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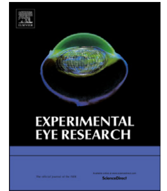

Identification of amacrine subtypes that express the atypical cadherin *celsr3*

Alaron A. Lewis, Joseph T. Mahoney, Neil Wilson, Susan E. Brockerhoff*

Department of Biochemistry, University of Washington, Seattle, WA 98195, USA

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ABSTRACT

We previously identified Celsr3, an atypical cadherin, as essential for normal inhibitory circuit formation in the inner retina. Its absence during retinal development leads to increases in GABA receptor numbers on ON-bipolar cell terminals and consequent changes in retinal physiology, but does not cause obvious cell spacing or synaptic lamination defects. This study focuses on defining the subset of amacrine cells that express *celsr3* during development of the wild type zebrafish retina. We have developed a BAC transgene expressing EGFP under the control of *celsr3* promoter, *Tg*(*celsr3:EGFP*). Similar to the retinal expression of the endogenous gene, the transgene is expressed in amacrine, ganglion and bipolar, but not horizontal or photoreceptor cells. We transiently expressed the BAC in zebrafish larvae and categorized 104 *celsr3* expressing amacrine cells based on their shape, arborization and lamination. Ten different amacrine cell types express *Tg*(*celsr3:EGFP*). These include narrow, medium and wide-field types of varicose cells. There are many multistratified cells, including one not previously identified and a few specific types of monostratified amacrine cells. Non-varicose amacrine cells do not express the transgene. We propose that *celsr3* expression in varicose amacrine cells is key to this molecule's function in circuitry formation during retinal development. The BAC transgene we have developed provides a useful tool to study Celsr3 function.

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1. Introduction

The development of neural networks is a complex and delicate process much of which is still not well understood. In particular, the molecular basis for proper circuit development is largely unknown (Zipursky and Sanes, 2010; Garrett and Burgess, 2011). Proper formation of a neuronal field like the eye requires accurate cell migration, dendritic and axonal targeting, synapse formation and refining, and maintenance of the system. Various cadherin family members are thought to play roles in all of these processes.

In the inner retina, amacrine cells, the main class of inhibitory interneurons, form complex connections with bipolar and ganglion cells (Masland, 2012). Anatomical studies have identified many amacrine subtypes and physiological studies have revealed some of the mechanisms of inhibitory modulation. Each of the subtypes is thought to be responsible for a particular aspect of normal vision and to have a non-random arrangement within the retina. Little is known about the molecular identity of the different amacrine subtypes.

Celsr3 is an atypical 7-pass cadherin receptor. The ectodomain is comprised of multiple cadherin domains, EGF repeats and also laminin A G-type repeats. A seven transmembrane domain connects this with a G-protein binding intracellular signaling domain. Celsr3 is present in both the developing and adult mouse brain (Ying et al., 2009). It has been functionally implicated in proper cell migration and axon targeting in several areas of the nervous system (Tissir et al., 2005; Zhou et al., 2008; Ying et al., 2009; Fenstermaker et al., 2010; Chai et al., 2014). *In vitro* work suggests that Celsr3 is also involved in dendritic targeting (Shima et al., 2007).

We have previously described a zebrafish mutant ($celsr3^{w65}$) with a defect in signal processing within the inner retina due to a premature stop codon within the first exon of the *celsr3* gene (Lewis et al., 2011). *In situ* experiments showed that *celsr3* is broadly expressed in many amacrine and ganglion cells as well as some bipolar cells in the retina. *Celsr3* mutants develop a super-normal b-wave in the electroretinogram due to an increase in GABA receptor number on the ON-bipolar cells. This specific alteration of GABAergic signaling suggests changes to subsets of the amacrine

* Corresponding author.
E-mail address: sbrocker@uw.edu (S.E. Brockerhoff).

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network in the *celsr3* mutant animals. In order to understand the role of *celsr3* in circuitry formation, it is necessary to understand which of the many amacrine cell subtypes express *celsr3* during the relevant developmental period, 3–5 days post fertilization (dpf).

To define the specific subtypes of amacrine cells that express *celsr3*, we developed a Bacterial Artificial Chromosome (BAC) transgene, *Tg*(*celsr3:EGFP*). Using a transient injection strategy, we identified isolated cells that express *celsr3* at 3 through 5 dpf and characterized them based on shape, arborization and lamination. *Celsr3* is expressed in narrow, medium and large amacrine cells, but only in the varicose varieties of these cells. We identified many of the broadly stratified cell classes, but only two monostratified narrow cell types. These data show that *celsr3* expression molecularly defines a wide-ranging but distinct subset of varicose amacrine cells, and suggest that changes in the connectivity of these cells account for the circuitry defects in the *celsr3* mutant retinas.

2. Material and methods

2.1. Zebrafish maintenance

Adult fish and larvae were maintained at 28.5 °C in reverse-osmosis distilled water reconstituted for fish compatibility by addition of salts and vitamins (Westerfield, 1995) on a 10/14 h dark/light cycle. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the IACUC of the University of Washington. Wild type animals used here were all $roy^{-/-}$.

2.2. BAC development and preparation

We used standard BAC recombineering strategies to place EGFP downstream of the *celsr3* promoter (Dhaka et al., 2007; Chatterjee and Lufkin, 2011). We used BAC CH73-42M24 obtained from Children's Hospital Oakland Research Institute (http://bacpac.chori.org/zebrafish71.htm), which is labeled as zH42M24T7 on the Sanger Welcome Trust web site. Briefly, PCR was performed to create a fragment containing EGFP and the kanamycin resistance gene flanked by LoxP sites. Engineered on either side of this construct were domains containing homology to *celsr3* exon1 in the BAC. This was done using primers forward (GGATTTCACTAGACTAATGGTGAGCAAGGGCGAGGAG) and reverse (CTTGCTCACCATTAGTCTAGTGAAATCCTTTCTCTCTC). Bacteria containing the BAC were electroporated with the fragment and then recombination was induced and selected for by kanamycin resistance. Colonies that were kanamycin resistant were grown and prepped using an Invitrogen Midi prep kit according to manufacturer instructions, except that DNA was pipetted a minimum number of times to avoid shearing. Colonies were checked by PCR and sequencing. After EGFP insertion was confirmed, the DNA was treated with Cre recombinase to remove the kanamycin resistance gene. The final DNA product was then re-electroporated into Bacteria.

2.3. BAC injections

Injections of the BAC into zebrafish eggs were done using a fresh DNA solution (no more than 3 weeks old) at 30 μM/ml. We injected approximately 1000 eggs to obtain approximately 30–40 fish with an average of 3 completely isolated amacrine cells/fish. Only isolated cells were analyzed by microscopy.

2.4. Cryosections and confocal microscopy

For live imaging, larvae were treated with 0.003% 1-phenyl-2-thiourea (PTU) in EM at ~24 h post fertilization to prevent melanization (Westerfield, 1995). At the desired age, larvae were anaesthetized in Tricaine (Sigma) and mounted in warm 0.5–1% low mount agarose. Embedded larvae were covered in EM containing PTU and Tricaine and imaged on an Olympus FV1000 using a 40× or 60× water immersion objective. To obtain cryosections, fish were grown to the indicated age in days, euthanized by immersion in ice, and then fixed in 4% paraformaldehyde (1 × PBS, 3% sucrose) for 2 h at room temperature (rt) or overnight (o/n) at 4 °C. Fixed fish were washed 1× in PBS and then immersed in 30% sucrose (1 × PBS) o/n at 4 °C. Fish were incubated in 50% OCT, 15% sucrose for 30 min at rt then frozen in 100% OCT on dry ice. 40 μM slices were cut using a Leica cm1850 cryostat. Slides were mounted in Vectashield (Vector Laboratories), and imaged using a 60× objective (N.A. 1.35) and an Olympus FV1000 scanning confocal microscope. Z-stacks were taken with a step size of 0.5 μM 3D images were prepared using the Image J software. Isolated individual cells were located in each image, and then rendered in 3 dimensions. Length measurements were done using the image J line tool at two different angles across the arbor of the cell.

2.5. Determining IPL boundaries

In live images, other nearby cells and the position of cell somas were used to visualize the boundaries of the IPL. Monostratified cells that laminated in the outer layers of the IPL generally had adjacently positioned somas. In slides, the edges of the IPL were also seen by auto-fluorescence. Sublaminae were assigned by visually dividing the IPL into sections.

3. Results

3.1. Developmental expression of Tg(celsr3:EGFP)

We constructed a BAC expression system to result in EGFP expression under the *celsr3* promoter. Our initial attempts to isolate *celsr3* promoter fragments suggested that proper expression required regulatory sequences located outside of the immediate region upstream of the gene. In order to drive fluorescent gene expression in cells that normally express *celsr3* we used a BAC that contains 15.5 kb of sequence upstream of the start of *celsr3* as well as the entire gene, all 39 exons and introns (Fig. 1A). We reasoned that this BAC likely contained the promoter as well as other important regulatory sequences possibly within introns that are required for proper *celsr3* expression.

Using recombination in bacteria mediated by homologous flanking sequences (Dhaka et al., 2007; Chatterjee and Lufkin, 2011), we inserted a PCR product containing the EGFP gene with its stop codon and a kanamycin resistance gene flanked by loxP sites at the start site of the *celsr3* gene. After successful insertion, the kanamycin resistance gene was removed through Cre recombination. The final BAC construct is diagrammed in Fig. 1A.

We injected this construct into wild type zebrafish embryos at the single cell stage. For optimal results, we found that the transgenic BAC construct needed to be freshly prepared and then used within a very narrow concentration range. Using these optimized injection conditions, we obtained up to 30% of the expected number of EGFP positive cells, as judged from our previous *in situ* experiments (Lewis et al., 2011). By labeling cells in a mosaic fashion, we were able to identify individual cells that expressed the transgene. The injected fish showed no morphological defects even at

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