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CRANIAL SENSORY GANGLIA NEURONS REQUIRE INTRINSIC N-CADHERIN FUNCTION FOR GUIDANCE OF AFFERENT FIBERS TO THEIR FINAL TARGETS

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Abstract—Cell adhesion molecules, such as N-cadherin (*cdh2*), are essential for normal neuronal development, and as such have been implicated in an array of processes including neuronal differentiation and migration, and axon growth and fasciculation. *cdh2* is expressed in neurons of the peripheral nervous system during development, but its role in these cells during this time is poorly understood. Using the transgenic zebrafish line, tg(p2xr3.2:eGFP^{sl1}), we have examined the involvement of *cdh2* in the formation of sensory circuits by the peripheral nervous system. The tg(p2xr3.2:eGFP^{sl1}) fish allows visualization of neurons comprising the trigeminal, facial, glossopharyngeal and vagal ganglia and their axons throughout development. Reduction of *cdh2* in this line was achieved by either crosses to the *cdh2*-mutant strain, *glass onion* (*glo*) or injection of a *cdh2* morpholino (MO) into single-cell embryos. Here we show that *cdh2* function is required to alter the directional vectors of growing axons upon reaching intermediate targets. The central axons enter the hindbrain appropriately but fail to turn caudally towards their final targets. Similarly, the peripheral axons extend ventrally, but fail to turn and project along a rostral/caudal axis. Furthermore, by expressing dominant negative *cdh2* constructs selectively within cranial sensory ganglia (CSG) neurons, we found that *cdh2* function is necessary within the axons to elicit these stereotypic turns, thus demonstrating that *cdh2* acts cell autonomously. Together, our *in vivo* data reveal a novel role for *cdh2* in the establishment of circuits by peripheral sensory neurons.

Key words: cadherin, axon, sensory, peripheral, ganglion, zebrafish.

The cranial nerves connect the CNS to the periphery, allowing vertebrates to sense and respond to their internal and external environment. Only four of the cranial nerves carry both sensory and motor information: the trigeminal (V), facial (VII), glossopharyngeal (IX), and vagal (X) nerves (Zhang and Ashwell, 2001). The sensory components of these four arise from the cranial sensory ganglia (CSG), which are clusters of neurons that reside outside the CNS in stereotypic positions. These ganglia transduce somatosensory, chemosensory and viscerosensory information to the brain from receptors in the head, throat, heart and viscera. While much is known about the anatomy and physiology of these ganglia in adult organisms, little is known about how these circuits are formed.

The process of peripheral sensory circuit formation is complex, requiring the interaction of multiple cues in the environment to position the neurons of each ganglia and guide the central and peripheral nerves to discrete targets [reviewed in Tessier-Lavigne and Goodman, 1996]. These cues may be diffusible or membrane-bound. Membrane-bound cues include not only receptors involved in bidirectional signaling such as the ephs/ephrins (Reber et al., 2007), but also proteins that participate in intercellular adhesion, such as the cadherins (Ranscht, 2000).

Cadherins are a family of calcium-dependent cell adhesion proteins. Their extracellular domains consist of cadherin repeats that selectively bind the extracellular domains of homotypic cadherins (i.e. cdh2 preferentially binds to other cdh2 proteins) (Ivanov et al., 2001). Type 1 cadherins, including N-, E- and R-cadherin have a single transmembrane and an intracellular domain that binds β- and α-catenin, to anchor the protein to the actin cytoskeleton (Ivanov et al., 2001). Additionally, the intracellular domain of cadherins interacts with many intracellular signaling paths affecting axon outgrowth, navigation and synaptogenesis [reviewed in Nollet et al., 2000; Wheelock and Johnson, 2003]. During neural circuit formation, axons expressing a set of cadherins will selectively contact neighboring cells or axons expressing the same cadherins (Redies et al., 1992; Ranscht, 2000). In such a way, cadherins can serve as molecular codes orchestrating connectivity of various peripheral sensory modalities to discrete targets in the CNS (Redies et al., 1997).

The expression pattern of N-cadherin (cadherin-2 or *cdh2*) has been studied extensively in mouse, chick, *Xenopus* and zebrafish. During development *cdh2* expression is detected in neural tissues, including subsets of the CSG (Simonneau et al., 1992; Bitzur et al., 1994; Redies, 1995; Liu et al., 2003). It has been shown that loss of *cdh2* function in animals disturbs the formation of cranial ganglia (Kerstetter et al., 2004); however, these studies were unable to clearly distinguish effects on CSG afferents from those on efferents. Here we show that disruption of *cdh2* function results in misguided CSG afferents to the periphery and the CNS. Furthermore, we show that these effects result from a cell autonomous role of *cdh2* within the CSG neurons.

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Abbreviations: cdh2, N-cadherin; CSG, cranial sensory ganglia; dpf, days postfertilization; gIX, glossopharyngeal ganglia; *glo*, *glass onion* mutant; gV, trigeminal ganglia; gVII, facial ganglia; gX, vagal ganglia; hpf, hours post-fertilization; mnV, trigeminal efferent; mnVII, facial efferent; mnX, vagal efferent; MO, morpholino oligonucleotide; mVII, facial nuclei; snIX, glossopharyngeal afferent; snVII, facial afferent; snX, vagal afferent; VII, facial nerve.

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EXPERIMENTAL PROCEDURES

Maintenance of fish

Fish were kept on a 14-h day, 10-h night schedule at a constant 28.5 °C, with feeding done twice daily. All animal husbandry was carried out as described by Westerfield (Westerfield, 2000). Embryos were staged according to hours post-fertilization (hpf) and morphological criteria (Kimmel et al., 1995). Embryos used for microscopy were treated with 0.003% phenylthiourea to reduce pigmentation.

Injections into embryos

For injections, plasmid DNA or morpholino oligonucleotides (MO) were dissolved in 0.1 M KCl, 20 mM Hepes (pH 7.4) containing 0.01% Phenol Red and injected into single-cell embryos; 2–5 nanoliters was injected using a Picospritzer III (General Valve Corporation, Fairfield, NJ, USA) attached to a broken glass capillary. The *cdh2* MO designed against base pairs −36 to −13 of N-cadherin cDNA was purchased from GeneTools (Open Biosystems, Huntsville, AL, USA). Two to 5 ng of the morpholino was injected into 1- to 4-cell-stage embryos. Postinjection, embryos were allowed to develop in fish water at 28.5 °C. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Saint Louis University Animal Care Committee approved the procedures used and all efforts were made to minimize the number of animals used and their suffering.

Zebrafish strains

Tg(UAS:kaede) and *glass onion* (*glo*^{m117}) embryos were obtained from the Zebrafish International Resource Center at the University of Oregon (Eugene, OR, USA). Tg(p2xr3.2:eGFP^{sl1}) embryos were described previously (Kucenas et al., 2006). *glo*^{m117} Heterozygotes were crossed to tg(p2xr3.2:eGFP^{sl1}) fish to generate tg(p2xr3.2:eGFP^{sl1}); *glass onion* mutant (*glo*) heterozygotes. Such heterozygotes were in-crossed to create homozygous *glo*^{m117} embryos containing the *p2xr3.2*:eGFP reporter. Tg(nkx2.2megfp) fish were a gift from Bruce Appel (Denver, CO, USA) (Ng et al., 2005), tg(isl1:eGFP) were a gift of H. Okamota (Tokyo, JPN) (Higashijima et al., 2000) and tg(PB4:*gvp*; UAS:kaede) are described below.

Generation of tg(PB4:gvp; UAS:kaede). The PB4 promoter is composed of the 4 kb of genomic sequence immediately upstream of the transcriptional start site of the *p2xr3.2* gene (Kucenas et al., 2006), and was obtained by PCR and subcloned into the pEGFP-1 vector (Clontech) using XhoI/EcoRI sites present in the PCR primers. Embryos injected with this construct expressed eGFP within the CSG in a pattern identical to the tg(p2xr3.2:eGFP^{sl1}) line fish, but had no detectable eGFP expression in the hindbrain. This PB4 promoter fragment was excised via NheI and AgeI sites and inserted upstream of a gal4VP16 element in the tol2 transposon vector pCH μs G4VP16 (a kind gift from Dr. Mike Nonet, St. Louis, MO, USA) yielding pCH.PB4:GVP. pCH.PB4:GVP (50 ng/μl) was coinjected with transposase RNA (30 ng/μl) (pCS-TP, a kind gift of Dr. K. Kawakami, Mishima, Shizuoka, JPN) into eggs from the AB genetic background. Transposase activity was verified by PCR analysis as described in (Kawakami et al., 1998). The pCH.PB4.GVP construct contains a second expression cassette that has the cardiac myosin light chain promoter in front of CFP, allowing us to identify carrier embryos by their cardiac CFP-expression. Carriers were raised to adulthood and intercrossed to identify founders. Approximately 10%–15% of carriers were identified as founders. The tg(PB4:*gvp*) fish were crossed to tg(UAS:kaede) fish to generate embryos for the tg(PB4:*gvp*; UAS:kaede) line.

Dominant negative construction and injection

Full length zebrafish *cdh2* (accession number: AF430842) was cloned from cDNA obtained from 4 days postfertilization (dpf) zebrafish by PCR (Phusion, New England Biolabs) and was verified by sequencing. The two dominant negative *cdh2* were constructed as follows. The C-terminal deleted *cdh2* (ΔC) was engineered by amplifying the coding region from the initiator ATG to amino acid $E_{739,}$ which was converted to a termination codon (TAG); this residue sits C-terminal to the transmembrane domain. The second dominant negative was a *cdh2* molecule that did not contain the extracellular cadherin domains (ΔN) and was constructed in a two-step process. The first fragment spanned amino acid M_1 through G_{30}: this sequence contains the signal peptide and terminates at the start of the pro-domain (Malicki et al., 2003). The 3′-primer used to generate this fragment included an extra 15 bp corresponding to 5 amino acids (S_{656}–S_{660}) situated 5′ of the transmembrane domain. A second PCR fragment was generated that began at these five amino acids and continued through the membrane spanning domain and ended at the native stop codon present in the 3′ primer. These two fragments were then used in an overlap PCR (Egan et al., 1998) such that the signal peptide was attached directly to a site 47 amino acids upstream of the transmembrane domain (Kintner, 1992). Both dominant negatives were then cloned into a UAS vector (a gift from Dr. R. Wong, Seattle, WA, USA; Mumm et al., 2006) at the Asp718/NotI sites. Primers used for PCR were as follows: full length: 5′-end: tataggtaccatcggtttctttatacagaac and 3′-end: tatagcggccgcctagtcgtcgttacctccgta; ΔC: 3′-end: tatagcggccgcctatttatcccgtctcttcat; ΔN: overlap forward: ccatgtcagcctggtagttacctggaaagt and overlap reverse: actttccaggtaactaccaggctgacatgg. All PCR was performed using the high fidelity DNA polymerase Phusion (New England Biolabs). PB4:GVP plasmid (50 ng/μl) was coinjected with either UAS:ΔC or UAS:ΔN (25 ng/μl).

Epifluorescent microscopy

Embryos were anesthetized with 0.02% tricaine in fish water and transferred to a 96-well plate. Epifluorescent images were taken using a Nikon TE200 inverted microscope equipped with a CoolSnap HQ digital camera. Metamorph Software (Universal Imaging Corp.) was used to acquire and process images. Cropping and rotating of images was carried out using Adobe Photoshop. For time lapse imaging, embryos were embedded in 0.5% low melting point agarose containing 0.02% tricaine. The embryos were maintained at 28–29 °C using a heated plate, and images were taken every 5 min.

Confocal imaging of embryos

Live or fixed embryos were embedded in 0.5% low melting point agarose containing 0.02% tricaine. Optical sections were taken using an Olympus FV1000 MPE and z-stacks were processed using Olympus Fluoview software and Adobe Photoshop. Kaede was photoconverted from red to green fluorescence under visual inspection by excitation with a 405 nm laser.

Scoring of phenotype

Offspring from *glo*^{m117}; tg(p2xr3.2:eGFP^{sl1}) adults were imaged under brightfield to identify homozygotes based on embryo morphology. At 4 dpf, GFP-positive embryos *glo*^{m117} embryos were imaged laterally under confocal microscopy as described above. The glossopharyngeal ganglia (gIX) central axon was not amenable to visualization due to its depth and the scattering of the epibranchial ganglia, so it was excluded from further analysis. The score of “no axon” was given only when no central facial ganglia (gVII) nerve (facial afferent, snVII) or vagal ganglia (gX) nerve (vagal afferent, snX) was visible. The score of “normally projecting” was given when the snVII axon terminated near its normal proximity in the hindbrain even when no plexus was formed, or when snX axons formed a plexus in the hindbrain, though this plexus was sometimes less defined. The score of “misrouted