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PURKINJE CELL PHENOTYPE RESTRICTS THE DISTRIBUTION OF UNIPOLAR BRUSH CELLS

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Abstract—Cerebellar unipolar brush cells (UBCs) are glutamatergic interneurons of the granular layer. Previous studies have identified three distinct UBC subsets in the mouse cerebellar cortex: one expressing the calcium-binding protein calretinin (CR), a second expressing both the metabotropic glutamate receptor (mGluR)1α and phospholipase C(PLC)β4, and a third expressing PLCβ4 but not mGluR1α. We have investigated UBC topography in two strains of mutant mice: early B-cell factor 2 (*Ebf2*) null and *scrambler*. In *Ebf2* null mice Purkinje cell topography is disrupted due to Purkinje cell death and ectopic gene expression. The topography of all three classes of UBCs is also abnormal: the CR$^+$ UBCs, which are normally aligned with zebrin II stripes, become homogeneously distributed; the numerical density of mGluRIα^+ UBCs is increased; and many PLCβ4$^+$ UBCs are located ectopically. The UBC ectopia is not a cell-intrinsic action of the *Ebf2* gene—analysis of the constitutive expression of a β-galactoside reporter under the control of the *Ebf2* promoter reveals no *Ebf2* expression in UBCs at any stage of cerebellar development. In *scrambler* (*Dab1^{scm}*), most Purkinje cells are ectopic but nevertheless have normal adult gene expression patterns. In *scrambler*, UBCs associate with specific ectopic Purkinje cell clusters. Finally, similar associations with specific Purkinje cell clusters are seen during normal cerebellar development. These data suggest that UBCs become regionally restricted during development through a non–cell-autonomous mechanism involving embryonic interactions with different Purkinje cell subtypes.

Key words: zebrin II, *Ebf2*, *scrambler*, pattern formation, cerebellar development.

Cerebellar unipolar brush cells (UBCs) are glutamatergic interneurons of the granular layer, with small somata, mossy fiber-like axon terminals and brush-like dendrites (Altman and Bayer, 1977; Mugnaini and Floris, 1994; Dino et al., 1999; Nunzi and Mugnaini, 2000; Nunzi et al., 2001, 2002). The distribution of UBCs reflects the topography of the cerebellum as a whole (e.g., Hawkes et al., 1992; Hawkes, 1997; Hawkes and Eisenman, 1997; Herrup and Kuemerle, 1997; Oberdick et al., 1998; Armstrong and Hawkes, 2000). First, the cerebellum is subdivided into four transverse zones: anterior (~lobules I–V), central (~lobules VI–VII), posterior (~lobules VIII–dorsal IX), and nodular (~ventral lobule IX+lobule X) (e.g., Ozol et al., 1999; Armstrong et al., 2000; Sillitoe and Hawkes, 2002). Each transverse zone is further subdivided into parasagittal stripes. For example, zebrin II (Brochu et al., 1990; reviewed in Hawkes et al., 1992: an epitope on aldolase C—Ahn et al., 1994) is expressed in parasagittal stripes that are highly reproducible between individuals and across species (Brochu et al., 1990; Ahn et al., 1994; Oberdick et al., 1998; Armstrong and Hawkes, 2000; Sillitoe et al., 2005).

Both mossy fiber (e.g., Akintunde and Eisenman, 1994; Ji and Hawkes, 1994) and climbing fiber (Gravel et al., 1987; Wassef et al., 1992; Voogd et al., 1996, 2003) afferents are restricted with by Purkinje cell zone and stripe boundaries. This topography is likely established during development through direct interactions between afferent axonal growth cones and the nascent cerebellar Purkinje cell stripes (e.g., Sotelo and Chédotal, 2005). Less is known about the topographic restriction of cerebellar interneurons (e.g., Hawkes and Turner, 1994; Gao et al., 2006; Sillitoe et al., 2008). To explore this for UBCs we have examined two strains of mutant mice—*Ebf2* null *scrambler*. *Ebf2* is an atypical helix–loop–helix transcription factor that has been implicated in neural development (Dubois et al., 1998; Garel et al., 1999). In the *Ebf2* null cerebellum, the normal alternating zebrin II$^{+/-}$ stripes are replaced by a field of uniform zebrin II$^+$ Purkinje cells, due to a combination of selective Purkinje cell death and the ectopic expression of zebrin II$^+$ markers (Croci et al., 2006; Chung et al., 2008). The data show that UBCs are also abnormally distributed in the *Ebf2* null cerebellum. This effect is not UBC cell-autonomous. Secondly, *scrambler* is a mutation of *Disabled homolog 1* (*Dab1*), which encodes the *Dab1* adaptor protein (Howell et al., 1997; Sheldon et al., 1997). During cerebellar development, *Dab1* mediates Purkinje cell dispersal into a monolayer. *Dab1* disruption results in the failure of dispersal and a loss of foliation (disabled—Howell et al., 1997; Gallagher et al., 1998; *scrambler*—Rice et al., 1998) but Purkinje cell stripe phenotypes are apparently normal (e.g., Chung et al., 2007b). In *scrambler*, UBCs are malpositioned in the cerebellar core in association with ectopic zebrin II$^+$ Purkinje cell clusters. A similar association is seen during normal cerebellar development. Taken together, these data suggest that UBCs become regionally restricted through interactions with the embryonic array of Purkinje cell subtypes.

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Abbreviations: Dab1, disabled homolog 1; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; UBCs, unipolar brush cells.

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EXPERIMENTAL PROCEDURES

Mice

All animal procedures conformed to institutional regulations and the Guide of the Care and Use of Experimental from the Canadian Council of Animal Care, and the provisions of the San Raffaele Scientific Institute Institutional Animal Care and Use Committee. Every effort was made to minimize the number of animals used, and their suffering. C57/BL6J and CD1 mice were obtained from Charles River Laboratories (St. Constant, QC, Canada). Time pregnant wild-type females were used for the developmental studies. Noon of the day the vaginal plus was seen was designated E0.5 and the day of birth was designated as P0.

Ebf2 null. The *Ebf2* targeting construct, described in Corradi et al. (2003), contained a *lacZ* cDNA, and *lacZ* expression during CNS development is in full agreement with the results of *in situ* hybridization studies (Garel et al., 1997). The cerebellar expression pattern of *Ebf2* has been described in detail (Croci et al., 2006; Chung et al., 2008). All experiments were carried out on F1 hybrids obtained by crossing Ebf2$^{+/-}$ FVB/N (N9) females with Ebf2$^{+/-}$ C57BL/6J males. This hybrid strain was chosen to obviate the low fertility and poor maternal behavior of C57BL/6J heterozygous mothers. Coisogenic littermates were used as controls.

Ebf2–Cre–lacZ. The RP24–283N8 BAC clone, consisting of 32 kb of 5′ flanking sequence plus the first six exons of the murine *Ebf2* gene, was obtained from the BACPAC Resource Center at Children's Hospital Oakland Research Institute, Oakland, CA, USA. By BAC recombineering (Copeland et al., 2001; Warming et al., 2005) the genomic clone was modified to insert a cDNA encoding a fusion of EGFP and the improved Cre recombinase followed by the SV40 polyadenylation signal in the first coding exon of the *Ebf2* gene (Badaloni et al., unpublished observations). The BAC DNA was microinjected into the pronucleus of FVB/N oocytes. Pups were analyzed by Southern blotting for the presence of the transgene. Founders were isolated and a transgenic line was established mating the founders with FVB/N mice. The distribution of the iCre transcript was compared with that of the *Ebf2* transcript during brain and cerebellar development. Our results indicate that iCre distribution accurately recapitulates the distribution of endogenous *Ebf2* at all locations and stages examined (Badaloni et al., unpublished observations). In all experiments described here, *Ebf2*: GFPiCre +/0 were crossed with the reporter line *Rosa26LacZ* (Soriano, 1999) that expresses the *lacZ* gene in a Cre-inducible manner.

Scrambler. *Scrambler* mice (*Dab1*^{scm}−/−: Sweet et al., 1996) and normal littermates were obtained from The Jackson Laboratory, Bar Harbor, ME, USA.

Antibodies

The following primary antibodies were used:

- Anti-zebrin II is a mouse monoclonal antibody produced by immunization with a crude cerebellar homogenate from the weakly electric fish Apteronotus (Brochu et al., 1990): it was used directly from spent hybridoma culture medium at a concentration of 1:1000. In the cerebellum, zebrin II immunoreactivity is restricted to a Purkinje cell subset (Brochu et al., 1990) together with weak uniform expression in some glial cells (e.g., Walther et al., 1998).
- Mouse monoclonal anti-calretinin (CR: 1:1000) was raised against full-length recombinant human CR (Swant Inc. Bellinzona, Switzerland: 7699/4). Antibody specificity is described in detail in Schwaller et al. (1994).
- Two different mouse monoclonal anti-calbindins were used—one from Sigma, St. Louis, MO, USA (anti-calbindin-D-28K, clone CB-955, ascites fluid, IgG1 isotype, raised against bovine kidney calbindin: used diluted 1:1000) and the other from Swant, Bellinzona, Switzerland (McAb 300, lot #18(F): raised against chicken calbindin and specifically stains the 45Ca-binding spot of calbindin D-28k (apparent molecular weight 28K, isoelectric point 4.8) in a two-dimensional gel of mouse brain homogenate (manufacturer's information); used here diluted 1:1000). Both antibodies yielded Purkinje cell specific staining identical to that reported often before (e.g., Baimbridge and Miller, 1982; Wassef et al., 1985; Ozol et al., 1999).
- Affinity purified rabbit anti-Tbr2 was raised against mouse Tbr2 synthetic peptide (Englund et al., 2006; Chemicon, 1:1000).
- Anti-phospho-gamma-aminobutyric acid B receptor 2 (anti-GABABR2) was raised in rabbit against a synthetic peptide surrounding serine 892 of the 110 kDa rat receptor 2 subunit (1:1000: Invitrogen) and epitope-affinity purified. On Western blots of mouse cerebellar homogenates it recognizes a 120 kDa band, consistent with the 892-phosphorylated GABA B receptor R2 subunit, and the specificity of the antibody was confirmed in GABABR2 knockout mice (Luján and Shigemoto, 2006).
- Rabbit polyclonal anti-ß-galactosidase (anti-β-gal: 1:700), generated by injecting a recombinant full-length *E. coli* ß-galactosidase protein, was purchased from Abcam (Cambridge, MA 02139, lot # ab616, USA).
- Rabbit anti-phospholipase Cß4 (anti-PLCß4; 1:1000: the gift of Dr. M. Watanabe: Hokkaido University, Japan) was raised against amino acids 15–74 of the mouse PLCβ4 protein fused to glutathione-S-transferase and expressed in bacteria. Control immunohistochemistry using either antibody preabsorbed with antigen polypeptides or cerebellar sections from a PLCß4 knockout mouse yielded no significant immunostaining (Nakamura et al., 2004; Sarna et al., 2006). The antiserum recognizes a single polypeptide band of 134 kDa apparent molecular weight on Western blots of mouse (Nakamura et al., 2004) and human (unpublished observations) cerebellar homogenates. The band is absent from Western blots of cerebellar homogenates from a PLCß4 null mouse (Jiang et al., 1996; Nakamura et al., 2004).
- Guinea-pig anti-mouse mGluR1α antibody was raised against a bacterially expressed peptide representing amino acid residues 945–1127 of the rat metabotropic glutamate receptor 1α (mGluR1α; 1:500, Tanaka et al., 2000: the generous gift of Dr. Masahiko Watanabe, Hokkaido University, Japan).

Perfusion sectioning and immunohistochemistry

Mice were deeply anaesthetized with sodium pentobarbital (100 mg/kg i.p.) and transcardially perfused with 0.9% NaCl in 0.1 M phosphate buffer (PBS: pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were then removed from the skull and post-fixed in 4% paraformaldehyde at 4 °C for 48 h. The cerebella were then cryoprotected through a series of buffered sucrose solutions: 10% (2 h), 20% (2 h) and 30% (overnight). Series of 40 μm thick transverse sections were cut through the extent of the cerebellum on a cryostat and collected for free-floating immunohistochemistry.

Peroxidase immunohistochemistry was carried out as described previously (Sillitoe et al., 2003). Briefly, tissue sections were washed thoroughly, blocked with 10% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and then incubated in 0.1 M phosphate buffered saline (PBS) containing 0.1% Triton-X and the primary antibody for 16–18 h at 4 °C. Secondary incubation in horseradish peroxidase (HRP) conjugated goat anti-rabbit, HRP-conjugated goat anti-mouse or HRP-conjugated goat anti-guinea pig antibodies (all diluted 1:200 in PBS; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was for 2 h at room temperature. Diaminobenzidine (DAB,