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Conserved Sensory-Neurosecretory Cell Types in Annelid and Fish Forebrain: Insights into Hypothalamus Evolution

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SUMMARY

Neurosecretory control centers form part of the forebrain in many animal phyla, including vertebrates, insects, and annelids. The evolutionary origin of these centers is largely unknown. To identify conserved, and thus phylogenetically ancient, components of neurosecretory brain centers, we characterize and compare neurons that express the prohormone vasotocin (vasopressin/oxytocin)-neurophysin in the developing forebrain of the annelid *Platynereis dumerilii* and of the zebrafish. These neurons express the same tissue-restricted microRNA, *miR-7*, and conserved, cell-type-specific combinations of transcription factors (*nk2.1*, *rx*, and *otp*) that specify their identity, as evidenced by the specific requirement of zebrafish rx3 for *vasotocin-neurophysin* expression. *MiR-7* also labels another shared population of neurons containing RFamides. Since the vasotocinergic and RFamidergic neurons appear to be directly sensory in annelid and fish, we propose that cell types with dual sensory-neurosecretory properties were the starting point for the evolution of neurosecretory brain centers in Bilateria.

INTRODUCTION

Animal brains integrate and process sensory stimuli such as light and chemicals. They elicit body-wide responses via two major output systems: direct innervation with synaptic transmission and neurosecretion. Neurosecretory brain centers secrete neuropeptides and nonpeptidergic neuromodulators (for example, see Norris, 1997) into the surrounding tissue or into the vascular system that distributes them through the body. They control major develop-

mental and physiological processes such as growth, metabolism, gonad maturation, or reproduction (Lovejoy, 2005).

Vertebrate neurosecretory brain centers are located in the hypothalamus and preoptic area regions of the forebrain (green in Figure 1A). The hypothalamus forms the major part of the ventral diencephalon, and the preoptic area forms between ventral telencephalon and diencephalon (Norris, 1997). Outside vertebrates, neurosecretory systems have been studied in molecular detail in insects, but similar brain centers also exist in crustaceans and spiders and in other invertebrate groups such as annelids and mollusks (e.g., Hartenstein, 2006; Matsumoto and Ishii, 1992).

Despite the importance and widespread occurrence of neurosecretory brain centers, their evolutionary origin is only beginning to be unraveled (e.g., Matsumoto and Ishii, 1992; Hartenstein, 2006). Based on structural, functional, and developmental evidence, it has recently been proposed that the vertebrate hypothalamus and the insect pars intercerebralis trace back to a simple brain with neurosecretory cells that existed in the common bilaterian ancestors (De Velasco et al., 2007; Hartenstein, 2006). Corroborating this, hypothalamus and pars intercerebralis secrete similar neuropeptides, such as insulin-like peptides, RFamides, and tachykinins (De Velasco et al., 2007). However, genes encoding other hypothalamic neuropeptides such as vasopressin, oxytocin, or gonadotropin releasing hormone (GnRH) have so far not been detected in the sequenced insect and nematode genomes, although their receptors are conserved in the fly genome (Hewes and Taghert, 2001).

A different picture has emerged from limited analyses of gene collections from annelids and mollusks. These have preserved a larger fraction of vertebrate-type neuropeptides, including also those that are lost in *Drosophila* and in *C. elegans*, such as a vasotocin (vasopressin/oxytocin)-neurophysin preprohormone homolog present in annelids (Oumi et al., 1994), gastropods (Van Kesteren et al., 1992), and cephalopods (Takuwa-Kuroda et al., 2003), a
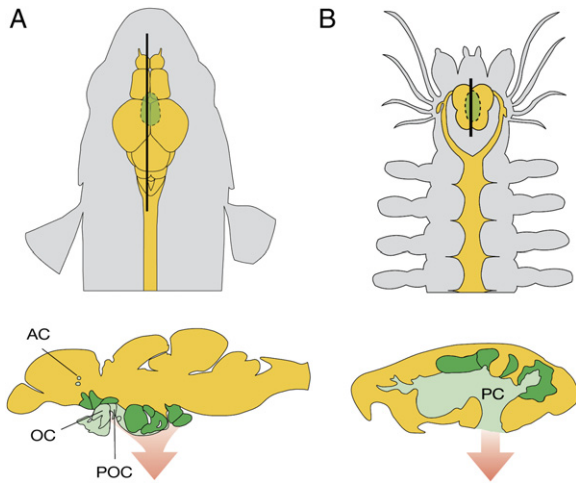
Figure 1. Neuroendocrine Centers in the Fish and Nereidid Forebrain

(Top) Schematic dorsal views of adult zebrafish (A) and nereidid (B) heads, indicating the CNS (yellow) and the main medial neurosecretory brain centers (green). Black lines: positions of sections. (Bottom) Respective parasagittal sections, showing main neurosecretory brain nuclei (green) and main sites of neurosecretory release (red arrow). Characteristic commissures and neuropil, light green; AC, anterior commissure; OC, optic chiasm; POC, postoptic commissure; PC, pre-oral comissure. Data integrated from Matsumoto and Ishii, 1992; Thorndyke and Goldsworthy, 1988; Wullimann et al., 1996.

GnRH-GAP preprohormone in a cephalopod (Iwakoshi et al., 2002), and a POMC preprohormone in leeches (Salzet et al., 1997). This is in line with recent gene-inventory comparisons suggesting that the annelid lineage has evolved at a slower evolutionary pace (Raible et al., 2005) and indicates that a large set of vertebrate-type neuropeptides already existed in Urbilateria.

Here we characterize neurosecretory cells containing *vasotocin-neurophysin* in the developing neurosecretory brain center of the annelid *Platynereis dumerilii* (Nereididae) and of the zebrafish *Danio rerio* as a vertebrate reference system. Nereidids develop elaborate neuroendocrine brain centers, as is characteristic for other annelids (e.g., Hofmann, 1976) (Figure 1B). We find that the overall positioning of the developing neurosecretory centers with respect to the molecular topography of the forebrain and to the early axonal scaffold is the same in *Platynereis* and vertebrates. We also show that, within these centers, early differentiating vasotocinergic neurons, as well as RFamidergic neurons, are similar across species in sensory modality, morphology, and in cell-type-specific gene expression, including the conserved expression of a tissue-specific microRNA, *miR-7*. Guided by our "molecular fingerprint" comparison, we establish that *vasotocin-neurophysin* expression specifically depends on one of the coexpressed transcription factors, *rx3*, in fish. We propose that light-sensitive vasotocinergic and chemosensory RFamidergic neuron types in the medial head region formed part of a primitive urbilaterian brain, directly

conveying sensory cues from the ancient marine environment to changes in body physiology.

RESULTS

Conserved Molecular Anatomy of the Developing Forebrain

During development, the spatially restricted expression of transcription factors subdivides the animal body into regions that show a surprising degree of conservation (Lowe et al., 2003). As a first step toward the comparative analysis of neurosecretory brain centers, we thus compared the "molecular anatomy" of the forebrain in *Platynereis* and fish, in order to determine whether similar cell types, such as the vasotocinergic cells, originate from corresponding regions.

In vertebrates from lamprey to mouse, the transcription factors nk2.1 and pax6 subdivide the forebrain anlage into a medial and a lateral region starting at open neural plate stages (e.g., Corbin et al., 2003; Murakami et al., 2001), as validated for zebrafish in Figure 2A. Neurulation transfers this medial-lateral subdivision of the neural plate (Figure 2A) into a ventral-dorsal subdivision of the brain vesicle (Figure 2B). The *nk2.1*+ region gives rise to the hypothalamus and preoptic region (Varga et al., 1999; Rohr et al., 2001) including the vasotocin-secreting cells, and hypothalamus development is severely affected in *nk2.1* mutant mice (Kimura et al., 1996). We determined and spatially related the expression of the *Platynereis* orthologs of *nk2.1* and *pax6*, *Pdu-nk2.1* and *Pdu-pax6*, in the prostomium of the *Platynereis* trochophora larva (Figures 2C and 2D). The prostomium gives rise to the paired cerebral ganglia, the polychaete forebrain equivalent. We found that medial expression of *Pdu-nk2.1* is laterally bounded by *Pdu-pax6* expression, from 15 hpf onward throughout development (Figure 2C and data not shown). This indicates evolutionary conservation of the molecular mediolateral subdivision of the anterior body regions in annelid and vertebrate.

To substantiate this, we studied additional transcription factors involved in forebrain specification. Vertebrate r̲etina homeobo̲x (*rx*) and v̲entral a̲nterior homeobo̲x (*vax*) genes are expressed exclusively in the developing medial forebrain, including prospective hypothalamus and preoptic area, and in its developmental derivative, the eye (Chuang et al., 1999; Take-uchi et al., 2003). For zebrafish, we determined expression overlap of these genes with *nk2.1a* at open neural plate (*Dr-rx3*, Figure 2E) and early neurula stages (*Dr-vax1*, Figure 2F). In *Platynereis*, we found *Pdu-rx* expression restricted to the developing prostomium (Arendt et al., 2004), partly overlapping with *Pdu-nk2.1* expression (Figure 2G). Unlike *Drosophila* and *C. elegans*, *Platynereis* also possesses a clear *vax* ortholog (see Figure S1B in the Supplemental Data available with this article online) that indeed exhibits highly restricted expression in the medial prostomial region, partly overlapping that of *Pdu-nk2.1* (Figure 2H). We conclude that both *Platynereis* and vertebrate express
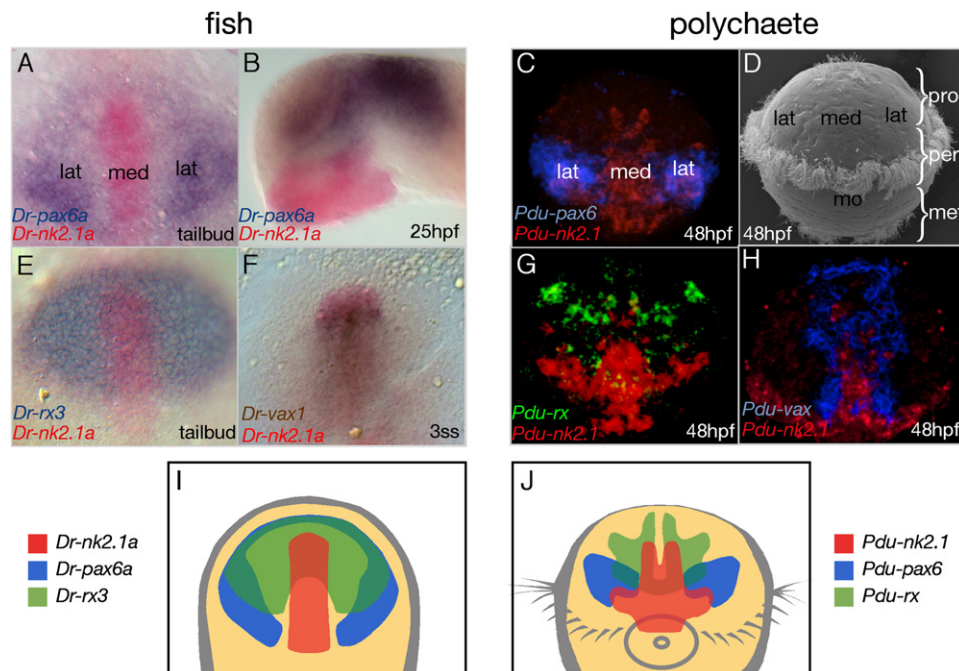
Figure 2. Conserved Regionalization of the Zebrafish and *Platynereis* Forebrain
(A, E, and F) Dorsal and (B) lateral views of zebrafish embryos hybridized with the indicated riboprobes; stages as indicated. (A, E, and F) Anterior to the top and (B) to the left, (B) eyes removed. (C, G, and H) Apical views of *Platynereis* embryos hybridized with the indicated riboprobes. (D) SEM picture of a *Platynereis* larva. Pro, prostomium; per, peristomium; met, metastomium; mo, mouth. (I and J) Expression synopsis of zebrafish *nk2.1a*, *pax6.1*, and *rx3* (I) and of the polychaete orthologs (J). Yellow, anterior neuroectoderm; med, medial; lat, lateral forebrain.

nk2.1, *rx*, and *vax* orthologs in the prospective medial forebrain region as part of a conserved molecular anatomy (Figures 2I and 2J). Since this region gives rise to major neurosecretory forebrain centers in vertebrates, we next tested whether this is also the case in *Platynereis*.

A Neurosecretory Fiber Plexus Located at the Anterior End of the Early Axonal Scaffold

We found that in the developing *Platynereis* forebrain many of the early differentiating neurons (expressing *synaptotagmin* as neuronal differentiation marker, Figure 3A) also express *prohormone convertase 2* (Figure 3B) and thus can be identified as neurosecretory cells (Lovejoy, 2005). Most of these early cells emerge from the anterior *nk2.1*, *rx*, and/or *vax* expressing region (compare Figure 3B to Figure 2J, and data not shown), providing evidence that this region also gives rise to neurosecretory brain centers in *Platynereis*. Using an antibody directed against stabilized acetylated microtubules that labels dendrites, cilia, and axonal projections, we determined that these neurons are positioned at the anterior end of the early axonal scaffold (Figure 3C), close to the preoral commissures (that include axons outgrowing and crossing from the larval and adult eyes; Arendt et al., 2004; blue arrows in Figure 3C). Embedded in the preoral commissure, we detected a dense medial plexus of interdigitating basal neurites (red asterisk, Figure 3C), reminiscent of the

neurosecretory plexus described for other annelid and mollusk larvae (Kempf et al., 1997; Lacalli, 1984). Electron optics revealed that the cellular processes of this plexus contain dense-core vesicles (black arrowheads, Figures 3D–3G; exocytosis in Figure 3E; confirming neurosecretory activity) and that they are interwoven with fluid-filled lacunae of the primary body cavity (Figures 3F and 3G), strongly suggesting a direct release of neuropeptides from these cells into the larval hemolymph. Therefore, the early differentiating neurosecretory cells in the *Platynereis* and vertebrate (Wilson et al., 2002) medial forebrain share their localization at the anterior end of the early axonal scaffold (close to the optic commissures) and their projection into an underlying medial neurosecretory fiber plexus surrounded by body fluid (Figure 3O).

Vasotocinergic and RFamidergic Neurons Differentiate in the Medial Forebrain Region in *Platynereis* and Zebrafish

We next compared selected neuron types that differentiate early in the neurosecretory brain centers. We cloned the gene encoding *Platynereis* vasotocin-neurophysin (*Pdu-vtn*) (Figure 3H and Figures S1E and S2). In the larval medial forebrain region, transcripts were detected in a pair of cells (Figures 3I and 3J) directly medially adjacent to the large cilia (arrowheads, Figure 3J) of the deep brain extra-ocular photoreceptor cells (Arendt et al., 2004). Each *vtn*+
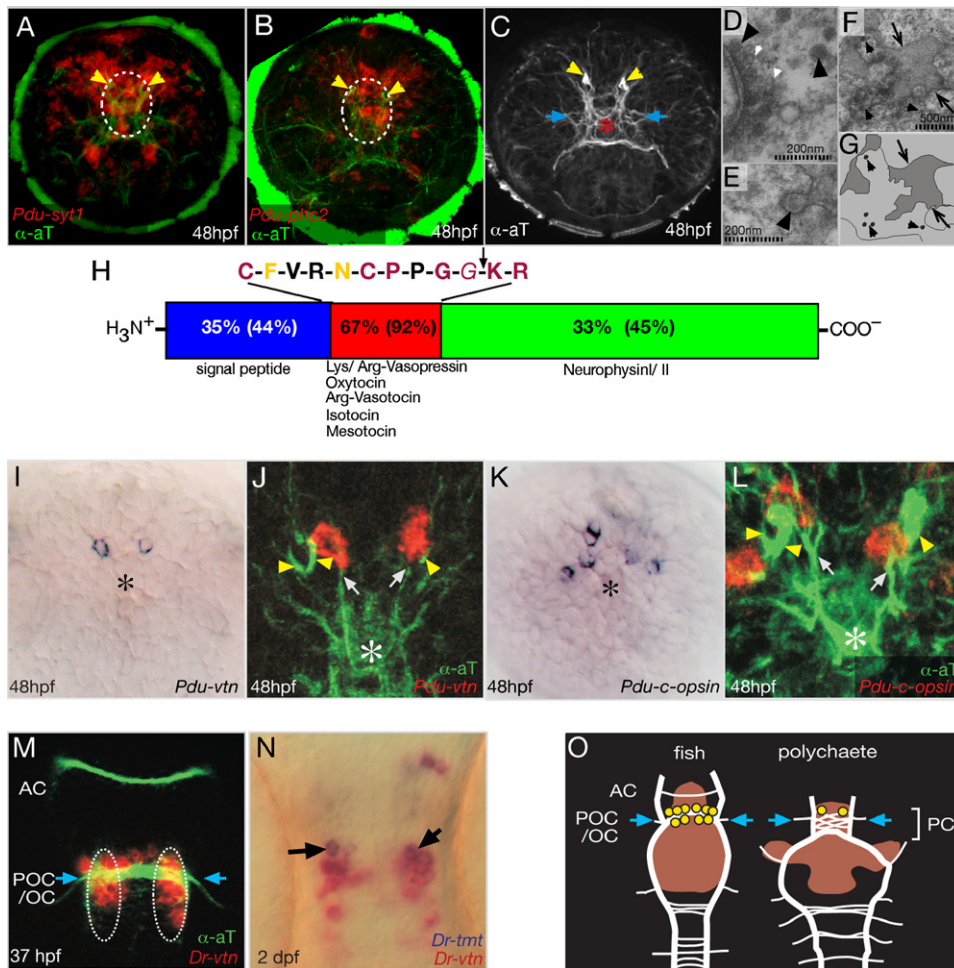
Figure 3. Vasotocinergic Cells in the Developing Medial Neurosecretory Forebrain

(A–C) Differentiating neurosecretory cells in the *Platynereis* medial forebrain. Circles demarcate the location of the studied vasotocinergic and RFamidergic cells in comparison to axonal scaffold and position of sections shown in (D)–(F).

(D–F) Transmission electromicrographs of the *Platynereis* medial forebrain plexus; black arrowheads, dense core vesicles (DCV); white arrowheads, synaptic vesicles (SV); scales as indicated. (D) Synapse containing DCV and SV. (E) DCV release, indicating larval neurosecretory activity. (F) Primary body cavity (black arrows) reaching into the plexus.

(G) Scheme of (F).

(H) Scheme of *Platynereis*-Vasotocin-Neurophysin (*Pdu*-Vtn), based on Figure S2. Percentages: identity of *Mus* ArgVasopressin-Neurophysin to *Pdu*-Vtn (in brackets: to zebrafish Vtn). Arrow demarcates the predicted cleavage site behind the nonapeptide. Different colors indicate the degree of conservation to other lophotrochozoans: complete (red), partial (yellow), none (black).

(I–L) *Pdu-vtn* and *Pdu-c-opsin* expression localizes to the same landmarks (axons and large cilia of extraocular PRCs). (Note that these cells do not appear to bear the prominent cilia themselves.) Coexpression could not be assayed directly due to technical limitations (Tessmar-Raible et al., 2005).

(M and N) Zebrafish *vtn* and *tmt-opsin* are coexpressed (arrows) by cells adjacent to the postoptic commissure/optic chiasm (blue arrows).

(O) Summary schemes of zebrafish and *Platynereis* larval brains, indicating the position of vasotocinergic cells (yellow) with respect to the axon tracts (white) and *nk2.1* expression (red). Anterior to the top. Blue arrows indicate positions of optic commissures (POC/OC).

Stages, riboprobes, and antibodies as indicated. Asterisks demarcate the position of the neurosecretory forebrain plexus; yellow arrowheads, large cilia of deep brain photoreceptor cells; white arrows (J and L), projections of *vtn+* cells into the plexus; blue arrows, entry of optic fibers into the axonal scaffolds of both *Platynereis* and zebrafish. (A–C and I–L) Apical view, ventral down; (M–O) ventral view, anterior to the top. AC, anterior commissure; POC/OC, postoptic commissure/optic chiasm; PC, preoral commissure. Depth of confocal reconstructions: (A) 31 μm, (B) 35 μm, (C) 30 μm, (J) 15 μm, (L) 18 μm.

cell sends out an axon projecting into the center of the medial neurosecretory plexus (arrows and asterisk, Figure 3J). The immediate vicinity to the brain ciliary photoreceptors prompted us to test for coexpression with *Pdu-c-opsin*, an ortholog of zebrafish *tmt-opsin* and of other vertebrate ciliary opsins (Arendt et al., 2004). We indeed found *c-opsin+* cells located at the same position (Figures 3K and 3L) as the *Pdu-vtn+* cells and connecting to the same axons (white arrows, Figure 3L). Guided by this observation, we tested for coexpression of *tmt-opsin*
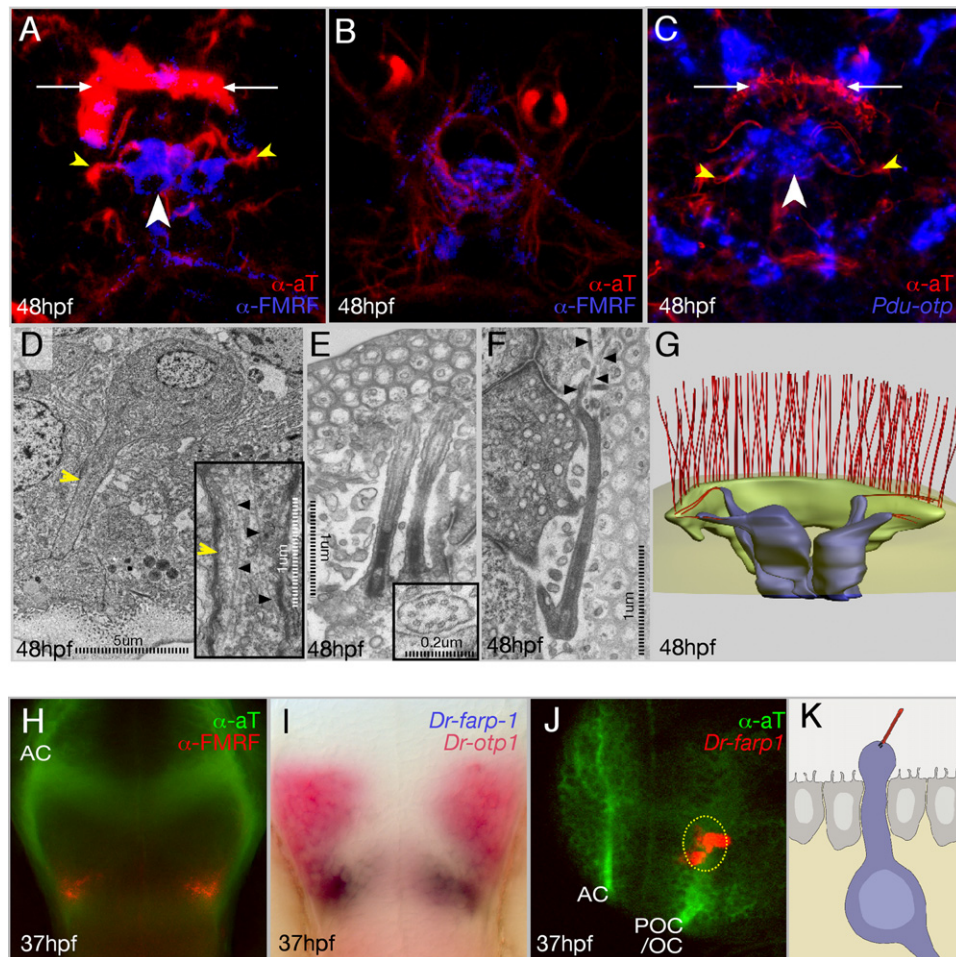
Figure 4. RFamidergic Neurons in *Platynereis* and Zebrafish Medial Forebrains

(A–C) Immunostainings/in situ hybridizations of RFamidergic, *otp*+ cells in the apical plexus of *Platynereis* embryos. (A and B) Same embryo, different confocal projections, showing the RFamidergic plexus (B) below the contributing cells ([A], same level as [C]). (D–F) Transmission electromicrographs and derived reconstruction (G). (D) TEM view of a medial flask-shaped cell. Inset: enlargement of the dendrite rich in microtubules (black arrowheads). (E) Two cilia protruding from a medial flask-shaped cell to the subcuticular space. Inset: cilium in cross-section. (F) Cilium of the branching type; black arrowheads, ramifications extending underneath the cuticle. (H) zebrafish RFamidergic cells and (I and J) *Dr-farp1* transcript in relation to *Dr-otp1* (red in [I]) and the axon scaffold (green in [J]). (K) Forebrain CSF-contacting neuron (according to data from Leonhardt, 1980).

AC, anterior commissure; POC/ OC, postoptic commissure/optic chiasm. Views: (A–F) apical, (A–C) ventral to the bottom; (G) ventroapical; (H and I) ventral, anterior to the top; (J) lateral, anterior left. White arrows: position of large multiciliated crescent cell as landmark for orientation, yellow arrowheads: microtubule-rich dendrites bearing cilia. Depth of confocal reconstructions: (A and C) 7 μm, (B) 3 μm.

and of the zebrafish *vasotocin-neurophysin (vtn)* gene in fish. *Vtn* appears as early as 27 hpf in small clusters of cells in the ventral hypothalamus (Figures 3M and 6N), and a subset of these indeed coexpress *Dr-tmt-opsin* (Figure 3N). These observations indicate the existence of an ancient population of vasotocinergic extraocular photoreceptors in the medial forebrain region in both *Platynereis* and fish (Figure 3O).

We also studied neurons containing the neuropeptide RFamide (Hartenstein, 2006). In *Platynereis*, a small but conspicuous set of early differentiating RFamidergic neurons (white arrowhead in Figure 4A) is embedded in, and contributes to, a dense RFamidergic fiber plexus (Figure 4B) as part of the medial neurosecretory plexus (see

above). These neurons persist into the postlarval brain (data not shown). Electron microscopy revealed apical dendrites that reach the surface and each bear two sensory cilia that protrude into the subcuticular extracellular space, a fluid-filled lumen underneath the cuticle in direct contact with the surrounding seawater (Figures 4D–4F). The cilia of the dorsal pair of cells are unbranched with a 9 × 2 + 0 axoneme (Figure 4E, inset), while the remaining cilia branch into several long and thin projections (black arrowheads, Figure 4F). The overall morphology of these cells conveys them the shape of a flask (Figure 4G). In vertebrates, RFamidergic forebrain neurons originate from two restricted populations, one of which is situated in the preoptic area/hypothalamus (Pinelli et al.,
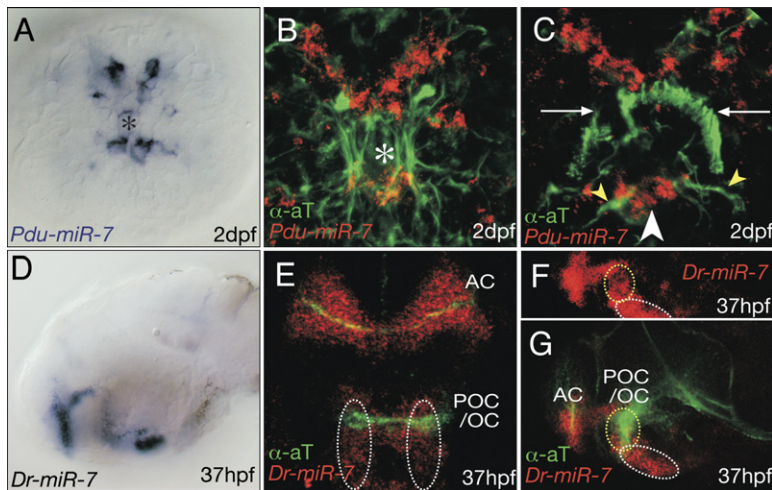
2000). We cloned and analyzed the *Dr-fmrfamide-related peptide 1* (*farp1*) gene, encoding a zebrafish RFamide family member (see also Supplemental Data). It is expressed in an early differentiating group of cells in the developing medial forebrain, in a position identical to the hypothalamic population of neurons stained by the FMRFamide antibody (Figures 4H–4J). Notably, in various vertebrates, including fish, the RFamidergic neurons of the hypothalamus have been described as central-spinal fluid (CSF)-contacting neurons (e.g., Castro et al., 2001, Figure 4K). Typically, these exhibit a slender dendritic process that expands into the CSF and bears one or more sensory cilia with 9 × 2 + 0 structure (Vigh et al., 2004), very reminiscent of the morphology of the *Platynereis* flask-shaped RFamidergic neurons and indicative of a chemosensory function in both species.

MicroRNA-7 Demarcates Small Subsets of Medial Forebrain Neurons, Including Vasotocinergic and RFamidergic Neurons in *Platynereis* and Zebrafish

Cell types are defined by their "molecular fingerprint," the unique combinatorial expression of cell-type-specific genes. These include effector genes that implement cell-type-specific characteristics (such as *vasotocin-neurophysin* or *c-opsin*), but also regulatory genes. We therefore examined regulatory genes that convey cell-type-specific "regulatory signatures" to forebrain neurons in *Platynereis* and fish. MicroRNAs are small noncoding RNAs that posttranscriptionally regulate expression (e.g., Wienholds et al., 2005). Some microRNAs are expressed in a highly restricted, tissue- or even cell-type-specific manner (Wienholds et al., 2005) and thus convey specific signatures of target mRNA expression to cell types (Sood et al., 2006). We chose to investigate *miR-7* because it shows a conserved and highly restricted expression in the medial forebrain at differentiation stages in zebrafish and medaka (Ason et al., 2006) and because it is conserved across Bilateria (Prochnik et al., 2007).

We established a protocol for miRNA detection in *Platynereis* and found *miR-7* expression highly restricted in the medial larval forebrain region (Figure 5A). Using whole-mount reflection confocal microscopy, we covisualized *miR-7+* cells and acetylated-tubulin-immunopositive axons, dendrites, and cilia. By this, we determined that *miR-7* expression comprised the vasotocinergic neurons medially adjacent to the large photoreceptor cilia (compare Figure 5B to Figure 3J) and the RFamidergic neurons with their conspicuous apical dendrites and cilia (white arrowhead in Figure 5C and Figures 4A and 4C). We also spatially correlated the restricted population of *miR-7+* cells present at 37 hpf in the zebrafish forebrain (Figure 5D) with the early axonal scaffold and determined that, as in *Platynereis*, vasotocinergic neurons coexpress *miR-7* (compare white outline in Figures 5E and 3M). *MiR-7* expression was also overlapping with RFamidergic cells (compare yellow outline in Figures 5F, 5G, and 4J).

A Conserved Regulatory Code of Transcription Factors

To further compare the regulatory signatures of forebrain neurosecretory cell types, we turned toward candidate transcription factors differentially coexpressed at differentiation stages. In mouse, the transcription factor orthopedia (Otp) localizes to and is directly required for the terminal differentiation of a subset of the neuropeptidergic hypothalamic neurons, including vasopressin/oxytocin-containing cells (Acampora et al., 1999). We identified a *Platynereis* ortholog, *Pdu-otp*, and found it expressed in a limited number of cells in the developing medial forebrain region (Figure 6A) comprising both the vasotocinergic neurons medially adjacent to the large photoreceptor cilia (compare Figure 6B to Figure 3J) and the RFamidergic neurons, as identified by costaining of their conspicuous apical dendrites and cilia (white arrowhead in Figure 4C). By double WMISH, we further determined that both cell types also express *Pdu-nk2.1* (Figures 6D
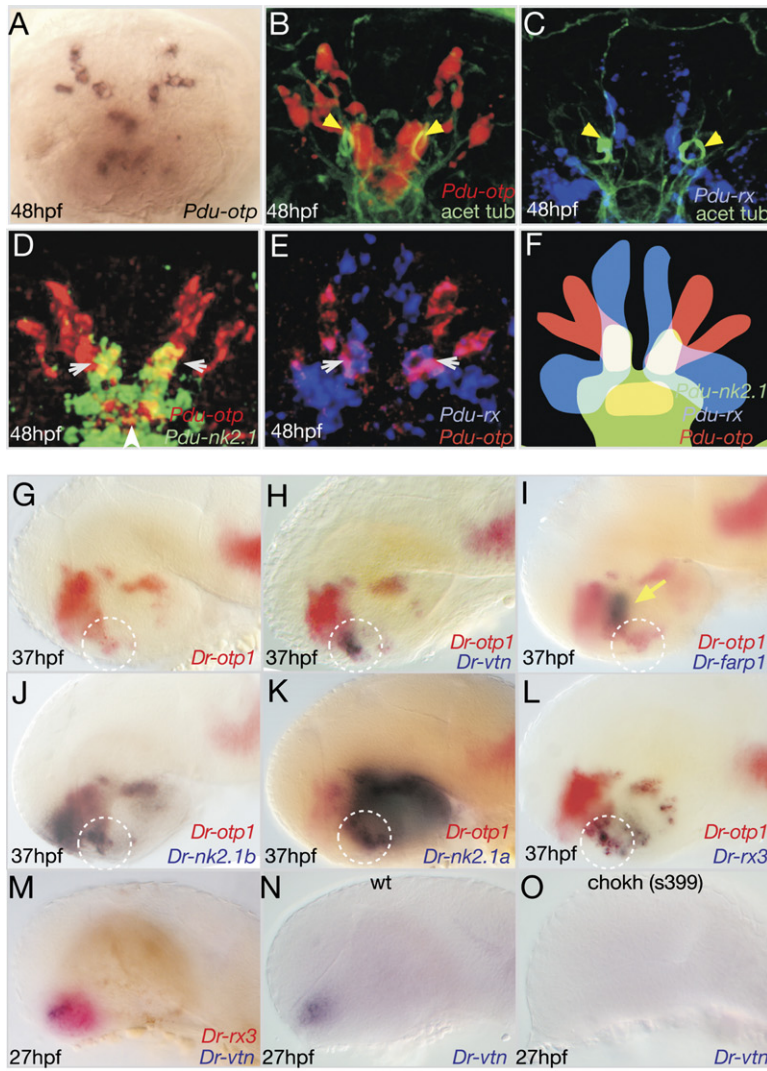
Figure 6. Regulatory Signature of *vtn+* and RFamidergic Cells and Dependence of *Dr-vtn* on *Dr-rx3*

Expression of indicated genes in (A–E) *Platynereis* and (G–O) zebrafish. (B and C) Expression in relation to the axonal scaffold (green), compare to Figures 3A–3C, 3J, 3L, and 5B for colocalization with *Pdu-vtn*, *Pdu-c-opsin*, *miR*-7. Yellow arrowheads: large cilia of deep brain ciliary photoreceptor cells. (F) Scheme of (D) and (E). The overlap of all three transcription factors (white) includes the medial *vtn+* cells (white arrows in [D] and [E]). Overlap of *nk2.1* and *otp* (yellow) in the medial RFamidergic cells (white arrowhead in [D]). (G–L) White circle demarcates same group of cells. Yellow arrow: *Dr-farp1+* cells. (N and O) Absence of *Dr-vtn* expression in wild-type (WT) versus chks399 (chokh) siblings. (P) Summary of regulatory analyses, all assayed at 27–28 hpf. Morpholino-assisted knockdown of *rx3* leads to dose-dependent reduction/absence of *vtn* expression at 27–28 hpf compared to controls (ctrl). Loss of *vtn* expression in early differentiating cells in two homozygous *rx3* mutant alleles (chk s399 and chk ne2611). Below each bar: numbers of investigated specimens and volume of injected morpholino solution/genotype of fish as judged by presence/absence of eyes. Views: (A–F) apical, ventral to the bottom, (G–O) lateral, anterior left.
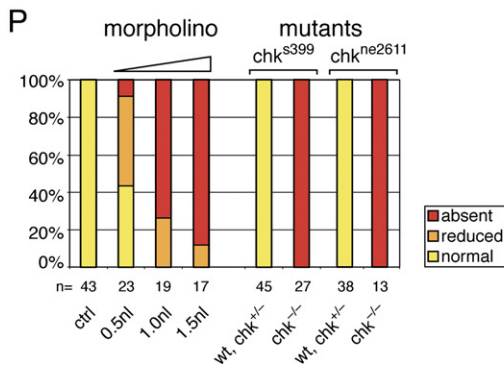
and 6F) and that the vasotocinergic neurons are positive for the three transcription factors, *Pdu-otp*, *Pdu-nk2.1*, and *Pdu-rx* (Figures 6C, 6E, and 6F, compare to Figure 3J). In the developing zebrafish brain, *Dr-otp1* is likewise expressed in the medial forebrain region in a restricted subset of cells (Del Giacco et al., 2006, and Figure 6G). We found that this expression comprises both the vasotocinergic (Figure 6H) and RFamidergic neurons (Figure 6I). As in the annelid, both neuron types are also positive for the zebrafish *nk2.1* genes (Figures 6J and 6K), and the early differentiating vasotocinergic neurons in addition coexpress *Dr-rx3* (Figure 6L).

Zebrafish *vasotocin-neurophysin* Expression Is Specifically Affected in *rx3* Mutants

The precise onset of zebrafish *vtn* expression in the overlap region of *rx3* and *otp* (Figures 6L and 6M and Figure S3) suggested that besides *otp* (Acampora et al., 1999), *rx3* should also play a role in the specification of the vasotocinergic cells. *Rx3* has so far been known for its role in teleost eye development (e.g., Rembold et al., 2006; Rojas-Munoz et al., 2005) and forebrain size regulation (Stigloher et al., 2006). However, despite the prominent expression of *rx3* in the developing hypothalamus, no specific hypothalamic target genes of *rx3* have yet been identified, except for the *pomc* gene that depends on *rx3* in a restricted population of cells (Dickmeis et al., 2007). We assayed expression onset and maintenance of *vtn* (and of various ventral forebrain markers) in zebrafish embryos in which Rx3 function was impaired. Indeed, injection of antisense-morpholino oligonucleotides designed to perturb *rx3* splicing and translation caused a reduction/absence of *vtn* transcripts in the early differentiating vasotocinergic cells in a dose-dependent manner (Figure 6P), consistent with a requirement of *rx3* for *vtn* expression. To test this more rigorously, we investigated two zebrafish mutants, *chk^{s399}* and *chk^{ne2611}*, that have been identified as likely null alleles of *Dr-rx3* (Loosli et al., 2003; Stigloher et al., 2006). In 100% of homozygous mutant embryos of both alleles, *Dr-vtn* expression failed to initiate and remained absent in the early differentiating vasotocinergic cells up to the latest time point analyzed (3 days of development) (Figures 6N–6P and Figures S4A and S4B). In contrast, the expression of various other transcription and differentiation factors remained unchanged or rather expanded (Figure S4C–S4T), as observed for example for *isotocin-neurophysin* (*itn*; Figures S4C and S4D), another *vtn* paralog expressed later in the preoptic nucleus (Unger and Glasgow, 2003). This indicates a novel and highly specific requirement of *rx3* for *vtn* expression in the vertebrate hypothalamus.

DISCUSSION

Identifying Ancestral Cell Types by Molecular Fingerprint Comparison

Our data reveal that neurosecretory brain centers in annelid and vertebrate form at similar molecular coordinates, as recently evidenced also for the *Drosophila* pars intercerebralis and pars lateralis and the vertebrate hypothalamus (De Velasco et al., 2007). On top of this, our data establish a link between a conserved molecular address (*miR-7+*, *nk2.1+*, *rx+*, *otp+*) and a conserved cell type (vasotocinergic extraocular photoreceptors). Based on this, we propose that this link is evolutionarily ancient and reflects the existence of a conserved vasotocinergic neuron type. Corroborating this, the actual number of *miR-7+*, *nk2.1+*, *rx+*, *otp+* coexpressing cells in fish and *Platynereis* is very small when compared to that of cells in the entire forebrain (∼1%, data not shown), and the *vtn+* population is similarly small, which makes it highly

unlikely that these cells coexpress *vtn* by chance. Also, it has been shown that vertebrate forebrain development involves extensive migration of hypothalamic (Varga et al., 1999) and telencephalic cell types (Corbin et al., 2001), reflecting the principle that forebrain neuron types are specified at stereotyped positions and later migrate over distances to their final destinations. This observation indicates that cell types do not easily arise de novo during evolution at any molecular address.

The conserved molecular fingerprints also allow the unraveling of cell-type-specific regulatory interactions, as we identified *Dr-vtn* as another hypothalamus-specific downstream gene of *Dr-rx3* beside *pomc* that depends on rx3 in another population of hypothalamic cells (Dickmeis et al., 2007). Since *vasopressin* and *oxytocin* expression is also abolished in mouse *otp* knockouts (Acampora et al., 1999) and since *otp* expression is not affected in *rx3* mutants (Figures S4K and S4L), we hypothesize that Otp and Rx transcription factor activities converge on the *vasotocin (vasopressin/oxytocin)-neurophysin* enhancers in vertebrates.

Vasotocinergic Extraocular Photoreceptors—An Ancient Module Coordinating Reproduction According to Light Cycles?

The identification of the *vasotocin-neurophysin* gene as an *rx3* target sheds new light on the yet enigmatic phenotype of *rx3* adult homozygous mutant medaka fish that exhibit absent or severely reduced gonads and disturbed outer sexual morphology (Ishikawa et al., 2001). Intriguingly, vasotocin-related nonapeptides are known to influence gonadal development, partly via an interaction with the androgen pathway (Adashi and Hsueh, 1981; Rodriguez and Specker, 1991), which in turn strongly influences sexual morphological dimorphisms (e.g., Oliveira et al., 2005). Furthermore, vasotocin orthologs govern proper reproductive behavior across Bilateria (in mollusks, Van Kesteren et al., 1995; annelids, Fujino et al., 1999; and vertebrates, Goodson and Bass, 2000). We thus propose that the described defects of the reproductive system in *rx3* mutants are due to a dysfunction of the vasotocinergic cells. Notably, the gonadotropin system, another key player in the control of reproductive functions, appears to be normal in *rx3* null mutant zebrafish, since the expression of the two known *gnrh* genes is unaffected during the examined developmental stages (Figure S4), as is the expression of α-*gsu*, the common subunit of *lh* and *fsh* (and *tsh*) (Dickmeis et al., 2007).

The conserved co-occurence of a ciliary opsin with vasotocin suggests an ancient minimal "module" to secrete the peptide in response to changes in light conditions. Indeed, in many vertebrates the hypothalamic content of vasotocin-related nonapeptides fluctuates throughout the daily light-dark cycle (for fish, see Gozdowska et al., 2006). Superimposed on this, there is a seasonal change in the daily vasotocin release that depends on long and short photoperiods (e.g., Gozdowska et al., 2006; Ota et al., 1999). Daily fluctuations of
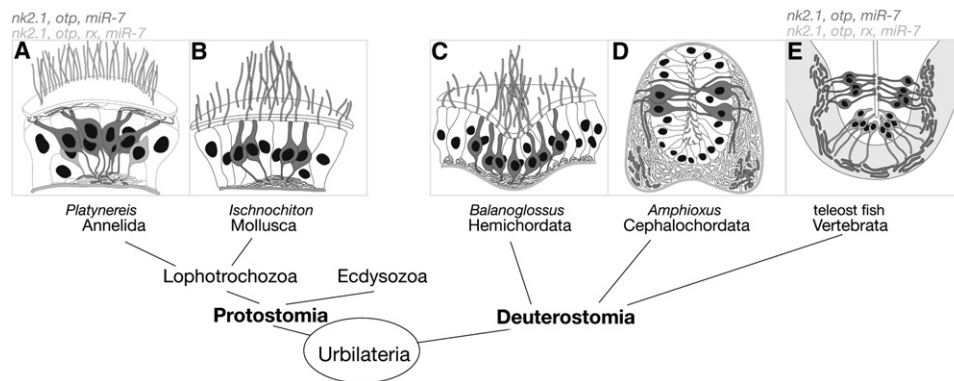
Figure 7. Evolutionary Continuity of Surface-Contacting RFamidergic Cells

(A–E) Schematized drawings of surface-contacting RFamidergic cells (dark gray) in the medial forebrain of different bilaterian groups (see Supplemental Data). Species and phylogenetic position as indicated. Schemes were based on the following primary data: (A) this work, (B) Voronezhskaya et al., 2002, (C) Nezlin and Yushin, 2004, (D) Lacalli, 1996, and Uemura et al., 1994, (E) this work and Pinelli et al., 2000. Schemes (A) and (E) include vasotocinergic cells (light gray) and transcription factor codes as reported in this study.

vasotocin-related nonapeptide release have also been recorded outside vertebrates: grasshopper diuretic hormone (a vasotocin-like nonapeptide; Proux et al., 1987) positive neurons are preferentially active in the dark (Thompson and Bacon, 1991). In addition to daily and seasonal changes, vasotocin release is also implicated in the control of semilunar and lunar reproductive cycles in the fish *Fundulus* (Taylor, 1986). We are currently exploring the role of Vtn in the adult *Platynereis* brain with respect also to its lunar reproduction (Hauenschild, 1955).

Flask-Shaped Surface-Contacting RFamidergic Neurons—Integrating Chemosensory Information for Locomotor Control?

Flask-shaped surface-contacting RFamidergic neurons are of widespread occurrence in the animal kingdom (Figure 7; Hartenstein 2006). What might have been their ancient function? The presence of ciliated dendrites contacting the environment is a strong indication for a direct sensory, possibly chemosensory function (Vigh et al., 2004). *Caenorhabditis elegans* possesses chemosensory RFamidergic neurons (the amphid neurons ASE, ADL, and ASH; Troemel et al., 1995) that control the locomotor pattern related to feeding (Rogers et al., 2003). This might reflect a more general function of RFamidergic sensory cells in locomotor control, given that the hypothalamic RFamidergic CSF-contacting neurons in nonmammalian vertebrates form part of the paraventricular organ (Vigh et al., 2004), lesions of which result in alteration of motor behavior (Dube et al., 1990). In amphioxus, dendrites of interneurons involved in major locomotor circuits are embedded in the neurosecretory plexus containing RFamidergic fibers (Lacalli and Kelly, 2000). Finally, RFamides are known to control the pattern of different kinds of movements of cnidarian planula larvae, most likely via the sensory RFamidergic cells (Plickert and Schneider, 2004; reviewed in Hartenstein, 2006).

Multifunctional Input-Output Neurons in Early Brain Evolution

Our data indicate that sensory-neurosecretory cell types have been present at the starting point of bilaterian brain evolution, extending the "protoneuron concept" (Vigh et al., 2004), which had postulated that surface-contacting sensory-neurosecretory neurons are ancestral for deuterostomes. Initially, such multifunctional cell types might have acted rather autonomously. Subsequent diversification into sensory, neurosecretory, and interneuron subtypes then optimized individual cell types toward a single function. At the end of this process, complex brain centers emerged, such as the vertebrate hypothalamus, that integrate multiple sensory input, such as light, temperature, osmolarity, and chemical stimuli, and orchestrate different neurosecretory output, such as the vasotocin/vasopressin/oxytocin, steroid, and gonadotropin systems, all of which likely share an ancestral involvement in reproduction control (Iwakoshi et al., 2002; Thornton et al., 2003; Twan et al., 2006). A broader survey of ancestral neuron types in other bilaterians, as well as in metazoan outgroups, will help to disentangle these crosstalks and further understand the evolution of the vertebrate hypothalamus.

EXPERIMENTAL PROCEDURES

Isolation of *Platynereis* and Zebrafish Genes

Fragments of *Pdu-vtn* and *Pdu-vax* were isolated by PCR after reverse transcription of embryonic total RNA (24, 48 hr). Total RNA was isolated from embryos using Trizol (GIBCO BRL). PCR conditions: Arendt et al., 2002. Fragments were expanded by using the SmartRACE kit (Clontech/BD Bioscience).

Dr-farp1 was identified blasting the *Mus* fmrfamide-related precursors (*Mus*: Q9WVA8) in the tblastn mode against the VEGA5 release of the zebrafish genome at the ENSEMBL database (release 37; http://www.ensembl.org/Danio_rerio/index.html). The best-scoring blast hit predicted the transcript ENSDART00000052627. 5′ and 3′ UTR sequence expansion by SmartRACE kit using adult head total RNA. (*Pdu-phc2* EF544394; *Pdu-vax* EF544395; *Pdu-otp* EF544396;

Pdu-syt EF544397; *Pdu-thl* EF544398; *Pdu-vtn* EF544399, *Dr-farp1* EF547661).

For primer sequences and for other genes, see Supplemental Experimental Procedures.

Whole-Mount In Situ Hybridizations with RNA and Locked Nucleic Acids Probes

Platynereis and zebrafish single and double WMISH were performed according to Tessmar-Raible et al., 2005, and Jowett and Lettice, 1994, with the modifications outlined in the Supplemental Experimental Procedures.

MiR-7 detection in *Platynereis* and zebrafish: basic protocol by Wienholds et al., 2005, with modification outlined in the Supplemental Experimental Procedures. Note that the mature *miR-7* sequence is identical in zebrafish and *Drosophila* (http://microrna.sanger.ac.uk/sequences/).

Morpholino Knockdown Experiments

We used an injection mixture containing 0.5 mM rx3ATG-MO (5′-GA GATCCAACAAGCCT<u>CATT</u>GAACG; see Rojas-Munoz et al., 2005), directed against the translational start site (position of ATG underlined), as well as 0.3 mM rx3E2I2-MO (5′-GTGTCTCTCAC<u>CTG TACTCGGACTT</u>), designed to prevent correct splicing of the 3′ end of the second exon (underlined), in a 200 mM KCl solution supplemented with 0.1%(w/v) phenol red. Freshly fertilized wild-type zebrafish eggs were injected using borosilicate glass needles. Injected embryos were kept in fresh embryo medium and analyzed at 27–28 hpf for the presence of early-born *vtn*+ cells via WMISH.

Immunocytochemistry

For antiacetylated tubulin stainings in zebrafish and in *Platynereis* larvae, a monoclonal mouse antibody (SigmaT6793) was used in 1:200 dilution to detect an interphyletically conserved epitope present in cilia and axons (Matsuyama et al., 2002). For staining of WMISH-treated embryos, the antibody was incubated together with the last anti-fluorescein or anti-digoxigenin antibody.

We ensured the presence of RFamides in the *Platynereis* developing medial forebrain by using two different antibodies, recognizing FMRFamide (Phoenix, H-047-29; 1:200) and RFamide (provided by N. Rebscher and G. Plickert; 1:30). Staining patterns were largely congruent (data not shown).

Secondary antibodies: Cy5, FITC, and TRITC from Jackson ImmunoResearch; 1:200.

Zebrafish: for anti-FMRF and antiacetylated tubulin staining, fish were treated as for WMISH until the postdigest/postfixation washes, incubated in primary antibody (in PTW), washed 5 × 10 min in PTW, incubated in the second antibody (in PTW), and washed 5 × 10 min in PTW. *Platynereis* fixations and treatment: Arendt et al., 2004.

Mounting and Microscopy

Zebrafish and *Platynereis* embryos were mounted in 100% or 87% glycerol, respectively. Fluorescently stained animals were mounted in 90% glycerol/10% PBS containing 2.5% DABCO (Sigma).

Confocal scans were taken on a Leica SP2 confocal microscope, using a 40× objective, with a resolution of 1024 × 1024. Scans were viewed and reconstructed using the ImageJ (version 1.34 s) program (http://rsb.info.nih.gov/ij/). NBT/BCIP reflection confocal microscopy: Jekely and Arendt (2007).

Electron Microscopy

EM on 48-hr-old larvae was done according to Hausen and Bartolomaeus, 1998, with modifications described in the Supplemental Experimental Procedures.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.cell.com/cgi/content/full/129/7/1389/DC1/.

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