Published in final edited form as:

J Comp Neurol. 2014 March ; 522(4): 861–875. doi:10.1002/cne.23449.

Connexin 35b expression in *Danio rerio* embryos and larvae spinal cord

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Abstract

Electrical synapses are expressed prominently in the developing and mature nervous systems. Unlike chemical synapses, little is known about the developmental role of electrical synapses, reflecting the limitations imposed by the lack of selective pharmacological blockers. At a molecular level, the building blocks of electrical synapses are connexin proteins. In this study, we report the expression pattern for neuronally expressed *connexin 35b* (*cx35b*), the zebrafish orthologue of mammalian *Connexin* (*Cx*) 36. We find that *cx35b* is expressed at the time of neural induction, indicating a possible early role in neural progenitor cells. Additionally, *cx35b* localizes to the ventral spinal cord during embryonic and early larval stages. We detect *cx35b* mRNA in secondary motor neurons (SMNs) and interneurons. We identified the premotor circumferential descending (CiD) interneuron as one interneuron subtype expressing *cx35b*. In addition, *cx35b* is present in other ventral interneurons of unknown subtype(s). This early expression of *cx35b* in SMNs and CiDs suggests a possible role in motor network function during embryonic and larval stages.

Keywords

cx35b; zebrafish; neurodevelopment

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Conflict of Interest Statement: The authors state no conflict of interest.

Role of Authors: All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: T.C.M. and A.B.R. Acquisition of data: T.C.M. Analysis and interpretation of data: T.C.M. Drafting of the manuscript: T.C.M. Critical revision of the manuscript for important intellectual content: T.C.M. and A.B.R. Statistical analysis: T.C.M. and A.B.R. Obtained funding: T.C.M. and A.B.R. Administrative, technical, and material support: A.B.R. Study supervision: A.B.R.

Introduction

Gap junctions between electrically excitable cells are the basis of electrical synapses (Bennett et al., 1963; Robertson et al., 1963; Furshpan, 1964). Vertebrate gap junction formations are composed of connexin proteins, which are plasma membrane proteins containing four transmembrane domains, intracellular amino- and carboxy-termini, a single intracellular loop, and two extracellular loops (Milks et al., 1988; Yeager and Gilula, 1992). Six connexin proteins oligomerize in a single membrane bilayer to create a connexon channel (Cascio et al., 1995), and two apposing connexon channels from abutting plasma membranes generate a pore connecting the two cells, or a gap junction (Goodenough and Revel, 1970; Chalcraft and Bullivant, 1970; McNutt and Weinstein, 1970). Through their pore, gap junctions allow for the transfer of second messengers and ions between the intracellular spaces of connected cells (Kumar and Gilula, 1996).

Gap junctions have been implicated in numerous neurodevelopmental processes, such as proliferation (Weissman et al., 2004; Kunze et al., 2009), differentiation (Chuang et al., 2007; Santiago et al., 2010), migration (Fushiki et al., 2003; Elias et al., 2007; Liu et al., 2010, 2012), and cell death (Cusato et al., 2003). However, the underlying mechanisms are largely unknown (for reviews, see Bruzzone and Dermietzel, 2006; Dere and Zlomuzica, 2012). The dynamic expression profiles of neuronal connexins during development have led to the hypothesis that electrical synapses may play transient developmental roles (Gulisano et al., 2000; Belluardo et al., 2000; Lee et al., 2005; Hansen et al., 2005; Cina et al., 2007). However, addressing this hypothesis experimentally faces two major challenges. First, existing gap junction blockers lack specificity and target many connexins in addition to other channels. Second, glial cells also express connexins, so gap junction blockers applied to nervous tissue would inhibit channels in glia as well as neurons.

Outside of the retina, only a subset of connexin genes are expressed by neurons (Söhl et al., 2005; Eugenin et al., 2012; Dere and Zlomuzica, 2012; Bruzzone and Dermietzel, 2006). In addition to *Cx36* in mammals, this subset may include *Cx30.2* (Kreuzberg et al., 2008), *Cx31.1* (Venance et al., 2004; Vandecasteele et al., 2006; Zheng-Fischhöfer et al., 2007), and *Cx45* (Maxeiner et al., 2003; Zlomuzica et al., 2010), but there are conflicting data for neuronal expression of *Cx26*, *Cx30*, *Cx32*, *Cx43*, and *Cx47* (Venance et al., 2004; Vandecasteele et al., 2006; Nadarajah et al., 1996, 1997; Rash et al., 2001 a; Odermatt et al., 2003). Unlike the other neuronally expressed connexins, *Cx36* orthologues are almost exclusively expressed by neurons (O'Brien et al., 1998; Condorelli et al., 1998; Rash et al., 2000; Rash et al., 2001 b; a, 2007; Meier et al., 2002; Degen et al., 2004). Additionally, evidence exists for developmentally regulated expression of *Cx36* orthologues in the retina (Al-Ubaidi et al., 2000; Hansen et al., 2005; Blankenship et al., 2011), brain (Söhl et al., 1998; Belluardo et al., 2000; Gulisano et al., 2000; Gildenagel et al., 2001; Cina et al., 2007), and spinal cord (Chang et al., 1999; Belluardo et al., 2000; Gulisano et al., 2000; Lee et al., 2005).

In this study, we focus on a zebrafish orthologue of mammalian *Cx36*, *connexin 35b* (*cx35b*; previously *cx35*, *cx35.1*, and *gja9*; McLachlan et al., 2003). We took an expression approach to determine the temporal and spatial expression patterns for zebrafish *cx35b* in the

embryonic and larval spinal cord. The zebrafish model provides easy access to early developmental stages for which there is limited access in other model systems. We find *cx35b* expression during neural induction, raising the possibility of an early role in the developing nervous system. We also find expression of *cx35b* in neurons of the developing motor network. This information identifies the developmental processes in which Cx35b may play a role and cellular networks that may require Cx35b gap junctions in the zebrafish spinal cord.

Materials and Methods

Animal care

Adults were maintained within the zebrafish facility at the University of Colorado Center for Comparative Medicine at 28.5°C with a light/dark cycle of 10/14 hours according to established procedures (Westerfield, 1995). The University of Colorado Committee on Use and Care of Animals approved all animal protocols. The transgenic lines *Tg(isl1:GFP)rw0* (Higashijima et al., 2000) and *Tg(vsx2:Kaede)nns2* (Kimura et al., 2006) were kindly provided by Shin-ichi Higashijima (NIPS, Okazaki, Japan); *Tg(gata2a:GFP)zf81* (Wen et al., 2008) was kindly provided by Shuo Lin (UCLA, Los Angeles, CA); *Tg(-8.1gata1:gata1-EGFP)zf100* (Batista et al., 2008) was kindly provided by Katherine Lewis (Syracuse University, Syracuse, NY); and *Tg(mnx1:GFP)ml2* (Flanagan-Steet et al., 2005) was obtained from ZIRC (<http://zebrafish.org>). Embryos and larvae were staged according to external morphological features (Kimmel et al., 1995).

Semi-quantitative RT-PCR

Total zebrafish RNA was isolated from whole embryos (1 cell, 256 cell, 50% epiboly, 90% epiboly, 19 hpf, 24 hpf, 36 hpf, and 60 hpf), larvae (72 hpf), and adult eye using the TRIzol® Reagent (Invitrogen, Grand Island, NY) method. RNA quality was determined by formaldehyde gel electrophoresis with ethidium bromide staining. Reverse transcription (RT) of total RNA was performed using a 1:1 mixture of oligo-dT and random hexamer primers and the SuperScript® III Reverse First-Strand Synthesis System (Invitrogen). Polymerase chain reaction (PCR) was performed using the Expand High Fidelity PCR System (Roche Applied Science, Indianapolis, IN) with a modified buffer (8.7 mM Tris-HCl, 35 mM MgCl₂, and 750 mM KCl) and cDNA comprising 10% of the reaction volume; primer concentrations were 0.3 and 0.04 µM for the *cx35b* and *actin, beta2 (actb2)* reactions, respectively.

The *cx35b* and *actb2* primers spanned genomic introns to distinguish between PCR products resulting from contaminating genomic DNA versus cDNA. The reference gene, *actb2*, was chosen based on its constant mRNA expression during zebrafish development (Casadei et al., 2011). The *cx35b*-specific forward and reverse primers were 5'-GAA TGG ACA ATT CTC GAG CGT CTC-3' (T_m 57.6°C) and 5'-GCA AAT GAA TCG GAA GTT CCA AAA C-3' (T_m 55.6°C), producing a 1072 bp product for cDNA and a 3021 bp product for genomic DNA amplification. The *actb2* primer sequences were 5'-ATG GGA CGG AAA GAC AGC TAC GTT-3' (T_m 60.3°C) and 5'-TCT CCT TCT GCA TCC TGT CAG CAA-3' (T_m 60.2°C), resulting in 811 and 1184 bp products for cDNA and genomic DNA

amplification, respectively. A touchdown PCR protocol was used for amplification of *cx35b* (Korbie and Mattick, 2008). The number of cycles for the PCR protocols for both *cx35b* and *actb2* were determined by performing standard curves to determine the exponential steps for each primer pair.

RT was performed without reverse transcriptase present followed by PCR to test for genomic contamination; additionally, each primer was used alone with cDNA samples to test for non-specific PCR product produced by single primer reactions. The *actb2* RT-PCR products and *cx35b* RT-PCR products from adult eye, 19 hpf embryos, and 72 hpf larvae samples were sequenced to confirm identity (CU Cancer Center DNA Sequencing and Analysis Service).

For gel electrophoresis, equal volumes of cDNA stage- or tissue-matched *cx35b* and *actb2* samples were mixed and analyzed on 1% agarose gels containing ethidium bromide. Gel images were digitally captured (Kodak Digital Science™ Image Station 440CF) and then analyzed with ImageJ (Rasband, ImageJ, <http://imagej.nih.gov/ij/>, 1997-2011; Abràmoff et al., 2004). The intensity of each *cx35b* band was normalized to that of its matched *actb2* band.

RT-PCR was performed independently three times and the mean and standard deviation (error bars) were calculated. Statistical analysis was done using ANOVA and post-hoc Tukey honestly significant difference test.

RNA in situ hybridization and immunohistochemistry

To produce the *cx35b* probe, a template sequence was amplified from cDNA using the primer pair 5'- CAC CAT GGG GGA ATG GAC AAT TC-3' (T_m 60.3°C) and 5'- TGC TCA TCC GCG GTA AGT CCT T-3' (T_m 60.2°C), resulting in a product that begins four nucleotides 5' to the *cx35b* start codon and ends within the second exon at nucleotide 868. This PCR product was cloned into the PCR-Script vector (Thermo). The *cx35b* mRNA sense and antisense probes were synthesized with digoxigenin labeled RNA (Roche) with T7 and T3 RNA polymerases (Promega) from the PCR-Script construct containing the *cx35b* template sequence.

We used the NCBI basic local alignment search tool (BLAST; Altschul et al., 1990), to predict the specificity of the probe for the *cx35b* mRNA sequence, for which the probe is 100% identical over the entire 868 nucleotide length. The next highest results were all for only partial coverage with identities ranging from 71-80% (NCBI Reference Sequence XM_002667310, NM_001128766 [*cx34.1*; Eastman et al., 2006], and XM_002666541 on chromosomes 17, 5, and 15, respectively). Evidence from short-length probes (50- and 70mers) supports the use of the highest acceptable sequence identity to off-target sequences as 85% (He et al., 2005). These results combined with the 70°C hybridization temperature (see below) support specificity of our probe for the *cx35b* mRNA sequence.

Fluorescent RNA *in situ* hybridization alone or in combination with fluorescent immunohistochemistry was performed as previously described (Novak and Ribera, 2003). Briefly, embryos were fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline

(PBS, pH 7.4) for 2 hours at 23°C or overnight at 4°C, dehydrated via standard methanol series, and stored in 100% methanol at -20°C for at least overnight. Just prior to hybridization, embryos were rehydrated, permeabilized using 10 µg/mL proteinase K (Invitrogen), blocked in hybridization buffer containing torula RNA and heparin (Hyb+) at 70°C, and hybridized in either sense or antisense probe diluted to 0.7 µg/mL in Hyb+ overnight at 70°C. Then, embryos were extensively rinsed with 50% formamide/2× saline sodium citrate with 0.1% Tween-20 (SSCT), 2×SSCT, 0.2×SSCT, and Maleic Acid Buffer (MAB, pH 7.5) and then blocked in a solution of MAB with 2% Boehringer Mannheim Blocking Reagent (BMBR) followed by an incubation in anti-digoxigenin-AP Fab fragments (Roche 11093274910) at 1:2000 in 2% BMBR in MAB overnight at 4°C. Embryos were then rinsed extensively in MAB and 100 mM Tris (pH 8.4), incubated in FastRed (Sigma), and fixed for 30 minutes in 4% PFA at 23°C.

When RNA *in situ* hybridization was performed in combination with fluorescent immunohistochemistry, the above protocol was followed and then embryos were blocked in 10% heat-inactivated goat serum (HIGS) in PBS with 0.2% Tween (PBST). Embryos were then incubated in either polyclonal rabbit anti-EGFP (Invitrogen A-6455) or rabbit anti-kaede (MBL International PM012) primary antibodies at 1:750 and 1:100, respectively (Table 1), followed by Alexa Fluor® 488 goat anti-rabbit (Invitrogen A-11034) secondary antibody at 1:1000 in 10% HIGS/PBST.

Antibody characterization

The primary antibodies used during this study are limited to anti-digoxigenin(DIG)-AP Fab fragments (Roche 11093274910), polyclonal rabbit anti-EGFP (Invitrogen A-6455), and rabbit anti-Kaede (MBL International PM012; Table 1). For the anti-DIG-AP Fab fragments, anti-DIG polyclonal antibodies are isolated from serum of sheep immunized against the entire digoxigenin molecule, processed for Fab fragments, and conjugated to alkaline phosphatase (AP; Roche 11093274910). Samples incubated in anti-DIG-AP in the absence of DIG-conjugated nucleotides did not show specific immunoreactivity following FastRed development (this study).

The rabbit anti-EGFP polyclonal antibody raised against the full-length amino acid sequence (Invitrogen A-6455) has been previously characterized, such that immunoreactivity is present in the brains of transgenic mice that express GFP as a transgene but not in the brains of wildtype mice that do not have GFP expression (Scott et al., 2009).

The anti-kaede (MBL International PM012) antibody was raised in rabbits exposed to the full-length kaede protein. This antibody's specificity was confirmed by a lack of immunoreactivity in the spinal cord of *Tg(vsx2: Kaede)nns2* embryos that were 19 hpf, when the *vsx2* promoter does not drive expression of kaede in the spinal cord, combined with positive immunoreactivity in the spinal of *Tg(vsx2: Kaede)nns2* larvae at 72 hpf, when the *vsx2* promoter does drive spinal cord expression of kaede (this study).

Generation of transient transgenics

The transgenic construct, *cx35b:mEGFP*, was assembled using the RP71-1C22 bacterial artificial chromosome (BAC; BACPAC Resources Center), which contains the full-length *cx35b* coding sequence and additional surrounding sequence. The Transgenic and Gene Targeting Core of the Rocky Mountain Neurological Disorders Center Core at University of Colorado at Anschutz Medical Center (P30 NS048154) performed recombineering to introduce mEGFP into the final BAC using the RP71-1C22 BAC and a construct containing a membrane-bound EGFP (mEGFP) coding region flanked by 5' *cx35b* sequence and *cx35b* exon 1 sequence. Transient transgenics were generated by injecting 1-4 cell embryos with 200 or 400 ng/uL *cx35b:mEGFP* DNA in injection buffer (1% FastGreen, 0.1 M KCl). For the data reported here, 50-100 embryos were injected in three different clutches of wild type embryos. Embryos were examined at 48 hpf for GFP expression, and the data reported here were obtained from eight embryos.

Confocal imaging

Fixed embryos were mounted in 0.5% low melting point (LMP) agarose (Gibco) in PBS in a modified squash-mount that utilizes electrical tape as a spacer between coverslips allowing for better anatomical integrity. Live embryos were mounted in a drop of 0.5% LMP agarose in embryo media containing 0.0002% tricaine (Sigma-Aldrich) in an uncoated Petri dish submersed in embryo media also containing 0.0002% tricaine.

All imaging was performed on a Zeiss LSM5 Pascal Confocal Upright Microscope using a 10× or 40× water-immersion lens. The pinhole was set to 1.00 Airy unit for the *cx35b* RNA *in situ* hybridization expression studies. For whole-mount embryos with *cx35b* RNA *in situ* hybridization alone, confocal z-stacks were acquired and the images were processed as z-stacks using ImageJ to display the full extent of the FastRed signal (Fig. 2). For transgenic embryos with *cx35b* RNA *in situ* hybridization and immunohistochemistry, confocal z-stacks were acquired in multi-track mode and the images were processed as single optical slices to determine overlap of the FastRed and Alexa Fluor® 488 signal (Figs. 3-6). Images were modified for brightness, contrast, and hue using ImageJ and figures were prepared using iWork Pages (Apple, Cupertino, CA).

Results

cx35b expression begins at the time of neural induction

Using RT-PCR, we tested for *cx35b* expression at various stages ranging between 1 cell to 72 hpf (Fig. 1). RNA extracted from fertilized eggs (1 cell) and blastula stage (256 cell) embryos did not produce detectable *cx35b* RT-PCR bands, suggesting *cx35b* mRNA is not maternally provided. We first detect *cx35b* expression at 50% epiboly (5.2 hpf), when gastrulation begins (Kimmel et al., 1995). In zebrafish, neural induction begins as early as the shield stage (6 hpf) followed by neural tube formation at the bud stage (10 hpf) during gastrulation (Woo and Fraser, 1995). We continue to detect *cx35b* mRNA by RT-PCR at all later stages examined. Therefore, *cx35b* is detectable by RT-PCR near the time of neural induction and continues to be expressed to early larval stages (72 hpf; Fig. 1).

Statistical analysis of the data indicates that the level of *cx35b* expression at both the 1 and 256 cell stages differ ($p < 0.05$) from the expression levels found at 90% epiboly, 19 hpf, 24 hpf, 60 hpf, and 72 hpf. Additionally, at 50% epiboly there is less *cx35b* expression than at 60 hpf and 72 hpf ($p < 0.05$).

In the spinal cord, *cx35b* expression predominates in ventral and intermediate regions and not the dorsal regions

As early as 17 hpf, fluorescent RNA *in situ* hybridization in whole-mount embryos detects *cx35b* mRNA expression in the spinal cord. At this stage, *cx35b* localizes to the ventral spinal cord where motor circuits are forming. Interestingly, spontaneous coiling begins around 17 hpf; additionally, we continue to see ventral expression of *cx35b* mRNA at 24 hpf, a developmental stage when embryos begin to respond to touch (for a review, see Brustein et al., 2003). *cx35b* expression is maintained in the ventral and intermediate spinal cord to 72 hpf, the latest stage investigated (Fig. 2).

In the *Tg(mnx1:GFP)* line, a subset of GFP-positive interneurons express *cx35b* between 19 to 72 hpf

To identify the spinal neurons expressing *cx35b*, we took advantage of several transgenic lines that express fluorescent reporter proteins in identified cell types. Given the expression of *cx35b* in the ventral spinal cord, we began these analyses with the *Tg(mnx1:GFP)ml2* line, characterized by expression of GFP in all motor neurons and a subset of unidentified interneurons (Flanagan-Steet et al., 2005). In 19 hpf *Tg(mnx1:GFP)ml2* embryos, GFP expression is limited to primary motor neurons (PMNs) and early born interneurons. However, at later stages, *Tg(mnx1:GFP)ml2* embryos and larvae also express GFP in a wider variety of interneuron subtypes and secondary motor neurons (SMNs). Fluorescent *cx35b* RNA *in situ* hybridization of *Tg(mnx1:GFP)ml2* embryos and larvae displays co-expression of GFP and *cx35b* for every stage investigated (Fig. 3).

PMNs do not express detectable *cx35b* between 19 to 72 hpf

To determine whether the *mnx1:GFP* neurons that express *cx35b* mRNA are PMNs, we used the *Tg(nrp1a:GFP)js12* line. *Tg(nrp1a:GFP)js12* embryos and larvae express GFP in the early born PMNs but not the later born SMNs (Sato-Maeda et al., 2008). In *Tg(nrp1a:GFP)js12* embryos and larvae ranging between 19 and 72 hpf, we do not detect *cx35b* signal in GFP-expressing PMNs, suggesting that PMNs do not express this connexin at detectable levels (Fig. 4). Given that *Tg(mnx1:GFP)ml2* embryos express GFP in PMNs as well as SMNs and a subset of unidentified interneurons, we next examined these neuronal subtypes.

Between 24 to 72 hpf, SMNs that project dorsally or ventrally express *cx35b*

The *Tg(-7.3gata2a:GFP)zf35* and *Tg(isl1:GFP)rw0* lines express GFP in ventrally projecting secondary motor neurons (vSMNs) and dorsally projecting secondary motor neurons (dSMNs), respectively (Meng et al., 1997; Uemura et al., 2005). *Tg(-7.3gata2a:GFP)zf35* and *Tg(isl1:GFP)rw0* embryos and larvae display co-expression of

GFP and *cx35b* mRNA in vSMNs (Fig. 5A-I) and dSMNs (Fig. 5J-R), respectively, at the three stages investigated.

Comparison of these data to those of Fig. 3 leads to additional insights. Although SMNs are born as early as 16 hpf, they do not express GFP in 19 hpf *Tg(mnx1:GFP)ml2* embryos. Since PMNs do not express detectable levels of *cx35b*, the most likely identity for the neurons positive for *cx35b* expression at 19 hpf are GFP-expressing early born interneurons. At subsequent stages in the *Tg(mnx1:GFP)ml2* line, GFP-positive neurons include SMNs.

Ventral longitudinal descending (VeLD) inhibitory interneurons do not express detectable levels of *cx35b* during the stages investigated

Early born spinal neurons that are located ventrally but slightly dorsal to motor neurons with initial growth cone extension around 17 hpf are known as VeLDs (Bernhardt et al., 1990; Kuwada et al., 1990). VeLDs function as inhibitory GABAergic interneurons with ipsilaterally descending axons (Hale et al., 2001). Since GFP expression of *Tg(mnx1:GFP)ml2* embryos is limited to PMNs and early born interneurons at 19 hpf and PMNs were already ruled out as the *cx35b* expressing cells (Fig. 4), VeLD interneurons were prime candidates for the *cx35b* expressing interneurons based on their ventral location and large soma size.

In both the *Tg(mnx1:GFP)ml2* line (Seredick et al., 2012) and the *Tg(-8.1gata1:gata1-EGFP)zf100* line (Batista et al., 2008), VeLDs express GFP. Our analysis of *cx35b* expression in the *Tg(-8.1gata1:gata1-EGFP)zf100* line began at 24 hpf, when we can first detect spinal cord expression of GFP. However, we do not detect *cx35b* in any GFP-positive cells in 24 hpf *Tg(-8.1gata1:gata1-EGFP)zf100* embryos, suggesting that the interneurons expressing detectable levels of *cx35b* are not VeLDs (Fig. 6A-C). Additionally, *cx35b* is not expressed at a detectable level in GFP-positive cells of 48 hpf *Tg(-8.1gata1:gata1-EGFP)zf100* embryos (Fig. 6D-F).

Circumferential descending (CiD) excitatory premotor interneurons express *cx35b* beginning at 24 hpf

CiDs are present both in the embryonic and the larval spinal cord (Bernhardt et al., 1990; Hale et al., 2001). CiD neurons are excitatory glutamatergic premotor interneurons with ipsilateral descending axons and somas located in the ventral and intermediate spinal cord slightly dorsal to VeLD interneurons (Kimura et al., 2006). CiDs do not express GFP in the *Tg(mnx1:GFP)ml2* line, thus placing CiDs as a possible candidate for the GFP-negative, *cx35b* positive cells present in 24 hpf *Tg(mnx1:GFP)ml2* embryos (Fig. 3D-F).

A small number of spinal cord cells, including CiDs, express *visual system homeobox 2* (*vsx2*), as early as 20 hpf (Kimura et al., 2006). In the *Tg(vsx2:Kaede)nns2* line, CiDs express Kaede (Kimura et al., 2006). In 24 and 48 hpf *Tg(vsx2:Kaede)nns2* embryos, Kaede and *cx35b* show co-expression (Fig. 6G-L). Combined with the data of Fig. 5, the results indicate that a subset of SMNs and a subset of premotor CiDs express *cx35b*, suggesting that the circuit formed between these neuronal types might involve Cx35b-containing electrical synapses.

Transient *cx35b:mEGFP* transgenics support expression of *cx35b* by SMNs, CiDs, and additional interneuron subtypes

We analyzed the pattern of transient mEGFP expression in eight 48 hpf embryos, resulting from injection of the *cx35b:mEGFP* construct into three different clutches. In the transient transgenic embryos, we detected mEGFP expression in eight cells identified as motor neurons on the basis of their peripherally projecting axons (Fig. 7A-C). Additionally, their relatively small somas indicate a SMN rather than a PMN identity. Consistent with mEGFP expression in cells with peripheral axons that project ventrally (Fig. 7A), we detected *cx35b* mRNA in GFP-positive SMNs with ventrally projecting axons in the *Tg(-7.3gata2a:GFP)zf35* line.

The lateral axon projection of another SMN (Fig. 7B) is similar to the morphology of a dorsal rostral primary motor neuron (dRoP) that has been described recently by Menelaou and McLean (2012). However, our analyses of *Tg(nrp1a:GFP)js12* embryos did not reveal *cx35b* expression in PMNs (Fig. 4). In this regard, we note that Menelaou and McLean (2012) proposed that dRoPs may be earlier born SMNs rather than PMNs. The third type of motor neuron showing mEGFP expression in the transient *cx35b:mEGFP* transgenics has peripheral axons that project dorsally as well as ventrally (Fig. 7C), which are defining characteristics of a newly described SMN subtype (dvSMNs; Menelaou and Mclean, 2012).

We also detect mEGFP expression in four cells that have a CiD-like morphology (Fig. 7D), which is consistent with the expression analyses of *cx35b* in the *Tg(vsx2:Kaede)nns2* line (Fig. 6). Less frequently, we observe mEGFP expression in other interneuron subtypes (Fig. 7E), including two cells with a commissural local (CoLo) morphology, two cells with a commissural longitudinal ascending (CoLA) morphology, and one cell with a unipolar commissural descending (UCoD) morphology. However, our direct immunohistochemical analyses of *cx35b* expression do not identify these interneurons as cells that express this gene. Given the reliability of expression patterns in transient transgenics, our detection in CoLos, CoLAs, and UCoDs points to interneurons that merit further study for direct analysis of *cx35b* expression.

Discussion

Our data indicate *cx35b* begins to be expressed around the time of neural induction and localizes to the ventral spinal cord during embryonic and larval stages. Expression of *cx35b* is not detected in PMNs or VeLD interneurons, but is detected in a subset of SMNs, a subset of CiDs, and other unidentified interneurons (summarized in Fig. 8). These data suggest *cx35b* may be a connexin contributing to electrophysiologically evident electrical synapses in the developing zebrafish spinal cord. Additionally, given our lack of detection in PMNs and VeLDs, the data raise questions regarding the identity of connexin(s) involved in electrophysiologically evident electrical synapses formed by these neurons.

Direct electrophysiological analyses of zebrafish spinal neurons have demonstrated electrical coupling between PMNs, VeLDs and PMNs, CiDs and PMNs, and CiDs and SMNs (Saint-Amant and Drapeau, 2000, 2001; Kimura et al., 2006). However, the molecular identities of the connexin proteins that participate in these electrical synapses

have yet to be identified. Although pharmacological agents combined with electrophysiological recording has proven useful for molecular identification of subunits comprising many ion channel types, this is not the situation for gap junctions. For example, heptanol is commonly used to inhibit gap junctions, but heptanol does not distinguish between gap junctions on the basis of their connexin composition. In addition, heptanol affects other ion channels, thus imposing significant caveats for data interpretation. Using general gap junction blockade is particularly problematic when trying to determine the role of electrical synapses in the developing nervous system as there are multiple connexin proteins expressed by neurons as well as glial cells.

Our lack of detection of *cx35b* in PMNs and VeLDs appears at odds with the electrophysiological data of Saint-Amant and Drapeau (2000, 2001). However, there are several explanations that reconcile this apparent discrepancy. First, *cx35b* mRNA may be present in PMNs and VeLDs at levels below the sensitivity of our methods. Second, due to a genome-wide duplication event during the teleost divergence, many zebrafish genes appear in duplicate when compared to mammalian genomes (Amores et al., 1998; Ekker et al., 1995; Holland and Williams, 1990; Stock et al., 1996; Vandepoele et al., 2004). *cx35b* has the highest similarity to mammalian Cx36 at the level of the primary sequence (Jabeen and Thirumalai, 2013), but the zebrafish genome contains at least three additional Cx36 orthologues. These additional orthologues may participate in the gap junctions that have been detected electrophysiologically between PMNs and between PMNs and VeLDs. Third, in addition to Cx36 homologues, neurons express other connexins that may underlie the electrical synapses detected electrophysiologically.

Our detection of *cx35b* in SMNs and CiDs is consistent with the prior demonstration of electrical coupling between these neurons (Kimura et al., 2006). However, the electrophysiological analyses did not provide information about the molecular identities of the connexin proteins that underlie the electrical coupling. Our data place *cx35b* as a prime candidate for the relevant connexin.

Our results complement immunohistochemical studies demonstrating Cx35 immunoreactivity in the brain and spinal cord of embryonic and larval zebrafish (Satou et al., 2009; Jabeen and Thirumalai, 2013). For these studies, Satou et al. (2009) and Jabeen and Thirumalai (2013) used a monoclonal antibody directed against perch Cx35 that recognizes Cx36 orthologues in several species. Cx35 immunoreactivity was detected, albeit weakly, throughout the brain as early as 1 day post fertilization (dpf); Cx35 immunoreactivity increased until around 4 dpf and persisted to the oldest stage examined at 15 dpf (Jabeen and Thirumalai, 2013). Interestingly, the immunohistochemical analyses revealed a broad expression pattern for Cx35-immunoreactive puncta in contrast to the restricted expression pattern for *cx35b* mRNA we find in the spinal cord. The goal of our study was to identify neurons that express *cx35b*, therefore our focus was on the soma where detection of mRNA and soluble fluorescent reporter proteins is most reliable. In contrast, Jabeen and Thirumalai (2013) analyzed where the immunoreactivity was present within the central nervous system without identifying the immunoreactive cells. The broad distribution pattern detected using the Cx35 antibody could reflect expression of immunoreactive protein in many neurons or in a subset of neurons that have elaborate processes that extend

throughout the examined region. Further work is required to identify neurons that contain Cx35 immunoreactivity.

Satou et al. (2009) examined Cx35 immunoreactivity in the larval spinal cord. Widely distributed immunoreactive puncta were found throughout the spinal cord, but the most intensely labeled sites corresponded to appositions of the Mauthner axons to CoLo inhibitory interneurons (Satou et al., 2009). Although we used several different transgenic lines to identify neuron subtypes, none of them specifically identified CoLos. However, in the transient transgenic embryos, neurons with a CoLo-like morphology were observed to express mEGFP (Fig. 7). These considerations combined with our detection of *cx35b* mRNA in spinal neurons we did not identify (e.g., Fig. 5) nominate CoLos as candidates for at least some of the unidentified neurons that express *cx35b* in our study.

***cx35b* expression begins to increase coincidentally with neural induction**

We find that *cx35b* is expressed as zebrafish neural induction begins at 50% epiboly (Fig. 1). Similarly, in the embryonic day 7.5 mouse, Cx36 mRNA is expressed in the forebrain as neurogenesis begins (Gulisano et al., 2000). On the basis of the developmentally dynamic Cx36 expression pattern, Gulisano et al. (2000) suggested Cx36 might initially mediate aspects of cortical cell neurogenesis during embryogenesis and later play a different role in the cerebral cortex when the postnatal mouse begins to process outside stimuli. The results of *in vitro* experiments support a role in neurogenesis such that overexpression and knockdown of Cx36 leads to an increase and decrease in neuronal differentiation, respectively, in embryonic rat hippocampus neurosphere cultures (Hartfield et al., 2011). Since zebrafish *cx35b* is expressed during neurogenesis and before post-mitotic neurons are present, our data similarly suggest an early role for Cx35b connexins in differentiation of neurons.

In the spinal cord, *cx35b* expression predominates ventrally and centrally but not dorsally

At stages subsequent to neurogenesis, *cx35b* expression localizes to the ventral and intermediate spinal cord (Fig. 2). The organization of the spinal cord is well conserved across vertebrates such that neuronal subtypes arise from specific domains of the spinal cord established by morphogen gradients and solidified by the resulting differential transcription factor expression (Diez del Corral and Storey, 2001; Melton et al., 2004; Goulding and Pfaff, 2005; Lewis, 2006). Grossly, this results in a dorso-ventral spinal cord organization in the adult with motor information processed ventrally and sensory information processed dorsally (Melton et al., 2004). Therefore, the predominantly ventral and intermediate expression pattern of *cx35b* raises the possibility that Cx35b electrical synapses play a role in motor output circuits rather than sensory circuits of the spinal cord.

Expression of *cx35b* in SMNs and CiDs suggest a role in motor circuits

Our data reveal expression of *cx35b* in SMNs, a motor neuron population that has received less attention (but see, McLean et al., 2007; Menelaou and McLean, 2012) even though they are more homologous to mammalian motor neurons (Beattie et al., 1997). SMNs with more ventrally located somas are active at low frequency swimming and those with more dorsally located somas are active during faster swimming episodes (McLean et al., 2007). Similarly,

the *cx35b* expression pattern that we found within the SMN population was not uniform such that not every cell within a SMN class had detectable levels of *cx35b* mRNA. This differential expression pattern raises the possibility that Cx35b-mediated electrical synapses are important for only specific components of the zebrafish swimming repertoire. However, whether this non-uniform detection of *cx35b* mRNA reflects technical issues or true biology is unknown.

CiDs express *alx*, the zebrafish orthologue of *Chx10* (Kimura et al., 2006). On this basis, CiDs may be the functional orthologue of mammalian V2a premotor interneurons (Kimura et al., 2006; Batista et al., 2008). Similar to CiDs, V2a cells are glutamatergic and ipsilaterally projecting interneurons (Lundfald et al., 2007). Our expression studies provide evidence for *cx35b* expression by CiDs. However, similar to SMNs, we do not detect *cx35b* in every CiD that expresses Kaede in the *Tg(vsx2:Kaede)nns2* line. As for SMNs, whether this reflects technical limitations versus biology has yet to be determined. However, it is interesting that CiDs appear to be a heterogeneous population on the basis of both morphological and electrophysiological criteria (Hale et al., 2001; McLean and Fetcho, 2009; Kimura et al., 2006). Overall, there is evidence for at least three different CiD subtypes (McLean and Fetcho, 2009). On this basis, it is possible that the subset of CiDs that express *cx35b* may represent a functional subtype. Therefore, determining whether the non-uniform detection of *cx35b* mRNA is biological will provide insight into whether *cx35b* plays a role in CiD subtype functional differences.

Moving forward to understand the role of electrical synapses

Zebrafish are an ideal model organism for studying the role of electrical synapses both in development and in behaving animals. Here, we provide the initial expression studies needed to investigate the role of Cx35b-containing electrical synapses in embryonic and larval stage zebrafish. These studies raise the possibility that Cx35b participates in the motor network. Additionally, these initial studies suggest that other connexins are involved in the electrical synapses detected electrophysiologically in the developing zebrafish spinal cord, which is another possibility warranting further investigation. The zebrafish toolbox that has emerged recently allows for strategic development of genetic mutants and transgenic lines. These tools will allow us to move beyond general pharmacological gap junction blockade and towards selective gene targeting of a specific connexin gene to gain a better understanding of the role electrical synapses play in the development and function of the nervous system.

Acknowledgments

We thank Drs. Shin-ichi Higashijima, Ph.D., Shuo Lin, Ph.D., Katherine Lewis, Ph.D., and the Zebrafish International Resource Center (ZIRC) for providing transgenic lines, Wallace Chick of the Transgenic and Gene Targeting Core of the Rocky Mountain Neurological Disorders Center Core (NS048154) provided expert assistance and generated the *cx35b:mEGFP* transgenic construct (Grant NIH/NS048154), and members of the Ribera group whom provided valuable comments on the progress of the work as well as the manuscript.

Grant Information: Tara C. Carlisle: 1F31NS076010, TL1RR025778, (Ronald J. Sokol), Angeles B. Ribera: 5R01NS38937, 5P30NS048154.

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Figure 1. *cx35b* mRNA begins to be expressed around the time of neural induction before post-mitotic neurons are present

A representative ethidium bromide stained agarose gel displaying RT-PCR results for *connexin 35b* (*cx35b*) and the reference gene, *actin, beta2* (*actb2*). The bar graph reports the normalized averages of the ratio of *cx35b* to *actb2* RT-PCR product gel intensities for three parallel experiments; the error bars display the standard deviations. The labels for the bar graph also refer to the representative gel. The inset includes both the positive and negative RT-PCR controls performed using RNA isolated from the adult retina. Abbreviations: hpf = hours post-fertilization, -RT = without reverse transcription enzyme, +RT = with reverse transcription enzyme.

Figure 2. *cx35b* expression predominates in the ventral and intermediate regions but not the dorsal region of the developing spinal cord

Fluorescent RNA *in situ* hybridization of *cx35b* mRNA (magenta) in whole-mount zebrafish embryos and larvae (dorsal, up; rostral, left). All images are z-stack projections taken at 5 or 10 μm (10 $\times$ objective) or 3 μm (40 $\times$ objective) slice intervals. Solid white lines outline approximate dorsal aspect of spinal cord and dotted white lines outline the approximate ventral aspect of the spinal cord. Grey boxed areas were imaged at higher magnification and displayed in grey inset. **A.** 17 hpf embryo. **B.** 19 hpf embryo (scale bar in B also for A = 200 μm ; in B inset also for A inset = 30 μm). **C.** 24 hpf embryo. **D.** 36 hpf embryo. **E.** 48 hpf embryo. **F.** 60 hpf embryo. **G.** 72 hpf larva (scale bar in G also for C-F = 200 μm ; in G inset also for C-F insets = 50 μm).

Figure 3. Ventral interneurons express *cx35b* beginning at 19 hpf and continuing to 72 hpf
 Fluorescent RNA *in situ* hybridization of *cx35b* mRNA (magenta) and GFP (green) immunohistochemistry of whole-mount *Tg(mnx1:GFP)ml2* embryos and larva (dorsal, up; rostral, left). All images are single optical slices taken from 3 μ m z-stacks. In the merge panels (C, F, I, and L), yellow arrowheads indicate cells with both *cx35b* signal and GFP immunoreactivity and white asterisks indicate cells with detectable *cx35b* signal that do not have detectable GFP immunoreactivity. **A-C.** 19 hpf embryo. **D-F.** 24 hpf embryo. **G-I.** 48 hpf embryo. **J-L.** 72 hpf larva (scale bar in L for all panels = 15 μ m).

Figure 4. Primary motor neurons do not express detectable levels of *cx35b*

Fluorescent RNA *in situ* hybridization of *cx35b* mRNA (magenta) and GFP (green) immunohistochemistry of whole-mount *Tg(nrp1a:GFP)js8* embryos and larvae (dorsal, up; rostral, left). All images are single optical slices taken from 3 μ m z-stacks. In the merge panels (C, F, I, and L), white asterisks indicate cells with detectable *cx35b* signal that do not have detectable GFP immunoreactivity. **A-C.** 19 hpf embryo. **D-F.** 24 hpf embryo. **G-I.** 48 hpf embryo. **J-L.** 72 hpf larva (scale bar in L for all panels = 15 μ m). Abbreviations: PMN = primary motor neuron.

Figure 5. Secondary motor neurons express *cx35b* as early as 24 hpf and continue expression to 72 hpf

Fluorescent RNA *in situ* hybridization of *cx35b* mRNA (magenta) and GFP (green) immunohistochemistry of whole-mount *Tg(-7.3gata2:GFP)zf35* (A-I panels) or *Tg(isll:GFP)rw0* (J-R panels) embryos and larvae (dorsal, up; rostral, left). All images are single optical slices taken from 3 μ m z-stacks. In the merge panels (C, F, I, L, O, and R), yellow arrowheads indicate cells with both *cx35b* signal and GFP immunoreactivity and white asterisks indicate cells with detectable *cx35b* signal that do not have detectable GFP immunoreactivity. **A-C.** 24 hpf *Tg(-7.3gata2:GFP)zf35* embryo. **D-F.** 48 hpf *Tg(-7.3gata2:GFP)zf35* embryo. **G-I.** 72 hpf *Tg(-7.3gata2:GFP)zf35* larva. **J-L.** 24 hpf *Tg(isll:GFP)rw0* embryo. **M-O.** 48 hpf *Tg(isll:GFP)rw0* embryo. **P-R.** 72 hpf *Tg(isll:GFP)rw0* larva (scale bar in R for all panels = 15 μ m). Abbreviations: SMN =

secondary motor neuron, vSMN = ventrally projecting SMN, dSMN = dorsally projecting SMN.

Figure 6. Ventral lateral descending interneurons do not express detectable levels of *cx35b* whereas circumferential descending interneurons do express *cx35b*
 Fluorescent RNA *in situ* hybridization of *cx35b* mRNA (magenta) and GFP or Kaede (green) immunohistochemistry of whole-mount *Tg(-8.1gata1:gata1-EGFP)zf100* (A-F panels) or *Tg(vsx2:Kaede)nns2* (G-L panels) embryos (dorsal, up; rostral, left). All images are single optical slices taken from 3 μ m z-stacks. In the merge panels (C, F, I, and L), yellow arrowheads indicate cells with both *cx35b* signal and Kaede immunoreactivity and white asterisks indicate cells with detectable *cx35b* signal that do not have detectable GFP or Kaede immunoreactivity. **A-C.** 24 hpf *Tg(-8.1gata1:gata1-EGFP)zf100* embryo. **D-F.** 48 hpf embryo *Tg(-8.1gata1:gata1-EGFP)zf100*. **G-I.** 24 hpf *Tg(vsx2:Kaede)nns2* embryo. **J-L.** 48 hpf *Tg(vsx2:Kaede)nns2* embryo (scale bar in L for all panels = 15 μ m).
 Abbreviations: CiD = circumferential descending, VeLD = ventral lateral descending.

Figure 7. *cx35b:EGFP-CAAX* transient transgenic has mEGFP expression in motor neurons and two interneuron subtypes

Confocal z-stack projections taken at 2-3 μm slice intervals of *cx35b:EGFP-CAAX* injected embryos and larvae imaged live (dorsal, up; rostral, left), ranging from 48 to 72 hpf. Dotted lines are an approximate of the notochord boundaries. Based on morphology, mEGFP is expressed in **A.** ventrally projecting secondary motor neuron (vSMN), **B.** dorsal-ventral secondary motor neuron (dvSMN), **C.** motor neuron with a dorsal rostral primary (dRoP) morphology, and **D.** circumferential descending (CiD) excitatory premotor interneuron (scale bar in D for all panels = 50 μm). **E.** The graph summarizes the number of times various cell types were observed to be positive for mEGFP expression in a total of three independent experiments. mEGFP-positive spinal cord cells are classified as motor neuron (MN), CiD, commissural local (CoLo) interneuron, commissural longitudinal ascending (CoLA) interneuron, and unipolar commissural descending (UCoD) interneuron based on axonal morphology. mEGFP-positive somas within the spinal cord lacking visible axons for proper cell type identification are tallied in the “No ID” category.

Figure 8. Summary of *cx35b* spinal cord expression

This cartoon of the zebrafish spinal cord (dorsal, up; rostral, left) summarizes our results. Neurons with green somas represent those for which our expression studies do support *cx35b* expression: dSMNs, vSMNs, and CiD interneurons. The *cx35:EGFP-CAAX* transient transgenics may also support expression of *cx35b* by dvSMNs, CoLA, CoLo, and UCoD interneurons, which are labeled with a light green soma. Neurons with red somas represent those for which our expression studies do not support *cx35b* expression: Kolmer Agduhr (KA) interneurons, VeLD interneurons, or the PMNs, rostral primary (RoP), middle primary (MiP), and caudal primary (CaP). As our studies have not identified all interneurons that may express *cx35b* (i.e., *Tg(mnx1:GFP)ml2* GFP-positive interneurons), we have also included all other interneurons labeled with grey somas for which we do not have direct evidence to conclude whether these cells express *cx35b* mRNA: commissural bifurcating longitudinal (CoBL), dorsal longitudinal ascending (DoLA), ventral medial (VeMe), commissural secondary ascending (CoSA), circumferential ascending (CiA), and commissural primary ascending (CoPA). Adapted with permission from Goulding, 2009.

Table 1

Antibodies.

Antibody	Immunogen	Species	Source/Catalog Number
Digoxigenin-AP	The entire digoxigenin molecule originally isolated from the flowering plant <i>Digitalis purpurea</i>	Sheep	Roche 11093274910 Polyclonal Fab fragments
EGFP	The full-length amino acid sequence (246 aa) of GFP from the jellyfish <i>Aequoria victoria</i>	Rabbit	Invitrogen A-6455 Polyclonal
Kaede	The full-length amino acid sequence (225 aa) of kaede protein from stony coral <i>Trachyphyllia geoffroyi</i>	Rabbit	MBL International PM012 Polyclonal