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PURKINJE CELL SUBTYPE SPECIFICATION IN THE CEREBELLAR CORTEX: EARLY B-CELL FACTOR 2 ACTS TO REPRESS THE ZEBRIN II-POSITIVE PURKINJE CELL PHENOTYPE

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Abstract—The mammalian cerebellar cortex is highly compartmentalized. First, it is subdivided into four transverse expression domains: the anterior zone (AZ), the central zone (CZ), the posterior zone (PZ), and the nodular zone (NZ). Within each zone, the cortex is further subdivided into a symmetrical array of parasagittal stripes. The most extensively studied compartmentation antigen is zebrin II/aldolase c, which is expressed by a subset of Purkinje cells forming parasagittal stripes. Stripe phenotypes are specified early in cerebellar development, in part through the action of early B-cell factor 2 (*Ebf2*), a member of the atypical helix–loop–helix transcription factor family Collier/Olf1/EBF. In the murine cerebellum, *Ebf2* expression is restricted to the zebrin II-immunonegative (zebrin II−) Purkinje cell population. We have identified multiple cerebellar defects in the *Ebf2* null mouse involving a combination of selective Purkinje cell death and ectopic expression of multiple genes normally restricted to the zebrin II− subset. The nature of the cerebellar defect in the *Ebf2* null is different in each transverse zone. In contrast to the ectopic expression of genes characteristic of the zebrin II+ Purkinje cell phenotype, phospholipase Cβ4 expression, restricted to zebrin II− Purkinje cells in control mice, is well maintained, and the normal number of stripes is present. Taken together, these data suggest that *Ebf2* regulates the expression of genes associated with the zebrin II+ Purkinje cell phenotype and that the zebrin II− Purkinje cell subtype is specified independently.

Key words: zebrin II, *Ebf2*, Purkinje cell, pattern formation.

Although the mammalian cerebellar cortex appears histologically uniform, it is subdivided rostrocaudally and mediolaterally into a complex array of transverse zones and parasagittal stripes (reviewed in Hawkes et al., 1992; Hawkes, 1997; Hawkes and Eisenman, 1997; Herrup and Kuemerle, 1997; Oberdick et al., 1998; Armstrong and Hawkes, 2000). This subdivision is not just of speculative relevance; in fact, it closely reflects the pattern of afferent and efferent connections linking the cerebellum to the rest of the central nervous system CNS (e.g., Akintunde and Eisenman, 1994; Ji and Hawkes, 1994). First, the cerebellar vermis can be subdivided into four transverse zones based on differential protein expression: the anterior zone (AZ; ~lobules I–V), the central zone (CZ; ~lobules VI–VII), the posterior zone (PZ; ~lobules VIII–dorsal IX), and the nodular zone (NZ; ~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. The most extensively studied marker for antigenic cerebellar compartmentation is the antigen zebrin II (Brochu et al., 1990; reviewed in Hawkes et al., 1992), later shown to be an epitope on the respiratory isoenzyme aldolase c (Aldoc; Ahn et al., 1994). Zebrin II is expressed by a subset of Purkinje cells forming parasagittal stripes in the AZ and PZ 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). Although the CZ and NZ express zebrin II uniformly, stripes in these regions can be revealed by using other markers (e.g. HSP25; Armstrong et al., 2000). In general, stripe markers can be categorized into at least three groups:

i) markers that have an identical expression pattern to that of zebrin II (P+ markers); for example, the sphingosine kinase (SPHK) 1a isoform (Terada et al., 2004), the GABA B receptor 2 (GABABR2; Fritsch et al., 1999; Chung et al., 2007a), and phospholipase C (PLC) β3 (Sarna et al., 2006).

ii) those with a complementary expression pattern to that of zebrin II (P− markers); for example, PLCβ4 (Sarna et al., 2006) and neuroplastin (Marzban et al., 2003) are expressed predominantly in the zebrin II-immunonegative (zebrin II−) Purkinje cell subset.

iii) those that identify subsets within the zebrin II+/− populations. For example, the small heat shock protein (HSP) 25 is constitutively expressed in stripes solely in the (uniformly zebrin II+) CZ, NZ and flocculus (Armstrong et al., 2000); the human natural killer cell antigen HNK-1 is expressed in zebrin II+ stripes in the PZ but not elsewhere (Marzban et al., 2004); an L7/pcp2-lacZ transgene is expressed in subsets of both the zebrin II+ and zebrin II− Purkinje cells (Ozol et al., 1999); PLCβ4, as well as being expressed in all zebrin II− Purkinje cells is also co-expressed with zebrin II in an array of stripes restricted to the PZ (Sarna et al., 2006).

Early B-cell factor 2 (*Ebf2*; Malgaretti et al., 1997; Wang et al., 1997) is the first regulatory gene identified that

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Abbreviations: AZ, anterior zone; CZ, central zone; E, embryonic day; *Ebf2*, early B-cell factor 2; GABABR2, GABA B receptor 2; HSP, heat shock protein; NZ, nodular zone; OMP, olfactory marker protein; PLC, phospholipase C; PZ, posterior zone; SPHK, sphingosine kinase; zebrin II−, zebrin II-immunonegative.

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is involved in Purkinje cell subtype specification. *Ebf2* is part of the Collier/Olf1/EBF family of atypical helix–loop–helix transcription factors, which has been implicated in various aspects of neural development (Dubois et al., 1998; Garel et al., 1999) and neuronal function (Kudrycki et al., 1993), including axon navigation (Garel et al., 1999), neuronal migration (Garel et al., 2000) and differentiation (Dubois et al., 1998; Pozzoli et al., 2001). Several *in vivo* studies have disclosed genetic interactions between *Ebf2* and important regulators of neural development, including *hedgehog* (Vervoort et al., 1999) and *notch* (Crozatier and Vincent, 1999). Mice carrying a null mutation of *Ebf2* have been generated (Corradi et al., 2003). *Ebf2* null mice have a characteristic "pigeon-toed" gait, and display severe motor deficits. Recently we identified defects in cerebellar development and topography in this mutant (Croci et al., 2006). In the null cerebellum (i) the number of Purkinje cells is markedly decreased, most dramatically in the AZ; (ii) zebrin II is uniformly expressed in the null PZ, while it is expressed in stripes in the heterozygote; (iii) some surviving Purkinje cells, which were destined to become zebrin II−, are transdifferentiated to zebrin II+. In the present study, we have used a panel of molecular markers to understand better the phenotypic abnormalities and altered antigenic compartmentation of the *Ebf2* null cerebellum. Our data now reveal that abnormal cerebellar topography in the *Ebf2* null mice is specific for each transverse zone: Purkinje cells are missing from the PZ and AZ vermis but not the CZ and NZ vermis and Purkinje cell subtype ectopic gene expression occurs in the posterior hemispheres and most of the AZ vermis, but not in the PZ vermis. Several P+ stripe markers in the *Ebf2* null cerebellum show the same ectopic expression pattern as that of zebrin II. In contrast, P− stripe markers are expressed relatively normally in the PZ and AZ. These data suggest that *Ebf2* acts to suppress the zebrin II+ phenotype independently of positive regulation of the zebrin II− phenotype.

EXPERIMENTAL PROCEDURES

Mice

All animal procedures conformed to institutional regulations and the Guide of the Care and Use of Experimental Animals from the Canadian Council of Animal Care. All experiments conformed to international guidelines on the ethical use of animals. Every effort was made to minimize the number of animals used and their suffering. The targeting construct, described in Corradi et al. (2003), contained a *lacZ* cDNA and the distribution of *lacZ* expression during CNS development is in full agreement with the results of *in situ* hybridization studies (Garel et al., 1999). All experiments were carried out on F_1 hybrids obtained by crossing *Ebf2*$^{+/-}$ FVB/N (N_9) 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 (+/+ and +/− yielded identical immunostaining).

Perfusion and sectioning

All mice were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused with 0.9% NaCl in 0.1 M phosphate buffer (pH 7.4) followed by 4% paraformaldehyde with 0.02% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were then removed, post-fixed in 4% paraformaldehyde fixative at 4 °C for 24 h and stored in Millonig's solution (pH 7.6). Series of 40 μm thick transverse or sagittal sections were cut through the extent of the cerebellum on a cryostat.

Section immunohistochemistry

The following 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 diluted 1:1000. Spent culture medium from myeloma cells gives no immunostaining.

Rabbit polyclonal anti-HSP25 (1:5000) was purchased from StressGen (Victoria, BC, Canada; SPA-801, lot #B111411). It gave a staining pattern identical to that reported previously, where antibody absorption controls using HSP25 also abolished all immunostaining (see Armstrong et al., 2000). On Western blots of cerebellar homogenate it recognizes a single band, apparent molecular weight 25 kDa (Armstrong et al., 2000).

Anti-SPHK1a (1:1000) was the gift of Dr. N. Terada (Yamanashi University, Japan). Rabbit polyclonal anti-SPHK1a was generated by injecting a synthetic oligopeptide, corresponding to the 16 C-terminal residues of the mouse SPHK1a sequence (Murate et al., 2001) that was glutaraldehyde-conjugated to keyhole limpet hemocyanin. The antiserum was affinity purified over a cyanogen bromide-activated agarose gel bound to the same synthetic peptide. On Western blots of adult mouse cerebellar homogenate it recognizes a single band, apparent molecular weight 49 kDa (Terada et al., 2004).

Anti-phospho-GABABR2 was raised in rabbit against a synthetic peptide surrounding serine 892 of the 110 kDa rat receptor 2 subunit (1:1000; Invitrogen Inc., Carlsbad, CA, USA) 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 (Waldvogel et al., 2004). It is selectively expressed in zebrin II-immunopositive Purkinje cells in the adult mouse cerebellum (Chung et al., 2007a).

Anti-β-gal (1:700; Abcam Inc., Cambridge, MA, USA) was raised in rabbit against *E. coli* recombinant full-length beta-galactosidase protein. This antibody reacts with beta-galactosidase from *E. coli* showing a 464 kDa band (tetramer) under non-reducing conditions of SDS-PAGE and a 116 kDa band under reducing conditions.

Anti-PLCβ4 (anti-PLCβ4; 1:1000) was the gift of Dr. M. Watanabe (Hokkaido University, Japan). Rabbit anti-PLCβ4 was raised against a synthetic peptide representing amino acids 15–74 of the mouse PLCβ4 protein fused to glutathione-S-transferase and expressed in bacteria. Control immunohistochemistry using either antibodies pre-absorbed with antigen polypeptides or cerebellar sections from a PLCβ4 knockout mouse yielded no significant immunostaining (Nakamura et al., 2004; Sarna et al., 2006). An identical staining pattern was also obtained with another anti-PLCβ4 antiserum, raised in guinea pig (M. Watanabe, unpublished observations). Both antisera recognize a single polypeptide band of 134 kDa apparent molecular weight on Western blots of mouse (Nakamura et al., 2004) and human (Hassan et al., unpublished data) cerebellar homogenates. The band is absent from Western blots of cerebellar homogenates from a PLCβ4 null mouse (Jiang et al., 1996; Nakamura et al., 2004).

Immunohistochemistry was carried out on free-floating sections as described previously (Marzban 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 PBS buffer containing 0.1% Triton X-100 and 5% bovine serum albumin (blocking solution) and the primary for 16–18 h at room temperature. Finally, sections were washed and incubated in biotinylated goat anti-rabbit or