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Gene Expression Patterns

Characterization of *tweety* gene (*ttyh1-3*) expression in *Xenopus laevis* during embryonic development

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ABSTRACT

The *tweety* family of genes encodes large-conductance chloride channels and has been implicated in a wide array of cellular processes including cell division, cell adhesion, regulation of calcium activity, and tumorigenesis, particularly in neuronal cells. However, their expression patterns during early development remain largely unknown. Here, we describe the spatial and temporal patterning of *ttyh1*, *ttyh2*, and *ttyh3* in *Xenopus laevis* during early embryonic development. *Ttyh1* and *ttyh3* are initially expressed at the late neurula stage are and primarily localized to the developing nervous system; however *ttyh1* and *ttyh3* both show transient expression in the somites. By swimming tadpole stages, all three genes are expressed in the brain, spinal cord, eye, and cranial ganglia. While *ttyh1* is restricted to proliferative, ventricular zones, *ttyh3* is primarily localized to postmitotic regions of the developing nervous system. *Ttyh2*, however, is strongly expressed in cranial ganglia V, VII, IX and X. The differing temporal and spatial expression patterns of *ttyh1*, *ttyh2*, and *ttyh3* suggest that they may play distinct roles throughout embryonic development.

First identified as a transcriptional unit adjacent to the *Drosophila flightless I* gene, the *tweety* genes comprise a highly conserved, evolutionarily ancient family that encode large-conductance chloride channels (Campbell et al., 1993, 2000). Displaying a broad phylogenetic distribution indicative of important cellular functions, *tweety* orthologs have been identified in plants, slime molds, protozoa, and a wide range of animals (Matthews et al., 2007). In vertebrates, the *tweety* gene family has three members, *tweety homolog 1 (ttyh1)*, *tweety homolog 2 (ttyh2)*, and *tweety homolog 3 (ttyh3)*, all of which are reported to encode large-conductance transmembrane chloride channels. Predicted to have either five (Ttyh1, Ttyh2) or six (Ttyh3) transmembrane domains, Ttyh1 is activated by cellular swelling while Ttyh2 and Ttyh3 appear to be activated in response to elevated Ca^{2+} concentrations (He et al., 2008; Suzuki, 2006; Suzuki and Mizuno, 2004).

The association of *tweety* genes with fundamental cellular processes as well as several pathological conditions has created a heightened interest in the members of this gene family. Primarily localized to neural tissues, *ttyh1* encodes a calcium-binding

endoplasmic reticulum protein that may be involved in regulating Ca^{2+} sequestration and concentration during mitosis. Inactivation of both copies of this gene in mice leads to very early embryonic lethality (Kumada et al., 2010). Ectopic expression of *ttyh1* in human epithelial kidney cells results in long, branched filopodia while overexpression in cultured rat hippocampal neurons leads to intense neuritogenesis and elaborate dendritic trees (Matthews et al., 2007; Stefaniuk et al., 2010). The latter observation is consistent with the upregulation of *ttyh1* in epilepsy and epileptogenesis (Lukasiuk et al., 2003; Stefaniuk and Lukasiuk, 2010; Stefaniuk et al., 2010). *Ttyh1* expression is also strongly upregulated in an array of childhood brain tumors; fusion of the *ttyh1* promoter to the large microRNA cluster C19MC drives the development of a particularly aggressive form of pediatric cancer (Kleinman et al., 2014; Matthews et al., 2007). While less is known about *ttyh2,* it is strongly upregulated in both colon and renal cell carcinoma (Rae et al., 2001; Toiyama et al., 2007), suggesting a possible role in cell division and tumor growth. Although the *ttyh3* gene product has been associated with calcium dynamics in excitable tissues such as skeletal muscle, brain, and spinal cord, research has focused almost exclusively on its biochemistry rather than expression or function (He et al., 2008; Suzuki, 2006).

Despite the importance of the *tweety* genes in development and disease, relatively little is known about their expression outside of select adult human tissue samples, tumor biopsies, a few cell lines, and adult rodent tissues (Al-Jumaily et al., 2007; Matthews et al., 2007; Rae et al., 2001; Suzuki, 2006; Suzuki and Mizuno, 2004;

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Toiyama et al., 2007). The only information on the expression of any of the *tweety* genes during embryonic development is Kumada et al. (2010) who analyzed expression of *ttyh1* in the E7.5 and E14.5 mouse brain and showed its essential role during embryogenesis. There has been no comprehensive analysis of the expression pattern of this important gene family during embryonic development. Here, we have chosen to characterize the spatial and temporal expression pattern of the three vertebrate *tweety* homologs throughout embryogenesis in *Xenopus laevis,* a particularly suitable model system given the ability to access and manipulate all stages of development (Sive et al., 2000). Using *Xenopus laevis*, we found that each of the *tweety* genes has a unique temporal and spatial expression pattern localized primarily to the developing nervous system.

1. Results and discussion

1.1. Cloning of Xenopus tweety orthologs

Using available sequence information from GenBank (GenBank accession numbers **BC073236.1**; **BC073236.1**; **BC073236**, respectively), *ttyh1*, *ttyh2*, and *ttyh3* were cloned from *X. laevis* tadpole (stage 35) cDNA using reverse transcriptase polymerase chain reaction (RT-PCR) (all stages are according to Nieuwkoop and Faber, 1994). Sequencing confirmed identity (99–100% at the nucleotide level) for all three clones. Pairwise comparisons between each clone and the GenBank sequence for the other two homologs showed minimal identity, the highest being 24%. This ensured that the probe for one family member would not cross-hybridize with endogenous mRNA from another family member under the conditions employed during whole mount *in situ* hybridization (ISH).

1.2. Expression of Xenopus ttyh1

Using whole mount *in situ* hybridization from early blastula stages onward, signal for *ttyh1* was first detected at neurula stages throughout the developing central nervous system. By late neurula stages (stage 19), *ttyh1* transcripts become particularly abundant in the midbrain in the presumptive eye region (Fig. 1A). During tailbud stages mRNA levels increased throughout the nervous system following an anterior (strongest signal) to posterior (weaker signal) gradient, with distinct expression in the somites also present (Fig. 1B–D). Signal increased dramatically at swimming tadpole stages by which point it extended to the posterior end of the spinal cord (Fig. 1E,F). Histological analysis revealed that *ttyh1* was distributed along the entire dorsal–ventral axis of the neural tube with the exception of the roofplate in the forebrain, hindbrain and spinal cord and the spinal cord floorplate. Notably, throughout the entire central nervous system *ttyh1* is tightly restricted to the ventricular, mitotic zone of the neural tube with little expression in the more lateral zones (Fig. 2A–E). Otic vesicle expression was apparent beginning at early tailbud stages, and continued through the swimming tadpoles stage (Figs 1C–F, 2C). There is strong *ttyh1* expression in the inner and outer layers of the retina by swimming tadpole stages (Figs 1E,F, 2B). *Ttyh1* transcripts appeared in cranial ganglia VII and IX beginning at the swimming tadpole stages (Figs 1F, 2C).

Since gene expression would not be detectable using whole mount *in situ* hybridization if transcripts were uniformly distributed at low levels, qRT-PCR was employed to determine if any of the *tweety* genes had earlier, particularly maternal, expression. While very low levels of *ttyh1* mRNA were detectable prior to the onset of transcription at mid-blastula stages, these levels were 100-fold less than at neurula stages and over a thousand fold less than at swimming tadpoles stages making it questionable whether the low level of early expression is physiologically relevant (Fig. 3).

1.3. Expression of Xenopus ttyh2

As with *ttyh1*, qRT-PCR revealed trace amounts of *ttyh2* transcripts throughout pre-gastrula stages followed by a hundred fold increase at neurula stages and another dramatic rise in mRNA abundance at swimming tadpole stages (Fig. 3). This result was consistent with whole mount *in situ* hybridization. Using *in situ* hybridization, *ttyh2* transcripts are first detectable at hatching stages in the cranial ganglia; by swimming tadpoles stages *ttyh2* is prominently expressed in cranial ganglia V, VII, IX and X as well as the outer layer of the eye (Figs 1K,L, 2G–I). To further investigate the expression of *ttyh2* in the cranial ganglion, co-localization with a known cranial nerve marker *xVGlut1* (Gleason et al., 2003) was assayed. Co-expression was evident in cranial ganglia V, VII, and X (Fig. 4B,C). By swimming tadpoles stages, *ttyh2* is also expressed weakly and diffusely throughout the neural tube (Fig. 1K,L), but there is strong expression in the dorsal aspect, particularly in the roofplate of the posterior neural tube (Fig. 2J).

1.4. Expression of Xenopus ttyh3

As with *ttyh1, ttyh3* was detectable using whole mount *in situ* hybridization at neurula stages when strong, localized signal appeared in the anterior regions of the nervous system, particularly the forebrain and developing eye (Fig. 1M,N). Like *ttyh1*, *ttyh3* also had transient signal in developing somite regions beginning at neurula stages (stage 20) (Fig. 1M), signal that gradually disappeared by the tailbud stage (stage 25) (Fig. 1O). Beginning at hatching stages (stage 30), *ttyh3* was also detected in the developing pharyngeal arches (Fig. 1P). By hatching stages, expression remained constant in the anterior nervous system and become stronger in the spinal cord (Fig. 1P). At swimming tadpole stages, *ttyh3* continued to be expressed throughout the central nervous system (Fig. 1Q,R). In contrast to *ttyh1, thyh3* was located preferentially in the postmitotic, lateral regions of the neural tube throughout its anterior–posterior axis (Fig. 2K–O). Interestingly, the expression patterns of *ttyh1* and *ttyh3* appear to be somewhat mutually exclusive; *ttyh1* is localized medially in highly mitotic domains along the inner edges of the brain ventricle while *ttyh3* is expressed throughout the more lateral regions of the neural tube. Through swimming tadpole stages, *ttyh3* transcripts are detected in ganglia V, VII, IX, and X (Figs 1R, 2M). Similar to *ttyh1* and *ttyh2, ttyh3* qRT-PCR showed an extremely low, but nevertheless detectable, level of expression during neurula stages and peaked at tailbud stages.

1.5. Discussion

The *tweety* gene family displays expression patterns highly suggestive of an important role during embryonic development. While delineation of the specific function of the three genes awaits further investigation, previous studies have suggested they play a critical role in dynamically regulating ion concentrations within the cell, particularly calcium (Kumada et al., 2010; Suzuki, 2006). The regions in which the genes are expressed – the developing nervous system and somites – are known to be highly dependent on tightly regulated calcium activity for proper development during embryogenesis. Ca^{2+} activity has been widely shown as a governing factor in nearly every aspect of neural development (Leclerc et al., 2006; Moreau et al., 2008; Rosenberg and Spitzer, 2011), from cell division (Li et al., 2008) to specification of neurotransmitter phenotype (Borodinsky et al., 2004, 2012), and also in somite formation and muscle regeneration (Chernoff and Hilfer, 1982; Tu and Borodinsky, 2014; Webb and Miller, 2006).

Although each of the genes displays some overlapping regions of expression during neural development, each possesses a unique temporal and spatial pattern suggesting distinct functions. *Ttyh1* for
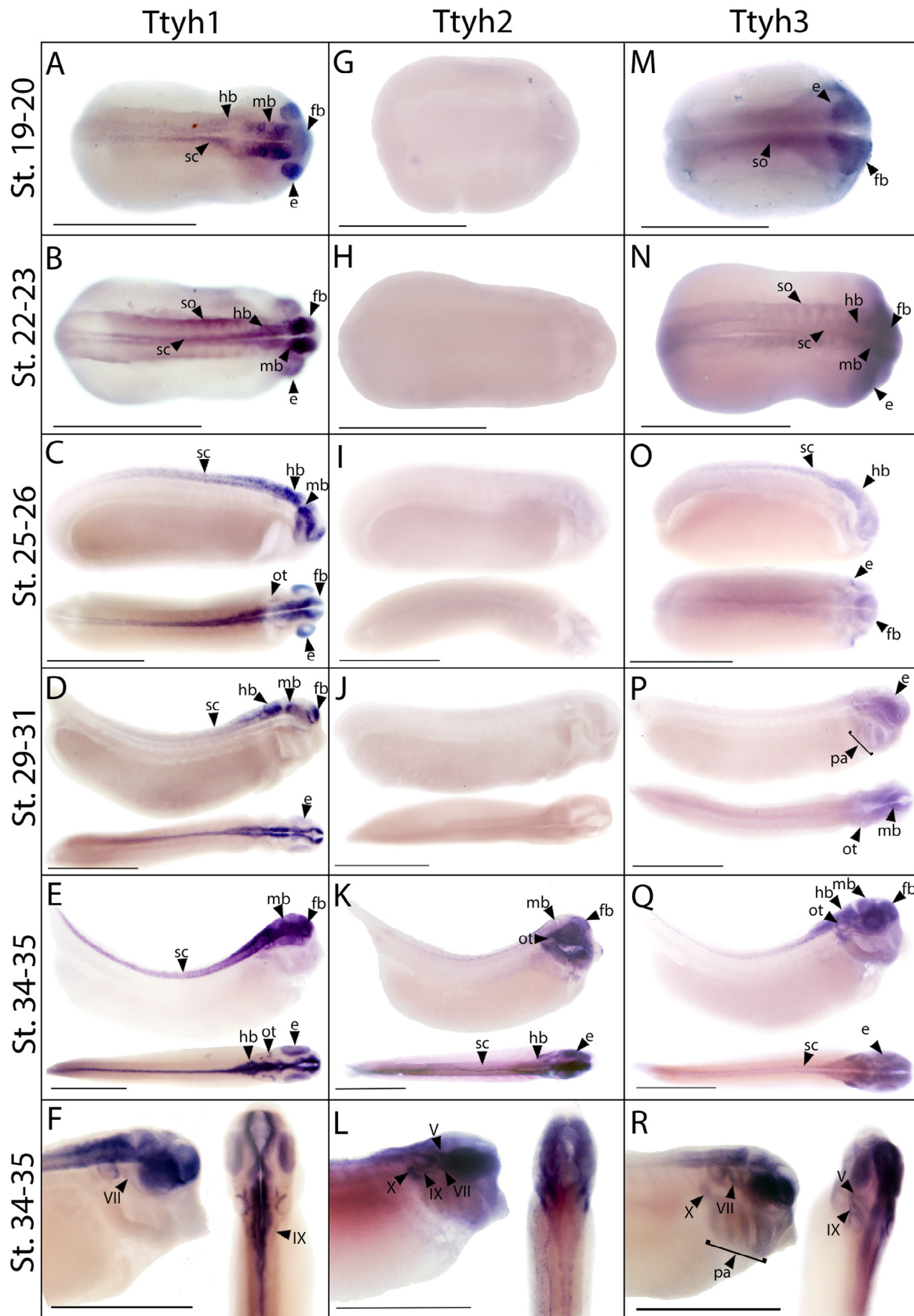
Fig. 1. Whole mount *in situ* hybridization showing spatial and temporal expression of *tweety* homologs in developing *X. laevis* embryos. Row 1 and 2: dorsal views. Rows 3, 4, 5: both lateral (top embryo, with dorsal side on top) and dorsal (bottom embryo) views. Anterior is oriented to the right for all embryos. Row 6: close up of anterior part of the embryo, with a lateral view (dorsal side on top) and a dorsal view (with anterior directed upwards). (A, G, M) Stages 19–20 (late neurula). (B, H, N) Stages 22–23 (early tailbud). (C, I, O) Stages 25–26 (mid-tailbud). (D, J, P) Stages 29–31 (late tailbud). (E, K, Q) Stages 34–35 (swimming tadpole). (F, L, R) Stages 34–35 (swimming tadpole, close up). (A–F) *Ttyh1* expression. (G–L) *Ttyh2* expression. (M–R) *Ttyh3* expression. Arrowheads indicate regions of expression. (fb, forebrain; e, eye; hb, hindbrain; mb, midbrain; ot, otic vesicle; pa, pharyngeal arches; sc, spinal cord; nt, neural tube; so, somites; Roman numerals: cranial ganglia.) Scale bars = 1.0 mm.
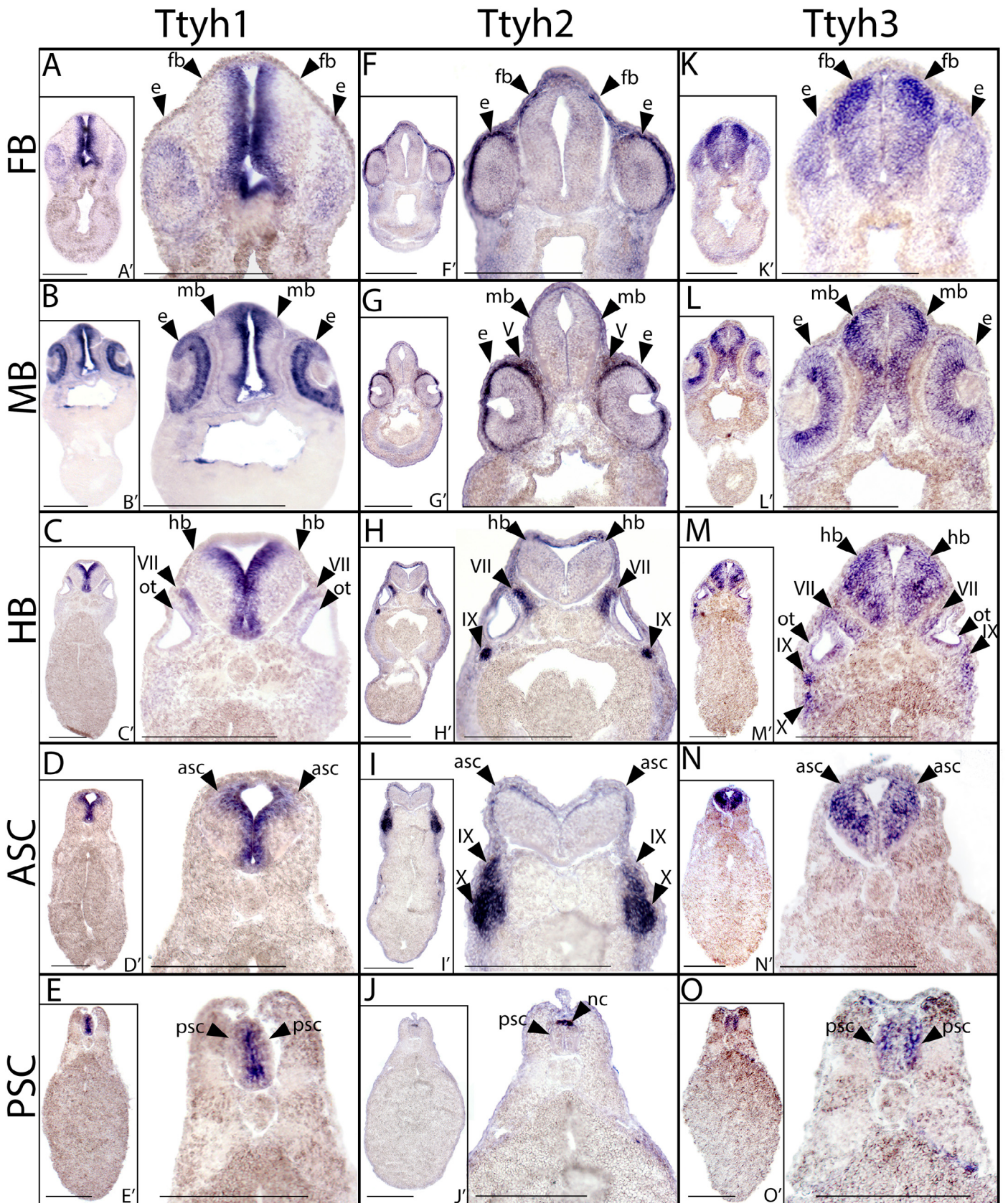
Fig. 2. Histological analysis of *tweety* homolog expression in *X. laevis* stages 35–37 (swimming tadpole) using whole-mount *in situ* hybridization. Transverse 18 µm sections along the anterior–posterior axis with dorsal to the top. (A–O) 10× magnification images focused on neural regions with signal. (A′–O′) 4× magnification images to show to the absence of signal in other regions. (A, F, K) Sections showing forebrain. (B, G, L) Sections showing midbrain. (C, H, M) Sections showing hindbrain. (D, I, N) Sections showing anterior spinal cord. (E, J, O) Sections showing posterior spinal cord. (A–E) *Ttyh1* expression. (F–J) *Ttyh2* expression. (K–O) *Ttyh3* expression. Arrowheads indicate regions of expression. (asc, anterior spinal cord; mb, midbrain; hb, hindbrain; fb, forebrain; e, eye; nc, neural crest; ot, otic vesicle; psc, posterior spinal cord; sc, spinal cord; Roman numerals: cranial ganglia.) Scale bars = 0.25 mm.
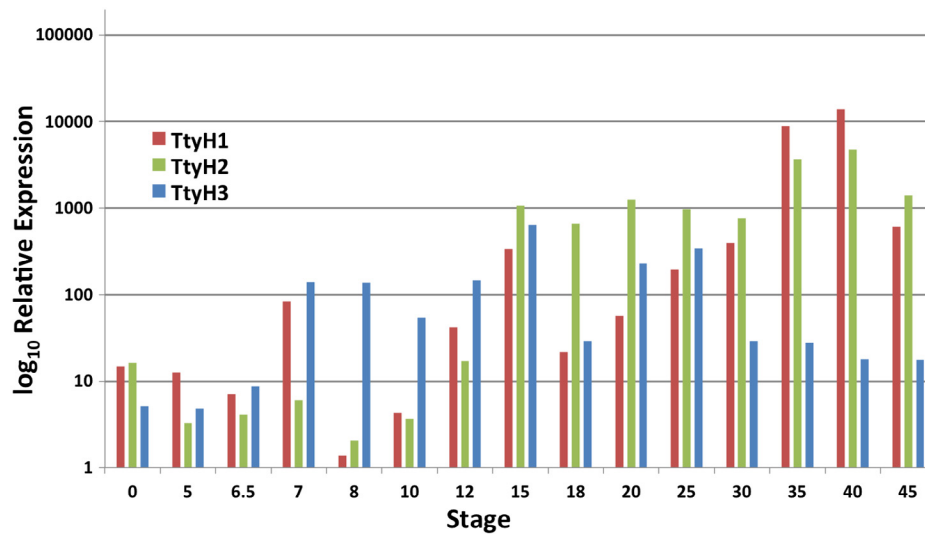
Fig. 3. qRT-PCR showing *ttyh* gene expression from single cell to stage 45. Stage 0 in the figure denotes unfertilized eggs. At each stage gene expression was quantitated using the $\Delta\Delta C_T$ method and corrected for expression of the control gene (*Drosha*). Relative expression is displayed $\log_{10}$ transformed.

example does not display significant maternal expression, but rather shows onset of expression at late neurula stages with dramatically increased neural-specific expression through swimming tadpole stages, a result also consistent with RNA-Seq results in *X. tropicalis* transcriptome database (Tan et al., 2013). *Ttyh1* is consistently and tightly localized to the ventricular regions of the developing nervous system, where cells are mitotic. The association with proliferative cells is consistent with the role of *ttyh1* in highly aggressive pediatric brain tumors (Kleinman et al., 2014; Matthews et al., 2007). *Ttyh1* has also been associated with neuritogenesis and dynamic filipodia-like protrusions in rat cell cultures and brain slices (Matthews et al., 2007; Stefaniuk and Lukasiuk, 2010). In *Xenopus*, the onset and timing of *ttyh1* expression coincides approximately with the timing of neurite formation (Hartenstein, 1989).

Like *ttyh1*, *ttyh2* is implicated in tumorigenesis (Rae et al., 2001; Toiyama et al., 2007), and it is almost exclusively restricted to the cranial ganglia during its initial phase of expression. *Ttyh3*, on the other hand, is expressed in postmitotic regions in a nearly mutually exclusive manner to *ttyh1*. *Ttyh3* is also expressed far more weakly and broadly than *ttyh1*, although both genes are transiently expressed in the somites. While qRT-PCR results were overall consistent with those from *in situ* hybridization there were some apparent discrepancies. This discrepancy may also be attributable to splice

variants known to occur in the *tweety* gene family. *Ttyh3*, for example, showed prominent expression throughout the nervous system at swimming tadpole stages while qRT-PCR revealed peak expression at tailbud stages. It should be noted, however, that the color reaction took considerably longer to produce distinct signal, a result that lends validity to the qRT-PCR data. Notably Ttyh1 and Ttyh2 share more similarity at the amino acid and predicted protein level than with Ttyh3 which actually has a different transmembrane structure. Predicted evolutionary relationships suggest that *ttyh3* homologs are more distant, having diverged prior to a duplication event that led to the *ttyh1* and *ttyh2* genes (Matthews et al., 2007). Although previous research on *tweety* gene expression has been limited primarily to cell lines and adult tissues, for the one example of embryonic expression for *ttyh1* in mice, the results presented here remain consistent with that study. The growing recognition of the key role that ion fluxes play in normal development, regenerative processes, and pathogenesis, make these genes intriguing candidates for further investigation (Borodinsky et al., 2012; Levin, 2014; Rosenberg and Spitzer, 2011). The broad phylogenetic distribution of this gene family, their proposed role in calcium dynamics and the discrete expression patterns of the *tweety* genes in the developing nervous system presented here suggest an important role for *tweety* genes in neural proliferation and differentiation.

Ttyh2

Fig. 4. Due to the concentrated expression of *ttyh2* in the cranial ganglia, double fluorescent *in situ* hybridization was carried out to determine its co-localization with another known cranial ganglia marker. Histological analysis of *tweety* homolog 2 (*ttyh2*) (Cy3-tyramide) and *xVGlut1* (FITC-tyramide) expression in *X. laevis* stage 35 (swimming tadpole) using whole-mount fluorescent *in situ* hybridization. Transverse 25 µm sections along the anterior–posterior axis with dorsal to the top. (A) Section showing forebrain. (B) Section showing midbrain. (C) Section showing hindbrain. (mb, midbrain; hb, hindbrain; fb, forebrain; e, eye; Roman numerals: cranial ganglia.) Scale bars = 0.25 mm.

2. Experimental procedures

2.1. Animal usage

Embryos were obtained from matings between adult albino *X. laevis* following injection of human chorionic gonadotropin as detailed by Sive et al. (2000). Embryos were staged according to Nieuwkoop and Faber (1994). All animal care and procedures were performed in accordance with the regulations set forth by the Institutional Animal Care and Use Committee (IACUC) at the College of William and Mary.

2.2. Cloning and sequence analysis

Primers for PCR were designed from cDNA clone sequences of *X. laevis* mRNA from *tweety* homologs. Sequences were obtained from NCBI Reference Sequences (NCBI accession numbers: *ttyh1*, NM_001093946; *ttyh2*, NM_001087123; *ttyh3*, NM_001092244) and used to design primers that produced PCR products approximately one kilobase in length.

Primer pairs for cloning were designed to be both unique to each homolog and highly conserved to ensure recognition of *X. laevis* sequences. Primers used for cloning were as follows: *ttyh1*, (5′-CCAAGAGCCAAAGGGGCTTA-3′) and (5′-AATCACTCAGTCCCACAGCC-3′); *ttyh2*, (5′-GACACTGCCAATGGGTTCTT-3′) and (5′-AGAGATTTCCGTCTGCCTCA-3′); *ttyh3*, (5′-TGATGCTTCTGGCTTGTGTC-3′) and (5′-AACCACGGCAATCTACCAAG-3′).

Total RNA was extracted from *X. laevis* stage 35 embryos with RNeasy Mini Kit (Qiagen). cDNA for PCR was synthesized using the Qiagen iScript cDNA Synthesis kit. PCR was performed using the Ambion Supertaq Plus DNA Polymerase Kit with annealing temperatures between 55 and 57 °C. PCR products were then cloned into the Agilent pSC-A-amp/kan PCR Cloning Vector and transformed into One Shot DH5α Competent *Escherichia coli* cells. PCR products within plasmid DNA were purified using the PureYield Plasmid Midiprep kit from Promega followed by sequencing to confirm each clone's identity. Sequencing was performed with ABI 3130 Genetic Analyzer sequences using the BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems). Using BLAST, sequence results were compared for both total nucleotide and amino acid similarities across all homologs to ensure stringency of probes. Sequences were aligned among orthologs including *X. tropicalis* and *H. sapiens* using ClustalW2 multi sequence alignment tool.

2.3. qRT-PCR

At each of stages 0, 5, 6.5, 7, 8, 10, 12, 15, 20, 25, 30, 35, 40 and 45 10 embryos were frozen in liquid nitrogen. Frozen embryos were homogenized by grinding with a pestle in 700 µL TRIzol (Life Technologies). RNA was extracted from homogenate with 4-bromoanisole and purified with the RNeasy Mini Kit (Qiagen). cDNA was synthesized from total RNA using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad). qRT-PCR was performed using the TaqMan Gene Expression Master Mix (Life Technologies) on a StepOne Real-Time PCR System. Cycling conditions were: 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min. TaqMan probes were labeled at the 5′ end with FAM dye and at the 3′ end with the NFQ quencher. Primers used were: *ttyh1,* (5′-GTGTCACCTGGAGCTGTGT-3′) and (5′-GCTGTTTCCGTAGAAACCAATGC-3′); *ttyh2,* (5′-CTGGACTGCTGCTGTGTCT) and (5′-CATTGGTCTCACTGTTCCCATAGAA-3′); *ttyh3*, (5′-TGGAAGAATCATTACTTGCTGGATGAA-3′) and (5′-CAGAAGCAGGCCCAGGTA-3′); *Drosha,* (5′-ACCCCGATCGCCTTCATG-3′) and (5′-GGCTTTCAAACTGCACTTACAGAGA-3′). Three technical replicates were performed for each run. Raw data were processed with StepOne Software v2.3. Data were normalized to Drosha RNA ($\Delta\Delta C_T$ analysis).

2.4. Expression analysis

Plasmid DNA was linearized with restriction enzymes, and mRNA probes were transcribed while incorporating digoxigenin-labeled uracil using the respective RNA polymerases [*ttyh1* sense – HindIII and T7; *ttyh1* antisense – Not1 and T3; *ttyh2* sense – HindIII and T7; *ttyh2* antisense – BamH1 and T3; *ttyh3* sense – HindIII and T7; *ttyh3* antisense – Not1 and T3]. Probes were then used for *in situ* hybridization of *X. laevis* embryos stages 15–40. Sense probes were used to verify the absence of nonspecific binding. Chromagenic *in situ* hybridization was performed using BCIP/NBT (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) alkaline phosphatase substrates as described by Harland (1991) with slight modifications. Double fluorescent *in situ* hybridization was performed to determine co-expression of *ttyh2* and *xVGlut1* (Gleason et al., 2003) in cranial ganglion. The multiplex fluorescent *in situ,* described by Vize et al. (2009) with some modifications, used *ttyh2* mRNA probes transcribed while incorporating fluorescein-labeled uracil and *xVGlut1* mRNA probes transcribed while incorporating digoxigenin-labeled uracil. The reaction was developed in Cy3-tyramide and FITC-tyramide (Davidson and Keller, 1999) for *ttyh2* and *xVGlut1,* respectively.

Whole mount patterning of spatial and temporal expression was visualized following dehydration and clearing of embryos according to Sive et al. (2000). Whole mount photography was obtained using an Olympus SZX7 dissecting scope attached to an Olympus DP71 camera. Histological data were obtained from embryos that were cryoprotected in a 1.6 M sucrose solution for at least 12 hours at 4 °C and subsequently fixed in Tissue Freezing Medium for a minimum of 2 hours at room temperature. Embryos were then embedded in TFM at –20 °C, cryosectioned into 18 µm slices, and transferred to slides for imaging. Transverse histological analysis was performed in accordance to Li et al. (2006). Histological photography was taken using an Olympus BX60 scope attached to a Media Cybernetics QCapture digital camera. For each stage and each gene, *in situ* hybridization was repeated at least three times with a minimum of 10 embryos for each stage.

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