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Zic1 LEVELS REGULATE MOSSY FIBER NEURON POSITION AND AXON LATERALITY CHOICE IN THE VENTRAL BRAIN STEM

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Abstract—Pontine gray neurons of the brain stem are a major source of mossy fiber (MF) afferents to granule cells of the cerebellum. Achieving this connectivity involves an early regionalization of pontine gray neuron cell bodies within the brainstem pontine nuclei, as well as establishing the proper ratio of crossed versus uncrossed MF projections to contralateral versus ipsilateral cerebellar territories. Here, we report expression of the transcription factor Zic1 in newly postmitotic pontine gray neurons and present functional experiments in embryonic and postnatal mice that implicate Zic1 levels as a key determinant of pontine neuron cell body position within the pons and axon laterality. Reducing Zic1 levels embryonically via *in utero* electroporation of short hairpin RNA interference (shRNAi) vectors shifted the postnatal distribution of pontine neurons from caudolateral to rostromedial territories; by contrast, increasing Zic1 levels resulted in the reciprocal shift, with electroporated cells redistributing caudolaterally. Associated with the latter was a change in axon laterality, with a greater proportion of marked projections now targeting the ipsilateral instead of contralateral cerebellum. Zic1 levels in pontine gray neurons, therefore, play an important role in the development of pontocerebellar circuitry.

Key words: pontocerebellar circuitry, precerebellar mossy fiber afferents, cerebellum, mouse, transcription factor.

Critical for coordination of skilled movements is a collection of brainstem structures known as precerebellar mossy fiber (MF) nuclei, aptly named because they provide major cerebellar afferents—MF axons—to granule cells of the cerebellar cortex and output neurons of the cerebellar nuclei (Palay and Chan-Palay, 1974; Altman and Bayer, 1987a; Altman and Bayer, 1987b; Altman and Bayer, 1997; Brodal and Bjaalie, 1992, 1997; Sotelo, 2004; Taber Pierce, 1966). Through their afferents, brainstem MF neurons transfer information to the cerebellum that is received from neurons residing in the cerebral cortex or spinal cord, the resultant circuitry having powerful means to control cerebellar activity (Altman and Bayer, 1997; Schmahmann and Pandya, 1997; Schwarz and Thier, 1999; Turner, 1941). MFs connect with the cerebellum via a stereotyped ratio of crossed (contralaterally-directed) and uncrossed (ipsilaterally-directed) axon pathways with respect to the brainstem midline (reviewed in Cicirata et al., 2005). While the nature of this ratio suggests an importance for cerebellar computations and motor control (Eccles, 1967; Ito, 1984), little is known about the molecular underpinnings regulating MF laterality. Similarly, little is known about the molecular determinants of MF cell body position within the brain stem and whether soma location and axon laterality are linked. Here we present findings implicating the transcription factor Zic1 in these processes. We focused on assembly and connectivity of the pontine gray nucleus (PGN) because, in mammals, it contributes the greatest number of MFs to the cerebellum, as compared to other classes of MF nuclei, and it appears to do so with a fixed laterality ratio (Brodal and Bjaalie, 1997; Cicirata et al., 2005; Mihailoff et al., 1981; Palay and Chan-Palay, 1974; Rosina and Provini, 1981; Serapide et al., 2001).

The PGN resides in the ventral brain stem (the pons) and is composed of bilaterally symmetrical lobes, one on each side of the midline (Altman and Bayer, 1987b; Taber Pierce, 1966) (Fig. 1A). Within each lobe are subclasses of MF neurons, categorized by cell body location—rostromedial or caudolateral (Azizi et al., 1981; Brodal and Bjaalie, 1992, 1997; Mihailoff et al., 1981; Rosina and Provini, 1981; Taber Pierce, 1966)—or on laterality of axon projection to the contralateral versus ipsilateral cerebellum (Cicirata et al., 2005). The majority (~80%) of MF axons extend medially from their respective PGN lobe, cross the ventral brainstem midline, and enter the contralateral cerebellum. A smaller cohort of MFs (~20%) project in the opposite direction to neurons situated in the ipsilaterally-located cerebellum, thus their axons do not cross the midline (Cicirata et al., 2005) (Fig. 1A, "contra" and "ipsi" labels are relative to the green lobe of the schematized PGN nucleus, the other lobe of the PGN is indicated in gray). It seems likely that at least some factors involved in determining PGN MF laterality and cell body position exert their effects during intermediate (postmitotic) stages of MF neuron development, after specification to a generic MF neuron fate has been made but before subtype identity manifests. Specification of generic MF neuron identity occurs early during development, in cycling progenitor cells of the dorsally situated germinal zone called the hind-brain rhombic lip (hRL), and requires the cell-autonomous activity of the basic helix–loop–helix (bHLH) transcription factor Math1 (mAtoh1) (Ben-Arie et al., 1997, 2000; Farago et al., 2006; Landsberg et al., 2005; Wang et al., 2005). Upon

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Abbreviations: Dcc, deleted in colorectal cancer; eGFP, enhanced green fluorescent protein; ems, extramural migratory stream; hRL, hind-brain rhombic lip; mcp, medial cerebellar peduncle; MF, mossy fiber; nßgal, nuclear-localized β-galactosidase; P, postnatal day; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PGN, pontine gray nucleus; shRNAi, short hairpin RNA interference.

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expression of Math1, committed MF cells emerge from the hRL as postmitotic cells that go on to circumnavigate the hind-brain ventrolaterally to take up residence flanking the ventral midline of the pons, from where they extend axons either contralaterally or ipsilaterally (schematized in Fig. 1A–1D). We refer to these early postmitotic cells as MF "precursor" cells because they are not fully differentiated, yet are no longer cycling as progenitor cells. Molecular programs implemented during this intermediate stage of MF neuron development (reviewed in Millen et al., 1999; Sotelo, 2004; Wingate, 2001) may determine, at least in part, where MF neurons settle in the brain stem and the pathway of projection to the cerebellum.

Molecular programs involved in MF neuron migration, nucleus assembly and MF laterality are just beginning to be characterized. Chemotropic cues secreted by the floor plate, like Netrin-1 (Kennedy et al., 1994), draw young MF neurons ventral (Alcantara et al., 2000; Yee et al., 1999) through activation of the transmembrane receptor deleted in colorectal cancer (Dcc) (Fazeli et al., 1997; Keino-Masu et al., 1996). Rig1/Robo3, a Slit receptor on young MFs, downregulates as leading processes cross the ventral midline (Marillat et al., 2004). Rig1/Robo3-deficiency leads to precocious settling of MF cell bodies laterally, well before reaching the ventral midline; it also results in the extension of processes only ipsilaterally, although it is unclear if these are axons and whether they reach cerebellar targets (Marillat et al., 2004). Rig1/Robo3 is thought to regulate Robo2, another Slit guidance receptor expressed by MF neurons (Marillat et al., 2004). The transcription factor Hoxa2 has recently been shown to regulate directly transcription of *Robo2*, and in so doing appears critical for maintaining the caudal-to-rostral migration of MF neurons as they traverse from the caudal hRL to the pons (Geisen et al., 2008). Also implicated in postmitotic stages of precerebellar afferent system development is the differential expression of cadherin-type cell adhesion molecules (Taniguchi et al., 2006) as well as intracellular Rho GTPases capable of inducing cytoskeletal changes critical for different migrations (reviewed in Bloch-Gallego et al., 2005). Toward identifying other factors involved in precerebellar afferent system organization as well as molecules involved in MF laterality choice—a process for which little is known—we have analyzed MF lineages for expressed transcription factors and then used gain- and loss-of-function experiments to ascertain how such factors influence MF neuron subtype identity, in particular, site of residence within and axon directionality from the PGN. Here, we present our findings regarding the zinc finger transcription factor Zic1.

Zic family transcription factors, relatives of the *Drosophila* Opa transcription factor (encoded by the pair rule gene *odd-paired*) (Aruga et al., 1994, 1996), are fitting candidates for consideration as regulators of MF axon laterality with respect to the brainstem midline. This is because Zic2 activity in mice has been implicated in directing retinal axon pathway choice at the optic chiasm (García-Frigola et al., 2008; Herrera et al., 2003; Lee et al., 2008) and Zic3 in establishing left–right body asymmetry (Purandare et al., 2002). While the role of Zic genes in early developmental processes such as cerebellar patterning and neuroectoderm differentiation has been well characterized (Aruga, 2004; Aruga et al., 1998, 2002; Grinberg et al., 2004), investigations into Zic protein functions in postmitotic cells are in their infancy.

Here, we present investigations by which we revealed Zic1 expression in postmitotic MF precursor cells, and then determined the consequences of altered Zic1 levels on MF nucleus formation and axon laterality. We used an electroporation method (Kawauchi et al., 2006; Okada et al., 2007; Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001; Takahashi et al., 2002) to perturb pontine gray MF precursor cells molecularly and acutely by delivering either *Zic1*-specific RNA interference or overexpression vectors along with reporter plasmids to the pontine gray MF lineage during *in utero* development. In this way, we bypassed the earlier requirement for Zic1 in neurogenesis and patterning and studied, for the first time, Zic1 functions in later phases of brainstem development. Because the electroporation strategy allowed for directing manipulations to just one side of the embryo, our analyses were also able to include visualization of axon laterality arising from MF neurons situated within a single lobe of the PGN on one side of the brain stem—an axon projection feature otherwise obscured by the passing fibers from homologous PGN neurons of the contralateral side. We found that reducing Zic1 levels shifted the postnatal distribution of the transfected neurons within the PGN, from caudolateral to rostromedial territories. By contrast, increasing Zic1 levels shifted the distribution reciprocally, from rostromedial to caudolateral territories. Associated with this caudolateral shift was a shift in relative proportion of the marked axons projecting to the ipsilateral cerebellum, thus axon laterality was also altered. Zic1, therefore, influences two essential processes during MF system development: where pontine neurons settle in the ventral brain stem and to which cerebellar side they project. Thus, we reveal a new role for Zic1 as a cell-autonomous regulator of key aspects of nucleus formation and axon pathway choice in the ventral brain stem.

EXPERIMENTAL PROCEDURES

Animals

Timed pregnant CD1 mice were obtained from Charles River Laboratories (Cambridge, MA, USA). Vaginal plug detection was considered 0.5 dpc. All experiments conformed to our institutional guidelines (IACUC) on the ethical use of animals-guidelines that meet all standards of the American Veterinary Medical Association.

Expression vectors

Complete *nlacZ* and *Zic1* coding regions (Open Biosystems, image # 6813617) were cloned into *pCAGGS* (Niwa et al., 1991) yielding *pCAG-nlacZ* and *pCAG-Zic1*, respectively. *Zic1* shRNAi constructs were amplified from oligonucleotides and cloned into *pBS/U6* (Sui et al., 2002). Oligonucleotides: mouse Zic1 (coding region 1407–1429), 5′-TGCTGTTGACAGTGAGCGAGG GCTGGAGCCTTCTTCCGCTTAGTGAAGCCACAGATGTAAGCG-