are mean±s.e.m.; n=10 for each group. Asterisks indicate significant differences between groups; ***P<0.001 (Student’s unpaired t-test).
A lower dose of Actinomycin D (1×10^{-7} M) did not affect AVT and IT release into perfusion media (Fig. 1A,B), and this dose was used subsequently. Actinomycin D (1×10^{-7} M) significantly inhibited the response of AVT (P<0.01; P<0.001) (Fig. 1A) to E2 (3.67×10^{-8} M) during the spawning-capable phase. The response of IT, induced by E2, was also significantly inhibited (P<0.05; P<0.01) (Fig. 1B) by Actinomycin D during this phase.

The influence of Fulvestrant on AVT and IT in the regressing phase
A higher dose of Fulvestrant (1×10^{-6} M) caused results comparable to those obtained during the spawning-capable phase so this dose was not used in further in vitro experiments (data not shown). A lower dose of Fulvestrant (1×10^{-7} M) did not influence AVT and IT release into perfusion media (Fig. 2A,B) and this dose was used subsequently. Fulvestrant (1×10^{-7} M) significantly inhibited the response of both AVT (P<0.01) (Fig. 2A) and IT (P<0.05; P<0.01) (Fig. 2B) to E2 stimulation (1.8×10^{-8} M) during the regressing phase.

The influence of Actinomycin D on AVT and IT during the regressing phase
A higher dose of Actinomycin D (1×10^{-6} M) caused results comparable to those obtained during the spawning phase and so this dose was not used in further in vitro experiments (data not shown). A lower dose of Actinomycin D (1×10^{-7} M) did not affect the AVT and IT release (Fig. 2A,B) into perfusion media and this dose was used in subsequent experiments. Actinomycin D (1×10^{-7} M) significantly inhibited the response of both AVT (P<0.01) (Fig. 2A) and IT (P<0.05; P<0.01) (Fig. 2B) induced by E2 (1.8×10^{-8} M) during the regressing phase.

Histological analysis of ovaries
Ovaries of female round gobies collected during the spawning-capable phase contained follicles at the end of vitellogenic oocyte growth and during oocyte maturation after germinal vesicle migration (GVM) but before germinal vesicle breakdown.
Fig. 3A). In contrast, ovaries collected during the regressing phase were regressed and reproductively inactive, containing primary growth oocytes (PG), cortical alveoli (CA), vitellogenic oocytes (Vtg1, Vtg2), atretic oocytes (A) and postovulatory follicle complexes (POFs) (Fig. 3B).

**DISCUSSION**

This study presents, for the first time, a presumable mechanism of 17β-oestradiol action on the AVT/IT system in female round goby during the spawning-capable phase and the regressing phase. Our results suggest that changes in oestradiol level related to the phases of the reproductive cycle can affect AVT and IT levels by their ERs. Available information is related only to seasonal changes in gene expression and immunoreactivity of the nonapeptides, which are probably linked with changes in steroid hormones in fish (Ota et al., 1999; Ohya and Hayashi, 2006; Maruska et al., 2007). Based on our studies in stickleback (Gozdowska et al., 2006; Kleszczyńska et al., 2012; Kulczykowska and Kleszczyńska, 2014) and round goby (Sokołowska et al., 2015), we presumed that there is probably a functional link between oestradiol and the AVT/IT system during different phases of the reproductive cycle.

In our experiments, E2 was applied at doses mimicking the plasma levels of this hormone in nature during the spawning-capable phase and regressing phase. The doses of E2 were established based on their concentrations measured in plasma samples collected during the spawning-capable phase (10±1.2 ng ml\(^{-1}\)) and the regressing phase (5±1.6 ng ml\(^{-1}\)). Our findings show that a high level of oestradiol appears at the end of vitellogenic oocyte growth and during oocyte maturation, within the spawning-capable phase in female round goby. In asynchronous spawners, both in vivo and in vitro studies have shown that the increase in E2 production by fully grown ovarian follicles occurs during oocyte maturation (Sakai et al., 1987; Trant and Thomas, 1989). In the study presented here, E2 shows a stimulatory effect on AVT during the spawning-capable phase. The secretion of AVT induced by E2 increased by ~140%. It has been noticed that AVT-IR in the preoptic–hypothalamic regions during the peak of the spawning phase was stronger than during the post-spawning phase in female halfspotted goby (*Asterropteryx semipunctata*) and catfish (*Heteropneustes fossilis*) (Maruska et al., 2007; Singh and Joy, 2008). Gozdowska et al. (2006) have demonstrated that the high AVT concentration in female brain in the spawning period was coincident with active reproduction and probably also with high...
levels of sex hormones. Subsequently, it was indicated that the high
AVT level observed before egg laying in female three-spined
sticklebacks probably induces the final oocyte maturation
(Kulczykowska and Kleszczynska, 2014). In female catfish, AVT
can induce not only the final oocyte maturation but also ovulation
through the influence on the production of the maturation-inducing
steroid (17α, 20β-dihydroxy-4-pregnen-3-one) (Singh and Joy,
2011). Sokolowska et al. (2015) demonstrated that the high level of
brain AVT corresponded with the late, advanced vitellogenesis
during the spawning-capable phase in female round goby. Our
results showed the fast oestradiol effect (within 20 min) on AVT
secretion. The ER antagonists Fulvestrant and Actinomycin D
inhibited oestradiol action on AVT secretion during the spawning-
capable phase. It is known that in fish, Fulvestrant blocks both
genomic and rapid, non-genomic effects of oestradiol via classical
nuclear and membrane ERs, respectively (Celius et al., 1999;
Loomis and Thomas, 2000; Liu et al., 2011). Therefore, the results
presented here indicate that oestradiol most probably stimulates the
release of AVT through classical ERs via both genomic and non-
genomic pathways during the spawning-capable phase. Moreover, it
has been postulated that in fish, as in tetrapods, cross-talk between
the non-genomic and genomic pathways of oestradiol signalling
may be involved in the control and synchronized maturation and
ovulation at the hypothalamic and ovary level (Vasudevan and
Pfaff, 2008; Roepke et al., 2009; Cornil and Charlier, 2010;
Thomas, 2017). Briefly, rapid effects of E2 mediated through mER
resulting in the activation of protein kinases can lead to
phosphorylation of cAMP-responsive binding protein, which can
alter gene transcription through its interaction with the cAMP-
responsive element (Cornil et al., 2006, 2012).

During the spawning-capable phase, 17β-oestradiol exerted a
stimulatory effect on IT release from brain explants of female round
goby. Seasonal fluctuations of IT, which show a higher level during
the breeding period, was demonstrated in female three-spined
stickleback (Gozdowska et al., 2006). In this species, IT is probably
engaged in controlling the final oocyte maturation and/or egg
deposition in response to male courtship (Kulczykowska and
Kleszczynska, 2014). However, in female round goby, IT may be
related to ovulation rather than late advanced vitellogenesis and
oocyte maturation (Sokolowska et al., 2015). Our results showed
that, in contrast to AVT, IT secretion stimulated by oestradiol
appeared later, after 40 min, and showed a ∼50% increase. In female
round goby, oestradiol probably influenced IT secretion by classical
nuclear ERs via the genomic pathway only, because Fulvestrant and
Actinomycin D inhibited oestradiol action. It is of note that
oestrogen may stimulate OT mRNA expression in neuroblastoma
cells via the direct genomic pathway (Richard and Zingg, 1990).

The post-spawning gonadal regression phase has received less
attention than other phases of the reproductive cycle. The results
presented here indicate that the plasma level of E2 significantly
decreases in female round goby during this phase. The regression
phase is a reproductively inactive period, mainly characterized by
decreases in female round goby during this phase. The regressing
phase is a reproducitively inactive period, mainly characterized by
the decline of plasma E2 levels, a relatively stable and low amount of
ERs, and the low expression of ER transcripts in brain and gonads
of batch spawners (Burke et al., 1984; Methven et al., 1992; Rinchard
et al., 1993; Chen et al., 2011; Nagasawa et al., 2014).

During the regressing phase, E2 exerted a stimulatory effect on
AVT in female round goby. Nonetheless, the AVT revealed only a
∼70% increase in response to E2. In female medaka, halfspotted
goby and catfish, AVT-IR in the preoptic–hypothalamic regions was
weaker during the post-spawning phase than in the pre-spawning
and spawning phases (Ohya and Hayashi, 2006; Maruska et al.,
2007; Singh and Joy, 2008). In the non-spawn period, the
concentration of AVT in the brain of female three-spined
sticklebacks was significantly lower than that during spawning,
which is associated with low levels of sex hormones (Gozdowska
et al., 2006). Similarly, the low level of brain AVT coincided with
the quiescent phase of gametogenesis during the regressing phase in
female round gobies (Sokolowska et al., 2015). In rodents, it was
demonstrated that during the non-breeding season, the decline in
gonadal hormone level is accompanied by decreased AVP
expression in several brain regions (Buijs et al., 1986; Hermes
et al., 1990; Bittman et al., 1996). In this study, oestrogen-stimulated
AVT secretion occurred later, after 40 min of perfusion. Fulvestrant,
as well as Actinomycin D, inhibited AVT release affected by E2.
The results presented here suggest that in the regressing phase, the
effect of oestradiol on AVT release is mediated through the classical
nuclear ERs via a genomic pathway only.

17β-Oestradiol also had a stimulatory effect on IT in female
round goby in the regressing phase. As in the case of AVT, IT
displayed a moderate, ∼50%, increase in response to E2. It should be
noted that IT-IR in the preoptic–hypothalamic regions was weaker in the post-spawning phase than in the pre-spawning phase in female medaka and halfspotted goby (Ohya and Hayashi, 2006; Maruska et al., 2007). In female round goby, the regressing phase was accompanied by a low level of brain IT (Sokolowska et al., 2015). Furthermore, brain IT level declined after egg laying and remained low during winter in female three-spined sticklebacks (Gozdowska et al., 2006; Kulczyzkowska and Kleszczyńska, 2014). Similar to AVT, IT secretion stimulated by oestradiol appeared after 40 min of perfusion and was inhibited by Fulvestrant and Actinomycin D. Our results suggest that IT release is mediated through classical nuclear ERs via a genomic pathway during this phase of the reproductive cycle.

Conclusions
Our in vitro study presents, for the first time, feasible mechanisms for 17β-oestradiol action on the AVT/IT system in female fish during different phases of the reproductive cycle. During the spawning-capable phase, the effect of E2 on AVT release is mediated through classical nuclear and membrane ERs via both genomic and non-genomic pathways, while IT release is mediated through classical nuclear ERs via a genomic pathway only. In the regressing phase, release of both nonapeptides is mediated through classical nuclear ERs via a genomic pathway. The presented mechanism of oestradiol action on the AVT/IT system does not exclude the possibility of action via non-classical mERs, such as ER-X, GPER and Gq-mER.

MATERIALS AND METHODS

Experimental fish
Adult female round gobies (Neogobius melanostomus Pallas, 1814) (n=80) were caught in the Gulf of Gdańsk (Gdynia, Poland) during the spawning-capable phase (April–August) and the regressing phase (September–November). Fish were kept in tanks at the Institute of Oceanology PAS (Sopot, Poland) for 1 week before experimentation. The tanks’ water salinity was 8 ppt. Fish were maintained under a lighting regime and temperature according to conditions prevailing in the natural environment. Fish were fed frozen mussels ad libitum. Studies were performed on round goby due to their availability and proper size for in vitro studies and plasma sample collections and our experience with this species (Kalamarz-Kubiak et al., 2011, 2015; Sokolowska et al., 2015). Before conducting experiments, 20 randomly selected fish were anaesthetised in MS 222 (tricaine methanesulfonate) water solution (50 mg l−1) (Sigma-Aldrich) and blood samples were collected by cardiac puncture. After 40 min of perfusion and was inhibited by Fulvestrant and Actinomycin D. Our results suggest that IT release is mediated through classical nuclear and membrane ERs via both genomic and non-genomic pathways, while IT release is mediated through classical nuclear ERs via a genomic pathway only. In the regressing phase, release of both nonapeptides is mediated through classical nuclear ERs via a genomic pathway. The presented mechanism of oestradiol action on the AVT/IT system does not exclude the possibility of action via non-classical mERs, such as ER-X, GPER and Gq-mER.

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The gonadosomatic index (GSI) was calculated as (gonad weight/body weight)×100. The mean GSI (%) was 11.14±0.97 for the spawning-capable phase and 1.34±0.19 for the regressing phase. In multiple-spawning fish, GSI seems to be a less reliable indicator of fish maturity than the gonadal histology (Guerriero, 2007; Zeyl et al., 2014). Round goby is a batch spawner; therefore, a histological analysis provides a precise assessment of ovarian maturity and distinguishes between fish in an intermediate spawning stage and those in a post-spawning stage.

Histology
Ovaries fixed in 4% buffered formalin were dehydrated and embedded in paraffin using standard histological techniques. Embedded tissues were cross-sectioned into 6 μm slices using a Leica RM2245 microtome (Leica Microsystems, Wetzlar, Germany) and stained with Haematoxylin (MHS16; Sigma-Aldrich) and Eosin (861006; Sigma-Aldrich). Slides prepared from each gonad were examined with a Leica H1210 light microscope (Leica Microsystems). The developmental stage of ovaries was determined according to the terminology developed by Brown-Peterson et al. (2011). This standardized terminology is applicable to all fish regardless of reproductive strategy or gender, including batch-spawning species with asynchronous oocyte development, such as round goby. Batch spawners can exhibit various levels of asynchronous oocyte development and spawn multiple batches of oocytes during the reproductive season (Tomczak and Sapota, 2006; Farrell et al., 2012). The histological analysis was crucial for estimation of the stage of the reproductive cycle and allowed assignment of individuals to groups.

Perfusion system
All in vitro experiments were performed using the perfusion system from MINUCells and MINUTISSUE Vertriebs (Bad Abbach, Germany) according to the method developed by Kalamarz-Kubiak et al. (2011). The set used in the experiments consisted of storage medium bottles, a peristaltic pump (ISIMATEC, Wertheim, Germany), two gas exchange modules, a gradient perfusion container and plastic vials for the sampling medium after perfusion. The unique structure of the gradient perfusion container facilitates the simultaneous supply of the medium from the top and bottom. In our experiments, two brain explants were perfused simultaneously in a single gradient perfusion container. Brain explants were perfused with Ringer buffer (pH 7.4) prepared according to the composition previously described by Kalamarz-Kubiak et al. (2015). Explants were put on the 20 μm Nylon Net Filter (NY2000500; Merck Millipore, Dormstadt, Germany) placed between the base and tension rings of the tissue carrier (MINUSHEET; diameter of 13 mm) in a gradient culture container. The flowing medium was aerated inside the gas exchange modules by the gas mixture (95% O2 and 5% CO2) at a pressure of 315.03 mm Hg. Next, a peristaltic pump transported the aerated medium (0.1 ml min−1) to the gradient perfusion container and then to the sampling vials. Storage medium bottles, a gradient perfusion container and sampling vials were placed on ice and the media collected after perfusions were stored at −70°C, prior to AVT and IT assay. The perfusions were carried out in a laminar air flow (NUAIRE Biological Safety Cabinet Class II, Plymouth, MN, USA). All chemicals were obtained from Sigma-Aldrich.

Experimental design
During 240 min of perfusion, 12 fractions of 2 ml each were collected every 20 min. The first 40 min of perfusion was carried out to stabilize incubation conditions; the next 20 min was to establish the base release of AVT and IT (control). A further 180 min was carried out in a medium without any treatments or medium supplemented with different treatments [17β-oestradiol (3301; Merck Millipore), Fulvestrant (H409; Sigma-Aldrich) and Actinomycin D (1071001; SERVA Electrophoresis, Heidelberg, Germany)]. The brain explants were perfused in medium supplemented with E2 at doses mimicking the plasma levels of this hormone in nature. The 17β-oestradiol doses were established based on their concentrations measured in plasma samples collected during the spawning-capable phase (10±1.2 ng ml−1) and the regressing phase (5±1.6 ng ml−1). The dose of inhibitors was selected based on available data for Fulvestrant (Bowma et al., 2003; Pang and Thomas, 2009; Notch and Mayer, 2011) or our previous experiments for Actinomycin D (Kalamarz-Kubiak et al., 2015). Moreover, before experiments, different doses of inhibitors (1×10−6 M; 1×10−7 M) were tested (data not shown). Finally, E2 at 3.67×10−8 M and 1.8×10−8 M,
separately or in combination with Fulvestrant ($1 \times 10^{-7}$ M) or Actinomycin D ($1 \times 10^{-7}$ M), were used in the experiments. The reactivity of explants was checked according to the method described previously by Kalamzar-Kubiak et al. (2011). The high K\(^+\) concentration (56 mM) treatment caused a 480% increase in AVT release and a 350% increase in IT release (data not shown). Our results are consistent with available data (Juszczak, 2002; Orlowska-Majdak et al., 2003).

17β-Oestradiol assay

17β-Oestradiol was determined in plasma using a Spectria Estradiol radioimmunoassay (RIA) kit (86833; Orion Diagnostica, Finland) according to the method described previously by Kuczykowska et al. (2015). Concentrations of E\(_2\) were measured directly from 100 µl plasma without extraction. Iodinated E\(_2\) with 125I was used as a tracer. A standard curve was prepared using six standard dilutions of 50, 150, 500, 1500, 5000 and 15,000 pmol l\(^{-1}\). The assay was performed according to the kit manufacturer’s instructions with slight modifications. The samples were added to RIA tubes that had been precoated with polyclonal anti-rabbit antisem. After vortexing for 10 s, the tubes were incubated for 2 h at 37°C, decanted, washed with 1 ml TWEEN 20 solution and decanted again. The radioactivity in each tube was measured for 1 min using a Wallac 1470 gamma counter (PerkinElmer Life Science, Shelton, CT, USA). The detection limit of the assay was 37 pmol l\(^{-1}\). The intra-assay coefficient of variation was 6.5%. The inter-assay variation was not determined because all samples were measured in the same assay. The mean E\(_2\) concentrations in the plasma of round gobies during the spawning-capable phase and the regressing phase were 10±1.2 ng ml\(^{-1}\) and 5±1.6 ng ml\(^{-1}\), respectively.

AVT and IT analysis

Concentrations of AVT and IT in the media collected after perfusion (2 ml) were determined using HPLC with fluorescence and UV detection, preceded by solid-phase extraction (SPE), according to the modified procedure by Gozdowska et al. (2013). The media after perfusion were acidified with 1 M HCl (258148-M; Sigma-Aldrich) to pH 3 and loaded on SPE columns. SPE extraction was carried out on Strata-X (30 mg ml\(^{-1}\)) columns (8BS100-TAK; Phenomenex, Torrance, CA, USA). The extraction procedure for perfusion media was as follows: samples loaded on conditioned columns [1 ml of 100% methanol (8405; JT Baker Chemicals, Deventer, The Netherlands) then 1 ml H\(_2\)O], then 600 µl H\(_2\)O and 600 µl of 0.1% trifluoroacetic acid (TFA; 302031-M; Sigma-Aldrich) in 5% acetonitrile were passed through the columns to wash away impurities. Hormones were eluted with 2×600 µl of 80% acetonitrile (8149.2500; JT Baker Chemicals). Eluates were evaporated to dryness using TurboVap LVVTM (Caliper Life Sciences, PerkinElmer, Waltham, MA, USA). Afterwards, derivatization of peptides was performed using 4-fluoro-7-nitro2,1,3-benzoxadiazole (NBD-F; 47140; Sigma-Aldrich). Dried samples were reconstituted with 40 µl of 0.1% TFA in H\(_2\)O. For the derivatization reaction, 20 µl of the sample, 20 µl of 0.2 M phosphate buffer (pH 9) and 20 µl acetonitrile were mixed and later 3 µl NBD-F (30 mg ml\(^{-1}\) acetonitrile) was added. The mixture was heated at 60°C for 3 min, cooled on ice, acidified with 4 µl of 1 M HCl and passed through a HPLC column. Quantitative analyses were performed on the 1200 series HPLC (Agilent Technology, Santa Clara, CA, USA) with a fluorescence detector and a diode array detector. The chromatographic separation of peptides was carried out on ZORBAX Eclipse XDB-C18 (4.6 mm×150 mm, 5 µm) column. The following optimized chromatographic conditions were used: mobile phase A [0.1% TFA in H\(_2\)O], B [0.1% TFA in acetonitrile; H\(_2\)O (3:1)]; linear gradient system: 45–70% phase B in 12 min. The column temperature was 20°C and the flow rate was 1 ml min\(^{-1}\). Fluorescence detection was performed at 470 nm with emission at 530 nm, UV detection at 215 and 340 nm. Recovery of peptides was in the range 79–85% for AVT and IT. The limit of detection was determined to be 0.25 pmol ml\(^{-1}\) for AVT and 1.0 pmol ml\(^{-1}\) for IT. Intra-day repeatability expressed as relative standard deviation was 6.9–7.9% and 5.3–8.2% for AVT and IT, respectively; inter-day precision was in the range 8.2–9.9 and 5.5–8.5% for AVT and IT, respectively.

Statistical analysis

Statistical analysis was performed using STATISTICA 7.1 software (StatSoft, http://www.statsoft.pl). Nonapeptide values in media are presented as % of control. A one-way ANOVA followed by Duncan’s multiple range test were used to compare the different treatments within each time point of incubation and the same treatment across the time of perfusion. A two-way ANOVA followed by the Newman–Keuls post hoc test were used to compare two different treatments across the time of perfusion. Student’s unpaired t-test was used to detect differences in plasma E\(_2\) concentration and a base level of nonapeptides during the spawning-capable phase and the regressing phase (Table 1). P<0.05 was considered significant.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: H.K.-K.; Methodology: H.K.-K., M.G., T.G.; Validation: H.K.-K.; Formal analysis: H.K.-K., M.G., T.G.; Investigation: H.K.-K.; Data curation: H.K.-K.; Writing - original draft: H.K.-K.; Writing - review & editing: H.K.-K., M.G., E.K.; Visualization: H.K.-K.; Supervision: H.K.-K.; Project administration: H.K.-K.; Funding acquisition: H.K.-K.

Funding

This study was supported by the National Science Centre (Narodowe Centrum Nauki) [2012/05/B/NS9/01024 to H.K.-K.].

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