

Androgen Receptor Gene Expression in the Developing and Adult Zebrafish Brain

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Androgens play a central role in the regulation of male sexual differentiation and behavior in many vertebrates, including zebrafish. Their signaling is mediated by activation of the androgen receptor. A single *androgen receptor* (*ar*) gene was recently identified in zebrafish, which encodes a protein that binds androgens in vitro. However, the tissue-specific expression pattern of this receptor in vivo has not been described. Using whole-mount RNA in situ hybridization, we characterized expression of the *ar* gene in developing zebrafish and in the adult brain. In embryos, transcripts were found in the presumptive pronephros and in olfactory placodes. By 3–5 days postfertilization, *ar* transcripts were also detected in the pineal organ anlage and the retina. In the adult brain, *ar* was expressed in discrete regions of the telencephalon, in the preoptic area, and throughout the periventricular hypothalamus, regions previously implicated in the regulation of sexually dimorphic behaviors in mammals. *Developmental Dynamics* 237:2987–2995, 2008. © 2008 Wiley-Liss, Inc.

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INTRODUCTION

Androgens regulate sexual differentiation, tissue development, and social and reproductive behaviors in mammals (Arnold and Breedlove, 1985; Hart and Leedy, 1985; Werner et al., 1996; Cooke et al., 1998; Morris et al., 2004). They also play similar roles in fish (Borg, 1994). For example, testosterone and 11-ketotestosterone increase muscle size in the plainfin midshipman fish *Porichthys notatus* (Brantley et al., 1993) and regulate the size of gonadotropin-releasing hormone-expressing neurons in the brain in the cichlid *Astatotilapia burtoni* (Soma et al., 1996). Moreover, higher levels of circulating androgens in cichlids are associated with social dominance (Parikh et al., 2006). Androgens also induce masculinization of the gonads in a variety of fish (Pandian and Sheela, 1995). In the ze-

brafish *Danio rerio*, increasing androgen levels during larval and juvenile stages by administering testosterone or by inhibiting the testosterone-metabolizing enzyme aromatase induces male sexual differentiation (Westerfield, 1993; Orn et al., 2003, 2006; Fenske and Segner, 2004; Holbech et al., 2006).

Androgens act by binding to cytosolic androgen receptors (AR), ligand inducible transcription factors that, once activated, translocate to the nucleus, bind DNA, and regulate gene expression (refer to Gao et al., 2005). Although AR localization has been extensively characterized in mammals from the time of sexual differentiation through adult stages of development, little is known about AR expression in embryos before gonad differentiation. The optical clarity and external development of zebrafish provide a unique opportunity to

study *ar* expression in a vertebrate embryo at developmental stages before gonad formation. In fish, the principal androgens are testosterone and 11-ketotestosterone (Borg, 1994), which were recently shown to bind to and activate a zebrafish androgen receptor in vitro (Hossain et al., 2007; Jorgensen et al., 2007; de Waal et al., 2008). Although real-time polymerase chain reaction (PCR) analysis demonstrated the presence of *ar* transcripts in zebrafish embryos, larvae, and in multiple tissues in adults (Hossain et al., 2007), the specific sites of *ar* expression during development and within the adult brain have not been described.

Using whole-mount RNA in situ hybridization, we analyzed expression of the zebrafish *androgen receptor* gene during development and in the adult brain. In adult zebrafish, *ar* was ex-

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pressed in the hypothalamus and pre-optic area, consistent with expression in goldfish and cichlid brains (Gelinis and Callard, 1997; Harbott et al., 2007) and with the mammalian brain (Simerly et al., 1990; Shah et al., 2004). In embryos and larvae, several unexpected sites of expression were found in the brain and pronephros, at developmental stages for which information is not yet available for mammals.

RESULTS

Identification of the Zebrafish *ar* Gene

We performed a BLAST search of the Ensembl zebrafish genomic sequence database using the human AR protein sequence as a query. A single gene was found (ENSDARG00000067976) whose putative protein product overall was 41% identical and 53% similar to the human AR. Within particular subregions, however, similarity was much higher. The putative DNA- and ligand-binding domains were 72% and 68% identical to the homologous regions of the human receptor. A striking difference was found in the N-terminal domain, where the putative zebrafish AR lacks the glutamine repeats present in the human AR.

Repeated BLAST searches using different parameters failed to uncover additional genes with high homology to ENSDARG00000067976, suggesting that there is only one *ar* in the zebrafish genome. Two reports recently appeared that supported our analysis by identifying a single zebrafish *ar* gene with sequence identical to ENSDARG00000067976 (Hossain et al., 2007; Jorgensen et al., 2007). These reports demonstrated that the in vitro synthesized protein binds testosterone and 11-ketotestosterone (Jorgensen et al., 2007) and activates transcription from an androgen response element promoter (Hossain et al., 2007). This strongly suggests that the zebrafish gene encodes the true orthologue of the human AR gene.

The *ar* Gene Is Expressed in Endocrine Regions of the Adult Brain

Androgen signaling is enriched within the preoptic area and hypothalamus,

regions of the brain important for regulating endocrine functions and reproductive behaviors. Therefore, expression of the putative zebrafish *ar* is expected to be robust in these brain regions as well. To detect *ar* gene expression rapidly throughout the adult zebrafish brain, we optimized the standard whole-mount in situ hybridization technique used on embryos (Thisse and Thisse, 2008) for dissected, whole adult brains (Fig. 1; see the Experimental Procedures section). In adult zebrafish brains of both sexes, robust *ar* expression was observed throughout the pre-optic area (POA) and hypothalamus, regions that control endocrine hormone secretion and regulate reproductive behaviors in vertebrates (Crews and Silver, 1985). In the POA, *ar* was expressed in the anterior and posterior parts of the parvocellular preoptic nuclei (PPa, PPp, Fig. 1D,D',E,E',E'') and in the adjacent periventricular nucleus of the posterior tuberculum (TPp; Fig. 1E'). In the hypothalamus, robust *ar* expression was observed near ventricles, especially in the dorsal, ventral, and caudal zones of the periventricular hypothalamus (Hd, Hv, Hc) surrounding the lateral recess (LR) and diencephalic ventricle (DiV; Fig. 1F,F',G,G',H,H'). The *ar* gene was also expressed in the lateral hypothalamic nuclei (LH) and paraventricular organ (PVO, Fig. 1F'). Robust *ar* expression in the zebrafish POA and hypothalamus is consistent with studies in the rat and mouse.

In rats, the androgen receptor is present in several nonendocrine regions of the brain, such as the visual cortex (Nunez et al., 2003) and hippocampus (Simerly et al., 1990). Similarly, *ar* transcripts were detected in nonendocrine regions of the zebrafish brain. In the optic tectum, *ar* transcripts localized to the periventricular gray zone (PGZ, Fig. 1F,F''). In the dorsal telencephalon, *ar* was robustly expressed in a lateral region (Fig. 1D,D'') thought to correspond to the mammalian hippocampus (Wullimann and Mueller, 2004). Expression was not observed in the olfactory bulbs (not shown) and was not assayed in olfactory epithelia, pineal or pituitary glands because these structures were not retained in all brain preparations.

The *ar* Gene Is Expressed in the Brain of Zebrafish Embryos and Larvae

At 1 day postfertilization (d), *ar* was expressed in the olfactory placodes, as well as in cells in the midbrain (Fig. 2A–C). At 2 d, transcripts were observed in a region of the medial diencephalon that corresponds to the presumptive hypothalamus (Fig. 2D,E).

In 3- to 5-d larvae, *ar* transcripts persisted in the olfactory placodes and the developing olfactory organ (Fig. 2F,G). Beginning at 3 d, robust expression was also observed in the presumptive pineal organ (Fig. 2H). Double-label in situ hybridization for *ar* and *otx5*, a gene expressed in the pineal complex (Gamse et al., 2002), demonstrated *ar* expression in the pineal but not the parapineal organ (Fig. 2I). At 3 d, *ar* expression was first observed in the pectoral fin buds (Fig. 2F) and persisted through 5 d. At 4 d, weak expression was detected in

Fig. 1. Expression of the *ar* gene in the adult zebrafish brain. **A:** Schematic of brain (modified from Wullimann et al., 1996), lateral view, anterior to the left, illustrating position of coronal sections shown in subsequent panels. **B,C:** low magnification of ventral brains hybridized with antisense (B) or sense probes (C) before sectioning. **D–H:** Whole brain brightfield images. Panels labeled D', D'', E', and so on, are high magnification differential interference contrast (DIC) images of the boxed regions from the corresponding sections. Dorsal to the top. **D:** Expression in the anterior parvocellular preoptic nucleus (PPa, D') and in the lateral zone (DL) of dorsal telencephalon (Tel, D''). **E:** Cells lining the diencephalic ventricle (DiV, E'), including the periventricular nucleus of posterior tuberculum (TPp) and the ventral zone of periventricular hypothalamus (Hv, E'') express *ar*. **F–H:** Expression in the periventricular gray zone of the optic tectum (PGZ, F') and throughout the hypothalamus (F',G',H'). **I,J:** Brains from *Tg(h2afv:GFP)^{kca66}* adults were analyzed for GFP fluorescence (I) or *gfp* gene expression (J) to confirm that RNA probes penetrated deep regions of the hypothalamus. I, epifluorescence image overlaid onto DIC image. CC, crista cerebellaris; CCe, corpus cerebelli; Hc, caudal zone of the periventricular hypothalamus; Hd, dorsal zone of periventricular hypothalamus; IL, inferior lobe of hypothalamus; LH, lateral hypothalamus; LR, lateral recess of DiV; OB, olfactory bulb; ON, optic nerve; PVO, paraventricular organ; TeO, optic tectum; TH, tuberal hypothalamus. Panels D, F–H are of female brains, panel E are male brains. Scale bars = 200 μ m in D–H, D', D'', E', and so on, 50 μ m in I,J.

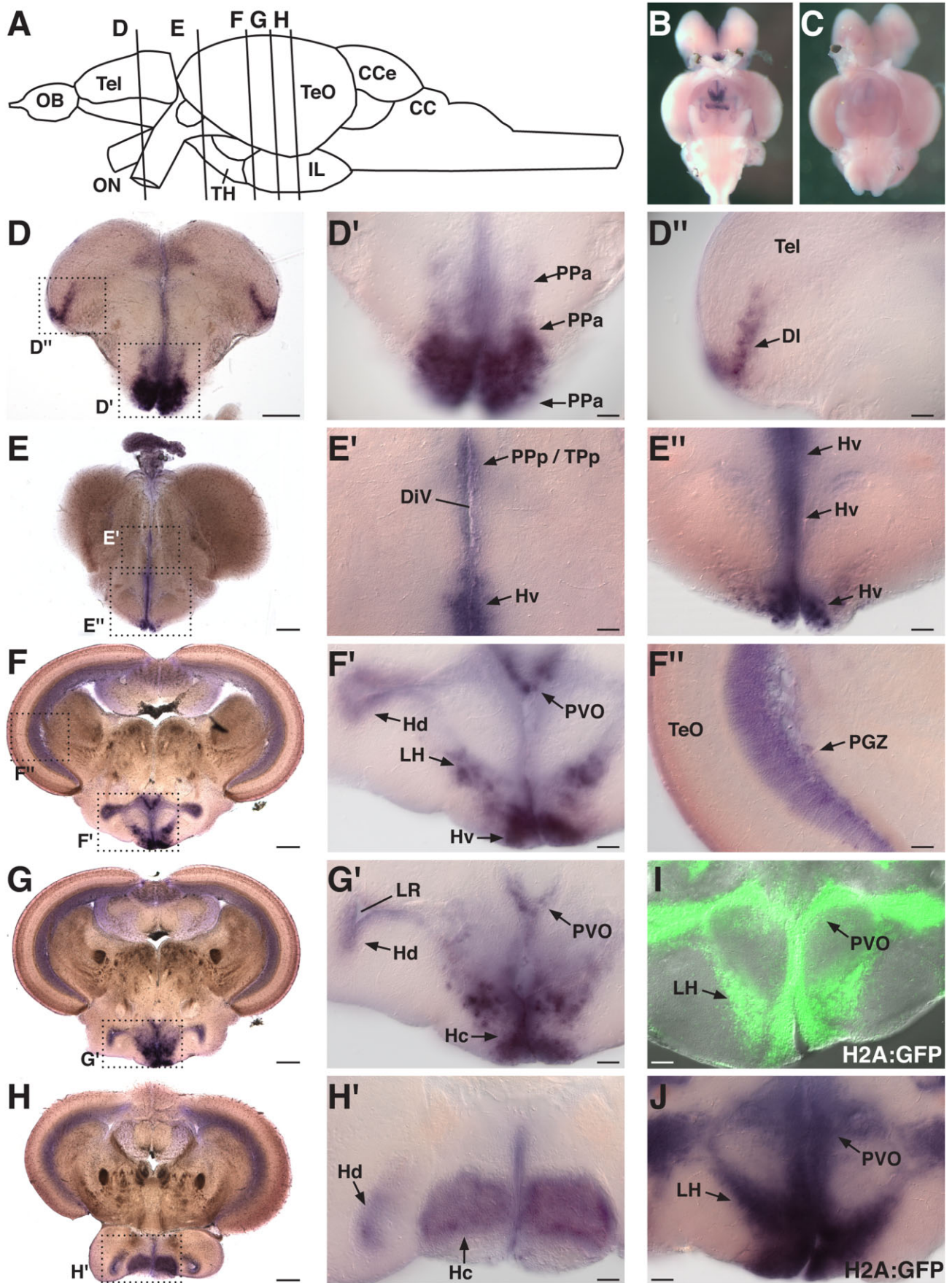


Fig. 1.

the retina (data not shown), but increased over time and was clearly visible in the inner nuclear cell layer of the retina by 5 d (Fig. 2G).

The *ar* Gene Is Expressed in the Pronephros Adjacent to Primordial Germ Cells

In embryos younger than the 14-somite stage, we failed to observe specific expression of the *ar* gene (data not shown). Between the 14- and 18-somite stages, *ar* transcripts were concentrated in a discrete population of cells in two bilateral rows lateral to the somites (Fig. 3A,B). By 24 hours postfertilization (h), these cells appeared to have migrated posteriorly along the body axis to a region immediately dorsal to the anterior yolk extension (Fig. 3C). Primordial germ cells (PGCs) are known to migrate to this region by 24 h (Pelegri et al., 1999; Weidinger et al., 1999). Because several reports suggest that the androgen receptor is active in mouse germ cells (Zhou et al., 1996; Cupp and Skinner, 2001; Merlet et al., 2007a,b), we assessed whether *ar* expression localized to zebrafish PGCs by performing double in situ hybridization for *ar* and *vasa*, a gene expressed exclusively in PGCs (Pelegri et al., 1999; Weidinger et al., 1999). The *ar* gene was expressed in a row of cells immediately posterior to the cluster of *vasa*-expressing PGCs (Fig. 3D,E). This analysis revealed that *ar* and *vasa* expression did not localize to the same cells, demonstrating that zebrafish embryonic germ cells do not express the *ar* gene.

In mammals, the androgen receptor is required for proper gonad differentiation (Werner et al., 1996). The close positioning of *ar*-expressing cells and PGCs in zebrafish embryos suggested that PGCs might influence *ar* expression or the migration of *ar*-expressing cells. To test this hypothesis, we examined expression in embryos that lack PGCs. PGCs were destroyed by injecting one-cell embryos with morpholino antisense oligonucleotides that block expression of the *dead end* gene (*dnd*-MO). *dnd* gene expression is required for PGC survival. By 24 h, PGCs in *dnd*-MO injected embryos fail to migrate to the presumptive gonad and die (Weidinger et al., 2003). In situ hybridization studies demonstrated that *ar* gene expression was unaffected at 24 h

in embryos that had received *dnd*-MO (data not shown), indicating that PGCs are not required for *ar* expression or for the normal posterior migration of *ar*-expressing cells.

PGCs migrate along the pronephros, bilateral tubular epithelial cells that extend posteriorly from the anterior end of the yolk extension and join together at the cloaca (Drummond, 2003). Because *ar* transcripts were detected in cells directly adjacent to PGCs, we examined whether *ar* expression is associated with the pronephros. In one-somite-thick transverse sections of the area along the yolk extension of 24-h embryos, *ar* transcript was detected only in cells adjacent to the pronephric lumen (Fig. 3F). Therefore, we conclude that the position of *ar*-expressing cells at 24 h corresponds to the developing pronephros.

The zebrafish pronephros is comprised of several segments that can be distinguished based on selective expression of solute transporter genes (Wingert et al., 2007). From anterior to posterior these segments are: the proximal convoluted tubules, the proximal straight tubules, the distal tubules, and the pronephric ducts (Wingert et al., 2007). To identify specific regions of the pronephros where the *ar* gene is expressed, we performed double in situ hybridization for *ar* and *slc26a1*, a putative anion transporter expressed in the proximal convoluted tubule (Wingert et al., 2007). A subset of cells expressed both genes (Fig. 3G). However, *ar* expression was also detected more posteriorly, in cells that failed to express *slc26a1* (Fig. 3G). This result indicates that the *ar* gene is expressed in the posterior region of the proximal convoluted tubule and in the anterior region of the presumptive proximal straight tubule. Expression in the pronephros is transient, observed at lower levels in some embryos at 2 d, but absent thereafter (data not shown).

DISCUSSION

The Zebrafish Genome Contains a Single *ar* Gene Whose Expression Is Conserved in Neuroendocrine Regions of the Adult Brain

The *ar* gene we describe is identical to one recently reported that encodes a

protein product that binds androgens and activates transcription through an androgen response element DNA sequence in vitro (Hossain et al., 2007; Jorgensen et al., 2007). Due to a whole-genome duplication that occurred in the teleost lineage (Jaillon et al., 2004; Postlethwait et al., 2004), many teleost species possess two *ar* genes, termed AR α and AR β (Ikeuchi et al., 2001; Harbott et al., 2007). The zebrafish genome, however, contains a single *ar* gene, possibly arising from the loss of a duplicate gene (Force et al., 1999). The sequence of the zebrafish *ar* gene appears more similar to the AR β family of teleost androgen receptors than to the AR α family. For example, a multiple sequence alignment between androgen receptors from zebrafish, cichlid (*Astatotilapia burtoni*) and Nile tilapia (*Oreochromis niloticus*) reveals that zebrafish AR is 58% similar to AR β from *A. burtoni* or *O. niloticus* but only 41% similar to AR α from *A. burtoni* or *O. niloticus*. In general, the AR β subtype is more similar to mammalian AR than is the AR α subtype (Harbott et al., 2007).

Expression of the *ar* gene in the zebrafish preoptic area and hypothalamus (Fig. 1) is similar to that described for other fish (Gelinas and Callard, 1997; Harbott et al., 2007) and mammals (Simerly et al., 1990; McAbee and DonCarlos, 1998; Shah et al., 2004). In both fish and mammals, the preoptic area is implicated in the regulation of reproductive behaviors (Koyama et al., 1984; Crews and Silver, 1985; Hart and Leedy, 1985; Pfaff and Modianos, 1985; Simerly, 2002), suggesting a functional conservation for androgen receptors in vertebrate brains. Taken together, the in vitro androgen binding studies (Hossain et al., 2007; Jorgensen et al., 2007) and our in vivo gene expression results strongly suggest that the putative zebrafish *ar* gene encodes a functional orthologue of the mammalian androgen receptor.

Because of the prominent role played by testosterone in masculinizing the developing mammalian brain and in regulating sex differences in behavior (Arnold and Breedlove, 1985; Hart and Leedy, 1985; Cooke et al., 1998; Morris et al., 2004), we hypothesized that adult male and female zebrafish would display differences in *ar* expression in the central nervous sys-

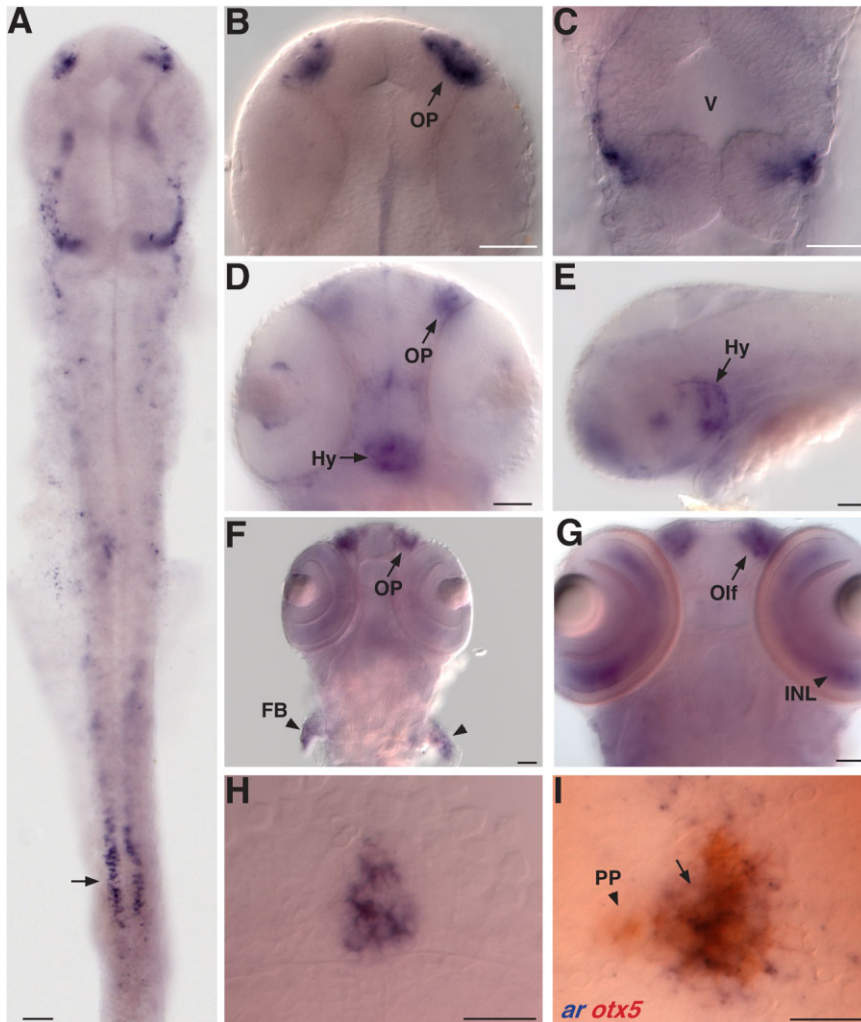


Fig. 2.

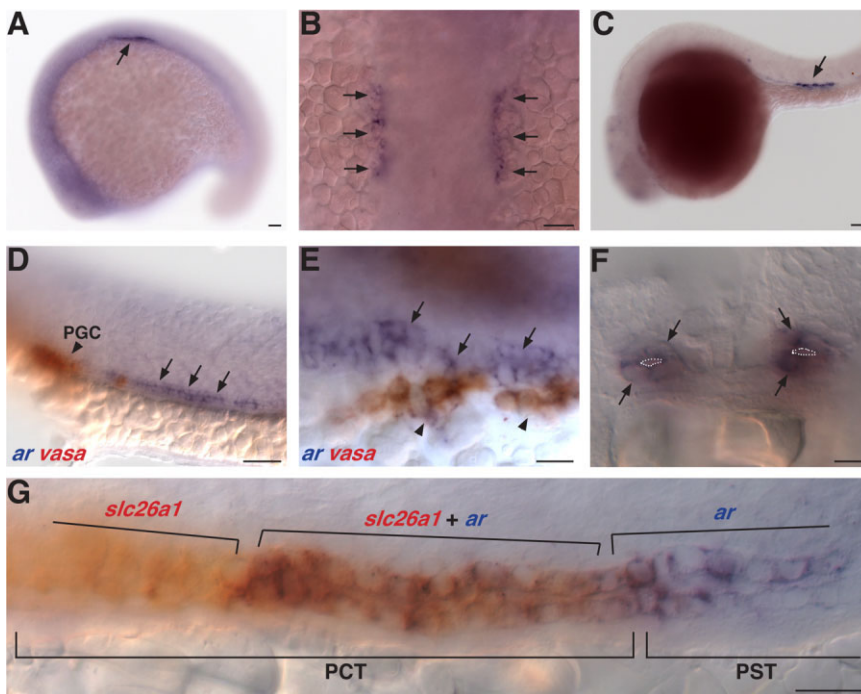


Fig. 3.

tem. Moreover, *Ar* gene expression is sexually dimorphic in the adult mouse brain (Shah et al., 2004). However, we did not detect any obvious sexual dimorphisms in *ar* expression in the adult zebrafish brain. It is possible that subtle differences in cell number or size may not be detected in 100- μ m tissue sections. Even with thinner sections, RNA in situ hybridization may not be sensitive enough to discern differences among small populations of cells in the brain. It may be necessary to generate transgenic fish that robustly express a readily detectable reporter gene, such as *lacZ*, under the control of *ar* regulatory elements. This strategy was used in the mouse, where it was difficult to detect sex differences in *Ar* expression by in situ hybridization (Shah et al., 2004). It is

Fig. 2. Expression of the *ar* gene in the embryonic brain. **A:** Overview of expression at 1 day (d). Arrow indicates expression in pronephros, detailed in Figure 3. **B,C:** Higher magnification images of expression in olfactory placodes (OP) and midbrain. V, midbrain ventricle. **D,E:** Expression in the presumptive hypothalamus (Hy) appears at 2 d. **F:** OP expression persists at 3 d and transcripts are detected in the pectoral fin buds (FB, arrowhead). **G:** Robust expression in the maturing olfactory organ (Olf, arrow) and the inner nuclear layer of the retina (INL, arrowhead) by 5 d. **H:** High magnification image of expression in the dorsal diencephalon at 3 d. **I:** Double-label in situ hybridization shows *ar* gene expression colocalizes with transcripts of *otx5* (red), a gene expressed in the pineal complex (arrow), *ar* is not expressed in the parapineal organ (PP, arrow head). All panels are dorsal views, anterior to the top except E, lateral view, anterior to the left. Scale bars = 50 μ m in A–G, 25 μ m in H,I.

Fig. 3. Expression of the *ar* gene in the embryonic pronephros. **A,B:** *ar* transcripts are detected in a bilateral row of cells (arrows) lateral to the somites at the 18 somite stage. **C:** *ar* expression is displaced posteriorly (arrow) by 24 hours (h). **D,E:** Double-label in situ hybridization of *ar* (blue, arrows) and *vasa* (red, arrow heads), a marker of primordial germ cells, at 24 h. **E:** Close association between *vasa* and *ar* expressing cells. **F:** Coronal section showing *ar* expression (arrows) in cells that form pronephric tubules at 24 h. Tubule lumen outlined in white, dorsal to the top. **G:** A subset of cells in the proximal convoluted tubule (PCT) express both *ar* (blue) and *slc26a1* (red), a gene expressed in the PCT but not in the adjacent proximal straight tubule (PST), at 24 h. *ar* expression is also present in the presumptive PST. A,C–E,G, lateral views anterior to the left, B, dorsal view, anterior to the top. Scale bars = 50 μ m in A–D, 10 μ m in F, 20 μ m in E,G.

also possible that differences in *ar* expression are dependent on reproductive behaviors, photoperiod, or olfactory and visual stimuli affected by population density and/or sex ratio per tank. Our results did not control for these parameters. Future studies are required to determine whether there are sex differences in the number of neurons expressing *ar*, their size or their connections during different behaviors.

***ar* Expression in the Embryonic Brain**

Because the *ar* gene is expressed in the adult preoptic area and hypothalamus, we expected to find expression in similar embryonic and larval brain regions. Expression was detected in the embryonic diencephalon, in a region that likely corresponds to the presumptive hypothalamus (Fig. 2D,E). Unexpectedly, we also detected robust *ar* gene expression in the olfactory placodes. In two other fish species, androgens were shown to modulate response to pheromones, chemical signals that act by means of the olfactory system to modulate behavior, suggesting that the androgen receptor plays a role in olfaction. Male round gobies *Neogobius melanostomus* increase their rate of ventilation (gil movement) in response to estrogenic pheromones. Females do not respond unless they are pretreated with methyltestosterone (Murphy and Stacey, 2002). Similarly, in response to the pheromone prostaglandin-F_{2a} olfactory epithelial cells in the male tinfoil barb *Puntius schwanenfeldi* produce a characteristic electrophysiological response whose magnitude and sensitivity is increased upon exposure to androgen (Cardwell et al., 1995). Direct connections between olfactory neurons and neurons in the preoptic area may allow androgens to modulate behavioral responses to olfactory stimuli. Dye-labeling and immunohistochemical studies in salmon, three-spined stickleback and trout reveal a population of olfactory neurons that bypass the olfactory bulb and project directly to the parvocellular preoptic nucleus and to the caudal hypothalamus (Bazer et al., 1987; Honkanen and Ekstrom, 1990, 1991; Becerra et al., 1994; Folgueira et al., 2004). The

robust *ar* expression in zebrafish olfactory placodes initially detected at 1 d (Fig. 2B) could indicate that *ar* is important for establishing connections between olfactory neurons and the preoptic area. However, olfactory–hypothalamic connections have not yet been described in zebrafish. Future studies are necessary to identify the targets of the *ar*-expressing cells in the developing zebrafish olfactory organ and to determine whether *ar* expression is required for formation of these connections.

***ar* Expression in the Embryonic Kidney**

In addition to expression in the embryonic brain, we also detected *ar* transcripts in the pronephros at 24 h. In zebrafish, specification of intermediate mesoderm to a pronephric fate is characterized by compartmental *wt1a*, *pax2.1*, and *sim1* gene expression (Serluca and Fishman, 2001). This occurs at the 8–10 somite stage of development, before *ar* gene expression is detected. Therefore, it is unlikely that *ar* plays a role in pronephric specification. Epithelialization of pronephric ducts and tubules occurs during later somitogenesis and is complete by 24 h (Drummond, 2003). Between 24 and 48 h, tubule and duct epithelia divide into distinct segments, marked by the restricted expression boundaries of solute transporter genes (Drummond, 2003; Wingert et al., 2007). The temporally restricted pattern of *ar* expression at 24 h may suggest a role in tubule formation. One possibility is that *ar* is regulating tubule segmentation by up-regulating solute transporter gene expression in a restricted region of the proximal tubule. In the proximal tubule of adult mice, androgens are known to up-regulate metabolic genes, such as ornithine decarboxylase, in an androgen receptor-dependent manner (Berger and Watson, 1989; Asadi et al., 1994). In a human cell line derived from the proximal tubule, the androgen receptor directly up-regulates α ENaC, an epithelial sodium channel subunit (Quinkler et al., 2005). The effects of androgens on renal function, however, are not well understood.

Androgen signaling plays a central role in the differentiation of the mammalian gonad, and is required for dif-

ferentiation of the male reproductive tract (Werner et al., 1996). It is not clear why the *ar* gene is expressed in the zebrafish embryo, weeks before sexual differentiation occurs. The zebrafish gonad is initially histologically apparent at 10 d, although at this stage it is bipotential, differentiating into either ovaries or testes (Takahashi, 1977). It is likely that *ar* gene expression is important for the sexual differentiation of the gonad that occurs at approximately 25 d (Takahashi, 1977; Rodriguez-Mari et al., 2005). In the adult zebrafish testis, the *ar* gene is expressed in a subset of Sertoli cells contacting spermatogonia (de Waal et al., 2008).

The kidney and gonad derive from the intermediate mesoderm and differentiate in adjacent regions of the embryo. In the male mouse, cells in the bipotential gonad primordium induce cells in the kidney precursor (mesonephros) to migrate into the gonad and contribute to its formation (Martineau et al., 1997; Capel, 2000). It is not known whether cells in the zebrafish pronephros contribute to the formation of the gonad. However, it seems unlikely that polarized, pronephric epithelial cells expressing the *ar* gene would depolarize and migrate away from the kidney tubule to form the gonad. One possibility is that *ar* expression in the pronephros influences adjacent cells, outside of the pronephric tubule, to undergo gonadogenesis.

Evidence That the Androgen Receptor Protein Is Active in Embryos

There are no studies directly measuring androgen receptor activity in zebrafish embryos or larvae. Levels of androgens in embryos and larvae are also not known. However, genes that code for steroid-synthesizing enzymes, required to convert cholesterol to androgens, are expressed in embryos (Hsu et al., 2002, 2003; Chai et al., 2003; To et al., 2007). The rate limiting enzyme in steroid synthesis is Cyp11a1 (P450scc). *cyp11a1* transcripts are maternally deposited into oocytes, and were detected in the yolk syncytial layer in embryos at 50% epiboly (Hsu et al., 2002). Between 24 and 28 h, interrenal cells, functional homologues of the androgen produc-

ing cells of the mammalian adrenal cortex, express *cyp11a1*, *star*, and β -*hsd*, genes required for the synthesis of androgens (Hsu et al., 2003; To et al., 2007). Moreover, β -hydroxysteroid dehydrogenase, the protein product of the β -*hsd* gene, is active at this stage in vivo (Chai et al., 2003). Taken together, these studies suggest that ligands are available to activate the androgen receptor during development.

In conclusion, we report the characterization of *ar* localization in a vertebrate embryo during developmental stages for which little information is available in mammals. The detailed localization studies presented here will enable a forward genetic screen to identify mutants with defective *ar* expression, as well as morpholino experiments to disrupt AR function. Because of the ability to readily visualize *ar* gene expression during embryogenesis, the zebrafish is a powerful system for examining the function of the androgen receptor in vertebrate development.

EXPERIMENTAL PROCEDURES

Animal Husbandry

Zebrafish were raised at 28.5°C on a 14/10 hour light/dark cycle and staged according to hours (h) or days (d) post-fertilization (Westerfield, 1993). The wild-type AB strain (Walker, 1999) was used for all studies unless mentioned otherwise. All protocols were approved by the institutional animal care and use committee.

Androgen Receptor Sequence Identification and Amplification

We performed a tBlastn search of the Ensembl zebrafish genomic sequence database, release Zv7 (www.ensembl.org), using the human AR protein sequence as a query. To ascertain similarity between human and zebrafish ARs, ClustalW multiple sequence alignments were performed using MacVector 9.0 (Accelrys Inc.). cDNA derived from total RNA from 3 and 5 d larvae was generated as a template for PCR (RetroScript, Ambion). Primers 5'-TGGAGTTTTTCCTTCCTCCA

and 5'-TCATTTGTGGAACAGGATT amplified an approximately 1,100-basepair (bp) region encoding part of the DNA-binding domain and the entire ligand-binding domain. Primers 5'-CCTCCCATTTGCCATTACATC and 5'-CAGCCCTTCTGTCCACTCTC amplified a 730-bp region containing 317-bp of 5'-untranslated region (UTR) followed by 413-bp encoding a portion of the N-terminal domain. PCR products were cloned into pCRII vectors using the TOPO-TA system (Invitrogen) and sequenced.

RNA In Situ Hybridization

Two RNA probes were generated from the *ar* cDNA fragments described above. One probe was 1,100 bp and contained part of the DNA-binding domain and the entire ligand-binding domain, the second probe was 730 bp and contained 317 bp of 5'UTR followed by 413 bp encoding a portion of the N-terminal domain. All images shown were generated using the 1,100 bp probe, although we obtained similar results using the 730 bp probe (not shown). No expression in either whole embryos or in adult brains was visible using sense RNA probes (not shown).

UTP-digoxigenin and UTP-fluorescein labeled RNA probes were generated using Roche Molecular Biochemicals reagents. Single and double-label whole-mount in situ hybridizations on embryos and larvae were performed as described (Liang et al., 2000) using *slc26a1* (Wingert et al., 2007) and *vasa* (Pelegrini et al., 1999) RNA probes. Hybridized probes were detected using alkaline phosphatase-conjugated antibodies and visualized by 4-nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) staining for single labeling, or NBT/BCIP followed by idonitrotetrazolium (INT) and BCIP staining for double labeling. To generate transverse sections at the level of pronephra, in situ hybridization for *ar* or *ar* and *slc26a1* was performed on whole embryos. Embryos were equilibrated in 50% glycerol in PBS and laid laterally on a slide. A 28 gauge needle was used to make cuts along the tail, perpendicular to the body axis. The one-somite thick sections were mounted on slides and imaged using differential interference contrast (DIC) microscopy.

To identify regions of the adult brain that express the *ar* gene, we optimized the standard whole-mount in situ hybridization technique used on embryos (Thisse and Thisse, 2008) for use on dissected, whole adult brains. Adult, 4.5-month-old male and female zebrafish were killed by rapid immersion in ice water and then decapitated. The body cavity was opened and the presence of ovaries or testes was used to determine sex. Brains were removed, fixed overnight at 4°C in 4% formaldehyde, then washed in PBS and stored in 100% methanol at -20°C. The whole-mount in situ hybridization protocol was performed (Liang et al., 2000) with the following modifications: brains were treated with 20 µg/ml proteinase K for 35 min and then fixed in 4% formaldehyde in PBS at room temperature for 1 h. Reactions were developed in NBT/BCIP for several hours to ensure that signal would be visible in deep regions of the brain. Brains were then fixed overnight in 4% formaldehyde in PBS at 4°C, washed in PBS, and embedded in 4% low melting point agarose (Cambrex) using a 10 mm × 10 mm × 5 mm disposable vinyl specimen mold (Sakura). Coronal sections (100 µm) were collected using a Vibratome (Leica VT1000S) and mounted on Superfrost Plus slides (VWR) in 50% glycerol in PBS. Agarose embedding and Vibratome sectioning preserve tissue morphology better than freezing and cryosectioning and is more rapid than embedding tissue in plastic or paraffin. Generating 100-µm-thick Vibratome sections provides approximately 25 sections per brain, allowing many brains to be sectioned and analyzed in the time it takes to analyze a single brain cut into 5- to 10-µm-thick sections.

To confirm that RNA probes are able to penetrate into deep regions of the brain (such as the dorsal zone of the periventricular hypothalamus and tuberal nucleus), we compared GFP fluorescence and *gfp* gene expression brains from adult *Tg(h2afv:GFP)^{kca66}* fish (Pauls et al., 2001; a generous gift from the Campos-Ortega laboratory). This transgenic line expresses a histone 2A-GFP fusion protein driven by a histone 2A promoter region. Because histone 2A is expressed ubiquitously, GFP is expressed in and labels all cell nuclei in embryos (Pauls et al., 2001) and is

thought to similarly label all cell nuclei in adults. Therefore, if RNA probes are able to penetrate into deep areas of whole, adult brains, then labeling of *gfp* transcripts using in situ hybridization should completely overlap with GFP fluorescence. Adult *Tg(h2afv:GFP)^{kca66}* fish (between 4 months and 1 year old) were killed and brains dissected and fixed as described above. To assay fluorescence, brains were washed in PBS, embedded in agarose and sectioned as described. To detect *gfp* transcripts, brains were incubated in methanol and the modified in situ hybridization protocol was performed as described. We observed robust fluorescence in cells of the paraventricular organ, lateral hypothalamus, and in cells bordering the lateral recess of the diencephalic ventricle (Fig. 1I). *gfp* transcripts were also detected in these regions (Fig. 1J), indicating that the modified whole-mount in situ hybridization protocol can detect gene expression in deep regions of the brain. We also observed corresponding patterns of GFP fluorescence and *gfp* gene expression throughout the telencephalon and cerebellum, regions where *ar* gene expression was not detected (not shown).

In situ hybridization images were collected using a Zeiss Axioskop microscope equipped with an AxioCam HRC digital camera. Fluorescence images were captured using a Zeiss Imager.Z1 microscope equipped with an AxioCam MRm digital camera. All images were captured using Zeiss Axiovision 4.5 software. Cropping, brightness-contrast adjustments, and imaging artifacts outside of the tissue were removed using Adobe Photoshop CS3. We assayed six male and six female brains, but observed no gross differences in the pattern of *ar* expression. Anatomical regions of the brain were identified based on comparison with a topological atlas of the adult zebrafish brain (Wullimann et al., 1996) and on subsequent revised information (Kaslin and Panula, 2001; Rink and Wullimann, 2001; Wullimann and Mueller, 2004).

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REFERENCES

- Arnold AP, Breedlove SM. 1985. Organizational and activational effects of sex steroids on brain and behavior: a reanalysis. *Horm Behav* 19:469–498.
- Asadi FK, Dimaculangan DD, Berger FG. 1994. Androgen regulation of gene expression in primary epithelial cells of the mouse kidney. *Endocrinology* 134:1179–1187.
- Bazer GT, Ebbesson SOE, Reynolds JB, Bailey RP. 1987. A cobalt-lysine study of primary olfactory projections in king salmon fry (*Oncorhynchus tshawytscha* Walbaum). *Cell Tissue Res* 248:499–503.
- Becerra M, Manso MJ, Rodriguez-Moldes I, Anadon R. 1994. Primary olfactory fibres project to the ventral telencephalon and preoptic region in trout (*Salmo trutta*): a developmental immunocytochemical study. *J Comp Neurol* 342:131–143.
- Berger FG, Watson G. 1989. Androgen-regulated gene expression. *Annu Rev Physiol* 51:51–65.
- Borg B. 1994. Androgens in teleost fishes. *Comp Biochem Physiol C* 109:219–245.
- Brantley RK, Marchaterre MA, Bass AH. 1993. Androgen effects on vocal muscle structure in a teleost fish with inter- and intra-sexual dimorphism. *J Morphol* 216:305–318.
- Capel B. 2000. The battle of the sexes. *Mech Dev* 92:89–103.
- Cardwell JR, Stacey NE, Lang SLC, Tan ESP, McAdam DSO. 1995. Androgen increases olfactory receptor response to a vertebrate sex pheromone. *J Comp Physiol [A]* 176:55–61.
- Chai C, Liu YW, Chan WK. 2003. Ff1b is required for the development of steroidogenic component of the zebrafish interrenal organ. *Dev Biol* 260:226–244.
- Cooke B, Hegstrom CD, Villeneuve LS, Breedlove SM. 1998. Sexual differentiation of the vertebrate brain: principles and mechanisms. *Front Neuroendocrinol* 19:323–362.
- Crews D, Silver R. 1985. Reproductive physiology and behavior interactions in nonmammalian vertebrates. In: Adler NT, Pfaff DW, Goy RW, editors. *Handbook of behavioral neurobiology*. Vol. 7. New York: Plenum Press. p xxi, 797.
- Cupp AS, Skinner MK. 2001. Actions of the endocrine disruptor methoxychlor and its estrogenic metabolite on in vitro embryonic rat seminiferous cord formation and perinatal testis growth. *Reprod Toxicol* 15:317–326.
- de Waal P, Wang D, Nijenhuis W, Schulz R, Bogerd J. 2008. Functional characterization and expression analysis of the androgen receptor in zebrafish (*Danio rerio*) testis. *Reproduction*.
- Drummond I. 2003. Making a zebrafish kidney: a tale of two tubes. *Trends Cell Biol* 13:357–365.
- Fenske M, Segner H. 2004. Aromatase modulation alters gonadal differentiation in developing zebrafish (*Danio rerio*). *Aquat Toxicol* 67:105–126.
- Folgueira M, Anadon R, Yanez J. 2004. An experimental study of the connections of the telencephalon in the rainbow trout (*Oncorhynchus mykiss*). I. Olfactory bulb and ventral area. *J Comp Neurol* 480:180–203.
- Force A, Lynch M, Pickett FB, Amores A, Yan YL, Postlethwait J. 1999. Preservation of duplicate genes by complementary, degenerative mutations. *Genetics* 151:1531–1545.
- Gamse JT, Shen Y-C, Thisse C, Thisse B, Raymond PA, Halpern ME, Liang JO. 2002. Otx5 regulates genes that show circadian expression in the zebrafish pineal complex. *Nat Genet* 30:117–121.
- Gao W, Bohl CE, Dalton JT. 2005. Chemistry and structural biology of androgen receptor. *Chem Rev* 105:3352–3370.
- Gelinas D, Callard GV. 1997. Immunolocalization of aromatase- and androgen receptor-positive neurons in the goldfish brain. *Gen Comp Endocrinol* 106:155–168.
- Harbott LK, Burmeister SS, White RB, Vagell M, Fernald RD. 2007. Androgen receptors in a cichlid fish, *Astatotilapia burtoni*: structure, localization, and expression levels. *J Comp Neurol* 504:57–73.
- Hart BL, Leedy MG. 1985. Neurological basis of male sexual behavior: a comparative analysis. In: Adler NT, Pfaff DW, Goy RW, editors. *Handbook of behavioral neurobiology*. Vol. 7. New York: Plenum Press. p xxi, 797.
- Holbech H, Kinnberg K, Petersen GI, Jackson P, Hylland K, Norrgren L, Bjerregaard P. 2006. Detection of endocrine disruptors: evaluation of a Fish Sexual Development Test (FSDT). *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 144:57–66.
- Honkanen T, Ekstrom P. 1990. An immunocytochemical study of the olfactory projections in the three-spined stickleback, *Gasterosteus aculeatus*, L. *J Comp Neurol* 292:65–72.
- Honkanen T, Ekstrom P. 1991. An immunocytochemical study of the development of the olfactory system in the three-spined stickleback (*Gasterosteus aculeatus* L., Teleostei). *Anat Embryol (Berl)* 184:469–477.
- Hossain MS, Larsson A, Scherbak N, Olsson PE, Orban L. 2007. Zebrafish androgen receptor: isolation, molecular, and biochemical characterization. *Biol Reprod* 78:361–369.
- Hsu H-J, Hsiao P, Kuo M-W, Chung B-C. 2002. Expression of zebrafish *cyp11a1* as a maternal transcript and in yolk syncytial layer. *Gene Expr Patterns* 2:219–222.
- Hsu HJ, Lin B, Chung BC. 2003. Parallel early development of zebrafish interrenal glands and pronephros: differential control by *wt1* and *ff1b*. *Development* 130:2107–2116.
- Ikeuchi T, Todo T, Kobayashi T, Nagahama Y. 2001. Two subtypes of androgen and progesterone receptors in fish testes.

- Comp Biochem Physiol B Biochem Mol Biol 129:449–455.
- Jaillon O, Aury J-M, Brunet F, Petit J-L, Stange-Thomann N, Mauceli E, Bouneau L, Fischer C, Ozouf-Costaz C, Bernot A, Nicaud S, Jaffe D, Fisher S, Lutfalla G, Dossat C, Segurens B, Dasilva C, Salanoubat M, Levy M, Boudet N, Castellano S, Anthouard V, Jubin C, Castelli V, Katinka M, Vacherie B, Biemont C, Skalli Z, Cattolico L, Poulain J, de Bernardinis V, Cruaud C, Duprat S, Brottier P, Coutanceau J-P, Guouzy J, Parra G, Lardier G, Chapple C, McKernan KJ, McEwan P, Bosak S, Kellis M, Volff J-N, Guigo R, Zody MC, Mesirov J, Lindblad-Toh K, Birren B, Nusbaum C, Kahn D, Robinson-Rechavi M, Laudet V, Schachter V, Quetier F, Saurin W, Scarpelli C, Wincker P, Lander ES, Weissenbach J, Roest Crollius H. 2004. Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. *Nature* 431:946–957.
- Jorgensen A, Andersen O, Bjerregaard P, Rasmussen LJ. 2007. Identification and characterisation of an androgen receptor from zebrafish *Danio rerio*. *Comp Biochem Physiol C Toxicol Pharmacol* 146:561–568.
- Kaslin J, Panula P. 2001. Comparative anatomy of the histaminergic and other aminergic systems in zebrafish (*Danio rerio*). *J Comp Neurol* 440:342–377.
- Koyama Y, Satou M, Oka Y, Ueda K. 1984. Involvement of the telencephalic hemispheres and the preoptic area in sexual behavior of the male goldfish, *Carassius auratus*: a brain-lesion study. *Behav Neural Biol* 40:70–86.
- Liang JO, Etheridge A, Hantsoo L, Rubinstein AL, Nowak SJ, Izpisua Belmonte JC, Halpern ME. 2000. Asymmetric nodal signaling in the zebrafish diencephalon positions the pineal organ. *Development* 127:5101–5112.
- Martineau J, Nordqvist K, Tilmann C, Lovell-Badge R, Capel B. 1997. Male-specific cell migration into the developing gonad. *Curr Biol* 7:958–968.
- McAbee MD, DonCarlos LL. 1998. Ontogeny of region-specific sex differences in androgen receptor messenger ribonucleic acid expression in the rat forebrain. *Endocrinology* 139:1738–1745.
- Merlet J, Moreau E, Habert R, Racine C. 2007a. Development of fetal testicular cells in androgen receptor deficient mice. *Cell Cycle* 6:2258–2262.
- Merlet J, Racine C, Moreau E, Moreno SG, Habert R. 2007b. Male fetal germ cells are targets for androgens that physiologically inhibit their proliferation. *Proc Natl Acad Sci U S A* 104:3615–3620.
- Morris JA, Jordan CL, Breedlove SM. 2004. Sexual differentiation of the vertebrate nervous system. *Nat Neurosci* 7:1034–1039.
- Murphy CA, Stacey NE. 2002. Methyl-testosterone induces male-typical ventilatory behavior in response to putative steroid pheromones in female round gobies (*Neogobius melanostomus*). *Horm Behav* 42:109–115.
- Nunez JL, Huppenbauer CB, McAbee MD, Juraska JM, DonCarlos LL. 2003. Androgen receptor expression in the developing male and female rat visual and prefrontal cortex. *J Neurobiol* 56:293–302.
- Orn S, Holbech H, Madsen TH, Norrgren L, Petersen GI. 2003. Gonad development and vitellogenin production in zebrafish (*Danio rerio*) exposed to ethinylestradiol and methyltestosterone. *Aquat Toxicol* 65:397–411.
- Orn S, Yamani S, Norrgren L. 2006. Comparison of vitellogenin induction, sex ratio, and gonad morphology between zebrafish and Japanese medaka after exposure to 17 α -ethinylestradiol and 17 β -trenbolone. *Arch Environ Contam Toxicol* 51:237–243.
- Pandian TJ, Sheela SG. 1995. Hormonal induction of sex reversal in fish. *Aquaculture* 138:1–22.
- Parikh VN, Clement TS, Fernald RD. 2006. Androgen level and male social status in the African cichlid, *Astatotilapia burtoni*. *Behav Brain Res* 166:291–295.
- Pauls S, Geldmacher-Voss B, Campos-Ortega JA. 2001. A zebrafish histone variant H2A.F/Z and a transgenic H2A.F/Z: GFP fusion protein for in vivo studies of embryonic development. *Dev Genes Evol* 211:603–610.
- Pelegri F, Knaut H, Maischein H-M, Schulte-Merker S, Nusslein-Volhard C. 1999. A mutation in the zebrafish maternal-effect gene *nebel* affects furrow formation and vasa RNA localization. *Curr Biol* 9:1431–1440.
- Pfaff D, Modianos D. 1985. Neural mechanisms of female reproductive behavior. In: Adler NT, Pfaff DW, Goy RW, editors. *Handbook of behavioral neurobiology*. Vol. 7. New York: Plenum Press. p xxi, 797.
- Postlethwait J, Amores A, Cresko W, Singer A, Yan Y-L. 2004. Subfunction partitioning, the teleost radiation and the annotation of the human genome. *Trends Genet* 20:481–490.
- Quinkler M, Bujalska IJ, Kaur K, Onyimba CU, Buhner S, Allolio B, Hughes SV, Hewison M, Stewart PM. 2005. Androgen receptor-mediated regulation of the α -subunit of the epithelial sodium channel in human kidney. *Hypertension* 46:787–798.
- Rink E, Wullimann MF. 2001. The teleostean (zebrafish) dopaminergic system ascending to the subpallium (striatum) is located in the basal diencephalon (posterior tuberculum). *Brain Res* 889:316–330.
- Rodriguez-Mari A, Yan YL, Bremiller RA, Wilson C, Canestro C, Postlethwait JH. 2005. Characterization and expression pattern of zebrafish Anti-Müllerian hormone (*Amh*) relative to *sox9a*, *sox9b*, and *cyp19a1a*, during gonad development. *Gene Expr Patterns* 5:655–667.
- Serluca FC, Fishman MC. 2001. Pre-pattern in the pronephric kidney field of zebrafish. *Development* 128:2233–2241.
- Shah NM, Pisapia DJ, Maniatis S, Mendelsohn MM, Nemes A, Axel R. 2004. Visualizing sexual dimorphism in the brain. *Neuron* 43:313–319.
- Simerly RB. 2002. Wired for reproduction: organization and development of sexually dimorphic circuits in the mammalian forebrain. *Annu Rev Neurosci* 25:507–536.
- Simerly RB, Chang C, Muramatsu M, Swanson LW. 1990. Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. *J Comp Neurol* 294:76–95.
- Soma KK, Francis RC, Wingfield JC, Fernald RD. 1996. Androgen regulation of hypothalamic neurons containing gonadotropin-releasing hormone in a cichlid fish: integration with social cues. *Horm Behav* 30:216–226.
- Takahashi T. 1977. Juvenile hermaphroditism in the zebrafish, *Brachydanio rerio*. *Bull Fac Fish Hokkaido Univ* 28:57–65.
- Thisse C, Thisse B. 2008. High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nat Protoc* 3:59–69.
- To TT, Hahner S, Nica G, Rohr KB, Hammerschmidt M, Winkler C, Allolio B. 2007. Pituitary-interrenal interaction in Zebrafish interrenal organ development. *Mol Endocrinol* 21:472–485.
- Walker C. 1999. Haploid screens and gamma-ray mutagenesis. *Methods Cell Biol* 60:43–70.
- Weidinger G, Wolke U, Kopranner M, Klingner M, Raz E. 1999. Identification of tissues and patterning events required for distinct steps in early migration of zebrafish primordial germ cells. *Development* 126:5295–5307.
- Weidinger G, Stebler J, Slanchev K, Dumstrei K, Wise C, Lovell-Badge R, Thisse C, Thisse B, Raz E. 2003. *dead end*, a novel vertebrate germ plasm component, is required for zebrafish primordial germ cell migration and survival. *Curr Biol* 13:1429–1434.
- Werner MH, Huth JR, Gronenborn AM, Clore GM. 1996. Molecular determinants of mammalian sex. *Trends Biochem Sci* 21:302–308.
- Westerfield M. 1993. *The Zebrafish book: a guide for the laboratory use of zebrafish (Brachydanio rerio)*. Eugene, OR: University of Oregon Press. 1 v. (various pagings) p.
- Wingert RA, Selleck R, Yu J, Song HD, Chen Z, Song A, Zhou Y, Thisse B, Thisse C, McMahon AP, Davidson AJ. 2007. The *cdx* genes and retinoic acid control the positioning and segmentation of the zebrafish pronephros. *PLoS Genet* 3:1922–1938.
- Wullimann MF, Mueller T. 2004. Teleostean and mammalian forebrains contrasted: evidence from genes to behavior. *J Comp Neurol* 475:143–162.
- Wullimann MF, Rupp B, Reichert H. 1996. *Neuroanatomy of the zebrafish brain*. Amsterdam: Birkhauser.
- Zhou X, Kudo A, Kawakami H, Hirano H. 1996. Immunohistochemical localization of androgen receptor in mouse testicular germ cells during fetal and postnatal development. *Anat Rec* 245:509–518.