Development/Plasticity/Repair

Regulation of Temporal and Spatial Organization of Newborn GnRH Neurons by IGF Signaling in Zebrafish

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When and how newborn neurons are organized to form a functional network in the developing brain remains poorly understood. An attractive model is the gonadotropin-releasing hormone (GnRH) neuron system, master regulator of the reproductive axis. Here we show that blockage of IGF signaling, a central growth-promoting signaling pathway, by the induced expression of a dominant-negative form of IGF1 receptor (IGF1R) or specific IGF1R inhibitors delayed the emergence of GnRH2 neurons in the midbrain and GnRH3 neurons in the olfactory bulb region. Blockage of IGF signaling also resulted in an abnormal appearance of GnRH3 neurons outside of the olfactory bulb region, although it did not change the locations of other olfactory neurons, GnRH2 neurons, or brain patterning. This IGF action is developmental stage-dependent because the blockade of IGF signaling in advanced embryos had no such effect. An application of phosphatidylinositol 3-kinase (PI3K) inhibitors phenocopied the IGF signaling deficient embryos, whereas the MAPK inhibitors had no effect, suggesting that this IGF action is mediated through the PI3K pathway. Real-time *in vivo* imaging studies revealed that the ectopic GnRH3 neurons emerged at the same time as the normal GnRH3 neurons in IGF-deficient embryos. Further experiments suggest that IGF signaling affects the spatial distribution of newborn GnRH3 neurons by influencing neural crest cell migration and/or differentiation. These results suggest that the IGF-IGF1R-PI3K pathway regulates the precise temporal and spatial organization of GnRH neurons in zebrafish and provides new insights into the regulation of GnRH neuron development.

Introduction

When and how newborn neurons are organized to form a functional network in the CNS remains poorly understood (Ayala et al., 2007). An attractive model involves the gonadotropin-releasing hormone (GnRH) neurons, master regulators of the vertebrate reproductive axis. During development, GnRH neurons conduct a long-distance migration, starting from the olfactory region and reaching the preoptic area (POA) and hypothalamus (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989). Many vertebrate species have multiple forms of GnRHs. For instance, there are two forms of GnRHs in zebrafish, namely GnRH2 and GnRH3. The zebrafish GnRH3 neurons are the counterparts of the mammalian hypophysiotropic GnRH1 neurons and are located at the preoptic area–hypothalamus and olfactory bulb–terminal nerve in the adult brain (Steven et al., 2003; Palevitch et al., 2007). Hypothalamic GnRH3 neurons are found to be originated from the olfactory area (Abra-

ham et al., 2008, 2010) and GnRH2 neurons are located in the midbrain in zebrafish (Abraham et al., 2010). Previous studies have suggested that both zebrafish GnRH3 neurons and GnRH2 neurons may originate from the cranial neural crest (Whitlock et al., 2003, 2005), although their precise embryonic origins and relationship are still under debate.

While the routes and timing of GnRH neuron migration have been elucidated, the underlying regulatory mechanisms are less clear (Tobet and Schwarting, 2006). Insulin-like growth factors (IGFs) are major embryonic growth-promoting factors (Duan and Xu, 2005). IGFs regulate many aspects of brain development in mice and zebrafish, such as neuron differentiation, survival, and migration (Bondy and Cheng, 2004; Schlueter et al., 2007; Joseph D'Ercole and Ye, 2008; Annenkov, 2009). Mammalian GnRH neurons coexpress IGF1 and the IGF1 receptor (IGF1R) in postnatal and adult stages *in vivo* (Daftary and Gore, 2005). IGF1 stimulates cell proliferation and GnRH gene expression in mammalian GnRH neuronal cell lines (Zhen et al., 1997; Longo et al., 1998) and modulates GnRH activities in cultured pituitary cells isolated from salmonid fish (Weil et al., 1999; Baker et al., 2000; Ando et al., 2006; Furukuma et al., 2008; Luckenbach et al., 2010). IGF1 has also been shown to regulate the migration of neural crest-derived melanoblasts in chick embryos (Schöfer et al., 2001). In young rats, pharmacological inhibition of brain IGF signaling impairs estrous cyclicity (Todd et al., 2007) and reduced brain IGF signaling in middle-aged female rats leads to luteinizing hormone surge dysfunction by affecting estrogen-dependent processes that influence GnRH neuron activation and/or GnRH

release (Todd et al., 2010). Despite these studies, it is unclear whether IGF signaling plays any role in GnRH neuron development during embryogenesis.

In this study, we used the zebrafish model to test the hypothesis that IGF signaling regulates the proper spatial and temporal organization of GnRH neurons in early development.

The free-living and transparent zebrafish embryos are well suited for investigation of the regulation of GnRH neuron development. The availability of the Tg(*GnRH3:EGFP*) line, in which GnRH3 neurons express EGFP (Abraham et al., 2008), provides a powerful system to visualize GnRH neurons in living embryos. Our results suggest that IGF signaling is critical for the timing of both GnRH2 and GnRH3 neuron emergence as well as for the spatial distribution of GnRH3 neurons.

Materials and Methods

Materials. All chemicals and reagents were purchased from Fisher Scientific unless noted otherwise. Superscript III reverse transcriptase and oligonucleotide primers were purchased from Invitrogen Life Technologies. The anti-total and Phospho-Akt antibodies were purchased from Cell Signaling Technology. The polyclonal anti-IGF1R antibody (C-20) was purchased from Santa Cruz Biotechnology and the fibroblast growth factor receptor 1a (FGFR1) antibody was from Ana Spec. The anti-phosphotyrosine antibody (4G10) was purchased from Millipore. A selective inhibitor of IGF1R kinase, NVP-AEW541 (García-Echeverría et al., 2004), was provided by Novartis Institutes. BMS-754807, another structurally distinct IGF1R inhibitor (Wittman et al., 2009), was purchased from Active BioChem. The phosphatidylinositol 3-kinase (PI3K) inhibitor LY-294002 and the MEK inhibitor U0126 were purchased from Biomol. The PI3K inhibitor wortmannin was purchased from Sigma. The olfactory marker protein (OMP) and transient receptor potential channel 2 (TRPC2) cDNAs were kindly provided by Dr. N. Miyasaka and Y. Yoshihara (RIKEN Brain Science Institute, Saitama, Japan) and Prof. R. Celik (Bogazici University, Istanbul, Turkey). The *sox10* and *crestin* cDNAs were kindly provided by Prof. P. Henion (Ohio State University, Columbus, Ohio).

Animals. Zebrafish (*Danio rerio*) were maintained at 28°C on a 14:10 h (light:dark) cycle and fed twice daily. Males and females were maintained in separate tanks until the day of natural crossing to generate embryos. The fertilized eggs were raised at 28.5°C and staged according to Kimmel et al. (1995). Phenylthiourea (0.2 mM) was added to prevent pigmentation. Tg(*hsp70:dnIGF1R-GFP*) and Tg(*hsp70:GFP*) transgenic lines, which express a dominant-negative form of IGF1R-GFP (dnIGF1R-GFP) or GFP under the control of the zebrafish *hsp*70 promoter, were generated and maintained as reported (Kamei et al., 2011). The generation of the Tg(*GnRH3:EGFP*) transgenic line that expresses EGFP in GnRH3 neurons has been reported previously (Abraham et al., 2008). To study the role of IGF signaling on GnRH3 neurons, the Tg(*hsp70: dnIGF1R-GFP*) line was crossed with the Tg(*GnRH3:EGFP*) line. As a control, the Tg(*hsp70: GFP*) line was crossed with the Tg(*GnRH3:EGFP*) line. The Tg(*a1-tubulin1a:EGFP*) fish, in which developing neurons are labeled with expression of EGFP (Goldman et al., 2001), were provided by Dr. D. Goldman's group (University of Michigan, Ann Arbor, Michigan). All experiments were conducted in accordance with guidelines approved by the University Committee on the Use and Care of Animals, University of Michigan.

Genetic and pharmacological blockade of the IGF signaling. To verify the effectiveness of the heat shock induction of dnIGF1R:GFP and its effect in blocking IGF signaling, Tg(*hsp70:dnIGF1R-GFP*) embryos were subjected to heat shock (HS) treatment. Briefly, the embryos were placed in a 0.5 ml tube containing 0.4 ml of embryo-rearing solution and then placed in a PCR machine at 37°C for 1 or 0.5 h. Wild-type and Tg(*hsp70: GFP*) embryos were treated at the same time and used as controls. For pharmacological inhibition experiments, drugs were dissolved in DMSO and added in the embryo-rearing solution directly.

In situ hybridization. To generate the GnRH2 and GnRH3 probes, their partial cDNAs were cloned using the following primers: GnRH2 forward primer: 5′-GCATGGAGTGGAAAGGAAGG-3′ and reverse

primer: 5′-GGAGTCCAAAAA CATGGTCT-3′; GnRH3 forward primer: 5′-ATGGTGCTGGTCTGCAGGCT-3′ and reverse primer: 5′-GAACTGCTGCA AATGGGTAC-3′. cDNA prepared from adult zebrafish brains was used as template. The PCR products were cloned into pGEM-T Easy Vector System I (Promega). The synthesis of digoxigenin-labeled RNA riboprobe and hybridization were performed as previously reported (Maures et al., 2002). Photographs were taken under a Leica MZ16F microscope (Leica Microsystems).

Reverse transcription PCR. Total RNA was extracted using TRIzol reagent (Invitrogen). After DNase treatment (Invitrogen), RNA samples were reverse transcribed with random hexamer primers and SuperScript III reverse transcriptase (Invitrogen). PCR was performed using TaqDNA polymerase (Biolabs) under the following conditions: 94°C for 2 min, 94°C for 30 s, 58°C for 30 s, 70°C for 30 s, 25 cycles for β-actin, and 36 cycles for GnRH3. The following primers were used: GnRH3 forward primer: 5′-CAGCACTGGTCATATGGTTGGC-3′ and reverse primer: 5′-CACTCTTCCCCGTCTGTCGG-3′; β-*actin* forward primer: 5′-AGGC-CAACCGCGAGAAGATGACC-3′ and reverse primer: 5′-GAAGTC CAGGGCGACGTAGCAC-3′.

Immunoprecipitation and Western immunoblotting analysis. Western immunoblotting was performed as described previously (Schlueter et al., 2007). Approximately 60 embryos for each group were dechorionated, deyolked, homogenized, centrifuged, and subjected to immunoblotting analysis. For phospho-IGF1R and FGFR1, equal amounts of proteins were subjected to immunoprecipitation using the G416 phosphotyrosine antibody and followed by Western immunoblotting as described previously (Kamei et al., 2011).

Developmental rate. The somite number was used as an indicator of developmental rate up to 26 h postfertilization (hpf). For this, embryos were anesthetized and photographed. Their somite number was determined manually.

Statistics. Data are shown as means $\pm$ SEM. Statistical significance among experimental groups was determined by one-way ANOVA, followed by the Tukey's multiple-comparison test. Fisher's exact test, followed by the χ^2 test, was used to compare the percentage of embryos with GnRH2 or GnRH3 neurons between test groups with the control group. Significance was accepted at $p < 0.05$ or better.

Results

Genetic and pharmacological blockade of IGF1R signaling in zebrafish embryos

Two transgenic zebrafish lines, Tg(*hsp70:dnIGF1R-GFP*) and Tg(*hsp70:GFP*), that express a dominant-negative form of the zebrafish IGF1Ra or GFP alone under the control of the heat shock-inducible *hsp70* promoter (Kamei et al., 2011) were used to investigate the role of IGF signaling in GnRH neuron development. Fluorescence microscopy indicated robust expression of dnIGF1R-GFP or GFP expression after the HS treatment (1 h at 37°C) (Fig. 1*A*). No GFP signal in somatic cells was observed in wild-type embryos after the HS treatment. Induction of dnIGF1R-GFP expression caused growth retardation and developmental delay, as indicated by a smaller body size (Fig. 1*A*) and reduced somite number (Fig. 1*D*). Somite number is used as a quantitative parameter for determining the developmental stage during 10–24 hpf (Kimmel et al., 1995). As shown in Figure 1*C*, HS treatment resulted in a major reduction in the levels of phospho-IGF1R and phospho-Akt. HS treatment of the control Tg(*hsp70:GFP*) embryos did not alter IGF1R or Akt phosphorylation or growth (data not shown). As previously reported (Kamei et al., 2011), induction of dnIGF1R-GFP expression did not decrease the levels of phospho-FGFR1 (Fig. 1*C*). Although the phospho-FGFR1 levels in the HS treatment group appeared to be higher, this was likely loading/transfer variation, as indicated by similar high levels of total Akt (Fig. 1*C*).

As an independent and complimentary approach to block IGF signaling, we used NVP-AEW541 and BMS-754807, two specific and structurally distinct IGF1R kinase inhibitors (García-

Echeverria et al., 2004; Wittman et al., 2009). Treatments of zebrafish embryos with either of these inhibitors led to marked reduction in IGF1R and Akt phosphorylation and developmental delay (Fig. 1 *B–D*). NVP-AEW541 or BMS-754807 treatment did not decrease the levels of phospho-FGFR1 (Fig. 1*C*). In fact, there was an increase in the levels of phospho-FGFR in the BMS-754807 1 μM group, but the increase was smaller in the BMS-754807 3 μM group (Fig. 1*C*). The levels of total FGFR were also higher in these groups.

Blockade of IGF signaling delays the timing of GnRH3 neuron emergence

We next examined the possible effect of the blockade of IGF signaling on the emergence/differentiation of GnRH3 neurons. For this, the Tg(*hsp70:dnIGF1R-GFP*) embryos were treated with HS from 4 to 5 hpf, and embryos were sampled at 26 and 32 hpf (Fig. 2*A*). Reverse transcription (RT)-PCR analysis showed a strong reduction in the levels of GnRH3 mRNA in the HS-treated Tg(*hsp70:dnIGF1R-GFP*) embryos (Fig. 2*B*). As shown in Figure 2*C*, two clusters of GnRH3 neurons were detected in the forebrain region in 24% of wild-type embryos and 20% of untreated Tg(*hsp70:dnIGF1R-GFP*) embryos at 26 hpf, but none were observed in HS-treated Tg(*hsp70:dnIGF1R-GFP*) embryos at this stage. At 32 hpf, GnRH3 neurons were found in 90% of the HS-treated wild-type embryos and 100% of the Tg(*hsp70:dnIGF1R-GFP*) embryos without HS treatment (Fig. 2*C*). In contrast, only 22% of the HS-treated Tg(*hsp70:dnIGF1R-GFP*) embryos were GnRH3-positive (Fig. 2*C*).

To further study the role of IGF signaling in GnRH3 neuron development, we generated Tg(*hsp70:dnIGF1R-GFP/GnRH3:EGFP*) double-transgenic embryos. Tg(*hsp70:GFP/GnRH3:EGFP*) embryos were also generated and used as a control. As shown in Figure 2, *D* and *F*, HS treatment significantly reduced the percentage of GnRH3/GFP neuron-positive embryos in the Tg(*hsp70:GFP/GnRH3:EGFP*) group at 32 hpf, but had no such effect in the control embryos. Likewise, addition of either NVP-AEW541 or BMS-754807 significantly reduced the number of GnRH3/GFP neuron-positive embryos at 32 hpf (Fig. 2*E,F*). These effects were gradually weakened as embryogenesis progressed. At 50 hpf, the HS-treated Tg(*hsp70:dnIGF1R-GFP*) embryos completely caught up (Fig. 2*G*). These results suggest that genetic and pharmacological blockade of IGF signaling delays the timing of GnRH3 neuron emergence in zebrafish.
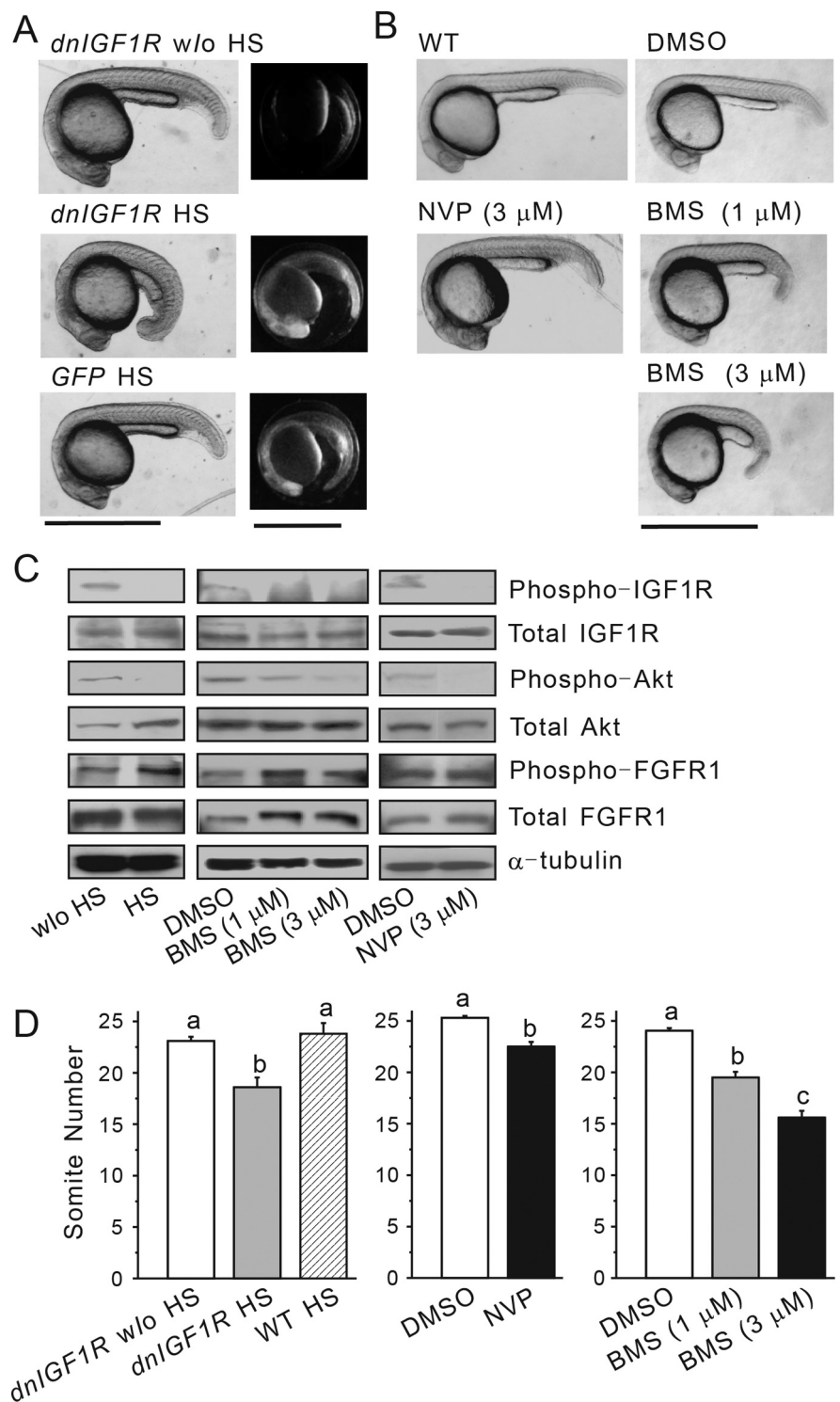

Figure 1. Temporally controlled genetic and pharmacological blockade of IGF signaling in zebrafish embryos. **A**, Inducible expression of a dominant-negative form of IGF1R. Tg(*hsp70:dnigf1r-GFP*) (dnIGF1R) or Tg(*hsp70:*GFP) (GFP) embryos were subjected to 37°C HS treatment at 4 hpf for 1 h and raised to 26 hpf and photographed. Phase-contrast views are shown in the left column and fluorescence views in the right column. Note GFP expression in somatic cells induced by HS. Yolk sac is autofluorescent. Scale bar, 1 mm. **B**, Pharmacological inhibition of IGF signaling. Wild-type embryos were treated with NVP-AEW541 (NVP), BMS-754807 (BMS), or the vehicle (DMSO) at the indicated concentrations from 4 to 26 hpf. A representative embryo from each group is shown. Scale bar, 1 mm. **C**, Efficient and specific blockade of IGF signaling. Embryos described in **A** and **B** were analyzed by Western blotting using the appropriate antibodies. For phosphorylated IGF1R and FGFR1, embryos were immunoprecipitated using an antiphosphotyrosine antibody followed by Western blotting using the appropriate antibodies. **D**, Effect of IGF signaling blockade on embryonic growth. Embryos were treated as described in **A** and **B** and raised to 26 hpf. Somite number was determined and shown. Data are mean ± SE, n = 7~20. Groups labeled with different letters are significantly different from each other ($p < 0.05$).
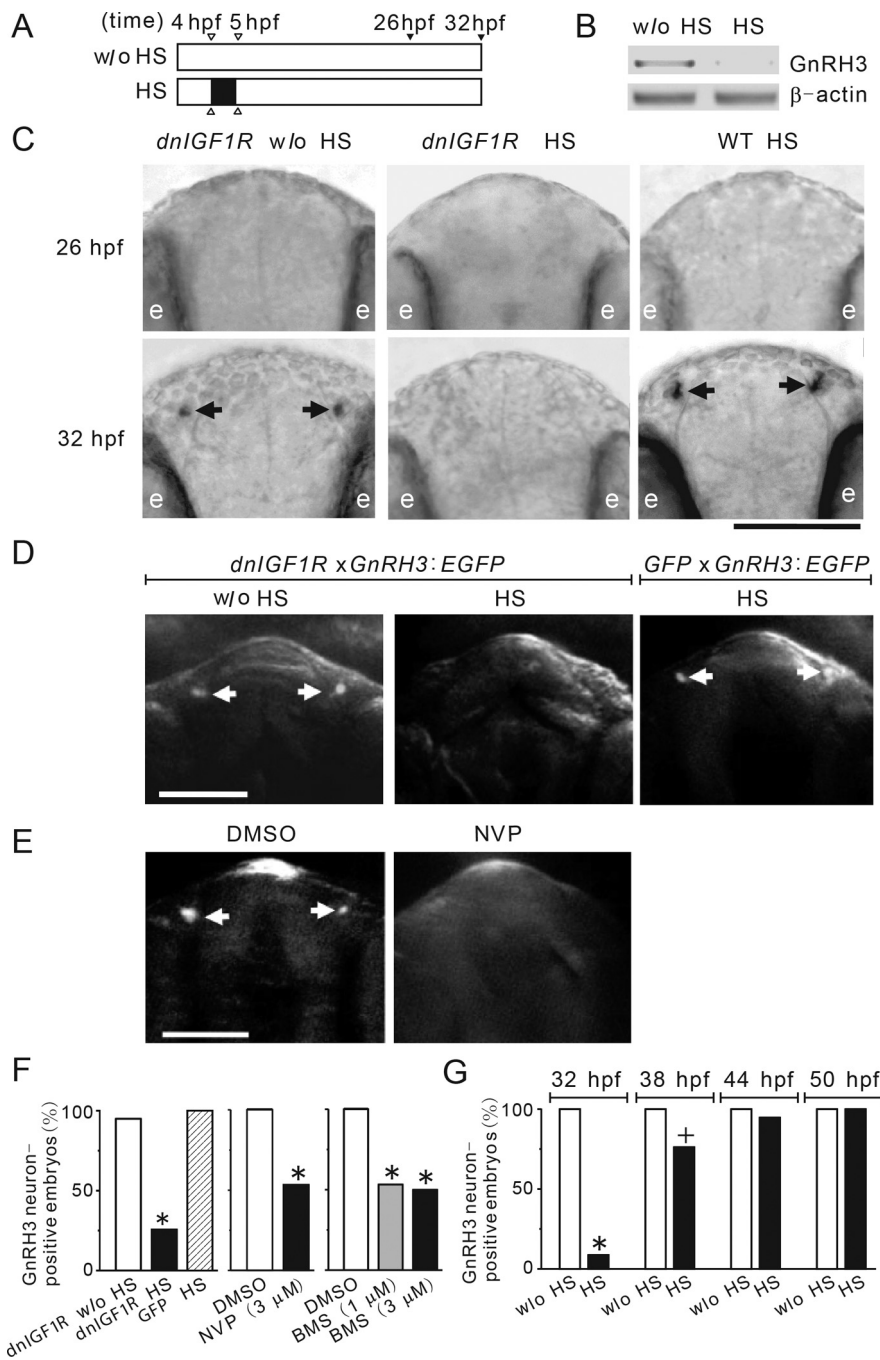
Figure 2. Blockade of IGF signaling delays the timing of GnRH3 neuron emergence. **A**, A schematic diagram illustrating the experimental design. Tg(*hsp70:dnigf1r-GFP*) embryos were subjected to 1 h 37°C HS treatment from 4 to 5 hpf or no HS treatment (w/o HS) and raised to 26 and/or 32 hpf and analyzed. **B**, RT-PCR result. Tg(*hsp70:dnigf1r-GFP*) described in **A** were raised to 26 hpf and analyzed by RT-PCR for GnRH3 and β-actin mRNA. **C**, Embryos described in **A** were analyzed by whole-mount *in situ* hybridization and representative views are shown in the bottom. Arrows indicate GnRH3 neurons. The locations of eyes are indicated by e. In this and all following figures, dorsal views are shown with anterior up unless stated otherwise. Scale bar, 0.1 mm. **D**, Tg(*hsp70:dnigf1r-GFP/GnRH3:EGFP*) and Tg(*hsp70:GFP/GnRH3:EGFP*) double-transgenic embryos were treated as described in **A**. GFP-positive GnRH3 neurons (arrows) were visualized in living embryos at 32 hpf. **E**, Tg(*GnRH3:EGFP*) embryos were treated with DMSO, 3 μM NVP-AEW541 (NVP) from 4 to 32 hpf and GFP-positive GnRH3 neurons (arrows) were visualized in living embryos at 32 hpf. **F**, Quantitative results of **D** and **E**. $n = 31 \sim 78$, *$p < 0.001$. **G**, Tg(*hsp70:dnigf1r-GFP*) embryos were subjected to one 1 h HS treatment at 4 hpf and two 0.5 h HS treatments at 26 and 38 hpf and raised up to the indicated time points. GnRH3 neurons were detected by whole-mount *in situ* hybridization. $n = 15 \sim 20$; +$p < 0.05$, *$p < 0.001$ compared with the without HS control group at the same time point. BMS, BMS-754807.

IGF signaling is required for the proper spatial organization of GnRH3 neurons

We next examined the role of IGF signaling on GnRH3 neuron spatial distribution. GnRH3 neurons formed two bilateral clusters in

the olfactory bulbs at 3 d postfertilization (dpf) and began to migrate caudally toward the telencephalon and hypothalamus until 7 dpf (Abraham et al. 2008). When IGF signaling was inhibited at 4 hpf by HS treatment and the distribution of GnRH3 neurons was examined at 3 and 7 dpf, many of them were detected in ectopic positions outside of the olfactory region (Fig. 3*A*). Two major abnormal phenotypes were observed. In phenotype 1, some GnRH3 neurons were observed outside the olfactory bulb region. In phenotype 2, the entire GnRH3 neuron clusters were dispersed outside the olfactory bulb region. Quantitative results indicate that the blockade of IGF signaling at the blastula stage (4 hpf) resulted in the appearance of ectopic GnRH3 in >60% of the embryos at 3 dpf (Fig. 3*A*,*C*). In contrast, inhibition of IGF signaling at 30 hpf had no effect on GnRH3 neuron distribution (Fig. 3*B*,*C*). Blockade of IGF signaling at 30 hpf resulted in growth retardation (data not shown). To ascertain that these ectopic GFP-positive cells are indeed GnRH3 mRNA-expressing cells, *in situ* hybridization analysis was performed. As shown in Figure 3*D*, GnRH3 mRNA was detected in normal and ectopic GFP-positive cells in all eight embryos examined, although the intensity of GnRH3 mRNA signals in these ectopic cells appeared to be weaker compared with that of the normal GnRH3 neurons (Fig. 3*D*).

We next investigated whether this action of IGF signaling is specific to GnRH3 neurons or represents a more general effect in the brain and/or the olfactory bulb region. As shown in Figure 3*E*, blockage of IGF signaling did not change in the following general brain marker genes: *emx1* expression in the forebrain; *pax2a* expression in the optic stalk, midbrain-hindbrain boundary, and hindbrain; *pax6a* expression in the eye, telencephalon, and diencephalon; *otx2* expression in the midbrain; and *krox20* expression in the third and fifth rhombomeres of the hindbrain. Likewise, no notable morphological change was found in the olfactory bulbs when IGF signaling was blocked in Tg(*α1-tubulin1a: EGFP*) embryos, in which EGFP is expressed in developing neurons under the control of the *α1-tubulin1a* promoter (Fig. 3*F*). Genes encoding the OMP and TRPC2 are known to be expressed in two distinct populations of neurons in the developing olfactory bulbs (Sato et al., 2005). As shown in Figure 3*G*, block-

age of IGF signaling did not change the locations of *omp*- and *trpc2* mRNA-expressing neurons. These results suggest that IGF signaling plays a specific role in regulating the proper spatial organization of GnRH3 neurons and that there is a
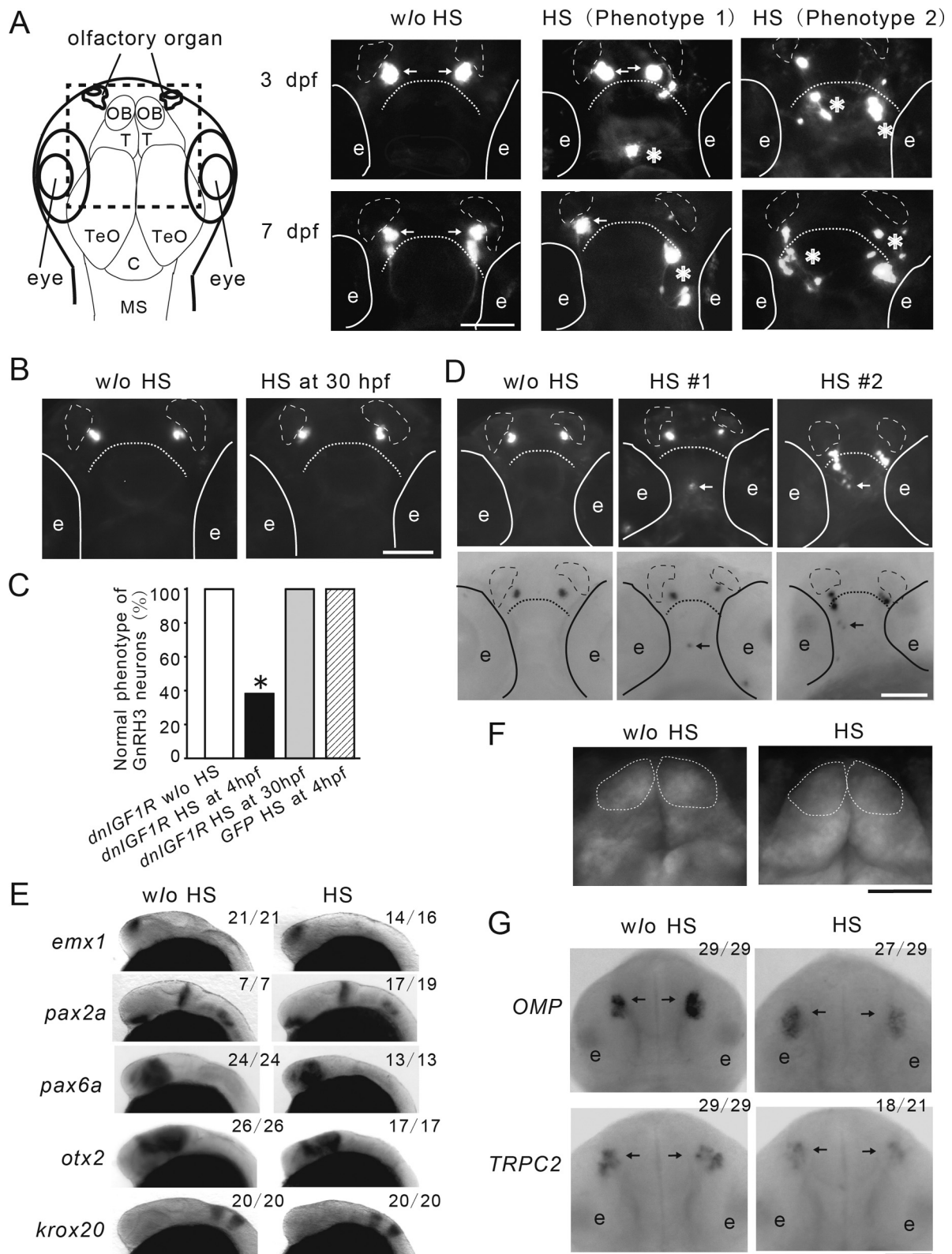
Figure 3. IGF signaling regulates the spatial organization of GnRH3 neurons; this action is developmental stage-dependent. ***A***, Blockade of IGF signaling in early embryos results in abnormal distribution of GnRH3 neurons in the larval brain. Left, Diagram illustrating the orientation and anatomy of zebrafish larval brain. OB, olfactory bulb; T, telencephalon; TeO, tectum opticum; C, cerebellum; MS, medulla spinalis. Tg(*hsp70:dnigf1r-GFP/GnRH3:EGFP*) double-transgenic embryos were treated as described in Figure 2*A* and raised to 3 and 7 dpf. The location of GFP-positive GnRH3 neurons was visualized and representative images are shown (right). GnRH3 neurons at the two normal locations are indicated by arrows. Aberrant GnRH3 neurons are indicated by asterisks. Solid lines indicate the locations of eyes (e). Broken lines represent olfactory organs. Dashed line denotes the boundary between the olfactory bulb and telencephalon. ***B***, Inhibition of IGF1R in advanced embryos has no effect. Tg(*hsp70:dnigf1r-GFP/GnRH3:EGFP*) double-transgenic embryos were subjected to 1 h HS treatment at 30 hpf. The embryos were raised to 3 dpf and the distribution of GnRH3 neurons was examined and shown. ***C***, Quantitative results of ***A*** and ***B***. n = 19∼41, *p < 0.001 compared with the no (w/o) HS group. ***D***, The ectopic GFP-positive cells are indeed GnRH3 neurons. Larvae (3 dpf old) described in ***A*** were subjected to *in situ* hybridization analysis for GnRH3mRNA. Top, GFP view; bottom, *in situ* hybridization results. (*Figure legend continues.*)

critical time window in early development for this action of IGF signaling.

IGF signaling regulates the timing of GnRH2 neuron emergence but not their locations

We next examined whether IGF signaling also regulates the temporal and spatial organization of GnRH2 neurons. In zebrafish, GnRH2 neurons emerge in the midbrain at ~26 hpf (Whitlock et al., 2003) (Fig. 4A). As shown in Figure 4, *A* and *B*, GnRH2 neurons were detected in the midbrain at 26 and 32 hpf in 100% of embryos in the HS-treated wild-type embryo group and the Tg(*hsp70:dnigf1r-GFP*)-without-HS treatment group. In comparison, only 25% of the HS-treated Tg(*hsp70:dnigf1r-GFP*) embryos had detectable GnRH2 neurons (Fig. 4A). RT-PCR analysis showed that the blockade of IGF signaling resulted in a major reduction in GnRH2 mRNA levels at 26 hpf (data not shown). At 32 hpf, however, GnRH2 neurons emerged in 100% of embryos in the HS-treated Tg(*hsp70:dnigf1r-GFP*) group. No ectopic GnRH2 neurons were detected in these IGF-deficient embryos (Fig. 4A). These data suggest that blockage of IGF signaling in early embryonic stage delays the timing of GnRH2 neuron emergence but has little effect on their location.

In zebrafish, the GnRH2 neurons migrate laterally and localize to the midline of the midbrain tegmentum by 72 hpf (Gopinath et al., 2004; Palevitch et al., 2007). We next determined the effect of the blockade of IGF signaling on GnRH2 neuron migration. For this, Tg(*hsp70:dnigf1r-GFP*) embryos were treated with two 0.5 h HS treatments at 32 and 44 hpf. The embryos were raised and GnRH2 neurons were detected by whole-mount *in situ* hybridization at 44 and 62 hpf (Fig. 4B). The migration index was determined and is shown in Figure 4C. While there was no difference between the groups at 32 hpf, the HS-treated Tg(*hsp70: dnigf1r-GFP*) group had a significantly greater value compared with the untreated transgenic embryo group or the HS-treated wild-type embryo group at 44 and 62 hpf. These data suggest that blockage of IGF signaling in advanced embryos slows down GnRH2 neuron migration.

IGF signaling regulates the spatial organization of newborn GnRH3 neurons by altering the migration and/or differentiation of neural crest cells

Next, we took advantage of the greater resolution of Tg(*GnRH3-GFP*) embryos to map the precise timing and locations of GnRH3 neurons in control and IGF-deficient embryos. For this, Tg(*hsp70:dnIGF1R-GFP/GnRH3:EGFP*) double-transgenic embryos were generated and used. In the control group, i.e., Tg(*hsp70:dnIGF1R-GFP/GnRH3:EGFP*) embryos without HS treatment, GnRH3 neurons began to emerge in two bilateral clusters in the olfactory bulb region at 26 hpf (Fig. 5A). At 32 hpf,

Figure 4. IGF signaling regulates the timing of GnRH2 neuron emergence but not their spatial locations. *A*, Blockage of IGF signaling delays the timing of emergence of GnRH2 neurons. Tg(*hsp70:dnigf1r-GFP*) embryos were treated as described in Figure 2*A*. GnRH2 neurons were detected by whole-mount *in situ* hybridization at the indicated time points. Ventral views are shown with anterior up. Arrows indicate GnRH2 neurons. The location of eyes are indicated by e. *B*, *C*, Blockage of IGF signaling in advanced embryos decreases GnRH2 neuron migration rate. Tg(*hsp70:dnigf1r-GFP*) embryos were treated with or without two 0.5 h HS treatments at 32 and 44 hpf. GnRH2 neurons were detected by whole-mount *in situ* hybridization the indicated time points. Ventral views are shown with anterior up. Arrows point to the positions of GnRH2 neurons (*B*). The migration index, defined as the length between right and left GnRH2 neuronal clusters divided by the length between the centers of the retinas, was quantified and shown in *C*. Values are shown as mean ± SE. n = 15–19, *p < 0.01 compared with the no (w/o) HS group of the same developmental age.

(*Figure legend continued.*) Aberrant GnRH3 neurons are indicated by arrows. Similar results were obtained in all eight embryos examined. *E*, Blockage of IGF signaling has no effect on global brain patterning. Tg(*hsp70:dnigf1r-GFP*) embryos were treated as described in *A*, raised to 32 hpf, and subjected to *in situ* hybridization analysis for the indicated marker genes. The frequency of embryos with the indicated expression patterns is shown in the upper right of each panel. Scale bar, 0.2 mm. *F*, Blockage of IGF signaling does not alter the morphology of olfactory bulbs. Tg (*α1-tubulin1a: EGFP/hsp70:dnIGF1R-GFP*) embryos are treated as described in *A*, raised to 3 dpf, and analyzed. Dashed line denotes the olfactory bulbs. *G*, Blockage of IGF signaling does not alter the location of specific sensory neurons. Tg(*hsp70:dnigf1r-GFP*) embryos were treated as described in *A*, raised to 32 hpf, and subjected to *in situ* hybridization analysis for the indicated marker genes. Arrows indicate locations of olfactory sensory neurons expressing *OMP* and *TRPC2*.
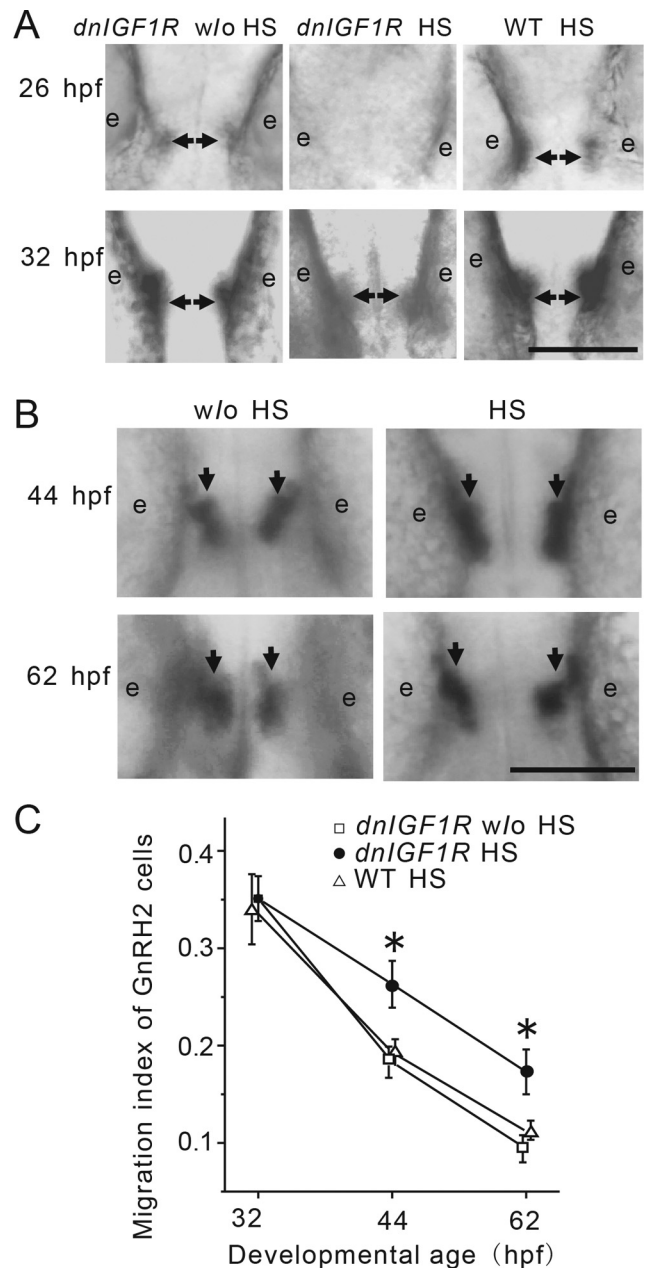
GnRH3 neurons were clearly seen in these two normal locations in all 14 individuals examined and remained so thereafter (Fig. 5A). When Tg(*hsp70:dnIGF1R-GFP/GnRH3:EGFP*) embryos were treated with 1 h HS at 4 hpf, the two clusters of bilateral GnRH neurons did not appear in any of these embryos at 26 hpf
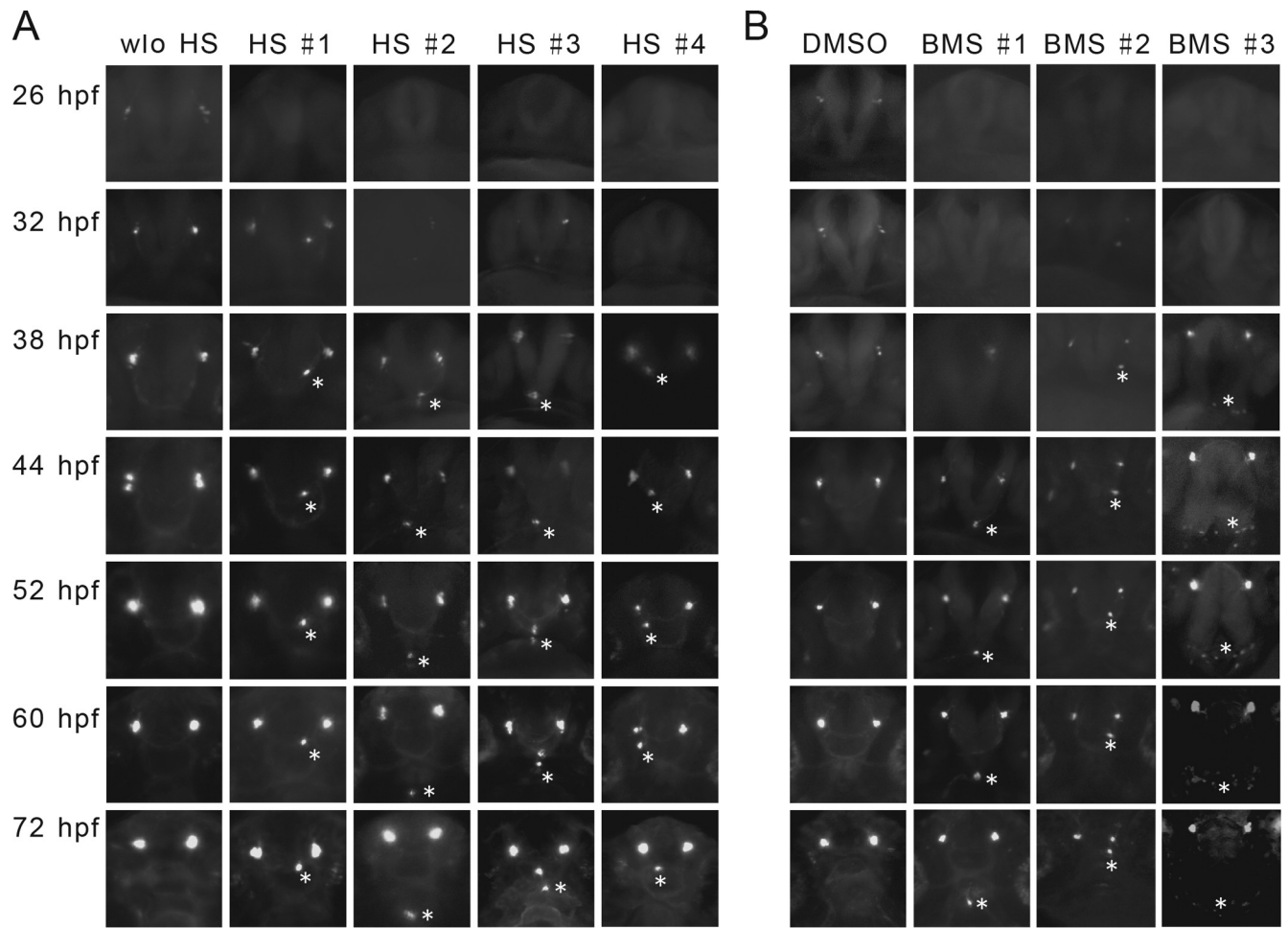
Figure 5. IGF signaling regulates the proper spatial distribution of newborn GnRH3 neurons. **A**, Time-lapse imaging of GnRH3 neurons in Tg(*hsp70:dnigf1r-GFP/GnRH3:EGFP*) double-transgenic fish. Embryos were treated as described in Figure 2A. The timing of emergence and spatial location of GnRH3 neurons were monitored in the same individuals at the indicated time points. Four HS-treated fish (#1–4) are shown. **B**, Time-lapse imaging of GnRH3 neurons in Tg(*GnRH3:EGFP*) fish. Embryos were treated with DMSO or BMS-754807 (BMS; 3 μM) from 4 to 32 hpf. The timing of emergence and spatial location of GnRH3 neurons were monitored in the same individuals at the indicated time points. Three treated fish (#1–3) are shown. Aberrant GnRH3 neurons are indicated by asterisks.

(Fig. 5*A*). At 32 hpf, they were detected in only one of the four embryos examined. At 38 hpf, GnRH neurons were finally detected in all four embryos. As shown in Figure 5*A*, in all cases, the ectopic GnRH3 neurons emerged at the same time as those normal GnRH3 neurons. Next, IGF signaling in Tg(*GnRH3:EGFP*) embryos was inhibited by adding BMS-754807. Treatment of Tg(*GnRH3:EGFP*) embryos with BMS-754807 resulted in essentially the same result (Fig. 5*B*): a delay in the timing of GnRH3 neuron emergence and a concurrent appearance of ectopic GnRH3 neurons in all embryos examined. This result suggests that IGF signaling regulates the spatial organization of newborn GnRH3 neurons.

GnRH3 neurons in the olfactory region are known to originate from the cranial neural crest in zebrafish (Whitlock et al., 2003, 2005). The concurrent appearance of the normal and ectopic GnRH3 neurons in IGF-deficient embryos led us to postulate that IGF signaling may regulate GnRH3 distribution by acting on their precursor cells in the cranial neural crest. To test this hypothesis, we examined the effect of IGF signaling blockade on neural crest cell migration/differentiation by analyzing *sox10* and *crestin* expression. In zebrafish, *sox10* is expressed in premigratory neural crest cells and extended caudally with the progress of embryogenesis (Dutton et al., 2001). Between 16 to 26 hpf, *sox10*

mRNA is maintained in migrating neural crest cells of the medial migration pathway but absent in the lateral pathway (Dutton et al., 2001). As shown in Figure 6*A*, the expression pattern of *sox10* in 16 hpf IGF-deficient embryos resembled that of the 12 hpf control embryos (Fig. 6*A*). Likewise, 20 and 32 hpf IGF-deficient embryos showed a similar *crestin* expression pattern to those of 16 and 26 hpf control embryos, respectively (Fig. 6*A*), indicating a 4–6 h delay in neural crest cell development. Importantly, the blockade of IGF signaling also resulted in the disappearance of *sox10* mRNA-expressing cells at 20, 26, and 32 hpf, most notably in the dorsal region (Fig. 6*A*). This is distinct from the *crestin* expression, which is a pan-neural crest marker and is expressed in all neural crest cells until 26 hpf, and is downregulated at 32 hpf (Luo et al., 2001). As shown in Figure 6*A* (middle), the expression pattern of *crestin mRNA* in 16 and 32 hpf IGF-deficient embryos resembled that of the 12 and 26 hpf control embryos, respectively. Differently from the case of *sox 10*, however, many *crestin* mRNA-expressing cells were observed in the migrating neural crest cells (likely those in the lateral pathways) at 16 and 20 hpf. We also examined the expression patterns of *pax2a*, a marker gene of optic stalk, mid-hindbrain boundary, and hindbrain. There was no major change between control and IGF-deficient embryos in all stages examined (Fig. 6*A*, right).
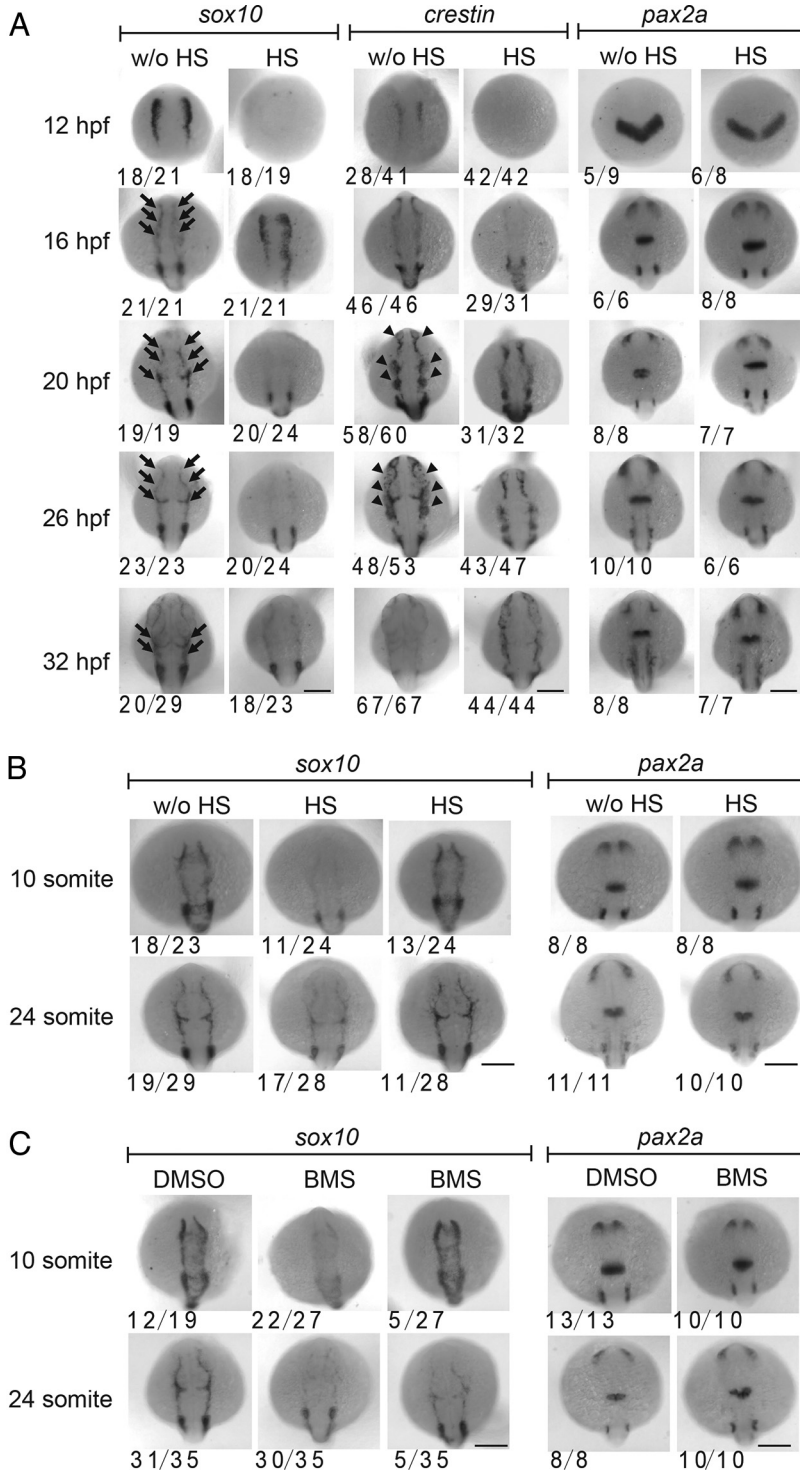
Figure 6. IGF signaling is important for neural crest cell differentiation and/or migration. ***A***, Blockade of IGF signaling affects neural crest cell development and/or migration. Tg(*hsp70:dnigf1r-GFP*) embryos were treated as described in Figure 2*A*, raised to the indicated time points, and subjected to *in situ* hybridization analysis for the indicated marker genes. The frequency of embryos with the indicated expression patterns is shown at the bottom of each panel. Note that while *sox10* mRNA-expressing cells in the medial migration pathway (arrows) disappeared in IGF-deficient embryos, *crestin* mRNA-expressing neural crest cells (arrowheads) were observed. ***B***, Blockade of IGF signaling decreases neural crest cell differentiation and/or migration. Tg(*hsp70:dnigf1r-GFP*) embryos were treated as described in ***A***. Control and experimental embryos of the same developmental stages were collected and analyzed as described in ***A***. ***C***, Wild-type embryos were treated with 3 μm BMS-754807 (BMS) or DMSO from 4 hpf to the indicated stages and analyzed by *in situ* hybridization analysis for the indicated marker genes. Scale bar, 0.2 mm.

To further rule out the possibility that the observed changes in *sox10* expression is an indirect consequence of the global developmental delay, we analyzed *sox10* expression using developmental stage-matched control and IGF-deficient embryos at the

10 and 24 somite stages. As shown in Figure 6*B*, 46% and 60% of HS-treated embryos at 10 and 24 somite stage had markedly reduced *sox10* expression in the dorsal region. We next tested the effect of BMS-754807. Eighty-one and 87% of BMS-treated embryos at 10 and 24 somite stage had markedly reduced *sox10* expression in the migrating neural crest cells. BMS-754807 treatment did not change the expression pattern of *pax2a* (Fig. 6*C*). These results suggest that the blockade of IGF signaling delays neural crest cell development and alters the migration and/or differentiation of the *sox10* mRNA-expressing neural crest cells in the medial migration pathway.

IGF signaling regulates GnRH3 neuron development via the PI3K but not the MAPK pathway

The IGF1R signaling is primarily mediated by the PI3K-Akt pathway and Mek-Erk MAPK pathway (Duan, 2002). Treatments of Tg(*GnRH3:EGFP*) embryos with LY294002, a PI3K inhibitor, phenocopied the IGF signaling-deficient embryos (Fig. 7*A*). The effect of LY294002 was concentration-dependent (Fig. 7*A*,*C*). Likewise, wortmannin, a structurally distinct PI3K inhibitor, also caused abnormal GnRH3 distribution (Fig. 7*B*). In contrast, U0126, a Mek inhibitor, had no effect (Fig. 7*A*,*B*). Together, our results suggest IGF signaling regulates spatial distribution of GnRH3 neuron via the PI3K-Akt, but not the MAPK, signaling pathway.

Discussion

In this study, we have unraveled an important role of IGF signaling in regulating the timing and location of newborn GnRH neurons using the zebrafish model. By a combination of genetic, biochemical, and pharmacological tools, and *in vivo* real-time imaging, we show that IGF signaling is critical for the timing of GnRH2 and GnRH3 neuron emergence and the spatial distribution of GnRH3 neurons. The action of IGF signaling on GnRH3 neurons is developmental stage-dependent and mediated through the PI3K-Akt, but not the MAPK, signaling pathway (Fig. 7*D*).

The GnRH neuronal system has long been used for studying tangential neuronal migration. During mammalian development, hypothalamic GnRH neurons conduct a long-distance migration, starting from the olfactory region and reaching the POA and hypothalamus (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989). Wierman et al. (2011) recently reviewed various regulators of GnRH neuron development and migration (both cell autonomous and nonautonomous

factors). Among them, two growth factor systems, FGF8/FGFR1 and hepatocyte growth factor, are important regulators of GnRH neuron proliferation and migration (Tsai et al., 2005; Giacobini et al., 2007; Falardeau et al., 2008; Chung and Tsai, 2010). The possible involvement of other embryonic growth factors, however, has not been well documented. In this study, we have provided several lines of evidence to support the notion that IGF signaling regulates the proper spatial and temporal organization of newborn GnRH neurons. First, specific inhibition of IGF1R by dnIGF1R-GFP delayed the timing of GnRH2 and GnRH3 neuron emergence and resulted in the mislocation of GnRH3 neurons. Another independent line of evidence came from the pharmacological inhibition experiments. Treatment of embryos with NVP-AEW541 and BMS-754807, two structurally distinct IGF1R inhibitors, resulted in similar changes: delaying GnRH2 emergence from 26 to 32 hpf and GnRH3 neurons emergence from 32 to ~38 hpf, and the appearance of ectopic GnRH3 neurons. Our finding on the role of IGF signaling in regulating the timing of GnRH neuron emergence is perhaps not surprising. Previous studies have shown that inhibition of IGF signaling results in global growth retardation and developmental delays and that IGF signaling plays an important role in accelerated developing rate in zebrafish embryos in response to oxygen availability (Schlueter et al., 2007; Kamei et al., 2011). IGF signaling has also been shown to regulate many aspects of the CNS development (Bondy and Cheng, 2004; Joseph D'Ercole and Ye, 2008; Annenkov, 2009). In zebrafish embryos, IGF signaling is implicated in the development of motoneurons and the notochord (Schlueter et al., 2007; Zou et al., 2009).

A new and potentially important finding is that IGF signaling regulates the spatial organization of GnRH3 neurons. Zebrafish GnRH3 neurons are the counterpart of the mammalian hypophysiotropic GnRH1 neurons, and ablation of embryonic GnRH3 neurons can lead to impaired reproduction in adult zebrafish (Abraham et al., 2010). GnRH3 neurons localize in the olfactory regions first and then start migration toward the hypothalamus (Abraham et al., 2008). In this study, we show that genetic and pharmacological blockades of IGF signaling in early embryogenesis result in abnormal spatial organization of the GnRH3 neurons in the larval brain. This action is specific to GnRH3 neurons, as no ectopic neurons were found with GnRH2 neurons. Likewise, blockade of IGF signaling had no effect in OMP neurons and TRPC2 neurons, two other neurons in the olfactory bulb region. In addition, inhibition of IGF signaling did not alter brain patterning or the size and structure of the olfactory bulbs. The emergence of ectopic GnRH3 neurons is not due to transient or leaky gene expression because
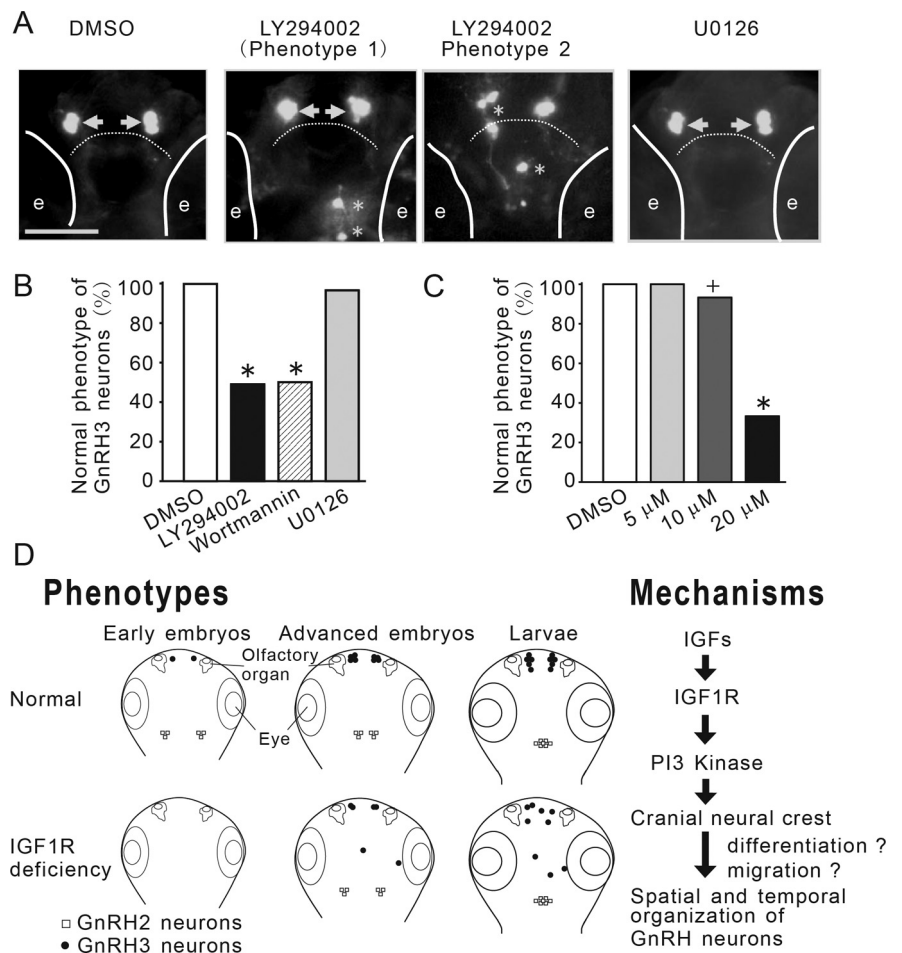

Figure 7. The IGF action on GnRH3 neurons is mediated through the PI3K, but not the MAPK, signaling pathway. **A**, Inhibition of PI3K, but not MAPK, phenocopies IGF-deficient embryos. Tg(*GnRH3:EGFP*) embryos were treated with DMSO or the indicated inhibitors. GnRH3 neurons were visualized at 3 dpf. GnRH3 neurons at the two normal locations are indicated by arrows. Aberrant GnRH3 neurons are indicated by asterisks. Solid lines indicate the locations of eyes (e). Dashed line denotes the boundary between the olfactory bulb and the telencephalon. **B**, Quantitative results of **A**. $n = 29 \sim 37$, $+p < 0.05$ and $*p < 0.001$ compared with the DMSO group. **C**, Dose-dependent effect of LY294002. **D**, A proposed model on the roles and underlying mechanisms of IGF signaling in regulating the temporal and spatial organization of newborn GnRH neurons in zebrafish embryos. Inhibition of IGF signaling delays the timing of GnRH2 and GnRH3 neuron development in early embryos and slows down the migration of GnRH2 neurons from lateral to the midline in advanced embryos. Inhibition of IGF signaling in early embryonic stage also results in abnormal distribution of GnRH3 neurons in the larval brain. Right, IGFs activate the IGF1R-PI3K pathway to regulate the migration/differentiation of cranial neural crest cells in early embryogenesis. This in turn results in altered temporal and spatial organization of newborn GnRH3 neurons.

these ectopic cells continued expressing GnRH3 mRNA until at least 7 dpf, albeit at reduced levels. To our knowledge, this is the first study demonstrating that the perturbation of IGF signaling in early embryonic stage has a long-lasting effect on the proper spatial distribution of GnRH neurons later in life.

The action of IGF signaling is developmental stage-dependent. While genetic or pharmacological inhibition at an early stage (4 hpf) resulted in the appearance of ectopic GnRH3 neurons, inhibition of IGF signaling at a later stage (30 hpf) had no such effect. It should be emphasized that inhibition of IGF signaling at 30 hpf was effective because it reduced embryonic growth and slowed GnRH2 neuron migration. This suggests that there is a critical time window in early embryogenesis during which IGF signaling regulates the emergence of GnRH3 neurons at the correct locations. We considered two possible reasons for the appearance of ectopic GnRH3 neurons in IGF-deficient embryos. First, the differentiated GnRH3 neurons may mismigrate

into abnormal locations. Second, GnRH3 neurons may be born in the wrong locations. Our *in vivo* time-lapse imaging analysis revealed that the ectopic GnRH3 neurons appeared at the same time as those in the normal place, thus supporting, although not necessarily proving, the second scenario. This is also in agreement with our finding that inhibition of IGF signaling at a later stage (30 hpf) did not result in the appearance of any ectopic GnRH3 neurons. Our findings in zebrafish may also explain previous findings made in the mouse model. While $Igf-1^{-/-}$ mice exhibited misplaced glutamergic neurons of the mitral cell layers in the olfactory bulb (Hurtado-Chong et al., 2009), mice with deleted IGF1R or the p85α regulatory subunit of PI3K in GnRH neurons only (under the control of the GnRH1 promoter) showed a normal number and spatial distribution of GnRH1 neurons, although they exhibited differences in GnRH morphology (Acosta-Martínez et al., 2009; Divall et al., 2010).

The precise origins of GnRH3 and GnRH2 neurons in zebrafish and their relationship are still under debate. Whitlock et al. (2005) proposed that the cranial neural crest-derived precursor cells migrate following two distinct routes: (1) anterior migration stream toward the olfactory region (presumably becoming GnRH3 neurons) and (2) lateral route passing ventrally to midbrain (presumably becoming GnRH2 neurons). In this study, we have shown that while IGF signaling regulates the timing of both GnRH2 and GnRH3 emergence, it also plays a highly specific role in determining the precise location of GnRH3 neurons. We discovered that blockade of IGF signaling in early embryos not only resulted in a delay in the neural crest cell development (as indicated by the delayed expression pattern of the pan-neural crest marker gene *crestin*), but also led to a major reduction in *sox10* mRNA-expressing cells in the medial migration pathway. These results imply that GnRH3 neurons may arise from neural crest cells, most likely those in the medial migratory pathway. This idea is in good agreement with recent cell fate-mapping studies in chicks and mice, indicating that neural crest cells give rise to a subpopulation of GnRH1 neurons and olfactory ensheathing glial cells (Barraud et al., 2010; Forni et al., 2011). Since *sox10* expression is maintained in the medial migration pathway and not on the lateral pathway, it is tempting to speculate that GnRH3 neurons may be derived from *sox10* mRNA-expressing neural crest cells in the medial migration pathway, while GnRH2 neurons are derived from other populations of neural crest cells. Whether IGF signaling controls the migration and/or differentiation of neural crest cells of the medial migration pathway and how the global blockade of IGF signaling leads to a reduction in *sox10*-expressing cells are not clear at present. How the global blockade of IGF signaling leads to a reduction in *sox10*-expressing cells is still unclear. Future studies are needed to answer these questions.

The IGF1R belongs to the receptor tyrosine kinase family and it uses two major intracellular signaling pathways, namely the PI3K-Akt cascade and Ras-MEK1/2-Erk1/2 cascade (Duan and Xu, 2005). How these downstream signaling pathways are used *in vivo* in different tissues and/or under different conditions is not well understood. Our results suggest that PI3K-Akt signaling, but not the MEK1/2-Erk1/2 signaling, mediated the actions of IGF-IGF1R in regulating GnRH neuronal system formation *in vivo*. While specific inhibition of the MEK1/2-Erk1/2 pathway using U0126 had no effect on the temporal and spatial organization of GnRH neurons, LY294002 and wortmannin, two distinct PI3K inhibitors, phenocopied the IGF signaling-deficient embryos. These results suggest that the IGF-IGF1R-PI3 kinase signaling

pathway regulates the precise timing and the spatial distribution of newborn GnRH neurons in zebrafish embryos.

In humans, disrupted GnRH neuronal ontogeny can lead to idiopathic hypogonadotrophic hypogonadism (IHH). For instance, mutations in KAL-1 cause X-linked Kallmann's syndrome, characterized by IHH, anosmia, synkinesis, and unilateral renal agenesis (González-Martínez et al., 2004). At present, only a small percentage (<30%) of IHH cases can be accounted for by known gene mutations, suggesting the existence of unexplored regulatory mechanisms (Falardeau et al., 2008). Therefore, our findings not only expand our knowledge of regulation of the temporal and spatial organization of GnRH neurons, but also may have implications in the understanding of the etiology of human reproductive disorders.

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