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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.

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 (Popesku 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% $KMnO_4$, respectively. Tanks were supplied with plants: Brazilian elodea (*Egeria densa*), coon's tail (*Ceratophyllum demersum*), angustifola (*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

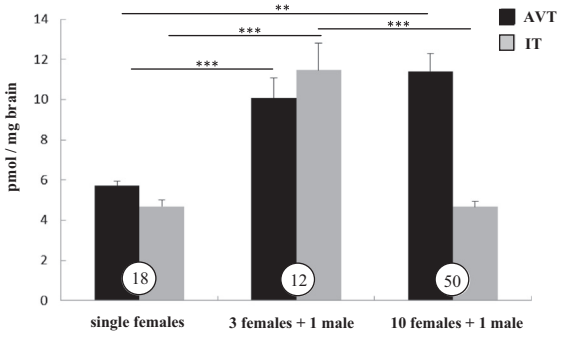

Fig. 1. Brain AVT and IT concentrations in females of three-spined stickleback while kept alone and in experimental schemes: 3 females + 1 male and 10 females + 1 male. Number (n) of females is given in the circles. The values are presented as means ± SEM. Significant differences are indicated as $^{**}P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