Involvement of IGF-1R-PI3K-AKT-mTOR pathway in increased number of GnRH3 neurons during androgen-induced sex reversal of the brain in female tilapia

Akari Oda1, Sakura Inoue1, Ryo Kaneko1, Yasuto Narita1, Suzuka Shiono1, Toyoji Kaneko2, Yung-Che Tseng3 & Ritsuko Ohtani-Kaneko1

The neuroplastic mechanism of sex reversal in the fish brain remains unclear due to the difficulty in identifying the key neurons involved. Mozambique tilapia show different reproductive behaviours between sexes; males build circular breeding nests while females hold and brood fertilized eggs in their mouth. In tilapia, gonadotropin-releasing hormone 3 (GnRH3) neurons, located in the terminal nerve, regulate male reproductive behaviour. Mature males have more GnRH3 neurons than mature females, and these neurons have been indicated to play a key role in the androgen-induced female-to-male sex reversal of the brain. We aimed to elucidate the signalling pathway involved in the androgen-induced increase in GnRH3 neurons in mature female tilapia. Applying inhibitors to organotypic cultures of brain slices, we showed that the insulin-like growth factor (IGF)-1 receptor (IGF-1R)/PI3K/AKT/mTOR pathway contributed to the androgen-induced increase in GnRH3 neurons.

The involvement of IGF-1 and IGF-1R in 11-ketotestosterone (11-KT)-induced development of GnRH3 neurons was supported by an increase in Igf-1 mRNA shortly after 11-KT treatment, the increase of GnRH3 neurons after IGF-1 treatment and the expression of IGF-1R in GnRH3 neurons. Our findings highlight the involvement of IGF-1 and its downstream signalling pathway in the sex reversal of the tilapia brain.

Sex reversal is a well-known phenomenon in teleost fish. Some teleosts naturally change their phenotypic sex during their lifetime, while others change it in response to environmental factors or treatments with hormones1–3. During sex reversal, sex change occurs not only in the gonads but also in the brain, which makes it possible to elicit the opposite sex-typical reproductive behaviours. The teleost brain and their gonads are known to be highly sexually labile throughout life4–6. However, the molecular and neuronal mechanisms of sex reversal in the teleost brain remain unclear.

There are some difficulties in elucidating the mechanism of sex reversal in teleost brains. First, male and female fish display the characteristic reproductive behaviours of their own species. When sex reversal occurs, reproductive behaviours change according to species-specific reproductive behaviours of the opposite sex. The species-specific difference in behavioural phenotypes could be mainly due to the evolutionary divergence of a common mechanism including a molecular system and neural circuitry. Additionally, the developmental, genetic, physiological, and neuromodulatory mechanisms may also bias the evolution of behaviour, thereby affecting the
evolvability of species-specific behaviour and the nervous system complexity. Second, sex reversal is induced in response to a variety of social, physical, environmental and hormonal stimuli. Thus, the regulatory mechanism related to sex reversal in the brain also depends on the type of key stimuli that induce it. These and other differences, such as the timing of sex reversal (e.g., during development or after maturation), make it difficult to identify the molecular and neuronal mechanisms of sex reversal in different species.

In this study, we investigated the regulatory mechanism of sex reversal in Mozambique tilapia (Oreochromis mossambicus). Tilapias are important species in aquaculture. Clemens and Inslse produced all male populations of Mozambique tilapia larvae by adding 17α-methyltestosterone (MT), an artificial potent androgen, into the diet. Since male tilapia grow approximately twice as fast as females, a monozone population of sex-reversed males is commercially produced using androgen-supplemented feed. The mechanisms underlying sex differentiation and hormone-induced sex reversal of the gonads during early development have been well studied in tilapia. However, the mechanisms underlying sex reversal in the tilapia brain remain unsolved.

Female and male tilapia exhibit vastly different reproductive behaviours; males fight each other and build a large circular breeding nest, while females hold and brood fertilized eggs in their mouth (mouth brooding). Treatments with androgens, such as a fish natural androgen 11-ketotestosterone (11-KT), were reported to induce male-specific reproductive behaviours in most mature females within two weeks, while their gonads still contained numerous vitellogenic oocytes and the plasma concentration of 17β-estradiol (E2) remained high. Thus, tilapia is a suitable model for investigating the regulatory mechanism of androgen-induced sex reversal in the brain.

Gonadotropin-releasing hormones (GnRHs) are neuropeptides responsible for reproduction in both vertebrates and non-vertebrates. Advanced teleost fishes, including tilapias, have three GnRH subtypes (GnRH1, GnRH2, and GnRH3). It has been shown that GnRH neurons in the terminal nerve (TN) of dwarf gouramis play a modulatory role in male-specific reproductive behavior. In addition, roles of TN-GnRH3 neurons have been demonstrated in modulation of mating preference for familiar males in medaka. Using Nile tilapia (Oreochromis niloticus), Ogawa et al. have demonstrated that GnRH3 is a potent neuromodulator of male sexual behaviours such as nesting and aggression. They injected antiserum against GnRH3 into the third ventricle of male Nile tilapia, which resulted in significantly decreased nest-building ability, nest size, and aggressive behavior. Mature male Mozambique tilapia have been shown to have more GnRH3 neurons in the TN than females. Treatment with 11-KT or MT, but not E2, increased the number of GnRH3 neurons in mature females to a level similar to that in males, and induced male-specific reproductive behaviors. Furthermore, 11-KT has also been shown to promote neurogenesis in female tilapia and generate newly proliferated GnRH3 neurons. The above-mentioned findings indicate the androgen-dependent regulation of GnRH3 neurons and their important role in male-specific reproductive behaviours in tilapia. However, the mechanism by which androgen affects GnRH3 neurons is yet to be understood.

We focused on GnRH3 neurons in Mozambique tilapia with respect to female-to-male sex reversal of the brain. The promoter of the GnRH3 gene in Nile tilapia contains binding sites for many receptors, including the glucocorticoid receptor (GR) and thyroid hormone receptor (TR), but not for the androgen receptor (AR). Although the promoter gene sequences of GnRH3 in Mozambique tilapia remains still unknown, the genomic similarity of GnRH gene between these two tilapias is about 98% identity in BLAST. It is highly likely that Mozambique tilapia also lacks a binding site for AR in the promoter region of the GnRH3 gene. It is thus suggested that androgen binding to AR does not directly affect GnRH3 gene expressions. Instead, the effects of androgen on GnRH neurons is likely mediated through other molecules, such as hormones or neurotransmitters. In human and experimental mammals, it is well-known that the combined action of the somatotropic [growth hormone—insulin-like growth factor 1 (IGF-I)] and gonadotropin (GnRH-LH/FSH—sex steroid including androgen) hormone axes play important roles in the reproduction, the latter of which stimulates the former to produce its downstream pathway in the androgen-induced effects on GnRH3 neurons. This study is the first to propose a molecular mechanism by which GnRH3 neurons involved in male reproductive behaviours are positively regulated through the IGF-1 and IGF-1R / phosphoinositide 3-kinase (PI3K)/AKT/ mechanistic target of rapamycin (mTOR) signalling pathway during androgen-induced female-to-male sex reversal in the tilapia brain.

To examine the effects of androgen in the tilapia brain without any influence from other organs such as the gonads and liver, we used an organotypic culture of brain slices at the TN level, and pharmacologically investigated the possible involvement of IGF-1 signalling pathways.

**Results**

**Effects of sex steroids on the number of GnRH3 neurons in cultured brain slices.** In order to examine whether 11-KT affects the number of GnRH3 neurons in vitro as previously observed in vivo, we first examined the effect of 11-KT at different concentrations and that of 10 nM E2 on their numbers in female tilapia brain slices. Cross-sectional slices of the brain were cut at the TN level, and the slices were further cut into left and right halves (Fig. 1). One half of each brain slice was treated with either 11-KT or E2 (11-KT- or E2-treated group) for three days, while the other half was treated with the solvent alone (control group). GnRH3 neurons were immunostained with anti-GnRH3 (Fig. 2a), and the total number of GnRH3 neurons per animal was counted from all the half slices (2–4 slices) of the brain at the TN level originating from the respective animal (Fig. 2b). Then, the mean number of GnRH3 neurons was compared between the control, E2-treated and 11-KT-treated groups. Fig. 2b shows that 11-KT treatments with 1nM and 10 nM increased the number of immunostained GnRH3 neurons, and there was a statistically significant difference between the 10 nM 11-KT-treated
and control groups (control vs. 11-KT (10 nM), p < 0.05, paired Student’s t-test). No increase was observed in the number of GnRH3 neurons in the 10 nM E2-treated group, compared to that in the control.

**Effects of IGF-1 on the number of GnRH3 neurons in cultured brain slices.** To examine the influence of IGF-1 on the number of GnRH3 neurons, the brain slices were treated with IGF-1 for three days. The number of GnRH3 neurons was compared between the control and IGF-1-treated slices originating from the same animal. Figure 3a, b show immunostained-GnRH3 neurons and the mean numbers of GnRH3 neurons per animal, respectively, in control and IGF-1-treated slices. Treatment with IGF-1 at 10 nM showed a tendency to increase the number of GnRH3 neurons compared to that of the controls. When 100 nM IGF-1 was added to the medium, the number of GnRH3 neurons was significantly greater than that in controls (control vs. 100 nM IGF-1, p < 0.05, paired Student’s t-test). These results revealed a stimulatory effect of IGF-1 on the number of GnRH3 neurons.

**Effects of 11-KT on IGF-1 mRNA expression.** *Igf-1* gene expression was examined in control and 11-KT groups using cultured halves of female brain slices originating from the same animal. The expression levels of *Igf-1* mRNA were measured 1 h after treatment with 10 nM 11-KT by real-time quantitative reverse transcription (qRT)-PCR, and compared between 11-KT-treated and control groups (Fig. 4). The expression of *Igf-1* mRNA was significantly higher in 11-KT-treated slices than in control slices (control vs. 11-KT, p < 0.05, paired Student’s t-test), indicating that 11-KT increased the expression of *Igf-1* mRNA in the brain.

**Effects of IGF-1R inhibitor on 11-KT-induced increase in GnRH3 neurons.** To examine the contribution of IGF-1R to 11-KT-induced increase in GnRH3 neurons, effects of IGF-1R inhibitor were studied. Micrographs in Fig. 5a show immunostained-GnRH3 neurons (red) in four brain slice groups: control, IGF-1R inhibitor (BMS-754807, 20 μM)-treated, 11-KT-treated, 11-KT- and IGF-1R inhibitor-treated groups. Control and inhibitor-treated animals originated from the same animal, as shown in Fig. 1. Similarly, 11-KT-treated and 11-KT- and IGF-1R inhibitor-treated groups originated from the same animal. The graphs in Fig. 5b indicate the average number of GnRH3 neurons per animal in each of the four groups.

As shown in Fig. 5b, two-way ANOVA revealed a significant effect of 11-KT (p = 0.033) but no significant effect of IGF-1R inhibitor (p = 0.21). There was no significant interaction between 11-KT and IGF-1R inhibitor (p = 0.074). Because a two-way ANOVA and commonly used post-hoc tests such as the Tukey test are not suitable for the comparison of individuals with the correspondence, we thus used a paired t-test for the statistical analysis of the numbers of GnRH3 neurons in the half brain slices originating from the same animal. Furthermore, in using Bonferroni correction to avoid the probability of committing a type I error, p < 0.025 was considered statistically significant for paired t-test. The difference in the number of GnRH3 neurons between the ‘11-KT’ and ‘11-KT & IGF-1R inhibitor’ groups was significant (p = 0.0043, paired t-test). The difference in the number of GnRH3 neurons between the ‘control’ and ‘IGF-1R inhibitor’ groups was not significant (p = 0.57, paired t-test). That is, IGF-1R inhibitor significantly suppressed the 11-KT-induced increase in GnRH3 neurons.
IGF-1R-immunoreactivities in GnRH3 neurons. To investigate whether GnRH3 neurons in 11-KT-treated females express IGF-1R, a pair of adjacent frozen sections with mirror images were subjected to immunocytochemical staining with rabbit polyclonal antibodies against GnRH3 and IGF-1R. GnRH-neurons (red in Fig. 6a) showed IGR-1R immunoreactivity (green in Fig. 6b), although IGF-1R immunoreactivity was also observed in the cells without GnRH3-immunoreactivities near the ventricle.

Effects of PI3K/AKT/mTOR pathway inhibitors on 11-KT-induced increase in the number of GnRH3 neurons. To examine the signalling pathway involved in the androgen-induced increase of GnRH3 neurons, one of the inhibitors examined was added to the medium simultaneously with 10 nM 11-KT, and their suppressive effect on the 11-KT-induced increase of GnRH3 neurons was examined by counting the number of immunostained GnRH3 neurons in the brain slices. The following inhibitors were examined in this study: PI3K
**Figure 3.** (a) Representative images of gonadotropin-releasing hormone 3 (GnRH3) neurons (red) in control (Cont) and insulin-like growth factor 1 (IGF-1)-treated (100 nM) slices. Cell nuclei (blue) were stained with Hoechst 33342. (b) The numbers of GnRH3 neurons in control (Cont) and IGF-1 (10 nM and 100 nM)-treated slices (scale bar, 50 μm). An asterisk indicates a significant difference at $p<0.05$ (paired Student’s $t$-test). Numerals in bars indicate the numbers of animals used for preparing slices. Data are expressed as the mean ± standard error of the mean.

**Figure 4.** Changes in expressions of insulin-like growth factor 1 ($Igf-1$) mRNA in control and 11-ketotestosterone-treated slices 1 h after the treatment. An asterisk indicates a significant difference at $p < 0.05$ (paired Student’s $t$-test). Numerals in bars indicate the numbers of animals used for preparing slices. Data are expressed as the mean ± standard error of the mean.
inhibitor (LY294002), AKT inhibitor (GDC-0068), and mTOR inhibitor (rapamycin). Micrographs in Fig. 7a–c show immunostained-GnRH3 neurons (red) in four brain slice groups: control, inhibitor-treated, 11-KT-treated, and 11-KT- and inhibitor-treated groups. Control and inhibitor-treated brain slices originated from the same animals. Similarly, 11-KT-treated and 11-KT- and inhibitor-treated brain slices originated from the same animal. Graphs in Fig. 7d–f indicate the average numbers of GnRH3 neurons per animal in the four groups.

Figure 5. (a) Representative images of gonadotropin-releasing hormone 3 (GnRH3) neurons (red) in the following four groups of brain slices: control, insulin-like growth factor 1 receptor (IGF-1R) inhibitor (20 μM BMS-754807)-treated, 11-ketotestosterone (11-KT)-treated (10 nM), and 11-KT- (10 nM) and IGF-1R inhibitor (20 μM BMS-754807)-treated groups. Control and inhibitor-treated slices originated from the same animals, while 11-KT-treated slices and 11-KT- and inhibitor-treated slices originated from the same animals. Cell nuclei (blue) were stained with Hoechst 33342. (b) The numbers of GnRH3 neurons counted in the four groups of brain slices. Results from two-way analysis of variance (ANOVA) are shown in the top. Numerals in bars indicate the numbers of animals used for preparing slices. *, a significant difference at \( p < 0.05 \) (two-way ANOVA). **, a significant difference at \( p < 0.005 \) [paired t-test, Bonferroni Correction (\( \alpha_{\text{new}} = \alpha_{\text{original}} / n = 0.01/2 \)]. Data are expressed as the mean ± standard error of the mean.

Figure 6. Representative images of gonadotropin-releasing hormone 3 (GnRH) neurons [red in (a)] and insulin-like growth factor 1 receptor immunoreactivities [green in (b)] on a pair of mirror-image frozen sections from 11-ketotestosterone-injected females. The same numerals in respective pictures indicate the same cells appearing on the two sections. Cell nuclei (blue) were stained with Hoechst 33342.
Figure 7. (a)–(c) Representative images of gonadotropin-releasing hormone 3 (GnRH3) neurons (red) in the following four groups of brain slices: control, inhibitor-treated, 11-ketotestosterone (11-KT)-treated (10 nM), and 11-KT- (10 nM) and inhibitor-treated groups. Cell nuclei (blue) were stained with Hoechst 33342. (d)–(f) The numbers of GnRH3 neurons in the four groups. Control and inhibitor-treated slices originated from the same animals, while 11-KT-treated slices and 11-KT and inhibitor-treated slices originated from the same animals. Inhibitors used in (a)–(c) and (d)–(f) were as follows: (a, d) phosphoinositide 3-kinase (PI3K) inhibitor (LY294002), (b, e) AKT inhibitor (GDC-0068), (c, f) mechanistic target of rapamycin (mTOR) inhibitor (rapamycin). Results from two-way analysis of variance (ANOVA) are shown in the top. Numerals in bars indicate the numbers of animals used for preparing slices. * and **, a significant difference at $p < 0.025$ and $p < 0.005$, respectively [paired $t$-test, Bonferroni Correction ($\alpha_{\text{new}} = \alpha_{\text{original}} / n = 0.05/2$ and 0.01/2]. Data are expressed as the mean ± standard error of the mean.
The effect of PI3K inhibitor (LY294002) was examined (Fig. 7a, d). A two-way ANOVA showed no significant effect of 11-KT (*p* = 0.18) and PI3K inhibitor (*p* = 0.24), but a significant 11-KT × PI3K inhibitor interaction (*p* = 0.037). The differences among four groups were not significant by a two-way ANOVA followed by Tukey post-hoc test. We next used a paired t-test for the statistical analysis of the numbers of GnRH3 neurons in the half brain slices originating from the same animal. Paired t-test showed that the difference in the number of GnRH3 neurons between the ‘11-KT’ and ‘11-KT & PI3K inhibitor’ groups was significant (*p* = 0.0029), but not significant between ‘control’ and ‘PI3K inhibitor’ groups (*p* = 0.19). That is, PI3K inhibitor significantly suppressed the 11-KT-induced increase in GnRH3 neurons.

Treatment with the AKT inhibitor (GDC-0068, pan-AKT inhibitor targeting AKT1, AKT2, and AKT3) was first examined at different concentrations (1, 10, and 20 μM). GDC-0068 at 10 and 20 μM significantly reduced the 11-KT-induced increase in the number of GnRH3 neurons. The effects of GDC-0068 at 20 μM were then examined in four brain slice groups (Fig. 7b and e). A two-way ANOVA showed no significant effect of 11-KT (*p* = 0.12), AKT inhibitor (*p* = 0.30), or 11-KT × AKT inhibitor interaction (*p* = 0.27). While AKT inhibitor alone did not affect the number of GnRH3 neurons (‘control’ vs ‘AKT inhibitor’ groups, *p* = 0.95, paired t-test), AKT inhibitor significantly suppressed the 11-KT-induced increase in the number of GnRH3 neurons (*p* = 0.0037, paired t-test) (Fig. 7e).

The effect of the mTOR inhibitor, rapamycin, (10 μM) was examined (Fig. 7c and f). A two-way ANOVA revealed a significant effect of 11-KT (*p* = 0.014) but no significant effect of mTOR inhibitor (*p* = 0.11). There was no significant interaction between 11-KT and mTOR inhibitor (*p* = 0.067). The effect of 11-KT on the number of GnRH3 neurons was confirmed. Paired t-test showed that the treatment with mTOR inhibitor alone did not affect the number of GnRH3 neurons (‘control’ vs ‘mTOR inhibitor’ groups, *p* = 0.85), but the treatment of mTOR inhibitor significantly suppressed the 11-KT-induced increase in the number of GnRH3 neurons (‘11-KT’ vs. ‘11-KT & mTOR inhibitor’ groups, *p* = 0.017) (Fig. 7f).

**Effects of ERK and JAK inhibitors on 11-KT-induced increase in GnRH3 neurons.** The effects of the extracellular signal-regulated kinase (ERK) inhibitor (U0126) and Janus kinase (JAK) inhibitor (InSolution™ JAK inhibitor 1) were similarly examined (Fig. 8). Micrographs in Fig. 8a, b show immunostained-GnRH3 neurons (red) in four brain slice groups: control, inhibitor-treated, 11-KT-treated, and 11-KT- and inhibitor-treated groups. Control and inhibitor-treated brain slices originated from the same animals. Similarly, 11-KT-treated and 11-KT- and inhibitor-treated brain slices originated from the same animal.

As for ERK inhibitor, two-way ANOVA revealed a significant effect of 11-KT (*p* = 0.014) but no significant effect of ERK inhibitor (*p* = 0.78). There was no significant interaction between 11-KT and ERK inhibitor (*p* = 0.36) (Fig. 8c). In addition, paired t-test showed no significant differences in the number of GnRH3 neurons between ‘11-KT’ and ‘11-KT & ERK inhibitor’ groups (‘control’ vs ‘ERK inhibitor’ groups, *p* = 0.56) (Fig. 8c).

As for JAK inhibitor, two-way ANOVA revealed a significant effect of 11-KT (*p* = 0.0003) but no significant effect of JAK inhibitor, InSolution™ JAK inhibitor I, (100 nM) (*p* = 0.57). No significant interaction was seen between 11-KT and JAK inhibitor (*p* = 0.46). In addition, paired t-test showed no significant differences in the numbers of GnRH3 neurons between ‘11-KT’ and ‘11-KT & JAK inhibitor’ groups (*p* = 0.14) or between ‘control’ and ‘JAK inhibitor’ groups (*p* = 0.65) (Fig. 8c).

Taken together, we showed that the inhibitors of IGF-1R, PI3K, AKT, and mTOR were highly effective in suppressing the 11-KT-induced increase of GnRH3 neurons in brain slices originating from mature females. Contrastingly, inhibitors of ERK and JAK did not significantly suppress the effect of 11-KT on the number of GnRH3 neurons.

**Discussion**

**Role of IGF-1 and IGF-1R/PI3K/AKT/mTOR signalling pathway with respect to the effects of 11-KT on GnRH3 neurons in the female tilapia brain.** According to the present results obtained with the TN-containing brain slices of mature females (Fig. 2), the *in vitro* treatment with 11-KT increased the number of GnRH3 neurons, indicating that 11-KT can increase the number of GnRH3 neurons without mediation via other organs, such as the gonad and liver. Since there are no AR binding sites in the promoter regions of GnRH3 gene in tilapia26, we considered that IGF-1 might mediate the action of 11-KT on GnRH3 neurons. Our hypothesis is strongly supported by the following four findings: (1) Treatment with IGF-1 increased the number of GnRH3 neurons (Fig. 3). (2) The expression of IGF-1 mRNA increased shortly after 11-KT treatment (Fig. 4). (3) The potent IGF-1R inhibitor, BMS-754807, strongly suppressed the 11-KT-induced increase of GnRH3 neurons (Fig. 5). (4) Immunohistochemical staining with anti-GnRH3 and anti-IGF-1R antibodies revealed the co-localization of IGF-1R and GnRH3 (Fig. 6). These results support the possibility that the effect of 11-KT on GnRH3 neurons is mediated by IGF-1 produced in the brain and IGF-1R on GnRH3 neurons. Furthermore, studies using inhibitors (Figs. 5, 7, and 8) clearly indicate the contribution of the IGF-1R downstream signalling pathway, PI3K/AKT/mTOR, to the effect of 11-KT on GnRH3 neurons. Therefore, our findings indicate that the effect of 11-KT on the number of GnRH3 neurons is mediated by the IGF-1 and its receptor, although it cannot be completely ruled out that 11-KT affects GnRH3 neurons directly.
The role of IGF-1 and IGF-1R/PI3K/AKT/mTOR signalling pathway in the regulatory process of GnRH neurons reported in other animals. IGF-1 is known to possess powerful neuroprotective effects in promoting neuronal survival, neuronal differentiation, neurite elongation, neurogenesis, and neurite regeneration in vivo and in vitro. It is a regulator of reproductive neuroendocrine function that affects the development and functions of GnRH neurons in the vertebrate brain, although this has been reported mainly in mammals. GnRH perikarya express IGF-1R before puberty in mice, suggesting a potential direct anatomical locus where IGF-1 can control reproductive development and function. Furthermore, the action of IGF-1 on IGF-1R in GnRH neurons regulates the onset of puberty in rodents. In fish like Mozambique tilapia, while IGF-1 and IGF-2 are mainly produced in the liver, their transcripts are expressed in various tissues including the brain. The absolute amounts of Igf-1 and Igf-2 mRNAs were also determined in many regions, such as the liver and brain of Nile tilapia. In yellow catfish, the expression of Igf-1 mRNA in the hypothalamus was higher in adult males than in adult females. Additionally, the effect of...
Reduced involvement of ERK and JAK in the 11-KT-induced increase of GnRH3 neurons. IGF-1R signalling can also activate the Ras/Raf/Mek/ERK pathway, which is broadly involved in the regulation of neuron proliferation, differentiation, and apoptosis. The co-activation of the ERK and AKT signalling pathways has been reported to promote the survival of Gn10 GnRH cells or GnRH neurons. Additionally, positive regulation of Gnrh mRNA levels induced by kisspeptin was mediated through the activation of ERK and AKT signalling pathways in mammalian GT1-7 (a clonal cell line of mature, differentiated GnRH neurons) and GN11 (a clonal cell line with GnRH expression) cells. JAK2 is necessary for the neuroendocrine control of female reproduction and development of GnRH neurons. In teleosts, there are only a few studies on IGF-1R downstream signalling pathways in GnRH neurons. Onuma et al. revealed that IGF-1 affects the development of GnRH2 and GnRH3 through IGF-1R in zebrafish embryos. They also examined whether PI3K inhibitors and/or ERK inhibitors disturbed the distribution of GnRH3 neurons in embryos. Treatment of embryos with a PI3K inhibitor (LY294002 or wortmannin) caused abnormal GnRH3 distribution similar to that in IGF signalling-deficient embryos. Contrastingly, U0126 had no effects. These results indicate that the influence of IGF-1 on the development and migration of GnRH2 and GnRH3 neurons was mediated through IGF-1R and the subsequent PI3K signalling pathway. According to findings regarding GnRH neurons in mammals and teleosts, it is highly likely that IGF-1 and the IGF-1R/PI3K/AKT/mTOR pathway play an important regulatory role in GnRH3 neurons in Mozambique tilapia.

In our previous studies, when 11-KT induced male-specific reproductive behaviours in mature female tilapia, GnRH3 neurons were increased in the TN region. Moreover, newly proliferated GnRH3 neurons have been found in the TN of 11-KT-treated mature females. Considering these findings with the present results, we conclude that IGF-1 and the IGF-1R/PI3K/AKT/mTOR signalling pathway are likely involved in the 11-KT-induced sex reversal of the female brain in terms of GnRH3 neurons.

Materials and methods

Animals. All experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee of Toyo University Itakura Campus and all experiments were performed in accordance with relevant guidelines and regulations.

Brain slice cultures. Preparation of brain slices. Brain slices at the level of the TN were prepared according to the protocol provided by Dr. Satoshi Ogawa in Prof. Ishwar S. Parhar’s laboratory (Monash University, Malaysia). Mature female tilapia fish were deeply anaesthetized with 0.2% 3-aminobenzoic acid ethyl ester (MS222, Sigma-Aldrich, St. Louis, MO, USA) and decapitated. The brain was removed and stored in cooled Ringer’s saline containing 124 mM NaCl, 3 mM KCl, 2 mM CaCl2, 2 mM MgSO4, 1.25 mM Na3HPO4, 26 mM NaHCO3, and 10 mM glucose (pH 7.35). The brain area of the olfactory bulb to the middle of the telencephalon was dissected and embedded in 4% low melting point agarose (Sigma-Aldrich) within embedding moulds. The embedding moulds were then placed on ice. After the agarose hardened (~5 min), the embedded brain and agarose were glued to the stage of a vibratome (Dosaka-EM Co, Ltd, Kyoto, Japan) and cut into 200-μm cross-sectional slices. The slices that covered all the GnRH neurons in the TN were collected in a dish containing Wash Medium [DMEM phenol free (Thermo Fisher Scientific, Waltham, MA, USA); supplemented with 134 U/ml penicillin (Cosmo Bio Co., Ltd, Tokyo, Japan), 0.25 glutamate (Sigma-Aldrich), 1 × B-27 serum-free (Thermo Fisher Scientific), 0.13 mM streptomycin (MeijiSeika Pharma, Tokyo, Japan), and 0.5 L-glutamine (Sigma-Aldrich)]. Each slice was then divided into equal halves by cutting at the midline (Fig. 1), and the halves were separately transferred to wells of a sterilised 96-well culture plate (Corning Inc., Corning, NY, USA) containing 200 μl of...
culture medium (CM) [Lebovitz's 15 (L-15) medium (Sigma-Aldrich) supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 mM HEPES buffer (Sigma-Aldrich), and 10 mM D-glucose (Wako Pure Chemical Co., Osaka, Japan)]. The plates were then incubated at 25 °C.

The CM was changed every 24 h. After brain slices were cultured for 72 h, they were fixed with 4% paraformaldehyde solution in phosphate buffered saline (PBS) for 1 h for immunocytochemistry.

**Treatment of brain slices with 11-KT or E2.** First, 10 mM 11-KT or E2 was prepared in 99.5% ethanol (Wako Pure Chemical Co.). In the experimental groups, after 11-KT and E2 were diluted with CM, they were added to a 96-well plate at final concentrations of 1, 10, 100, and 1000 nM for 11-KT and 10 nM for E2. For the control groups, ethanol was added at the same concentration as that of the corresponding experimental group. The slices at the TN were cut into left and right halves (Fig. 1). One half of the brain slice was treated with either 11-KT or E2 (11-KT or E2 group) and the other half was treated with ethanol (control group). We used 3–5 animals for the respective concentrations of 11-KT and four animals for 10 nM E2.

**Treatment of brain slices with IGF-1.** A solution of 50 μM IGF-1 (IGF-1 Gilthead Seabream, PROSPEC, Hamada, Israel) was prepared using CM. Then, IGF-1 was added to a 96-well plate at a final concentration of 10 or 100 nM. For the control group, an equal volume of CM was added to each well. Slices at the TN were cut into left and right halves, which were used for the IGF-1 and control groups, respectively. Thus, IGF-1-treated and control slices originated from the same fish. We used slices from 3 to 5 animals for the respective concentrations of IGF-1.

**Treatment of brain slices with 11-KT and/or one of the inhibitors.** The following inhibitors were used in this study: IGF-1R inhibitor (BMS-754807; Funakoshi Co. Ltd., Tokyo, Japan), PI3K inhibitor (LY294002; Wako Pure Chemical Co.), AKT inhibitor (GDC-0068; Funakoshi Co. Ltd.), mTOR inhibitor (Rapa-mycin, Funakoshi Co. Ltd.), ERK inhibitor (U0126; Cosmo Bio Co., Ltd.), and JAK inhibitor (InSolution™ JAK inhibitor I, Calbiochem, San Diego, CA, USA).

The right and left halves of brain slices were used. In ‘the control group’, the slice was incubated in the CM containing solvent (ethanol, DMSO, or saline) at the same concentration as that used to dissolve the respective inhibitor and 11-KT. In ‘the inhibitor group’, the slice was incubated in CM containing one of the inhibitors and the solvent to dissolve 11-KT. In ‘the 11-KT group’, the slice was incubated with 10 nM 11-KT, and the solvent was added at the same concentration as that used to dissolve the respective inhibitor. In ‘the 11-KT + inhibitor group’, the slices were incubated in CM containing 10 nM 11-KT and one of the inhibitors.

The slices for ‘control’ and ‘inhibitor’ groups originated from the same animal. Similarly, the slices for ‘11-KT’ and ‘11-KT + inhibitor’ groups originated from the same animal. We used 10–14 animals for the following four groups: the ‘control’, ‘inhibitor’, ‘11-KT’, and ‘11-KT + inhibitor’ groups.

**Immunostaining with anti-GnRH3 antibodies.** The brain slices at 72 h after cultivation were fixed with 4% paraformaldehyde. They were washed gently with PBS three times. Immunocytochemistry with an anti-GnRH3 antibody was performed using a custom-made rabbit anti-GnRH3 antibody (×7000, produced by MyBioSource, Com., San Diego, CA, USA). Since these two antibodies were polyclonal antibodies raised in rabbits, a pair of mirror-image frozen sections was immunostained separately with those antibodies. The sections were stained either with one of the antibodies against GnRH3 (×7000) and IGF-1R (×500, ThermoFisher Scientific, Ltd.). Hoechst 33342 solution (1000×) was used for nuclear staining. Finally, coverslips were mounted with ProLong Gold antifade reagent. Stained sections were observed using a confocal laser microscope.
Real-time qRT-PCR analysis. Nine animals were used in this experiment. Brain slices at the TN were cut into left and right halves, which were used for the 11-KT (10 nM) and control groups, respectively. Thus, 11-KT-treated and control slices originated from the same fish. The RNA samples were collected from the slices 1 h after the treatment. Reverse transcription and real-time qRT-PCR were performed as reported previously \(^{26,27}\). Cytoskeletal \(\beta\)-actin mRNA was used as a reference for qRT-PCR assays. The primer pairs used are as follows: CTGGTGGAGAGGAGGCTTTTT and TTGGGAGTCTTGACAGTGTC for \(Igf-1\), and AGCCCAACAGGAGAAGATGCTGCGAGATGACGATAC for \(\beta\)-actin.

Statistical analysis. Numerical data are presented as means ± standard error of the mean. The significance of differences between the control and experimental groups (Fig. 2, 3, and 4) was determined by the Student’s paired \(t\)-test, where \(p < 0.05\) was considered statistically significant. In these experiments, the half brain slices for experimental and control groups originated from the same animals. For the control groups, the solvent was added to the medium at the same concentration as the corresponding experimental group.

In the experiments involving treatments with 11-KT and/or inhibitors (Fig. 5, 7, and 8), the effect of two factors (11-KT and the inhibitor treatments) on the number of GnRH3 neurons was analyzed by a two-way analysis of variance (ANOVA). When an interaction between the two factors was significant, the Tukey–Kramer test was used as a post-hoc test. In both cases, \(p < 0.05\) was considered statistically significant. Because two-way ANOVA and commonly used post-hoc tests such as the Tukey test are not suitable for the comparison of individuals with the correspondence, we used a paired \(t\)-test for the statistical analysis of the numbers of GnRH3 neurons in the half brain slices originating from the same animal. Furthermore, we used Bonferroni correction to adjust the alpha \((\alpha)\) level for a family of statistical tests so that we control the probability of committing a type I error. The formula for a Bonferroni Correction is as follows: \(\alpha_{\text{new}} = \alpha_{\text{original}} / n\). Paired \(t\)-test was conducted two times (control group vs inhibitor group; 11-KT group vs \('11\)-KT and inhibitor’ group) for each inhibitor experiment. Thus, \(p < 0.025\), which was calculated from 0.05/2, was considered statistically significant in Figs. 5, 7, and 8.

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Received: 8 June 2021; Accepted: 24 January 2022

Published online: 14 February 2022

References

1. Liu, H. et al. Sexual plasticity: a fishy tale. Mol. Reprod. Dev. 84(2), 171–194. https://doi.org/10.1002/mrd.22691 (2017).
2. Godwin, J. Neuroendocrinology of sexual plasticity in teleost fishes. Front. Neuroendocrinol. 31(2), 203–216. https://doi.org/10.1016/j.yfrne.2010.02.002 (2010).
3. Senior, A. M., Nat Lim, J. & Nakagawa, S. The fitness consequences of environmental sex reversal in fish: a quantitative review. Biol. Rev. Camb. Philos. Soc. 87(4), 900–911 (2012). doi:https://doi.org/10.1111/j.1469-185X.2012.00230.x
4. Bass, A. H. & Grober, M. S. Social and neural modulation of sexual plasticity in teleost fish. Brain Behav. Evol. 57(5), 293–300. https://doi.org/10.1159/000047247 (2001).
5. Munakata, A. & Kobayashi, M. Endocrine control of sexual behavior in teleost fish. Gen. Comp. Endocrinol. 165(3), 456–468. https://doi.org/10.1016/j.ygcen.2009.04.011 (2010).
6. Okubo, K., Miyazoe, D. & Nishiike, Y. A conceptual framework for understanding sexual differentiation of the teleost brain. Gen. Comp. Endocrinol. 284, 113129. https://doi.org/10.1016/j.ygcen.2019.02.020 (2019).
7. Clemens, H. P. & Inglese, T. The production of unisexual broods by tilapia mossambica sex-reversed with methyltestosterone. Trans. Am. Fish. Soc. 97(1), 18–21. https://doi.org/10.1577/1548-8659(1968)77<18:TPOUSB>2.0.CO;2 (1968).
8. Kobayashi, T., Kajiiura-Kobayashi, H., Guan, G. & Nagahama, Y. Sexual dimorphic expression of DMRT1 and Sox9 during gonadal differentiation and hormone-induced sex reversal in the teleost fish Nile tilapia (Oreochromis niloticus). Dev. Dyn. 237(1), 297–306. https://doi.org/10.1002/dvdy.2409 (2008).
9. Ijiri, S. et al. Sexual dimorphic expression of genes in gonads during early differentiation of a teleost fish, the Nile tilapia Oreochromis niloticus. Biol. Reprod. 78(2), 333–341. https://doi.org/10.1095/biolreprod.107.064246 (2008).
10. Wang, D. S. et al. Foxl2 up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with and binding protein/steroidogenic factor 1. Mol. Endocrinol. 21(3), 712–725. https://doi.org/10.1210/me.2006-0248 (2007).
11. Wang, D. S. et al. Doublesex- and Mab-3-related transcription factor-1 repression of aromatase transcription, a possible mechanism favoring the male pathway in tilapia. Endocrinology 151(3), 1331–1340. https://doi.org/10.1210/en.2009-0999 (2010).
12. Sun, L. N. et al. Transdifferentiation of differentiated ovaries into functional testis by long-term treatment of aromatase inhibitor in Nile tilapia. Endocrinology 155(4), 1476–1488. https://doi.org/10.1210/en.2013-1939 (2014).
13. Shi, H. et al. Blockage of androgen and administration of estrogen induce transdifferentiation of testis into ovary. J. Endocrinol. 233(1), 65–80. https://doi.org/10.1530/JOE-16-0551 (2017).
14. Kuramochi, A., Tsutiyi, A., Kaneko, T. & Ohtani-Kaneko, R. Sexual dimorphism of gonadotropin-releasing hormone type-III (GnRH3) neurons and hormonal sex reversal of male reproductive behavior in Mozambique tilapia. Zoolog. Sci. 28(10), 733–739. https://doi.org/10.2108/zjs.28.733 (2011).
15. Goebbels, A. & Sower, S. A. Evolution of the role of GnRH in animal (Metazoan) biology. Gen. Comp. Endocrinol. 134(3), 207–213. https://doi.org/10.1016/j.ygcen.2003.09.018 (2003).
16. Morgan, K. & Millar, R. P. Evolution of GnRH ligand precursors and GnRH receptors in protocadherate and vertebrate species. Gen. Comp. Endocrinol. 139(3), 191–197. https://doi.org/10.1016/j.ygcen.2004.09.015 (2004).
17. Tsai, P. S. Gonadotropin-releasing hormone in vertebrates: structure, function, and evolution. Gen. Comp. Endocrinol. 148(1), 48–53. https://doi.org/10.1016/j.ygcen.2005.09.016 (2006).
18. White, S. A., Kasten, T. L., Bond, C. T., Adelman, J. P. & Fernald, T. D. Three gonadotropin-releasing hormone genes in one organism suggest novel roles for an ancient peptide. Proc. Natl Acad. Sci. USA. 92(18), 8363–8367 (1995). https://doi.org/10.1073/pnas.92.18.8363
19. Parhar, I. S., Pfaff, D. W. & Schwanzel-Fukuda, M. Gonadotropin-releasing hormone gene expression in teleosts. Brain Res. Mol. Brain Res. 41(1–2), 216–227. https://doi.org/10.1016/0169-328x(96)00099-x (1996).
20. White, R. B. & Fernald, R. D. Genomic structure and expression sites of three gonadotropin-releasing hormone genes in one species. *Gen. Comp. Endocrinol.* **112**(1), 17–25. https://doi.org/10.1016/j.gene.1998.7125 (1998).

21. Yamamoto, N., Oka, Y. & Kawashima, S. Lesions of gonadotropin-releasing hormone-immunoreactive terminal nerve cells on the reproductive behavior of male dwarf gourami. *Neuroendocrinology* **65**(6), 403–12 (1997).

22. Abe, H. & Oka, Y. Mechanisms of neuromodulation by a nonhypophysiotropic GnRH system controlling motility of reproducive behavior in the teleost brain. *J. Reprod. Dev.* **57**(6), 665–674. https://doi.org/10.1262/jrd.11-055e (2011).

23. Okuyama, et al. A neural mechanism underlying mating preferences for familiar individuals in medaka fish. *Science* **343**(6166), 91–4. https://doi.org/10.1126/science.1244774 (2014).

24. Ogawa, S. et al. Immunoneutralization of gonadotropin-releasing hormone type-III suppresses male reproductive behavior of cichlids. *Neurosci. Lett.* **403**(3), 201–205. https://doi.org/10.1016/j.neulet.2006.02.041 (2006).

25. Nanta, Y. et al. Androgen induced cellular proliferation, neurogenesis, and generation of GnRH3 neurons in the brain of mature female Mozambique tilapia. *Sci. Rep.* **8**(1), 16855. https://doi.org/10.1038/s41598-018-35303-9 (2018).

26. Kitahashi, T., Sato, H., Sakuma, Y. & Parhar, I. S. Cloning and functional analysis of promoters of three GnRH genes in a cichlid. *Biochem. Biophys. Res. Commun.* **336**(2), 536–543. https://doi.org/10.1016/j.bbrc.2005.08.122 (2005).

27. Veldhuis, J. D. et al. Somatotropin and gonadotropin axes linkages in infancy, childhood, and the puberty-adult transition. *Endocrinol. Rev.* **27**(2), 101–40. https://doi.org/10.1210/er.2005-0006 (2006).

28. Danziger, B. & Swanson, E. M. Mediation of vertebrate life histories via insulin-like growth factor-1. *Biol. Rev. Camb. Philos. Soc.* **87**(2), 414–429. https://doi.org/10.1111/1469-185X.12004.x (2012).

29. Neirijnck, Y., Papaioannou & Nef. The insulin/IGF system in mammalian sexual development and reproduction. *Int. J. Mol. Sci.* **20**(18), 4440–4499 (2019). https://doi.org/10.3390/ijms20184440

30. Zempo, B., Kanda, S., Okubo, K., Akazome, Y. & Oka, Y. Anatomical distribution of sex steroid hormone receptors in the brain of males and females of the dwarf gourami *Poecilia reticulata*. *J. Neuroendocrinol.* **33**(6), 579–587. https://doi.org/10.1111/jne.12225 (2021).

31. Ogawa, S. & Parhar, I. S. Single-cell gene profiling reveals social status-dependent modulation of nuclear hormone receptors in GnRH neurons in a male cichlid fish. *Int. J. Mol. Sci.* **21**(8), 2724. https://doi.org/10.3390/ijms21082724 (2020).

32. Dyer, A. H., Vahdatpour, C., Saneflu, A. & Tropea, D. The role of insulin-like growth factor 1 (IGF-1) in brain development, maturation and neuroplasticity. *Neurosci. Lett.* **525**(2), 89–99. https://doi.org/10.1016/j.neulet.2016.03.056 (2016).

33. Daftary, S. S. & Gore, A. C. IGF-1 in the brain as a regulator of reproductive neuroendocrine function. *Exp. Biol. Med. (Maywood)* **230**(5), 292–306. https://doi.org/10.1177/153537020230005003 (2005).

34. Wolfe, A., Divalle, S. & Wu, S. The regulation of reproductive neuroendocrine function by insulin and insulin-like growth factor-1 (IGF-1). *Front. Neuroendocrinol.* **35**(4), 538–572. https://doi.org/10.1016/j.yfrne.2014.05.007 (2014).

35. Daftary, S. S. & Gore, A. C. The hypothalamic insulin-like growth factor-1 receptor and its relationship to gonadotropin-releasing hormones neurons during postnatal development. *J. Neuroendocrinol.* **16**(2), 160–169. https://doi.org/10.1111/j.1365-2826.2004.01190.x (2004).

36. DiVall, S. A. et al. Divergent roles of growth factors in the GnRH regulation of puberty in mice. *J. Clin. Invest.* **120**(8), 2900–2909. https://doi.org/10.1172/JCI41069 (2010).

37. Dees, W. L., Hiney, J. K. & Srivastava, V. K. IGF-1 influences gonadotropin-releasing hormone regulation of puberty. *Neuroendocrinology* https://doi.org/10.1159/000514217 (2021).

38. Reinecke, M., Schmid, A., Ermatinger, R. & Loffing-Cueni, D. Insulin-like growth factor 1 in the teleost Oresochromis mossambicus, the tilapia: gene sequence, tissue expression, and cellular localization. *Endocrinologist* **13**(9), 3613–3619. https://doi.org/10.1007/s12039.03735 (1997).

39. Caelders, A., Berishvili, G., Meli, M. L., Eppler, E. & Reinecke, M. Establishment of a real-time RT-PCR for the determination of absolute amounts of IGF-I and IGF-II gene expression in liver and extrahepatic sites of the tilapia. *Gen. Comp. Endocrinol.* **137**(2), 196–204. https://doi.org/10.1016/j.ygcen.2003.09.006 (2004).

40. Ma, W. et al. Sex differences in the expression of GH/IGF axis genes underlie sexual size dimorphism in the yellow catfish (*Peleobagrus fulvidraco*). *Sci. China Life Sci.* **59**(4), 431–433 (2016). https://doi.org/10.1007/s11427-015-4957-6

41. Shao, P. et al. The interference of DEHP in precocious puberty of females mediated by the hypothalamic IGF-1/Pi3K/Akt/mTOR signalling pathway. *Ecotoxicol. Environ. Saf.* **181**, 362–369. https://doi.org/10.1016/j.ecoenv.2019.06.017 (2019).

42. Balint, F. et al. Insulin-like growth factor 1 (IGF-1) increases GABAergic neurotransmission to GnRH neurons via suppressing the retrograde tonic endocannabinoid signaling pathway in mice. *Neuroendocrinology* (2020). https://doi.org/10.1159/000514043

43. Online. Ahead of print.

44. Onuma, T. A. et al. Regulation of temporal and spatial organization of newborn GnRH neurons by IGF signaling in zebrafish. *J. Neurosci.* **31**(33), 11814–11824. https://doi.org/10.1523/JNEUROSCI.2980-11.2011 (2011).
Acknowledgements
We would like to acknowledge Dr. Satoshi Ogawa and Prof. Ishwar S. Parhar's (Jeffrey Cheah School of Medicine and Health Sciences and the Brain Research Institute, Monash University, Malaysia) for kindly giving us their protocol for cultivating brain slices of tilapia. We also thank Prof. Hwang (Academia Sinica, Taiwan) for his helpful supports and useful advice. We appreciate Prof. Junya Hiroi (St. Marianna University School of Medicine) for his help in performing data analysis.

Author contributions
R.O.-K. conceived and designed the experiments, and wrote the manuscript. A.O., S.I., R.K., Y.N. and S.S. carried out the experiments and data analysis. T.K. and Y-C.T contributed to sample preparation and aided in interpretation of results and writing of manuscript. All authors reviewed the manuscript.

Funding
This study was supported in part by grants from Toyo University (the Inoue Enryo Memorial Foundation, Research Institute of Industrial Technology, and Research Center for Biomedical Engineering).

Competing interests
The authors declare no competing interests.

Additional information
Correspondence
Correspondence and requests for materials should be addressed to R.O.-K.

Reprints and permissions information
Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access
This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022