Vasotocin/Isotocin-immunoreactive Neurons in the Medaka Fish Brain Are Sexually Dimorphic and Their Numbers Decrease After Spawning in the Female

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In teleosts, the distribution of neurons in the preoptic-hypothalamic region and their associated neurohypophysial hormones, such as vasotocin (VT), appears to be different among species. This differential distribution is thought to reflect the social and/or sexual status of individuals within a species. In the present study, we analyzed the number, size and the distribution of vasotocin/isotocin (VT/IT) neurons in the brains of both male and female medaka (Oryzias latipes) using immunohistochemistry. VT/IT neurons were similarly located in an inverted L-shape in the nucleus preopticus in both gender, as has been already reported in salmonids. However, computer-assisted image analysis revealed sexual dimorphism in the number of VT/IT-immunoreactive (ir) neurons, with greater numbers found in males as compared to females. Further, in the female brain, the number of VT/IT-ir neurons decreased significantly after spawning. In pre-spawning compared to post-spawning females, the small-sized VT/IT-ir neurons dominated. Sexual differentiation of the medaka is fully dependent upon the steroid status during the early developmental stages and steroids are also known to trigger gender-specific behavior in the adult medaka. Our findings strongly suggest that VT and/or IT neurons may be functionally related to ovulation and/or the reproductive axes through connection to their steroidal status.

Key words: medaka, vasotocin, isotocin, sexual difference, spawning, ovulation

INTRODUCTION

Arginine vasopressin (AVP) is a cyclic nonapeptide that exerts a variety of biological functions in mammals (Ruggles et al., 1985). The primary role of AVP involves the regulation of water and salt excretion from the kidney. The other physiological functions of AVP include control of blood pressure (Johnston, 1985), platelet aggregation, glycosylation and neuroglucogenesis in the liver (Eugenin et al., 1988), adenocorticotropic (ACTH) release from the adenohypophysis, and aldosterone secretion from the adrenal gland (Gillies et al., 1982).

AVP is also implicated in interneuronal communication in the central nervous system (CNS) and modulates several behavioral functions such as feeding, memory, thermoregulation and the control of adaptive, social and sexual processes (de Wied et al., 1993).

In amphibians, vasotocin (VT), a counterpart of AVP of mammals, is also expressed in the hypothalamus and have roles as a neurohormone and a neuromodulator as reported in mammals (Gonzalez and Smeets, 1992). At the periphery, VT regulates osmotic and electrolyte balance and steroid secretion from frog adenocortical cells (Warburg, 1995; Larcher et al., 1989). In the CNS, VT functions as a neuromodulator to control reproductive processes in teleosts (Goodson and Bass, 2000).

On the other hand, VT is considered as the ancestral peptide in the vertebrate neurohypophysial hormone family, especially AVP and oxytocin (OT) in mammals, and IT occurs in the teleost fish. The structures of VT and IT are very similar, with only positions 4 and 8 of the amino acid sequence being different. The physiological functions controlled by these hormones remain uncertain. By use of specific analogues for neurohypophyseal hormones, it was indicated that the effects of VT and IT were mediated by a new type of receptor, functionally similar to the mammalian neurohypophysial VT1a type (Guibbolini and Lahlou, 1990).

So far, no reports are available on the VT and/or IT neurons in correlation with reproductive behavior in medaka. In this report, we confirmed that there were differences in the number of VT/IT-immunoreactive (-ir) neurons in both sexes, and furthermore detected significant changes in the number and the sizes of the VT/IT-ir neurons along with spawning in the female medaka.

MATERIALS AND METHODS

Fish

We used mature medaka (4–6 months old) of the d-rR strain (generously provided by Dr. Shima A, University of Tokyo). Fishes were bred and kept in plastic containers (Nisso PC-1, Tokyo, Japan; 156W×151H×231D mm). In each container, two females were maintained with a single male. From our experience, when the fish were kept in a larger container (182W×128H×260D mm) in a
group, e.g., 5 males and 5 females together, only a few females spawned in a day, and maturation of the ovary is not completed in other females. Water temperature was maintained at 26°C. The illumination was turned on at 10:00 and off at 24:00. During the light period, additional sunlight was given at least for 6 hrs. They were fed brine shrimp (Sanders Brine Shrimp Company, Utah, USA) and tetratin (Tetra, Meile, Germany) three times a day.

**Sampling**

All females oviposited every morning. One day before sampling, the females and the male were separated by a transparent plastic board. On the next morning one hour after the illumination was turned on, i.e. at 11:00, the board was removed and the female fish were killed immediately and brains collected. In this group there was no physical contact between the male and females (pre-spawmed female samples). The other females, which were allowed to contact the male, spawned and were killed at 12:00 (post-spawmed female samples). All the males were killed at the same time the females in their tank were killed (regardless pre- or post-spawning) and their brains were collected. Body length of the males, the pre-spawmed females, and the post-spawmed females ranged 25.2-28.8, 28.4-33.3, and 28.8-33.3 mm, respectively. Body weight of the males, the pre-spawmed females, and the post-spawmed females ranged 34.9-52.5, 657-811, and 412-700 mg, respectively. Gonadosomatic index (GSI, Mean ± SE) of the males, the pre-spawmed, and the post-spawmed females varied: 0.677 ± 0.024, 10.6 ± 0.944, and 9.94 ± 0.636, respectively. The numbers of eggs ranged from 18-38 in the post-spawmed females.

**Immunohistochemistry (IHC) of the sectioned brains**

Total number of fish used in this experiment was 15 (5 mature males, 5 pre-spawmed mature females, and 5 post-spawmed females). The fish were anesthetized in ice cold water, then body length (mm) and body weight (mg) were measured. The brains were taken out immediately and immersed in Bouin’s fixative without acetic acid at 4°C for overnight. After fixation, samples were preserved in ice-cold 70% ethanol overnight, dehydrated and embedded in paraffin (m. p. 52°C). Serial sections of the brains were cut at the thickness of 10 μm. The sections were incubated with anti-vasotocin (VT) antiserum with the dilution of 1:10000 (kindly supplied from Dr. Urano A., Hokkaido University). The working dilution of the primary antiserum was determined by serial dilutions. Immunostaining protocols of Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) were followed with slight modifications (Jokura and Urano, 1987). After treatment with 0.3% H2O2 in methanol for 15 min, the sections were first incubated with the primary antiserum with dilution buffer (1% BSA, 0.05% NaN3, 0.05% skim milk, and 0.1% Triton-X containing 0.1M phosphate buffered saline, PBS pH 7.4) at 4°C for 72 hrs. The sections were then washed three times in 0.05M PBS containing 0.1% Triton-X (PBS-T) at 4°C each for 5 min, incubated with biotinylated anti-rabbit IgG (Vector Laboratories) at room temperature (RT) for 30 min, and washed in PBS-T three times. The sections were incubated in the avidin-biotin-peroxidase complex (ABC) in PBS-T at RT for 30 min, and rinsed three times with PBS-T. They were then incubated in a solution containing 0.05% 3,3-diaminobenzidine (DAB; Dojindo Co., Kumamoto, Japan) and 0.01% hydrogen peroxide in 0.05M tris buffer, pH 7.4 (DAB solution) at RT, and briefly washed in Tris buffer (pH 7.4). They were then dehydrated and cover-slipped with Permount® (Fisher, New Jersey, USA). Sections of the male, pre-spawmed, and post-spawmed females were mounted on the same glass slides, and reactions were repeated more than 5 times to reduce any differences in immunoreactive density among staining batches.

**Whole mount IHC**

More than 30 brains of both sexes were taken out immediately after anesthesia in ice water and immersed in Bouin’s fixative without acetic acid at 4°C for overnight. After fixation, samples were preserved in ice-cold 70% ethanol. The brains were cut at the mid-line. The half brain blocks were immunostained with anti-VT antisem as mentioned above. After treatment with 0.3% H2O2 in methanol for 30 min, the brains were first incubated with the primary antiserum at 4°C for 1 week. The brains were then washed three times in PBS-T at 4°C each for 10 min, incubated with biotinylated anti-rabbit IgG at RT for 30 min, and washed in PBS-T three times. They were incubated in the ABC kit PBS-T at RT for 30 min, rinsed three times with PBS-T, incubated in the DAB solution on ice, and then briefly washed in Tris buffer (pH 7.4) several times. The samples were then dehydrated and cover-slipped with Permount®.

**Specificity of antiserum**

The anti-VT antiserum used in the present study was raised against synthetic VT in a rabbit. Ota et al. (1996) incubated the salmon brain with the antiserum that had been pre-absorbed with CNBr activated Sephaloc 4B beads (Pharmacia) conjugated with synthetic IT, and detected positive signals in the cells in the NPO. They further detected another group of cells in the NPO with anti-OT antiserum that was pre-absorbed with CNBr activated beads conjugated with synthetic VT. Thus, essentially the VT-expressing cells and the IT-expressing cells seemed grouped in different population. We confirmed similar results in the medaka brain (data not shown). Furthermore, by immunoblotting tests, we detected cross reaction to IT at the concentration of 1:10000 that was the working dilution used in the present study (data not shown). Thus, even though the antiserum was raised against the synthetic VT, cross reactivity to IT cannot be excluded.

**Morphometrical analysis**

Digital images of the serial sections of each medaka brain were captured with a multi-purpose microscope (AxioScope, Zeiss, Jena, Germany) at magnification × 200. Then the images were morphometrically analyzed on PC and immunostained VT/IT-ir neuron number and sizes were measured from the whole images by ImageJ (http://rsb.info.nih.gov/ij/). In brief, the digital images of neurons stained in paraffin preparations were captured and traced by a pen tablet (Wacom, Saitama, Japan). The numbers and sizes of neurons were then measured in a computer-assisted system. Finally, total numbers of the analyzed neurons in each fish brain were obtained by counting the neurons from all serial sections of an individual brain.

**Statistics**

Differences in the number of neurons between both sexes were analyzed by ANOVA then Student's t-test. Differences between neuron numbers and sizes in the pre-spawmed and post-spawmed mature females were analyzed further by ANOVA then Student's t-test. When the p values were less than 0.05, difference was considered as significant.

**RESULTS**

Whole-mount IHC revealed an inverted L-shaped cluster of VT/IT-ir neurons only in the NPO. In this L-shaped cluster, smaller and larger neurons were distinguished (Fig. 1). Smaller neurons were found in the vertical portion of the inverted L-shaped cluster which project fibers with varicocities to neurohypophysis (preopticohypophysial tract, Fig. 1B, C). Fibers extending from smaller VT/IT neurons in the rostral direction were also observed as shown in Fig. 1C. Both VT/IT neurons and fibers were detected in the transverse sections of the NPO at the magnocellularis level (Fig. 2A). At this level, the VT/IT fibers mainly formed the
Decrease of VT/IT Neurons in Medaka

Fig. 1. Whole mount IHC of the NPO of a hemi-brain from a male fish killed at 16:00. The inverted capital L-shaped cluster of VT/IT-ir neurons is evident. A: The left side is rostral. Note that many small immunoreactive neurons (white arrows) are seen in the NPO. While some fibers turn ventrally and probably run to the hypophysial direction (black arrows), majority of the fibers take dorsal course and seem to project to extra-hypothalamic areas (an arrowhead). B, C: Another half of the brain focused in L-shaped neuron cluster (B), and on the projections from the smaller neurons that run ventral side (C). Right side is rostral. Fibers indicated with arrowheads contain varicocities and project to caudal direction. Note that rostral projections are also evident (white arrows in C). Scale bar 100 μm.

Fig. 2. Transverse sections of VT/IT neurons and fibers counterstained with cresyl violet. A: Neurons (white arrowheads) project to preopticohypophysial tracts (black arrowhead). B: A section of parvocellularis posteriores of the NPO (PPp) level. C: In the PPp, ir-positive fibers facing to the third ventricle indicated by arrows. D: A section of habenular (Hb) level. E: In the habenular portion, fibers extending caudally are indicated by arrows. The VT/IT fibers of pre-optico-hypophysial tract are indicated by black arrowheads in A, B and D. V3, third ventricle. Scale bars: 100 μm for A, B and D; 50 μm for C and E.
Fig. 3. Whole mount IHC of the medaka brain of the male, the extra-hypothalamic VT/IT-ir fibers are seen. The left side is rostral. A: forebrain. Fibers extending from NPO (arrowhead) to the dorsal telencephalon (D), the ventral telencephalon (V), and the habenular region (Hb). B: dorsal hypothalamus. Many fibers and terminals are seen. C: spinal cord. Fibers are extending caudally (arrowheads; to the right side). A plexus with VT/IT-ir signals is also observed (P). Scale bar 200 μm.

Fig. 4. Transverse views of the NPO in the mature medaka brains. Representative paraffin sections of the comparable levels from a mature male (A), pre-spawned (B) and post-spawned mature females (C). Note that only faintly stained VT/IT-ir fibers and neurons were detected in the post-spawned female (C), while the VT/IT-ir signals were the strongest in the male (A). Scale bar 50 μm.
preoptohypophyseal tract (black arrowheads). Caudal to the NPO magnocellularis, VT/IT fibers to the third ventricle (V3) were found (Fig. 2B, C). Caudal to this, at the habenular level, VT/IT fibers that projected in a caudal direction were evident (Fig. 2D, E). At the same time, the preoptohypophyseal tracts were detected ventrally and contained strong VT/IT signals (Fig. 2A, B, D, black arrowheads).

The major extra-hypothalamic projections from the larger neurons were traced caudally. These projections extended to structures in the diencephalon, the midbrain, and the hindbrain, e.g. the dorsal hypothalamus (Fig. 3B), and the plexus of the medulla (Fig. 3C, indicated by P).

Some fibers were also detected in the spinal cord (Fig. 3C, arrowheads). Most of the fibers extended caudally, but a few turned ventrally to project to the neurohypophysis (Fig. 1A, black arrows). It is intriguing that groups of VT/IT-ir positive fibers were found rostral to the NPO, in both the ventral and dorsal portions of the telencephalon (Fig. 3A marked as V and D, respectively).

The VT/IT staining found both in the neurons and fibers of the NPO region were the strongest in males, moderate in the pre-spawned females, and least in post-spawned females (Fig. 4A, B, C, respectively). The immunoreactive neurons in the mature male fish were significantly greater in number compared to the female fish (Mean ± SE: 352 ± 70.7, n=5; vs. 94 ± 15.9, n=10, p < 0.05, Fig. 5). In addition, the post-spawned female had significantly smaller numbers of VT/IT-ir neurons than that of the pre-spawned females (Mean ± SE: 127 ± 20.4 vs. 60 ± 12.2, n=5, respectively, p<0.05, Fig. 6). Significant differences were observed in the smaller neurons (0–10 μm² range) of pre-spawned and post-spawned females, whereas the numbers of neurons in the range 5–15 mm² were smaller in pre-spawned mature females but not statistically different (Fig. 7).

**DISCUSSION**

The present study analyzed the distribution and alternation of VT/IT in the medaka brain between both sexes and the influence of spawning in the female. The antibody we used detects both VT and IT in the neurons and fibers simultaneously, even though it was raised against the synthetic VT. The VT/IT signals found both in the neurons and fibers in the male were stronger than in the female. Moreover, the signals in the pre-spawned females were stronger than those in the post-spawned females. Decrease in the VT/IT
signals in post-spawned females may be due to abrupt release of the nonapeptides from storage which are evoked by physiological changes. Whether the production of the nonapeptides is suppressed by mating and/or spawning is unknown based on the present observations.

Sexual differences in the hypothalamic neurosecretory system have been well established in a wide range of vertebrate species. Most of these differences are deemed to be related to sex hormones secreted during development. In the medaka (Oryzias latipes), sex determination and differentiation is fully dependent on the steroid status during the early developmental stages (Yamamoto, 1968; Papoulias et al., 2000).

Grober and Sunobe (1996) found that when gobies (Trinma okinawae) underwent reversible sex change, the VT producing neurons changed their size. In bluehead wrasses (Thalassoma bifasciatum), the large territorial terminal phase males have greater hypothalamic VT mRNA levels than females and non-territorial males, but these levels in the female rise rapidly along with female-to-male sex change. In contrast Parhar et al. (2001) proposed the hypothesis that phenotypic and behavioral sex differences need not to be accompanied by structural differences of VT in goldfish.

Sex differences in VT/IT function have been found for the polymorphic plainfin midshipman fish (Goodson and Bass, 2000), although peptide functions in this case are associated with divergent social tactics and not with gonadal sex per se. Differential usage of vocalization between the midshipman morphi is tied to the differential expression of parental behavior, nest defense, and courtship. All adult morphs produce a train of grunts, courtship ‘hums’ and ‘grows’ (Bass et al., 2000; Brantley and Bass, 1994). Vocal-motor activity elicited by electrical stimulation of the NPO (predominantly grunt-like) is inhibited by local VT administration in the courting male morph, whereas IT is ineffective. This peptide sensitivity is reversed in females. However, sneak-spawning males, which neither make nest nor show courtship behavior and are female-typical in numerous vocal characteristics, share the IT-sensitivity of females and are insensitive to VT. Thus, sex differences in VT function in midshipman are dissociated from gonadal sex, suggesting that gonadal and peptidergic characteristics are independently differentiated and are potentially visible to natural selection as individual phenotypic characters (Goodson and Bass, 2001).

Haruta et al. (1991) reported the effect of osmotic stress to VT by using IHC followed by morphometry in medaka. Fresh water (FW)-adapted fish transferred to sea water (SW) showed a decrease in the number of VT-ir larger neurons, while the cell nuclear sizes were increased. Further, when SW-adapted fish were transferred to FW, the number of VT-ir larger neurons increased, but the cell nuclear size did not change. By comparing the population of VT-ir neurons to that of aldehyde fuchsin (AF)-positive neurons in the NPO, they postulated that the AF-positive neurons produce nonapeptides, e.g., IT, VT etc. However, they did not report any relationship between VT and spawning.

In the chicken, the expression of VT gene in the hypothalamus and localization of VT-ir neurons in the magnocellular paraventricular nuclei were studied immediately and 2 h after laying eggs. Simultaneous changes in the VT and VT1 receptor genes were reported in the shell gland, which responds to VT for smooth muscle contraction and expulsion of eggs (Seth et al., 2004).

Further, in white sucker, Hausmann et al. (1995) reported that oocytes express IT receptor after application of IT. They further found that IT receptor (ITR) was activated not only by IT but also by other nonapeptides such as VT, mesotocin, OT, and AVP, although at lower affinities to ITR than IT. White sucker ITR-encoding mRNA has been detected in brain, intestine, bladder, skeletal muscle, lateral line, gills, and kidney indicating that IT may mediate a variety of physiological functions in addition to ovulation. In the present experiment, we used the antiserum which detected both VT and IT. If the analogy from the mammals is applicable to medaka, IT would presumably not be involved in ovulation and spawning in the fish, since OT in mammals has a selective influence on contraction of uterine smooth muscles rather than ovulation.

In home-migrated chum salmon, levels of VT mRNA were higher in the males than the females. Changes in the levels of VT mRNA were markedly different between both sexes (Hiraoka et al., 1997). The present observation in the medaka seems in good agreement with their finding.

Whole-mount IHC revealed an inverted L-shaped cluster of VT/IT-ir neurons only in the NPO. In this L-shaped cluster, smaller and larger neurons were distinguished (Fig. 1). Smaller neurons were found in the vertical areas of the inverted L-shaped cluster. These neurons project fibers with varicosities to neurohypophysis (preopticohypophyseal tract, Fig. 1B, C). Moreover, fibers extending from the smaller VT/IT neurons to the telencephalon were also observed (Fig. 1C). On the other hand, both in the ventral and dorsal portions of the telencephalon, the immunoreactive fibers were detected (Fig. 3A). Whether the VT/IT fibers in the telencephalon originate from the smaller neurons requires further analysis by other techniques, such as retrograde track-tracing (Xue et al., 2004).

In this study, we found that the number of VT/IT-ir cells of smaller neuron size (10 μm²) showed a significant decrease in the post-spawned females (Fig. 7). Moreover, a tendency towards a decrease in number of the VT/IT-ir cells of less than 15 μm² seemed clear, even though no statistical significance was detected. In summary, we found there were gender differences in VT and/or IT neurons in the medaka brain. Additionally, in females decreased numbers of VT/IT neurons were observed after spawning. Moreover, we found that VT/IT neurons extend their projections not only to the pituitary, but also to the rostral and caudal directions. Since a decrease in the number of VT/IT neurons after spawning occurs mostly in the small-sized neurons, these components are most likely related to the physiology and behavior of spawning. Additionally, because many extrahypothalamic actions of neurohypophysial nonapeptides have been reported in various animal species, VT/IT neurons in other parts of the "inverted L-shaped" cluster of the NPO likely have an influence on reproduction through these extrahypothalamic regions in the medaka fish.
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