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Zebrafish transgenic constructs label specific neurons in *Xenopus laevis* spinal cord and identify frog V0v spinal neurons

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Abstract

A correctly functioning spinal cord is crucial for locomotion and communication between body and brain but there are fundamental gaps in our knowledge of how spinal neuronal circuitry is established and functions. To understand the genetic program that regulates specification and functions of this circuitry, we need to connect neuronal molecular phenotypes with physiological analyses. Studies using *Xenopus laevis* tadpoles have increased our understanding of spinal cord neuronal physiology and function, particularly in locomotor circuitry. However, the *X. laevis* tetraploid genome and long generation time make it difficult to investigate how neurons are specified. The opacity of *X. laevis* embryos also makes it hard to connect functional classes of neurons and the genes that they express. We demonstrate here that Tol2 transgenic constructs using zebrafish enhancers that drive expression in specific zebrafish spinal neurons label equivalent neurons in *X. laevis* and that the incorporation of a *Gal4:UAS* amplification cassette enables cells to be observed in live *X. laevis* tadpoles. This technique should enable the molecular phenotypes, morphologies and physiologies of distinct *X. laevis* spinal neurons to be examined together *in vivo*. We have used an *islet1* enhancer to label Rohon-Beard sensory neurons and *evx* enhancers to identify V0v neurons, for the first time, in *X. laevis* spinal cord. Our work demonstrates the homology of spinal cord circuitry in zebrafish and *X. laevis*, suggesting that future work could combine their relative strengths to elucidate a more complete picture of how vertebrate spinal cord neurons are specified, and function to generate behavior.

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Conflict of Interest/Competing Interests

The authors have no competing interests.

Author Contributions

J.M. performed all of the experiments and analyses in the paper, with the exception of some *X. laevis* embryo injections and *X. laevis in situ* hybridizations which were performed by R.M.D. A.R. and K.L designed and directed the study and provided expertise in frogs and zebrafish respectively. K.L and J.M wrote the paper with substantial input from A.R. All authors read, commented on and approved the final manuscript.

Keywords

V0v; V0; Interneuron; Evx; Xhox3; Xvglut1; Glutamatergic; RB; Islet1; *elavl3*; *slc17a7*

Introduction

The spinal cord is a crucial part of the central nervous system, responsible for controlling movements as well as receiving and processing sensory information from the trunk and the limbs. Despite its relative simplicity when compared to the brain, there are still fundamental gaps in our knowledge of how spinal cord neuronal circuitry is established and functions. Traditionally, there have been two main approaches that have addressed different aspects of this question. Developmental studies have investigated neuronal specification (how cells are instructed to differentiate into neurons of particular types), while physiological studies have concentrated on identifying different functional types of neurons and determining their roles in particular behaviors. These different approaches usually identify specific populations of neurons using different criteria: gene expression in developmental studies and morphology and electrophysiological characteristics in physiological studies. They have also often exploited the strengths of different model systems. For example, *Xenopus laevis* has been an invaluable model for elucidating the components and functions of spinal cord circuitry, whereas zebrafish and mouse have contributed more to our understanding of how different spinal cord neurons are specified.

Spinal cord circuitry controlling vertebrate locomotor behavior is probably best understood in the *X. laevis* tadpole. *X. laevis* tadpoles are relatively easy to manipulate and have robust tissues and cells, which makes them ideal for electrophysiology. The morphologies, physiological properties, synaptic connections and activities of most classes of spinal interneuron during the two main *X. laevis* tadpole locomotion behaviors, swimming and struggling, have been established (Li et al., 2001; Li et al., 2003; Li et al., 2004a; Li et al., 2004c; Li et al., 2004d; Li et al., 2004b; Li et al., 2006; Li et al., 2007a; Li et al., 2007b; Sautois et al., 2007; Roberts et al., 2008; Li et al., 2009; Soffe et al., 2009; Roberts et al., 2010; Roberts et al., 2012). However, the tetraploid genome and long generation time of *X. laevis* makes it hard to make mutants and investigate how different populations of neurons are genetically specified using this animal. Furthermore, the opacity of *X. laevis* tadpoles, due to the yolk in each cell, makes it difficult to analyze double labeling experiments and determine which genes are expressed by functionally-defined spinal neurons. Consequently, to date, only one molecularly-identified spinal cord population, *engrailed*-expressing V1 cells, has been correlated with a physiologically/morphologically identified population of cells, aIN neurons, in *X. laevis* (Li et al., 2004a).

In contrast, zebrafish are a powerful model system for investigating how spinal cord neurons are specified because loss-of-function and gain-of-function experiments are more easy to perform using mutant lines and antisense reagents (e.g. Lewis and Eisen, 2001; Varga et al., 2001; Lewis and Eisen, 2003; Lewis and Eisen, 2004; Lewis et al., 2005; Gribble et al., 2007; Batista et al., 2008; Batista and Lewis, 2008; Bonner et al., 2008; Gribble et al., 2009; Yang et al., 2010; England et al., 2011; Hilinski et al., 2016; Juárez-Morales et al., 2016).

Zebrafish embryos are also transparent making it relatively easy to identify genomic enhancers that drive expression in particular populations of zebrafish neurons (e.g. Higashijima et al., 2000; Bohm et al., 2016; Juárez-Morales et al., 2016) and use these to correlate gene expression with neuronal morphology (e.g. Kimura et al., 2006; Batista et al., 2008; Satou et al., 2012; Satou et al., 2013; Juárez-Morales et al., 2016). However, while it is possible to make electrophysiological recordings in zebrafish embryos (e.g. Higashijima et al., 2004; Kimura et al., 2006; Satou et al., 2009; Bohm et al., 2016) their small size and fragility makes these techniques very technically challenging, compared to *X. laevis*.

To really understand the genetic programs that regulate the specification and functions of neuronal circuitry, we need to be able to combine these different approaches so that the molecular phenotypes, morphologies and physiologies of spinal cord neurons can be examined together *in vivo*. Therefore, we decided to test if we could use genomic enhancers identified and validated in zebrafish spinal cord to drive expression of fluorescent proteins in equivalent *X. laevis* spinal cord neurons. We used the *Tol2* method of transgenesis as this is widely used in zebrafish (Kawakami, 2004; Kwan et al., 2007; Villefranc et al., 2007; Asakawa et al., 2008), therefore maximizing the chances that, in the future, additional zebrafish constructs will be available for *X. laevis* researchers to use. We also exploited the gateway cloning system so that future constructs could be easily assembled using the same middle entry and destination plasmids (Kwan et al., 2007; Villefranc et al., 2007) (for a similar system in frogs see (Love et al., 2011)).

While several different methods of transgenesis have been used to label specific cells in *X. laevis* (e.g. Ogino et al., 2006; Amaya and Kroll, 2010; Haeri and Knox, 2012; Ishibashi et al., 2012; Zuber et al., 2012; Takagi et al., 2013; Tam et al., 2013; Wang and Szaro, 2015), *Tol2* has only been used to test potential skeletal muscle enhancers (Loots et al., 2013). Therefore, before our study, it was unclear whether this method of transgenesis would be generally succesful in this animal. There have also been no reports where specific populations of transgenically-labeled spinal cord neurons have been observed in live tadpoles, which is what would be necessary to target specific molecularly-identified cells for electrophysiological analyses. Previous reports have usually used transgenic constructs to label and observe more superficially located cells in the skin, heart, eye, muscle or tail, where the opacity of *X. laevis* embryos presents less of a problem. (e.g. Moritz et al., 1999; Jansen et al., 2002; Lim et al., 2004; Smith et al., 2005; Scheenen et al., 2009; Yokoyama et al., 2011; Vivien et al., 2012; Haeri et al., 2013; Loots et al., 2013; Tam et al., 2013; Zhuo et al., 2013) (although also see (Love et al., 2011) and (L'Hostis-Guidet et al., 2009) as these authors used a few widely expressed neuronal promoters to test different types of transgenesis).

We tested different enhancers and promoters that label specific spinal cord neurons in zebrafish. The *elavl3* (formerly *HuC*) enhancer drives expression in most post-mitotic spinal neurons (Park et al., 2000a; Park et al., 2000b; Sato et al., 2006), the *islet1* enhancer drives expression primarily in skin sensory Rohon Beard cells (RBs) (Higashijima et al., 2000; Reyes et al., 2004) and the *evx1* enhancer drives expression in a population of interneurons that form in the most dorsal part of the ventral spinal cord called V0v cells (e.g. Briscoe et al., 2000; Moran-Rivard et al., 2001; Pierani et al., 2001; Lanuza et al., 2004; Griener et al.,

2015; Juárez-Morales et al., 2016) (also called V0e cells in zebrafish – see (Satou et al., 2012)) that develop into glutamatergic commissural interneurons in the zebrafish spinal cord (Juárez-Morales et al., 2016). We also identified a new *evx2* enhancer region that drives expression in a similar manner to *evx1*. We found that while we could successfully label the expected spinal cord cells as assayed by immunohistochemistry, it was very hard to detect fluorescent spinal cord neurons in live tadpoles due to the location of the spinal cord deep inside the tadpole and the opacity of the tadpoles at these stages of development. However, when we incorporated a *Gal4:UAS* amplification cassette into our constructs we were able to observe EGFP in live spinal cords.

Interestingly, despite the extensive characterization of *X. laevis* spinal cord circuitry, and the similarity between zebrafish and *X. laevis* spinal cord neurons, V0v neurons have not yet been identified in *X. laevis.* Therefore, to confirm that the cells labeled by our *evx* transgenic constructs were indeed V0v cells we tested whether they also expressed *X. laevis evx1* RNA and a marker of glutamatergic cells. We found that in all of the cases we examined, they did. This is important as it establishes that V0v neurons are present in the *X. laevis* spinal cord and further confirms the homology of spinal cord neurons and neuronal circuitry in fish, frogs and amniotes.

Methods

Xenopus laevis husbandry

X. laevis embryos were obtained following standard protocols from the Roberts lab colony at Bristol University, the Harris lab colony at the University of Cambridge and the Zuber lab colony at SUNY Upstate Medical University. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). All experiments were approved by either the Syracuse University IACUC committee or the UK Home Office.

Construction of Transgenic Constructs

The *elavl3* promoter was previously described (Park et al., 2000a; Park et al., 2000b). We PCR-amplified a 3.1Kb amplicon encompassing 2771bp upstream and 382bp downstream of the *elavl3* coding sequence from the *HuC cameleon 2.1 SV40poly(A)* plasmid (Higashijima et al., 2003) with the following primers: Forward primer: ATTCACTAATTTGAATTTAA, Reverse primer: TCTTGACGTACAAAGATGAT. This PCR product was cloned into the pDONR™ P4-P1R vector from Invitrogen using Gateway technology (Sasaki et al., 2004; Suzuki et al., 2005). Two reporter constructs were generated using the resulting 3.1Kb *elavl3* 5'pDONR vector. One was assembled using the pME-EGFP plasmid and the pCSDest2 vector (Kwan et al., 2007; Villefranc et al., 2007) to generate the *Tg(Tol2:3.1Kb5'zfish elavl3:EGFP:pA:Tol2)* construct. The other was made using the *Gal4VP16;UAS:EGFP* middle entry construct (Koster and Fraser, 2001; Juárez-Morales et al., 2016) and the pCSDest2 vector to generate the *Tg(Tol2:3.1Kb5'zfishelavl3:Gal4VP16;UAS:EGFP;pA:Tol2)* construct.

The *islet1* enhancer was also previously described (Uemura et al., 2005). We PCR-amplified this enhancer from zebrafish genomic DNA using the following primers: Forward primer:

TGCAGCTTTAGACATTTAAA; Reverse primer: TCCAGCACCATAATTCACCA. The 750bp PCR product was cloned into the pDONR™ P4-P1R vector from Invitrogen using Gateway technology (Sasaki et al., 2004; Suzuki et al., 2005). Two reporter constructs were generated by assembling the 750bp *islet1* 5′ pDONR with either the pENTRbasegfp plasmid (which contains the *βcarp* minimal promoter) or the *cfos* minimal promoter:*Gal4VP16;UAS:EGFP* middle entry construct (Koster and Fraser, 2001; Juárez-Morales et al., 2016) and the pCSDest2 vector to generate either the *Tg(Tol2:750bp3'zfish_islet1:βcarp minimal promoter:EGFP:pA:Tol2)* or the *Tg(Tol2:750bp3'zfish_isletl1:cfos minimal promoter:Gal4VP16;UAS:EGFP:pA:Tol2)* construct respectively.

We identified the *evx2* enhancer sequence through multispecies sequence comparisons using the global alignment program Shuffle-LAGAN (Brudno et al., 2003) and VISTA (Mayor et al., 2000) as described for *evx1* in (Juárez-Morales et al., 2016) (Fig 1A). We identified three Conserved Non-coding Elements (CNEs) in the vicinity of *evx2*. The first is located 419bp upstream of zebrafish *evx2* and extends for 97 bp. The other two CNEs are located downstream of *evx2*, one is 2052bp downstream of the stop codon and is 182bp long whereas the other is 2289bp downstream of the stop codon and extends for 700bp (Fig. 1A). We PCR-amplified one region encompassing the two 3′ CNEs and 3′ UTR. The forward primer was designed just after the stop codon. We used the BAC RP71-78H1 (BACPAC Resources Center) as a template with forward primer: GCGAGATGTAACGATGCTAT and reverse primer: CAAATGTGT TGAGGTGAGCA. The resulting amplicon was cloned into the pDONR™ P4-P1R vector from Invitrogen using Gateway technology (Sasaki et al., 2004; Suzuki et al., 2005). The final reporter construct was assembled using the pENTRbasegfp plasmid (which contains the *βcarp* minimal promoter) and the pCSDest2 vector (Villefranc., 2007). This produced the *Tg(Tol2:3.9Kb3'zfish_evx2:βcarp_minimal promoter:EGFP:pA:Tol2)* construct.

The *Tg(Tol2:1.3Kb 3'zfish evx1: βcarp minimal promoter:EGFP:Tol2)* and the *Tg(Tol2:1.3Kb 3'zfish evx1:cfos minimal promoter:Gal4VP16;UAS-EGFP:pA:Tol2)* constructs were previously described (Juárez-Morales et al., 2016). We have previously demonstrated that these constructs specifically drive expression in *evx1*-expressing spinal cord cells in both transient transgenics and two stable transgenic lines in zebrafish (Juárez-Morales et al., 2016).

DNA and *transposase* mRNA preparation and microinjection

Plasmid DNA was prepared using QIAfilter plasmid purification kit (Qiagen, 12743) and *transposase* mRNA was prepared using the pCS2FA transposase plasmid (Kwan et al., 2007). After the transposase plamid was linearized and purified using the QIAquick PCR Purification Kit (Qiagen, 28104), mRNA *in vitro* transcription was conducted using the Ambion mMessage mMachine SP6 kit (Ambion, AM1340) and manufacturer's protocols. Microinjection of DNA plus RNA was carried out using an air-pressure picospritzer II (General Valve Corporation). Glass microneedles were pulled in a P-2000 micropipette puller (Sutter Instruments Co.). Approximately 10 nl of a combination of Plasmid DNA

[33-20 ng/μL] and transposase mRNA [30ng/μL] was injected into both blastomeres of 2-cell stage de-jellied *X. laevis* embryos.

Analysis of gene expression and transgenic labeling

X. laevis embryos were incubated at 18^0C until they reached stage 41. Just before fixation, tadpoles were analyzed for EGFP expression by examining the hindbrain to rostral spinal cord region on an Olympus SZX16 stereomicroscope. By stage 41 most of the yolk has been consumed making these internal structures more visible. In our experience, if we observe neurons expressing EGFP in these structures there is a high chance that there will also be EGFP-expressing neurons more caudally in the spinal cord.

For *in situ* hybridization and immunohistochemistry, embryos were fixed in 4% PFA for 1 hr at room temperature. Embryos were then washed in PBS + 0.01% Tween-20 three times for 5 minutes each, followed by 50% MeOH in PBS + 0.01% Tween-20 and then stored in 100% MeOH at −20°C. Whole mount *in situ* hybridizations were performed as previously described (Zuber et al., 2003; Viczian et al., 2006) with the following modifications: tadpoles were bleached for 11 mins with 0.5% SSC, 10% H_2O_2 and 5% formamide and proteinase K treatment [10μg/mL] was performed for 5 minutes at room temperature. RNA probes were detected with Anti-Digoxigenin-AP, Fab fragments (Sigma Aldrich 11093274910) and BM purple AP (Roche 11442074001).

RNA *in situ hybridization* probes were prepared using the following templates: *evx1*, (previously called *Xhox3*) was kindly provided by Jonathan Slack (Beck and Slack, 1998) and *slc17a7* (previously called *XVGlut1)* was kindly provided by Margaret Saha (Gleason et al., 2003).

Primary antibodies used were rabbit anti-GFP (Invitrogen A6465, 1/500) or chicken anti-GFP (Abcam ab13970, 1/500) and secondary/tertiary antibodies were Alexa Fluor 488 goat anti-rabbit (Invitrogen A11034, 1/500) or Alexa Fluor 488 goat anti-chicken (Invitrogen A11039, 1/500) for fluorescent staining or goat anti-rabbit IgG (Covance SMI-5030C, 1/200) and rabbit PAP (Covance SMI-4010 L, 1/200) for DAB staining.

Tadpoles used for fluorescent immunohistochemistry were treated with Image-iT Signal Enhancer (Invitrogen, I36933) for 30 minutes, then incubated in block solution (2% goat serum, 1% BSA, 10% DMSO and 0.5% Triton) for 1 hour at room temperature followed by incubation in primary antibody in fresh block solution at 4°C overnight. Tadpoles were washed with PBT for 2 hours at room temperature and incubated with secondary antibody in block solution at 4°C overnight. Tadpoles were then washed with PBT for at least 2 hours at room temperature and stored in 2% DABCO (Acros Organics, AC11247-1000).

Tadpoles used for DAB immunohistochemistry were incubated at 4°C overnight with primary antibody and then incubated in fresh blocking solution with goat anti-rabbit IgG (Covance SMI-5030C, 1:200) at 4°C overnight. Embryos were then washed with PBT for 2 hours and incubated with rabbit PAP (Covance SMI-4010 L, 1:200) in block solution at 4°C overnight. Embryos were then washed in PBT for 2 hours. DAB staining was developed using SigmaFast™ 3,3′-diamino- benzidine tablets (Sigma, D4293-5set).

For *in situ* hybridization and immunohistochemistry on tissue sections, stage 42 tadpoles were fixed in 4% paraformaldehyde (PFA) for 1 hour at room temperature and cryoprotected in 30% sucrose in PBS at 4°C overnight. Tadpoles were embedded in Tissue Tek OCT (VWR, 25608-930) and cryostat sectioned at 12 μm. *in situ* hybridization was performed as previously described (Viczian et al., 2006; Martinez-De Luna et al., 2013) except that Proteinase K was used at a concentration of 10μg/ml and tissue sections were incubated in this solution for 1 min at room temperature. *in situ* hybridization was performed first and followed by immunohistochemistry as described above with the following modifications; tissue sections were fixed in 4% PFA for 30 min at room temperature, washed 3X 5 mins in PDT (1XPBS, 1% DMSO, 0.1% Triton X-100) then incubated in block solution (1XPBS, 1% DMSO, 0.1% Triton X-100, 2% goat serum, 1% BSA) for 1hour at room temperature, followed by incubation in primary antibody in fresh block solution at 4°C overnight. Sections were washed with PDT 3X 5 mins and then 4X 20 mins at room temperature and incubated with secondary antibody in block solution for 2 hours. After incubation, sections were washed with PDT 3X 5 mins and then 4X 20 mins at room temperature and stored in 2% DABCO (Acros Organics, AC11247-1000).

Image acquisition and processing

Whole-mount tadpoles were placed in a 1% agarose plate and covered in PBS for imaging using a Olympus SZX16 stereomicroscope and a Q-Imaging Micropublisher 5.0 RTV camera. Cross sections were mounted in 2% DABCO on microscope slides and brightfield pictures were taken using an AxioCam MRc5 camera mounted on a Zeiss Axio Imager M1 compound microscope. Fluorescent images were taken on a Zeiss LSM 710 confocal microscope. Images were processed using Adobe Photoshop software (Adobe, Inc) and Image J software (Abramoff et al., 2004).

Spinal cord dissections

After inmunohistochemistry, *X. laevis* tadpoles were placed in a dish which had a rotatable shaft with a sylgard platform on one side. The tadpoles were pinned down on this platform using two tungsten pins, one through the eye and the second through the muscle and notochord two thirds of the way down the body. Using a pair of dissecting pins, skin and muscle were removed from the tadpole to expose the spinal cord.

Results

To test whether we could use zebrafish enhancers and *Tol2* transgenesis methods to label *X. laevis* spinal neurons we constructed three different *Tol2* EGFP constructs with enhancers or promoters that had already been validated in zebrafish either by our lab or other groups and a fourth construct using a newly identified enhancer for *evx2*. In cases where we used enhancers, we combined these with either a *cfos* or *βcarp* basal promoter (Wang et al., 2000; Villefranc et al., 2007). In zebrafish, the *elavl3* (previously called *HuC)* promoter drives expression in most post-mitotic neurons (Park et al., 2000a; Park et al., 2000b; Sato et al., 2006), the *islet1* enhancer drives expression primarily in RB cells (Higashijima et al., 2000) and the *evx1* enhancer drives expression in V0v neurons (Juárez-Morales et al., 2016). Given that *evx1* and *evx2* are expressed in the exact same cells in the zebrafish spinal cord

we also investigated whether a similar enhancer to our previously identified *evx1* enhancer (Juárez-Morales et al., 2016) existed for *evx2.* Our bioinformatic analyses (see methods) identified a similar region of high conservation downstream of *evx2* (Fig. 1A and compare to Fig. 1D in (Juárez-Morales et al., 2016)) and, therefore, we also cloned and tested this enhancer. We injected these constructs into 1–2 cell stage *X. laevis* embryos. In most cases, we could not detect any obvious EGFP expression in the spinal cord of live tadpoles, but when we fixed the tadpoles and performed anti-GFP immunohistochemistry, detecting EGFP expression with a coloured substrate (3,3′-Diaminobenzidine; DAB), we could clearly observe expression in dissected spinal cords, in the types of spinal cord neurons that we would expect, based on the behavior of these enhancers/promoter in zebrafish (Fig. 1C–F and data not shown, for more detailed discussion of cell types observed see below). These immunohistochemistry results showed that the transgenesis method was working, suggesting that the EGFP was not bright enough to be detected in live spinal cords, probably due to the opacity of the *X. laevis* tadpoles at these stages of development.

To use transgenic constructs to label cells for electrophysiological experiments, we would need to visualize and identify individual cells in live tadpoles. To try and overcome the fact that we could not detect EGFP-labeled neurons in live *X. laevis* tadpoles using these initial constructs, we incorporated a *Gal4:UAS* amplification cassette (Koster and Fraser, 2001) into our *Tol2* constructs. Using these new constructs we could observe EGFP-labeled neurons in live whole tadpoles (Fig. 1G). These labeled cells became even clearer after overlying skin and muscle was dissected away to view the spinal cords (Fig. 1H–I) and they had the same specificity as the cells labeled with the non-amplified constructs (e.g. Fig 1C and 1H). In each case, as expected for transient transgenesis assays, there was variability in the number of cells labeled in each tadpole, with some animals containing many labeled cells (e.g. Fig. 1C, D, G & H) and others fewer labeled cells (e.g. Fig. 1E & F).

As in fish larvae, the *Tg(elavl3:EGFP)* constructs labeled a wide range of different neurons in the *X. laevis* spinal cord (Fig. 1C, G & H). While in some tadpoles so many cells were labeled that it was hard to distinguish individual cell morphologies (e.g. Fig. 1G & H), in other cases there were stretches of spinal cord where individual cells could be easily identified (Fig. 1C–F & I).

Consistent with its activity in zebrafish, the *islet1* enhancer drove expression mainly in RBs in the *X. laevis* spinal cord. We analyzed 7 tadpoles injected with *Tg(islet1:cfos:Gal4VP16;UAS:EGFP)* and identified 97 labeled cells. 72 of these (74%) were clearly RBs (Fig. 2A & B). Their cell bodies were located at the dorsal surface of the spinal cord and their axons extended in the dorsal tract. In more densely labeled animals, we observed labeled RBs along the whole spinal cord and into the caudal hindbrain. Interestingly, most of the non-RB cells labeled in these tadpoles had very similar morphologies to each other with oval somata and ventrally-directed axons (Fig 2C).

In addition, both the *evx1* and *evx2* enhancers drove expression mainly in *X. laevis* spinal cord neurons with pear shaped somata, located in a mid to dorsal dorsal-ventral position in the spinal cord. Several of these neurons had somata in a dorso-lateral position next to the dorsal tract of RB axons (e.g. Fig. 2D left 4 neurons). The labeled cells also had ventrally-

directed axons that continued to the ventral commissure and then turned to ascend on the other side of the spinal cord where they also sometimes formed a caudally directed branch. For example, we analyzed 6 tadpoles injected with *Tg(evx1:cfos:Gal4VP16;UAS:EGFP)* and identified 291 labeled cells. Of these, 232 (~80%) had this morphology and dorso-ventral location (Fig 1D–F and Fig. 2D–E). The labeled neurons with different morphologies included 8 RBs in the dorsal spinal cord, 26 cells in the ventral spinal cord, 5 cells with ipsilateral axons in the mid-region of the spinal cord and 20 cells with a horizontal pear-shaped cell body that were also located in the mid-region of the spinal cord.

The dorso-ventral locations and commisural axon trajectories of the major class of labeled cells was highly reminiscent of zebrafish V0v neurons. However, while RB cells can be unambiguously identified by their morphology, this is not the case for V0v cells as they are not the only commissural neurons in the spinal cord and, their morphologies can differ (Satou et al., 2012). The characteristic that unambiguously identifies V0v cells in zebrafish and amniotes is their expression of *evx1* and *evx2* (they are the only cells in the spinal cord that express these genes). In addition, in all animals examined so far, V0v cells express glutamatergic markers (Moran-Rivard et al., 2001; Satou et al., 2012; Juárez-Morales et al., 2016). Therefore, to test whether cells labeled by our *evx1* enhancer might be V0v cells, we performed double labeling experiments for EGFP and either *X. laevis evx1* (previously called *Xhox3)* or *slc17a7* (previously called *vglut1* and *Xvglut1*), which labels glutamatergic cells. In the vast majority of the cases that we analyzed, spinal cord cells that expressed EGFP in *Tg(evx1:cfos:Gal4VP16;UAS:EGFP)* injected tadpoles also expressed these RNAs. 21/23 cells that expressed EGFP co-expressed *evx1* RNA and in separate experiments 32/35 cells that expressed EGFP co-expressed the glutamatergic marker *slc17a7* (Fig 2F–H, J–L). In both cases, rare EGFP single-positive cells were mainly located very ventrally in the spinal cord, suggesting that these are likely to be cells that were ectopically expressing EGFP (3 cells were ventral and 1 was very dorsal).

Discussion

All of the evidence so far, suggests that mechanisms of spinal cord patterning and resulting neuronal circuitry are highly conserved in vertebrates (e.g. Goulding and Pfaff, 2005; Lewis, 2006). In particular, comparisons between zebrafish, *X. laevis* and mouse suggest that the bony fish and tetrapod ancestor had a basic plan of spinal cord circuitry, where distinct classes of neurons with particular functions, were specified in the embryo by different transcription factors (e.g. Roberts, 2000; Higashijima et al., 2004; Li et al., 2004a; Goulding and Pfaff, 2005; Lewis, 2006). Data suggest that both the properties of these neurons and their method of specification have been conserved throughout the evolution of bony fish and tetrapods; with the proviso that in mammals and potentially other amniotes, some classes of neurons may have diversified during development, into specialized sub-classes of related neurons (e.g. Lewis and Eisen, 2001; Higashijima et al., 2004; Sapir et al., 2004; Alvarez et al., 2005; Goulding and Pfaff, 2005; Gosgnach et al., 2006; Kimura et al., 2006; Griener et al., 2015). Taken together, these observations argue strongly that we can use the simpler spinal cords of anamniote embryos such as zebrafish and *X. laevis* to determine fundamental aspects of vertebrate spinal cord neuronal specification and function and that findings from one vertebrate will usually apply more widely to the whole vertebrate family.

In this paper, we further demonstrate the homology of vertebrate spinal cord circuitry by showing that transgenic constructs using four different zebrafish genomic enhancers or promoters that label particular spinal cord neurons in zebrafish, are expressed by equivalent neurons in *X. laevis* tadpoles (see discussion below). Combined with data from previous reports that used mammalian (e.g. Beck and Slack, 1999; Lim et al., 2004; Suzuki et al., 2007; Yokoyama et al., 2011; Loots et al., 2013) or zebrafish (Concha et al., 2003; Love et al., 2011) enhancers to label specific cell types in frogs, this suggests that enhancers and promoters from other vertebrates can be used successfully for *X. laevis* transgenesis.

In addition, we identify a neuronal class, V0v interneurons, that has not been previously identified in *X. laevis,* but is present in both amniotes and zebrafish (Moran-Rivard et al., 2001; Suster et al., 2009; Satou et al., 2012; Juárez-Morales et al., 2016). As discussed in more detail below, we show that these cells have similar properties in frog to V0v cells in other vertebrates. As well as adding to our understanding of frog spinal circuitry, this again confirms that spinal cord neurons are highly conserved across vertebrates. This conservation is particularly strong for *X. laevis* and zebrafish. Both of these animals develop fast from eggs into free moving larvae in a similar way and their hatchling larvae have a relatively small number of different classes of spinal cord neurons and similar locomotor behaviours. Comparisons of neuronal morphology and function suggest that spinal cord circuitry in these vertebrates is also very similar, reflecting the basic common vertebrate plan for early spinal cord organisation (Roberts, 2000; Goulding and Pfaff, 2005). This suggests the intriguing possibility that *X. laevis* and zebrafish larvae could be used in a complementary way to study spinal cord neuronal circuits, enabling a more powerful and complete analysis than would be possible with either alone, using *X. laevis* to characterize the physiology of particular neurons and zebrafish to decipher how those same neurons are specified.

The transgenesis method that we test in this study enables us to identify EGFP-labeled spinal neurons in live *X. laevis* tadpoles. We used Gal4 and UAS components to amplify the expression of EGFP so that it can be observed even in the spinal cord of live tadpoles. We have also demonstrated that these constructs work well in injected F0 animals/transient transgenics. This is important as making stable lines in *X. laevis* is very time-consuming and laborious due to the long generation time of this animal. These new genetic tools should enable researchers to label specific neurons for electrophysiological studies and hence facilitate studies correlating the molecular, morphological and physiological properties of cells. In fact, mosaically-labeled transient transgenic larvae are arguably more useful for these sorts of studies than stable transgenic animals as labeling just a small subset of a particular population of neurons makes it easier to identify and characterize individual cells. These and other zebrafish enhancers could also potentially be used for optogenetics and/or to drive expression of constructs that alter neuronal behavior by silencing, activating or ablating specific neurons to study the links between neuronal circuitry and behaviour, although in some of these cases, stable transgenics may be needed for robust conclusions to be drawn.

We observed a reasonably high level of selectivity for both the *islet1* and the *evx1* enhancers in our transient transgenesis assays in *X. laevis*. For example, over 74% of cells labeled with the *islet1* enhancer could be unambiguously identified as RB cells, by their unique cell

morphology and dorsal spinal cord location. Interestingly, most of the remaining cells had an oval soma and ventrally-directed axon. While in zebrafish, the *islet1* enhancer has only been reported to label RB cells (Higashijima et al., 2000; Reyes et al., 2004), *islet1* is also expressed by motoneurons and interneurons in zebrafish (Inoue et al., 1994; Appel et al., 1995; Tokumoto et al., 1995; Lewis and Eisen, 2001; Tamme et al., 2002; Lewis and Eisen, 2004). The non-RB labeled cells did not have the morphology or ventral spinal cord position of motoneurons. However, it is possible that these cells are an *islet1*-expressing population of interneurons. Interneuron expression could have been missed in the previous zebrafish analyses particularly if the interneuron somas were located close to RBs. Even if these non-RB cells in *X. laevis* are examples of ectopic expression, which is very common with transient transgenesis/injected F0 animals, the frequency of correct labeling is high enough for this construct to be a very useful tool for identifying RB cells for physiological analyses in live tadpoles. As RBs have a unique characteristic morphology, labeled RBs can easily be distinguished from non-RB cells. Therefore, this construct should be invaluable for future studies, for example for examining synapse formation between RBs and dorsal sensory pathway neurons (Li et al., 2003, 2004c).

As mentioned above, we also found that our constructs using the *evx1* enhancer that we identified in zebrafish (Juárez-Morales et al., 2016) labeled a population of interneurons with a unipolar pear-shaped cell body, a medial-dorsal dorsal-ventral spinal cord position and commissural ascending axons with no obvious dendrites. In all of these ways, they resemble zebrafish V0v cells which develop into excitatory Commissural Seconday Ascending (CoSA) neurons (Satou et al., 2012; Juárez-Morales et al., 2016). Approximately 80% of the labeled *X. laevis* cells had these characteristics. We have confirmed that these commissural cells do indeed express endogenous *evx1* and that, as in zebrafish, they express the glutamatergic marker *slc17a7*, which strongly suggests that they are V0v neurons. In addition, we also demonstrated that a newly identified *evx2* zebrafish enhancer sequence drives expression in cells with the same morphology (Fig. 1D–F), again consistent with these cells being *evx1* and *evx2*-expressing V0v cells. This is the first time that V0v neurons have been identified in *X. laevis*. The only excitatory commissural cells that have been described so far in *X. laevis* spinal cord are dorsolateral commissural (dlc) (Li et al., 2003) and excitatory commissural (ecINs) sensory pathway interneurons (Li et al., 2007b). However, dlc neurons are multipolar with dorsal dendrites (Li et al., 2003) and at least most ecINs also have dendrites although these are variable (Li et al., 2007b). In contrast, our labeled cells have no obvious dendrites. Therefore, it is possible that at least some of our labeled cells are a different population of glutamatergic commissural cells, probably equivalent to excitatory CoSA neurons, the cell type labeled by these enhancers in zebrafish (Juárez-Morales et al., 2016). However, we can not rule out the possibility that dendrites may not have been clearly visible in our experiments, some ecIN neurons may be unipolar like zebrafish CoSA neurons (Li et al., 2007b) and at least some of our labeled cells are in a similar dorso-lateral position to dlc and ecIN neurons. Therefore, it is possible that at least some dlc and ecIN neurons correspond to *evx*-expressing V0v INs.

In conclusion, our data suggest that V0v interneurons exist in *X. laevis* tadpoles, further confirming the similarity of spinal cord circuitry and spinal neuron development across vertebrates. This is only the second molecularly-defined spinal cord cell type to be

correlated with morphologically/physiologically defined neurons in *X. laevis*: the first was *engrailed*-expressing V1 cells that were shown to correspond to aIN neurons (Li et al., 2004a). In addition, we demonstrate that *Tol2* transgenesis using zebrafish enhancers and a *Gal4:UAS* amplification cassette labels equivalent spinal neurons in *X. laevis* to those labeled in zebrafish and that EGFP expression driven by these constructs is visible in live *X. laevis* tadpole spinal cords. This should facilitate electrophysiological studies of specific interneurons in *X. laevis* tadpoles by reducing uncertainty in the selection of candidate neurons for recording. It should also enable additional connections between molecularly-distinct cells and functionally-distinct neurons to be made in this model system. This is important as *X. laevis* is arguably the animal in which we have the most complete understanding of spinal cord circuitry. Genetically modified mice and zebrafish have already been produced with fluorescent spinal neurons, which can be recorded electrically to study their physiology. These methods clearly work, but the mouse spinal cord is very complex and making recordings in zebrafish remains very difficult. Increasing the ease of electrophysiological analyses in *X. laevis*, while also confirming the homology of specific *X. laevis* neurons with equivalent neurons in zebrafish and amniotes, in which much more is known about neuronal specification, should help us to achieve our ultimate aim, which is a complete, integrated picture of how vertebrate spinal cord neurons and neuronal circuits are specified, function and generate behavior.

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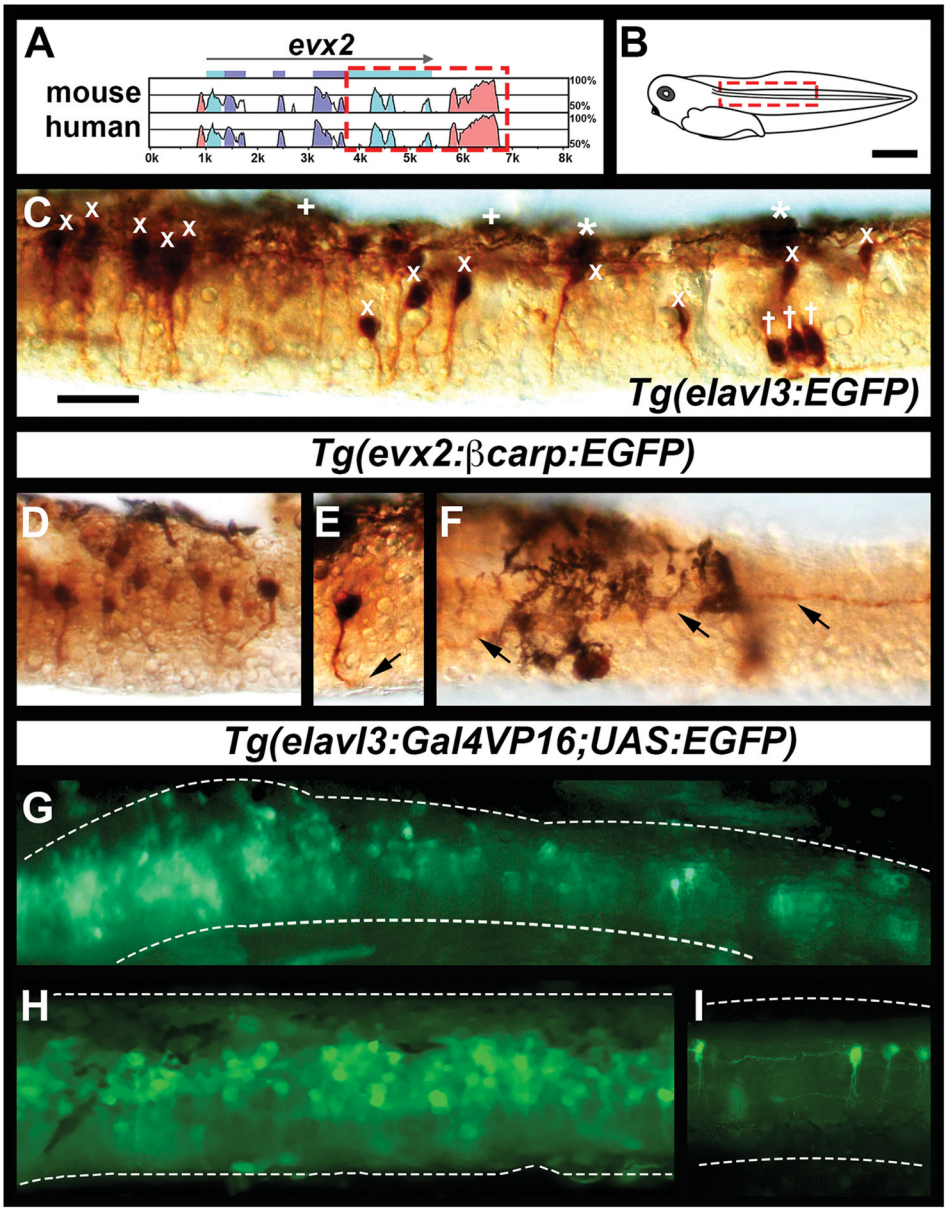

Figure 1. Zebrafish enhancers label appropriate neurons in *Xenopus laevis* spinal cord

(A) Schematic of shuffle-LAGAN analysis of *evx2* genomic region, with zebrafish *evx2* used as the baseline and compared to orthologous genomic regions in mouse and human. Conserved coding sequences are indicated in purple and conserved UTR regions are indicated in light blue. Percentage of sequence conservation is indicated by peak heights (scale is provided on RHS), grey arrow indicates 5′-3′ gene orientation, CNEs are indicated in pink. Red dotted box indicates region amplified to create *evx2* enhancer transgenic constructs. (B) Schematic of stage 41 *X. laevis* tadpole. Red box indicates the approximate spinal cord region shown in subsequent lateral views. (D–F and I) show only a small part of this region. (C – I) Lateral views of one side of stage 41 *X. laevis* spinal cord, dorsal top, rostral left. (C–F, H and I) show dissected spinal cords. The tissue shown is the full dorsal-

ventral extent of the spinal cord and no other tissue is included except for a few pigment cells. (C) DAB immunohistochemistry (dark brown staining) for EGFP in transient transgenic *Tg(elavl3:EGFP)* spinal cord showing several different labeled post-mitotic neurons. For example, we have indicated a couple of RB neurons (*), a group of three motoneurons in the ventral spinal cord on the RHS of the panel (†) and some commissural cells (x). The dorsal black cells are pigment cells (+). (D–F) DAB immunohistochemistry for EGFP in transient transgenic *Tg(evx2:βcarp:EGFP)* spinal cords. (D) shows a region of rostral spinal cord with several labeled cells. The cells have pear shaped somata approximately 4.7 μm wide along the rostral-caudal axis and 6 μm tall in the dorsal-ventral axis, they are located in the dorsal 48–68% of the spinal cord, and they all have axons that project to the ventral spinal cord and then cross the midline to become commissural. (E & F) show two different focal planes of the same spinal cord in a region with just one labeled cell, black arrows indicate axon trajectory, black cells in F and dorsally in E are pigment cells. The cell soma is visible in (E) and its axon is visible on the contralateral side of the spinal cord in (F). (E) is slightly more rostral than (F). (G–I) Live expression of EGFP in transient transgenic *Tg(elavl3:Gal4VP16;UAS:EGFP)* spinal cords. (G) shows expression on one side of the mid-trunk spinal cord of an intact tadpole. The white dotted lines show the dorsal and ventral limits of the spinal cord. The expression appears weaker/more diffuse because we are looking through the skin and muscle overlying the spinal cord. (H & I) show expression in dissected live spinal cords where these other tissues have been removed. (H) shows an example where many cells are labeled. (I) shows an example of more sparse labeling where individual cells and their axons can be observed and identified. Scale bar in C = 20 μm (panels C–F) and 30 μm (panels G–I). Scale bar in B = 1mm.

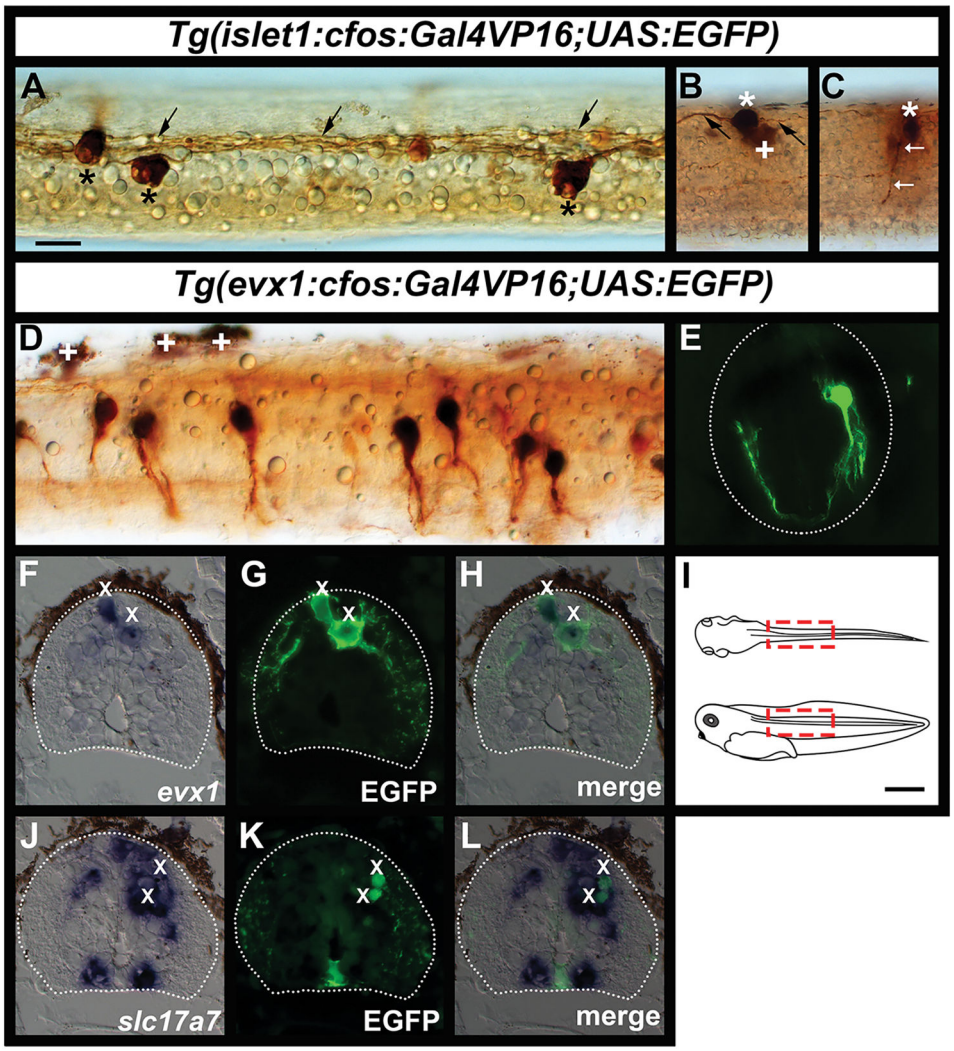

Figure 2. Zebrafish *islet1* enhancer predominantly labels Rohon Beard neurons and *evx1* enhancer labels V0v neurons in stage 41 *Xenopus laevis* spinal cord

(A, B & C) DAB immunohistochemistry for EGFP in transient *Tg(islet1:cfos:Gal4VP16;UAS:EGFP)* transgenic spinal cord. (A) Dorsal view of spinal cord, rostral left showing labeled RB neuron soma (*) and longitudinal axons in the dorsal tract (black arrows). The smaller, more weakly labeled cells are probably not RB neurons. (B & C) lateral views, rostral left, dorsal top. (B) RB neuron with characteristic large round soma in dorsal spinal cord (*) and ascending and descending longitudinal axons (black arrows), + indicates a pigment cell located ventral and lateral to the RB neuron. (C) shows a slightly more ventrally located neuron in the same embryo as (B) with an oval soma and ventral axon (arrows). (D) Lateral view with rostral left and dorsal top showing DAB immunohistochemistry for EGFP in transient *Tg(evx1:cfos:Gal4VP16;UAS;EGFP)* transgenic spinal cord. Labeled cells have pear-shaped soma, axons that extend ventrally and then become commissural and are located in the dorsal 48–68% of the spinal cord. White crosses indicate dorsal pigment cells. (E) Fluorescent immunohistochemistry for EGFP in transient *Tg(evx1:cfos:Gal4VP16;UAS:EGFP)* spinal cord; cross section (dorsal up)

showing a single labeled neuron soma and ventral commissural axon. White dotted lines show edge of spinal cord. (F–H & J–L) Double labels (*in situ* hybridization with BM purple plus fluorescent immunohistochemistry for EGFP) of *Tg(evx1:cfos:Gal4VP16;UAS:EGFP)* spinal cord cross sections. Spinal cord margins are delinated with dotted lines. (F) *X. laevis evx1* RNA expression (purple). (G) EGFP expression (green) in same cross section as (F). (H) merged image of F and G. Two EGFP-labeled cells (X) co-express *evx1* RNA. (J) *X. laevis slc17a7* RNA expression (purple). (K) EGFP expression (green) in same cross section as (J). (L) merged image of J and K. Two EGFP-labeled cells (X) co-express *slc17a7* RNA. (I) schematics showing approximate region of spinal cord shown in dorsal view in (A) and lateral views in (B–D). (B&C) show only part of this region. (A–L) are all stage 41. Scale bar in A = 20 μm (panels A–H & J–L) and scale bar in I = 1mm.