

Brain arginine vasotocin and isotocin in breeding female three-spined sticklebacks (*Gasterosteus aculeatus*): The presence of male and egg deposition



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ABSTRACT

Arginine vasotocin (AVT) and isotocin (IT) are fish hypothalamic nonapeptides involved in numerous social and reproductive behaviors. Vasotocinergic and isotocinergic fibers project to different brain areas where peptides act as neurotransmitters and/or neuromodulators. In this study, we measured whole brain levels of bioactive AVT and IT in breeding females of three-spined stickleback (*Gasterosteus aculeatus*) when they were kept with: (i) courting nest-owners, (ii) courting males that did not build the nest, (iii) non-courting males, and (iv) alone. Only some of the females kept with courting nest-owners deposited eggs. The highest and similar brain AVT levels were in those of females that did not deposit eggs, regardless of whether they were kept with non-courting or courting male, having the nest or not. The highest IT levels were in females that did not deposit eggs but only in those kept with courting male. We suggest that production of AVT in females' brain is stimulated by the presence of male in close proximity, irrespective of whether or not it displays courting behavior, but that of IT is stimulated by male courtship proxies. Moreover, presence of courting or non-courting male that stimulate IT or/and AVT producing neurones may be decisive for final oocyte maturation or egg deposition, because brain levels of both nonapeptides decrease after egg deposition. Similar AVT levels in brains of aggressive and non-aggressive individuals and lack of correlation between brain IT levels and aggressive behavior of females suggest that the nonapeptides are not related to females aggressiveness in three-spined sticklebacks.

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1. Introduction

Arginine vasotocin (AVT) and isotocin (IT), evolutionary predecessors of mammalian arginine vasopressin (AVP) and oxytocin (OT) are synthesized in separate preoptic and lateral tuberal nuclei of fish brain (Saito et al., 2004). These brain nonapeptides are involved in social and reproductive behaviors acting centrally as neurotransmitters and neuromodulators in many vertebrates, including fishes (Goodson, 2005; Goodson and Bass, 2001).

Our previous studies showed that brain AVT and IT are engaged in particular reproductive behavior in male three-spined sticklebacks (*Gasterosteus aculeatus*) (Kleszczyńska et al., 2012). The highest brain concentrations of AVT were observed in the most aggressive males that cared for eggs and nuptial colored subordinates that fought to change their social status. On the other hand, IT was significantly higher in brains of aggressive dominant males

that defended their territory or courted females. A link between brain nonapeptides and aggressive behavior of males in distinct phases of breeding has also been studied in other fish species. Brain AVT correlated positively with aggressive behavior in the butterflyfish (*Chaetodon multicinctus*) and monogamous cichlid fish (*Amatitlania nigrofasciata*) (Dewan and Tricas, 2011; Dewan et al., 2011; Oldfield and Hofmann, 2011). On the other hand, exogenous AVT diminished aggression in juvenile rainbow trout (*Oncorhynchus mykiss*) (Backström and Winberg, 2009) and territorial bluehead wrasse males (*Thalassoma bifasciatum*) (Semsar et al., 2001). In the round goby males (*Neogobius melanostomus*), higher brain AVT levels corresponded with lessening aggression (Sokołowska et al., 2013) and in males of the clown anemonefish (*Amphiprion ocellaris*), a large number of AVT-producing neurones in the preoptic area (POA) corresponded with non-aggressive behavior (Iwata et al., 2010). There is considerable evidence that nonapeptides are involved in social and reproductive behavior of males. For example, AVT regulated courtship behavior in the white perch (*Morone americana*) (Salek et al., 2002) and the peacock blenny (*Salaria pavo*) (Carneiro et al., 2003; Grober et al., 2002), as well

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as paternal brooding in two species of pipefishes (*Syngnathus fuscus* and *Syngnathus floridae*) (Ripley and Foran, 2010). What is more, AVT modulated vocalization in brown ghost knife fish (*Apteronotus leptorhynchus*) (Bastian et al., 2001) and the plainfin midshipman fish (*Porichthys notatus*) (Goodson and Bass, 2000), and triggered spawning reflex in the killifish (*Fundulus heteroclitus*) (Pickford and Strecker, 1977). Isotocin, the second teleostean nonapeptide engaged in reproduction (Popescu et al., 2008), induced social approach in the goldfish males (*Carassius auratus*) (Thompson and Walton, 2004) and affected paternal behavior in monogamous cichlid fish (O'Connell et al., 2012). However, the knowledge of specific function of brain AVT and IT in breeding females is lagging far behind that in males. In female fish, brain AVT and IT are suggested to be engaged in regulation of final oocyte maturation (FOM) and ovulation (Singh and Joy, 2010, 2011; Joy and Singh, 2013).

In this study, we aim to fill the gap in current knowledge of the roles of AVT and IT in female fish during breeding using our model species that is three-spined stickleback (Kleszczyńska et al., 2012; Kleszczyńska and Kulczykowska, 2013). The stickleback, because of its well-defined reproductive behavior, is a useful model in studies of sexual behavior and social interactions (Wootton, 1976; Fitzgerald, 1993). Briefly, in breeding season, males manifest nuptial coloration, fix territory, build nests, and court females. Females usually mate only with selected nest owner, guiding by its external features. The external characteristics that females seem to use in mate choice are courtship effort (spectrum of sexual behaviors, including zig-zag dance), nuptial coloration, symmetry of secondary sexual characters and condition (Fitzgerald, 1993; Cubillos and Guderley, 2000; Mazzi et al., 2003). After successful spawning, males defend their nests and care for eggs and offspring. Breeding females that reside in large groups usually display aggressive behavior towards males that have the nests. They raid nests and eat up deposited eggs trying to increase their own reproductive success (Fitzgerald, 1993).

The present study was designed to establish if the levels of bioactive nonapeptides in females' brain are just related to presence of the male, or they are also linked with male behavior and presence of the nest. To achieve the goal we measured AVT and IT concentrations in whole brains of breeding females kept with: (i) courting nest-owners, (ii) courting males that did not build the nest, (iii) non-courting males, and (iv) alone. We compared the brain nonapeptides' levels in females that did deposit eggs with those that did not.

2. Materials and methods

2.1. Animals and experimental design

Females of three-spined stickleback (1.066–3.489 g) used in this study were caught in the spawning season, i.e. during spring in the Vistula river (Northern Poland). Before experiments fish were acclimatized to the laboratory conditions for five days. Experiments were conducted in freshwater at room temperature, under natural photoperiod which was changing from 13L:11D to 16L:8D. Before experiment, fish and tanks were shortly disinfected with 0.05% and 1% KMnO₄, respectively. Tanks were supplied with plants: Brazilian elodea (*Egeria densa*), coon's tail (*Ceratophyllum demersum*), angustifolia (*Hygrophila angustifolia*) and crystalwort (*Riccia fluitans*). Fish were fed frozen food (*Chironomus plumosus*) *ad libitum*. After sectioning the spinal cord brains (average mass 20.15 mg) were removed, immediately frozen and stored in –70 °C until analysis.

The following experimental schemes were elaborated on basis of several years of our experience with this species (Sokołowska

et al., 2004; Kleszczyńska et al., 2012; Kleszczyńska and Kulczykowska, 2013).

2.1.1. Experiment 1

Breeding females kept alone or in presence of courting or non-courting male that did not build the nest: two experimental designs: 3 females + 1 male and 10 females + 1 male.

Experiment was carried out between April and June. At the beginning single males displaying nuptial color were put into 300-liter and 30-liter tanks. First dorsal spine of each male was cut to distinguish males from females in case they lose coloration. On the 7th day three females and ten females were introduced to a single male in 300-liter and 30-liter tanks, respectively. At the same time single females were placed in 30-liter tanks. In our experience, the situation where one male is kept with three females in 300-liter tank triggers male courting behavior, whereas the situation where one male is kept with ten females in 30-liter tank excludes courtship. Behavioral observations were made several times a day. Seven days after introducing females into the tanks fish were captured and brains were removed from 12 females (design: 3 females + 1 male), 50 females (design: 10 females + 1 male) and from females kept alone ($n = 18$). The results were presented in Fig. 1.

2.1.2. Experiment 2

Breeding females that did deposit or did not deposit eggs in presence of courting male that did build the nest: experimental design: 1 female + 1 male.

Experiment was carried out between May and July in 30-liter tanks. A single female was introduced to a single male after the male finished nest building. Experiment was repeated many times. All males courted females but only some of the females responded positively to courtship and followed the male to the nest and laid eggs. It is not unexpected, because stickleback females usually mate only with selected nest owners, guiding by their external features as it has been described in Introduction. Behavioral observations were made several times a day. Brains were removed from females one minute after egg deposition ($n = 33$) or from those that did not lay eggs two hours after courtship began ($n = 39$). The results were presented in Fig. 2.

2.2. Analysis of AVT and IT

Brains were defrosted, weighted and sonicated separately in 1 ml of distilled water using Microson™ XL 2000. Then the extraction of AVT and IT was performed in 0.25% (v/v) glacial acetic acid in a boiling water bath for 3.5 min. The extracts were cooled on ice, and then centrifuged at 15000 rpm for 30 min at 4 °C. Then the

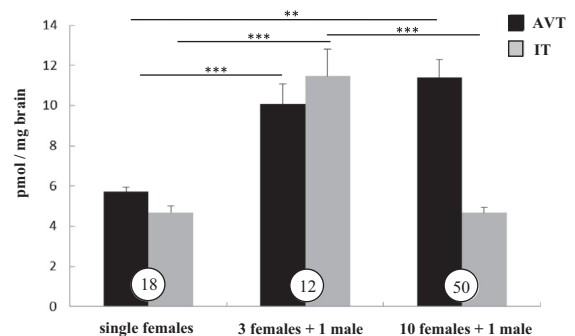


Fig. 1. Brain AVT and IT concentrations in females of three-spined stickleback while kept alone and in experimental schemes: 3 females + 1 male and 10 females + 1 male. Number (n) of females is given in the circles. The values are presented as means \pm SEM. Significant differences are indicated as *** $P < 0.001$, ** $P < 0.01$.

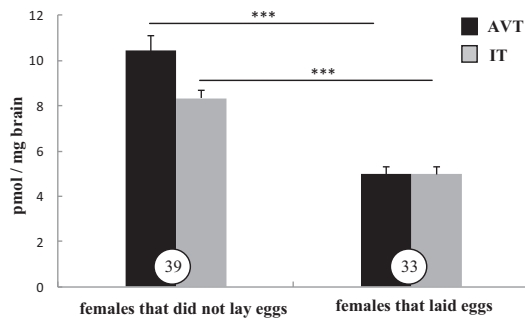


Fig. 2. Brain AVT and IT concentrations in females of three-spined stickleback that did not lay eggs and laid eggs. Number (n) of females is given in the circles. The values are presented as means \pm SEM. Significant differences are indicated as *** $P < 0.001$.

solid-phase extraction (SPE) combined with derivatization of the peptides was carried out according to the procedure described by Kleszczyńska et al. (2012). The derivatization was performed using NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole) in borate buffer (pH 9.5) for 20 min at room temperature. The derivatized peptides were injected directly to HPLC (Beckman modular HPLC system with Shimadzu spectrofluorometric detector RF-551). HPLC separation was done on Ultrasphere ODS column (Beckman, 250×4.6 mm, $5 \mu\text{m}$ particle) in linear gradient system: 48–80% phase B (0.1% trifluoroacetic acid in acetonitrile:water 3:1) in phase A (0.1% trifluoroacetic acid in water) for 20 min at flow rate 1 ml/min. Fluorescence detection was carried out at 530 nm with excitation at 470 nm. The concentration of the peptides was expressed in pmol per mg of brain.

2.3. Statistical analysis

Statistical analyses of data were carried out using Statistica 7.1 software. Values are expressed as means \pm standard error of the mean (SEM). For multiple comparisons of hormone levels, the analysis of variance (one-way ANOVA) was performed followed by post hoc test (Spjotvoll and Stoline's test for unequal numbers of cases). Significance was taken at $P < 0.05$.

3. Results and discussion

The current study was designed to examine if in female sticklebacks during breeding, the whole brain concentrations of AVT and IT are related to presence of the male, presence of the nest, or they are also influenced by male sexual behavior. Moreover, we checked if nonapeptide levels in females' brain are coupled with egg deposition.

In Experiment 1, immediately after introducing three females into the tank, the male began to court them. Females followed the male towards the corner of the tank where he simulated having the nest, but none of the females laid eggs, because none of the males built the nest. On the other hand, ten females in the tank swam in the group and were extremely aggressive towards the male chasing, hitting and biting him what excluded courtship. The males tried to hide away behind the water filter to avoid attacks. Twenty aggressive females did not survive until the end of the experiment. Females kept alone in tanks were calm and intensively fed. Significantly higher brain AVT concentration was measured in females kept with male, courting or non-courting, than in females kept alone (Fig. 1). It indicates that AVT production in female brain is probably stimulated just by the presence of the male in close proximity, regardless of whether it displays courting behavior or not. On the other hand, the highest IT levels were

found in those females that were kept with courting male (Fig. 1). Therefore, brain IT synthesis in females seems to be stimulated by male courtship proxies. Several studies confirm that in females of three-spined stickleback, visual cues play a pivotal role in male choice (Fitzgerald, 1993; Cubillos and Guderley, 2000; Mazzi et al., 2003). It should be emphasized that high levels of AVT and IT (Fig. 1, design: 3 females + 1 male) are not related to the presence of the nest, because in this experiment none of the males built the nest. Therefore, in females, the visual perception of the male or male courtship behavior, not the nest, makes important signal influencing their brain nonapeptide levels.

In Experiment 2, some of the females did not lay eggs even though the nests were present. In their brain, AVT and IT concentrations were significantly higher than in those, which deposited eggs (Fig. 2). In addition, the AVT levels were the highest and similar in all females that did not deposit eggs, regardless of whether they were kept in the presence of courting or non-courting male, having the nest or not (Figs. 1 and 2).

The higher brain concentrations of AVT detected in all breeding female before egg deposition may suggest that this nonapeptide is engaged in regulation of FOM, ovulation, or egg lying. Studies in the medaka (*Oryzias latipes*) indicate that females before egg lying possess more AVT-ir and IT-ir cells in preoptic area than females after spawning (Ohya and Hayashi, 2006). If the presence of more AVT-ir and IT-ir neurones is equivalent to higher synthesis of both neuropeptides in the brain our findings tally with those in medaka. In the catfish (*Heteropneustes fossilis*), *in vitro* studies reveal that AVT may induce FOM and ovulation (Singh and Joy, 2010, 2011; Joy and Singh, 2013). In teleosts, maturing oocytes undergo complex changes, including swelling due to water uptake before ovulation. Therefore, it is not unexpected that the oocyte hydration is affected by AVT (Singh and Joy, 2010), because AVT is this nonapeptide that is involved in osmoregulation in fish (Kulczykowska, 2007). Other studies in catfish report that AVT and to a lesser extent IT can induce FOM and ovulation through the regulation of ovarian steroidogenesis (Singh and Joy, 2011) and/or stimulation of secretion of prostaglandins (PGs) in ovary (Joy and Singh, 2013). The status of AVT and IT as reproductive neuropeptides has been confirmed in the pupfish (*Cyprinodon nevadensis amargosae*) (Lema, 2010) and medaka (Konno et al., 2010), in which the V2-type AVT receptor transcript has been shown in the ovary and in the white sucker (*Catostomus commersoni*), in which IT receptor mRNA has been quantified in different tissues of the reproductive axis, including brain and ovary (Hausmann et al., 1995). The lower levels of brain AVT and IT measured in females after egg deposition may result from suppression of nonapeptides' production after mating and spawning. It agrees with the results of our previous work in sticklebacks, where significantly lower brain AVT and IT concentrations were presented in females after breeding season (Gozdowska et al., 2006).

There are no examples of coupling between brain AVT or IT and an act of egg deposition in fish, but there is evidence for a regulatory role of hypothalamic nonapeptides in oviposition in birds. In the turkey, mesotocin (MT), the avian homolog of fish IT, probably is coupled with oviposition, because numbers of MT-immunoreactive neurons in hypothalamus are higher in late stage incubating hens compared to the layers (Thayananuphat et al., 2011). In birds, AVT and PGs are known to control the movement of the egg into the cloaca, as well as its extrusion into the nest (oviposition), because under the influence of these hormones uterine muscles contract leading to expulsion of the egg (Rzaşa, 1984; Rzaşa and Ewy, 1970). Moreover, it has been shown that AVT stimulates release of PG from the uterus in hens (Rzaşa, 1984). Also in fish, AVT can be engaged in egg deposition stimulating PG secretion in ovary. This mechanism of the nonapeptide action has been reported in catfish (Joy and Singh, 2013). The other mechanism

may include regulation of steroidogenesis in ovary by production of the maturation-inducing steroid 17, 20 β -dihydroxy-4-pregnen-3-one (MIS, 17, 20 β -DP) as has been shown in catfish again (Singh and Joy, 2011). The AVT may affect steroidogenesis in gonads also through regulation of gonadotropin-releasing hormone (GnRH) production in the brain, because there is evidence that the GnRH and AVT pathways overlap in Teleostei. The GnRH synthesizing neurones are widely distributed in the POA hypothalamus region adjacent to the AVT containing neurons in the brain of the green molly *Poecilia latipinna* (Batten et al., 1990).

In our study, we observe that females in larger group (10 individuals + 1 male) are aggressive towards the male that does not have the nest, although in the wild, breeding females living in large groups display aggressive behavior only towards nest owners (Fitzgerald, 1993). Brain AVT levels are similar in all females, those kept in larger and those kept in smaller group, irrespective of whether or not they display aggressive behavior (Fig. 1). Also brain IT levels is not coupled with aggressive behavior of females (Fig. 1). In general, little is known about relationship between brain AVT and aggressive behavior in female fish and just nothing about that of IT. Aggressive monogamous multiband butterflyfishes of both sexes have more AVT cells in the gigantocellular preoptic area (gPOA) than non-aggressive ones, however the size and the number of neurons in the parvocellular preoptic area (pPOA) correlate negatively with aggressive behavior in males and females of this species (Dewan and Tricas, 2011).

In summary, two different scenarios may be considered: (i) AVT alone, (ii) IT alone, and (iii) both AVT and IT are engaged in controlling of FOM and/or egg deposition in female three-spined sticklebacks. Provided FOM or egg deposition or both are regulated by IT, male courtship proxies are decisive for inducing these processes. Provided AVT is decisive, the presence of the male in close proximity is just enough. There are the first studies showing that the brain IT and AVT production in females' brain can be influenced by presence of the male, courting or non-courting, respectively. Similar AVT levels in brains of aggressive and non-aggressive individuals and a lack of correlation between brain IT levels and aggressive behavior of females suggest that the nonapeptides are not related to females aggressiveness in three-spined sticklebacks.

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